



Original Article

## Protective effects of *Eucommia ulmoides* Oliv. leaf extract on cisplatin-induced kidney injury in mice

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**ABSTRACT** — Cisplatin is a widely used chemotherapeutic agent; however, its clinical utility is limited by dose-dependent nephrotoxicity. Although hydration and diuretics are commonly employed to mitigate renal injury, their protective efficacy remains suboptimal. Correspondingly, *Eucommia ulmoides* Oliv. leaf extract (ELE) is a functional food ingredient with diuretic, antioxidant, and anti-inflammatory properties. Therefore, this study investigated the protective effects of ELE against cisplatin-induced nephrotoxicity (CIN) in mice and elucidated the underlying mechanisms. Mice were administered cisplatin (15 mg/kg) with or without ELE or furosemide (potent diuretic). Cisplatin administration markedly elevated blood plasma urea nitrogen levels and reduced creatinine clearance, thereby indicating renal dysfunction. Co-administration of ELE substantially ameliorated renal function markers and attenuated histological tubular damage. In contrast, furosemide failed to improve renal dysfunction despite increasing urine output. This suggests that diuresis alone is insufficient for renal protection. Furthermore, ELE markedly suppressed cisplatin-induced upregulation of proinflammatory cytokines (tumor necrosis factor- $\alpha$  and interleukin-1  $\beta$ ) and renal injury markers (kidney injury molecule-1 and lipocalin-2) in kidney tissues. These findings indicate that ELE exerts renoprotective effects against CIN through diuretic, anti-inflammatory, and antioxidant mechanisms. These mechanisms most likely involve modulation of inflammatory signaling pathways in the kidneys. Overall, ELE shows promising potential as an adjunctive therapeutic agent for patients undergoing chemotherapy.

**Key words:** Chemotherapy, Cisplatin, Diuresis, *Eucommia ulmoides*, Nephrotoxicity, Renoprotection



## INTRODUCTION

Although many cancer treatment options have been established in recent years owing to advances in cancer drug therapy, serious side effects associated with anticancer drugs continue to markedly impair patients' quality of life and represent a major factor limiting treatment continuation. Among these agents, cisplatin, a platinum-based chemotherapeutic, is widely used as a core treatment for lung, gastric, and ovarian cancers; however, nephrotoxicity is a common dose-limiting side effect of cisplatin (Chovanec *et al.*, 2017). Cisplatin-induced nephrotoxicity (CIN) primarily occurs due to cellular damage in the proximal tubules and may progress to irreversible renal dysfunction (Arany and Safirstein, 2003; Isnard-Bagnis *et al.*, 2005; Karasawa and Steyger, 2015; Digby *et al.*, 2020; Holditch *et al.*, 2019). Currently, hydration therapy and diuretics are used as supportive care to prevent CIN; however, neither approach is based on sufficient evidence and both strategies impose a heavy physical burden on patients (Santoso *et al.*, 2003; Cornelison and Reed, 1993). Therefore, the development of safer and more effective renal protection strategies is urgently needed. Recently, *in vitro* and *in vivo* studies have shown that natural products, such as polyphenols, can inhibit renal damage, and their use has attracted attention (Ashkar *et al.*, 2022; Liu *et al.* 2023; Rodrigo and Bosco, 2005; Rodrigo *et al.*, 2004; Jin *et al.*, 2009; He *et al.*, 2018; Wu *et al.*, 2014; Ong *et al.*, 2012). However, the efficacy of many of these products in humans has not been clearly demonstrated, and they have not yet reached clinical application.

Correspondingly, *Eucommia ulmoides* Oliv. is a deciduous tree that has long been used as a medicinal plant in China and other Asian countries (He *et al.*, 2013; Ishimitsu *et al.*, 2021). The bark of the plant is used as a crude drug, and its leaves are consumed as a health food. Moreover, *E. ulmoides* leaf extract (ELE), which is derived from *Eucommia* leaves, contains functional components, such as geniposidic acid, chlorogenic acid, quercetin, and asperuloside. The extract possesses various pharmacological activities, including hypotensive, diuretic, anti-inflammatory, and antioxidant effects (Hashikawa-Hobara *et al.*, 2020; Hosoo *et al.*, 2015; Kwan *et al.*, 2003; Fujikawa *et al.*, 2010; Hirata *et al.*, 2011; Fujikawa *et al.*, 2012). Notably, ELE has demonstrated these effects not only *in vitro* and in animal experiments but also in clinical studies in humans, thereby confirming its safety and efficacy (Greenway *et al.*, 2011). Unlike conventional natural product-derived ingredients, ELE is among the few functional materials whose efficacy in humans has been scientifically supported; hence, the extract rep-

resents a realistic and promising candidate for clinical application. Accordingly, this study investigated whether ELE, a naturally occurring functional food ingredient, exerts a preventive effect against cisplatin-induced renal injury and elucidated its efficacy and mechanism of action in renal protection. Based on the established safety and pharmacological effects of ELE in humans, this study presents a new supportive care option for cancer pharmacotherapy and a clinically implementable strategy for preventing renal injury.

## MATERIALS AND METHODS

### Preparation of ELE

ELE was provided by Kobayashi Pharmaceutical Co., Ltd. (Osaka, Japan) and prepared as previously described (Fujikawa *et al.*, 2010). Briefly, fresh *E. ulmoides* leaves were steamed at 100–110°C, dried, and roasted. The roasted leaves were extracted with hot water at 90°C for 1 hr. The extract was filtered, concentrated, and allowed to stand for one day. The supernatant was then filtered, concentrated, and vacuum-dried to obtain ELE powder.

### Quantification of asperuloside, geniposidic acid, and chlorogenic acid

The ELE was diluted with ultrapure water to a concentration of 0.4 mg/mL, mixed with an equal volume of acetonitrile to achieve a final concentration of 0.2 mg/mL, and filtered through a 0.22- $\mu$ m filter before analysis. Quantitative analysis was performed using a Shimadzu Nexera Ultra-High-Performance Liquid Chromatography (UHPLC) system coupled to a Triple Quad 3500 mass spectrometer (AB SCIEX, Framingham, MA, USA). Chromatographic separation was achieved using a Shim-pack VP-ODS column (150 mm  $\times$  2.0 mm i.d., 5  $\mu$ m; Shimadzu, Kyoto, Japan) maintained at 40°C, and the injection volume was 2  $\mu$ L. The gradient elution program was as follows: 10% solvent B at 0.0 min, increasing to 20% B at 10.0 min, with a flow rate of 0.4 mL/min. Solvent A consisted of 0.1% formic acid in water, and solvent B consisted of 0.1% formic acid in acetonitrile. The mass spectrometer was operated in the negative electrospray ionization mode with multiple reaction monitoring (MRM). The ion-source temperature was set to 550°C. The optimized MRM transitions and collision energies (CE) for each compound were as follows: asperuloside ( $m/z$  413.0  $\rightarrow$  147.0, CE –26.8 V), geniposidic acid ( $m/z$  373.0  $\rightarrow$  123.0, CE –26.1 V), and chlorogenic acid ( $m/z$  353.0  $\rightarrow$  191.0, CE –29.8 V). Data acquisition and processing were conducted using Analyst software (version 1.7.3).

### Animal model of CIN

All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee of Tokushima University Graduate School; the protocol was approved by the Institutional Review Board of Tokushima University Graduate School for Animal Protection (permit numbers T2021-62 and T2024-57). Nine-week-old C57BL/6 J male mice weighing 24–27 g were purchased from Nippon CLEA (Tokyo, Japan) and maintained with *ad libitum* access to water and food (NMF type; Oriental Yeast, Tokyo, Japan). For the treatment group, the NMF diet was mixed with ELE at a 15% (w/w) ratio. The control, cisplatin, and furosemide groups were fed an NMF diet without ELE. The relative humidity in the breeding room was set at  $50 \pm 10\%$ , and the room temperature was  $26 \pm 1^\circ\text{C}$ , with a 12-hr light/dark cycle (lights on at 8:00 AM; lights off at 8:00 PM).

### Sample collection and evaluation of renal damage

The mice were randomly divided into the following six groups ( $n = 9\text{--}11$  per group): group 1, vehicle-injected; group 2, ELE-treated; group 3, furosemide-injected (10 mg/kg); group 4, cisplatin-injected; group 5, cisplatin-injected with ELE; and group 6, cisplatin-injected with furosemide. The mice were intraperitoneally injected once with cisplatin (groups 4–6, 15 mg/kg) or vehicle (groups 1–3). ELE was administered *ad libitum* for four days before the cisplatin injection until sample collection (groups 2 and 5). Furosemide was administered intraperitoneally for four days starting from the day before cisplatin injection (groups 3 and 6). The doses were based on those used as diuretics in animal models (Goda *et al.*, 2021; Yoshioka *et al.*, 2023; Kanda *et al.*, 2022).

The mice were housed in metabolic cages (under the same conditions described above) for 24 hr between 48 and 72 hr after cisplatin administration to collect urine. At 72 hr after cisplatin administration, the mice were intraperitoneally anesthetized with a mixture of medetomidine, midazolam, and butorphanol and euthanized. After weighing, approximately 500  $\mu\text{L}$  of blood was collected from each mouse using a microcapillary tube. Subsequently, a thoracotomy was performed, and systemic blood was removed by perfusing saline from the apex of the left ventricle at 8 mL/min for approximately 15 min. Subsequently, a laparotomy was performed to confirm complete renal blood removal, and the kidneys were excised. The collected blood was incubated at  $20^\circ\text{C}$  for 1 hr and then centrifuged twice (Centrifuge 5417R; Eppendorf, Hamburg, Germany) at  $1,600 \times g$  and  $4^\circ\text{C}$  for

15 min to obtain the serum. Thereafter, serum and urine samples were sent to Oriental Yeast Co., Ltd. (Shiga, Japan) to measure blood urea nitrogen (BUN), serum creatinine, and urine creatinine levels. Creatinine clearance (Ccr) was calculated using the following formula:

$$\text{Ccr (mL min}^{-1} \text{ kg}^{-1}) = \frac{\text{urine volume (mL min}^{-1} \text{ kg}^{-1}) \times \text{urine Cr concentration (mg L}^{-1})}{\text{serum Cr concentration (mg L}^{-1})}$$

### Real-time polymerase chain reaction

Kidneys were collected from mice with cisplatin-induced kidney injury. Thereafter, RNA was extracted from the kidney samples using an RNA extraction solution (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) was reverse-transcribed using the PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan) and a Polymerase Chain Reaction (PCR) Thermal Cycler Dice (Takara Bio). The cDNA from each sample was mixed with forward and reverse primers and THUNDERBIRD SYBR quantitative PCR Mix (Toyobo, Osaka, Japan). PCR was performed using an Applied Biosystems StepOnePlus machine (Applied Biosystems, Waltham, MA, USA). Fold changes in gene expression relative to those in the control group were evaluated using mouse glyceraldehyde 3-phosphate dehydrogenase as the internal standard. The PCR primer sets are listed in Supplementary Table S1.

### Histological analysis

Renal tubular injury was assessed as previously described. Briefly, the kidney samples were fixed with 4% paraformaldehyde and embedded in paraffin. The samples were cut into 4- $\mu\text{m}$  sections and stained with hematoxylin and eosin. Tubular damage was scored blindly by  $\geq 3$  independent observers, other than the experimenter. Scoring was based on the percentage of damage, which was characterized by tubular necrosis, brush border loss, cast formation, tubular dilation, and tubular degeneration. The following scale was used: 0, normal; 1,  $<25\%$ ; 2, 25–50%; 3, 50–75%; and 4,  $>75\%$ . From each sample, eight images of the proximal tubules were captured at different locations, and the degree of damage was scored for each image. The average damage score of the eight images was used as the damage score for each sample.

### Statistical analysis

Statistical analyses were conducted using Student's *t*-test for comparisons between two groups. One-way analysis of variance was performed to compare three or more groups, followed by post hoc analysis using Tukey's test. Statistical analyses were performed using R version

3.2.1 for Windows (R Foundation for Statistical Computing, Vienna, Austria). Results were considered statistically significant at  $P < 0.05$  (two-tailed).

## RESULTS

### Active ingredient content in ELE

The concentrations of asperuloside, geniposidic acid, and chlorogenic acid in ELE were determined using UHPLC-tandem mass spectrometry. Quantitative analysis revealed that the extract contained 4.3 mg/g of asperuloside, 15.5 mg/g of geniposidic acid, and 29.2 mg/g of chlorogenic acid (mean of duplicate measurements). Representative MRM chromatograms for each compound are shown in Supplementary Data 1–3.

### Protective effect of ELE on cisplatin-induced kidney injury in mice

The effects of ELE on cisplatin-induced renal injury were investigated in mice. Body weight was lower in cisplatin-treated mice than in vehicle-treated mice; however, kidney weight remained unchanged (Table 1). The effects of ELE on cisplatin-induced renal dysfunction in mice were also examined. As shown in Fig. 1, cisplatin administration markedly increased plasma BUN levels and decreased Ccr compared with that in the vehicle group, thereby indicating severe renal impairment in the cisplatin group. However, co-administration of ELE considerably attenuated these changes and preserved renal function. A furosemide group was included to evaluate the role of diuresis. Furosemide alone increased urinary volume (Supplemental Data 4), thus confirming its diuretic effect at the administered dose (Mekonnen *et al.*, 2009). Although cisplatin tended to decrease urinary volume, co-administration of ELE markedly increased urinary volume compared with that in the cisplatin group. In contrast, co-administration of furosemide with cisplatin did not improve BUN or Ccr levels, despite its diuretic potential (Fig. 1).

### Suppression of tubular injury markers

Renal tubular injury was further assessed by measuring the mRNA expression levels of kidney injury molecule-1 (Kim-1) and lipocalin-2 (Lcn-2). Cisplatin administration markedly upregulated the expression of both markers. However, the co-administration of ELE considerably suppressed these increases compared with that in the cisplatin-only group (Fig. 2A, B).

### Histological improvement of renal tissue

Histological examination using hematoxylin and eosin staining revealed severe renal tubular damage in cisplatin-treated mice, which was characterized by nuclear loss, proximal tubular cell vacuolization, and dilation. In contrast, mice treated with ELE and cisplatin exhibited preserved renal architecture, with substantially less tubular necrosis and dilation (Fig. 2C). The histological damage score (evaluated blindly) was substantially lower in the ELE and cisplatin groups than in the cisplatin-alone group (Fig. 2D).

### Inhibition of inflammatory cytokine expression

The effects of ELE on renal inflammation were investigated. Quantitative real-time PCR analysis showed that the mRNA levels of proinflammatory cytokines, tumor necrosis factor- $\alpha$  (*Tnfa*) and interleukin-1 beta (*Il1b*), were upregulated in the kidney tissues 72 hr after cisplatin administration. Co-administration of ELE substantially downregulated the expression of these cytokines, thereby suggesting an anti-inflammatory effect (Fig. 3).

## DISCUSSION

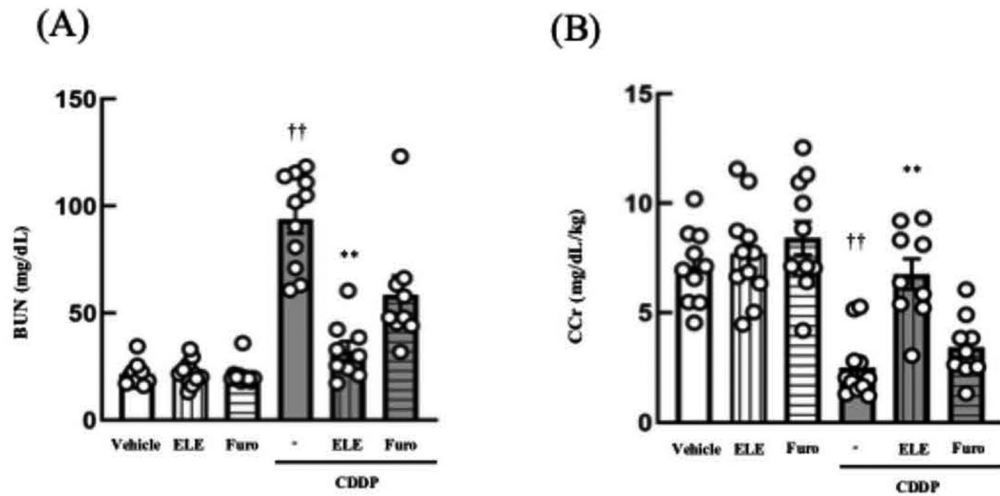
This study demonstrated that ELE exhibits a marked protective effect against CIN. These findings suggest that this effect is mediated by multifaceted pharmacological activities, including antioxidant and anti-inflammatory effects in addition to diuretic action. Drug repositioning research, using large medical databases and basic

**Table 1.** Body weight, kidney weight, and renal function in vehicle-treated mice and cisplatin-treated mice with or without ELE.

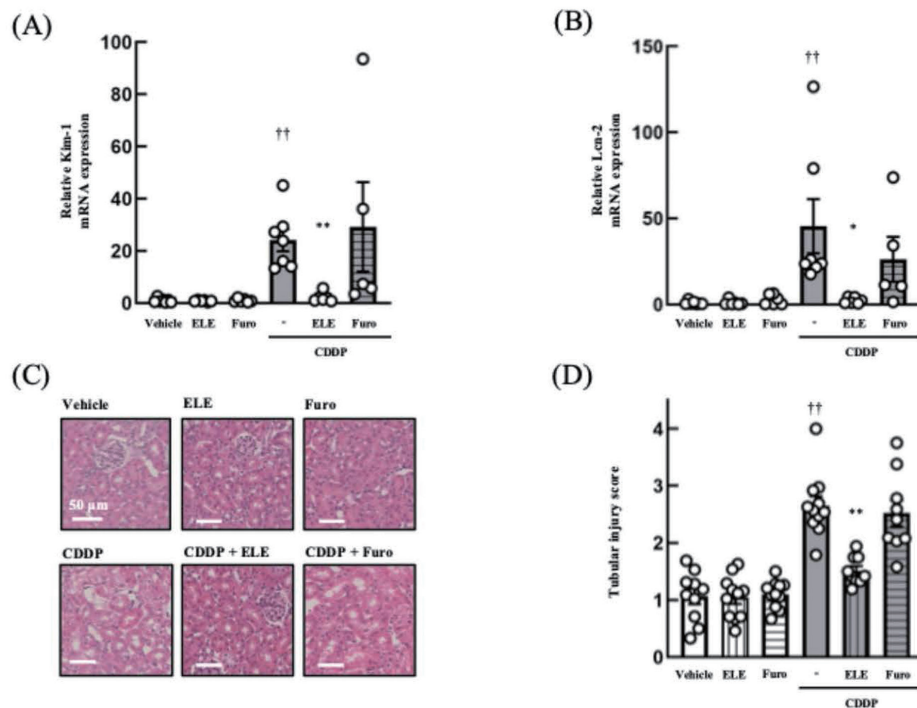
|                                    | Vehicle         | ELE15%          | Furo            | CDDP            | CDDP+ELE15%     | CDDP+Furo       |
|------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Initial body weight (g)            | 23.7 $\pm$ 0.2  | 23.7 $\pm$ 0.3  | 23.0 $\pm$ 0.4  | 22.9 $\pm$ 0.3  | 23.4 $\pm$ 0.6  | 22.9 $\pm$ 0.5  |
| Post body weight (g)               | 21.9 $\pm$ 0.4  | 19.5 $\pm$ 0.4  | 22.2 $\pm$ 0.7  | 19.8 $\pm$ 0.4  | 18.6 $\pm$ 0.7  | 18.6 $\pm$ 0.5  |
| Kidney weight (mg)                 | 136.1 $\pm$ 3.7 | 135.1 $\pm$ 4.1 | 132.9 $\pm$ 2.7 | 134.4 $\pm$ 3.0 | 122.8 $\pm$ 4.3 | 126.0 $\pm$ 4.4 |
| Kidney weight / body weight (mg/g) | 6.2 $\pm$ 0.2   | 6.9 $\pm$ 0.2   | 6.0 $\pm$ 0.2   | 6.8 $\pm$ 0.2   | 6.7 $\pm$ 0.2   | 6.8 $\pm$ 0.3   |

Note: Data are expressed as mean  $\pm$  SEM. SEM, standard error of the mean; CDDP, cisplatin; ELE, *Eucommia ulmoides* leaf extract, Furo, furosemide.

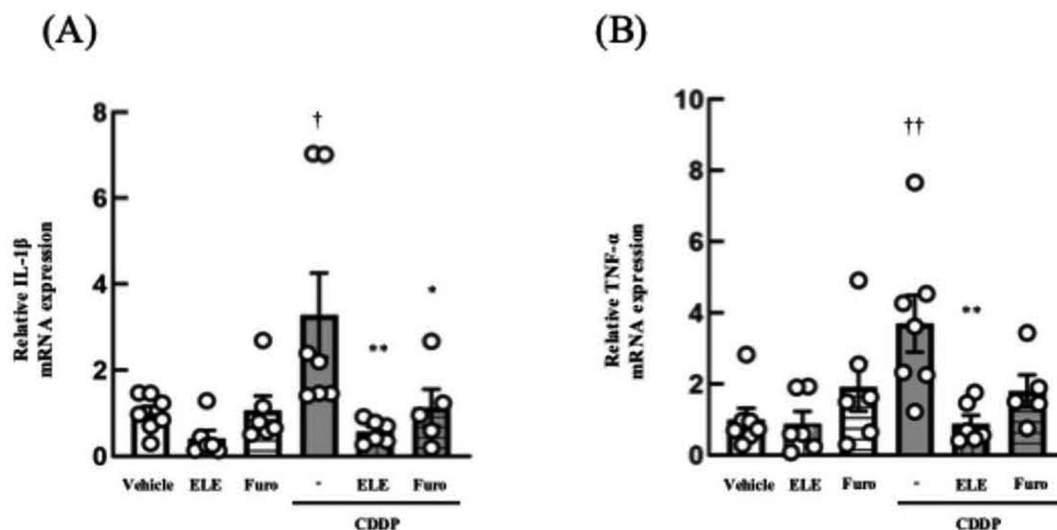
*Eucommia ulmoides* leaf extract alleviates kidney injury



**Fig. 1.** Effect of ELE on cisplatin-induced nephrotoxicity. Renal function was assessed using: (A) blood urea nitrogen (BUN) and (B) creatinine clearance (CCr) tests. Data are expressed as mean  $\pm$  SEM. SEM, standard error of the mean; Veh, vehicle; CDDP, cisplatin; ELE, *Eucommia ulmoides* leaf extract; Furo, furosemide. † $P$  < 0.05, †† $P$  < 0.01 vs. Veh group; \* $P$  < 0.05, \*\* $P$  < 0.01 vs. CDDP group.  $n$  = 9–11 per group



**Fig. 2.** Effect of ELE on cisplatin-induced kidney injury markers and histological damage. The mRNA expression levels of kidney injury markers (A) Kim-1 and (B) Lcn-2 in the kidney tissues of mice from each group. (C) Representative images of hematoxylin and eosin staining of kidney sections from vehicle-treated, cisplatin-injected, and cisplatin plus ELE-treated mice. Scale bar, 100  $\mu$ m. (D) Quantitative analysis of renal damage score. Data are expressed as mean  $\pm$  SEM. SEM, standard error of the mean; Veh, vehicle; CDDP, cisplatin; ELE, *Eucommia ulmoides* leaf extract; Furo, furosemide; Kim-1, kidney injury molecule-1; Lcn-2, lipocalin-2. † $P$  < 0.05, †† $P$  < 0.01 vs. Veh group; \* $P$  < 0.05, \*\* $P$  < 0.01 vs. CDDP group.  $n$  = 5–11 per group



**Fig. 3.** Effect of ELE on inflammatory cytokine expression in the kidneys. The mRNA expression levels of the inflammatory cytokines (A) IL-1 $\beta$  and (B) TNF- $\alpha$  in the kidneys of mice in each group. Data are expressed as mean  $\pm$  SEM. SEM, standard error of the mean; Veh, vehicle; CDDP, cisplatin; ELE, *Eucommia ulmoides* leaf extract; Furo, furosemide; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ , interleukin-1 beta. † $P < 0.05$ , †† $P < 0.01$  vs. Veh group; \* $P < 0.05$ , \*\* $P < 0.01$  vs. CDDP group.  $n = 5-7$  per group

research, has been actively pursued to discover novel preventive agents for CIN (Kanda *et al.*, 2022). Accordingly, agents such as diphenhydramine (Hamano *et al.*, 2021) and valproic acid (Yoshioka *et al.*, 2023) have shown promise; however, caution is required with supportive care drugs such as first-generation 5-hydroxytryptamine type 3 receptor antagonists, which may exacerbate CIN (Goda *et al.*, 2021). Therefore, this study focused on ELE, a safe and readily available functional food.

ELE markedly improved renal function markers (BUN and Ccr) in the murine CIN model. Notably, the group treated with furosemide, a potent diuretic, showed no improvement in renal dysfunction despite an increase in urine volume. This result strongly suggests that merely promoting cisplatin excretion via diuresis is insufficient for preventing CIN and direct protective effects on renal tissues are essential.

Histological evaluation and analysis of renal injury markers (Kim-1 and Lcn-2) confirmed that ELE suppressed cisplatin-induced tubular necrosis in the kidneys. Furthermore, ELE substantially downregulated the expression of proinflammatory cytokines (*Tnfa* and *Il1b*). Cisplatin-induced mitochondrial dysfunction leads to overproduction of reactive oxygen species (ROS), which activate the transcription factor nuclear factor kappa b (NF- $\kappa$ B), thereby amplifying the inflammatory response.

Geniposidic acid is a major component of ELE that inhibits the NF- $\kappa$ B signaling pathway in various inflammation conditions (Xia *et al.*, 2017; Kosaka *et al.*, 2012; Zhang and Yue; 2020). The study findings of suppressed cytokine expression align with these reports, which suggests that the anti-inflammatory effect of ELE is likely mediated by the downregulation of NF- $\kappa$ B activation, thereby blocking the inflammatory cascade.

ELE is rich in iridoids (e.g., geniposidic acid and asperuloside), phenylpropanoids, and flavonoids, which possess potent antioxidant properties. These components directly scavenge cisplatin-induced excess ROS and prevent oxidative stress-induced cellular damage through various mechanisms. Thus, ELE likely halts kidney injury progression through a dual mechanism of alleviating oxidative stress and suppressing the inflammatory response. Although the diuretic effect of ELE may contribute to reducing cisplatin retention time in the tubules, data indicates that its cytoprotective properties play a more pronounced role.

This study has some limitations. First, a detailed phytochemical analysis of the study ELE batch using high performance liquid chromatography was not performed; however, ELE contains stable amounts of bioactive compounds, such as geniposidic acid. Second, the molecular mechanisms, particularly direct inhibition of the

NF- $\kappa$ B pathway and protein-level changes in antioxidant enzymes, have not been fully elucidated. Therefore, further studies using specific inhibitors or knockout models are warranted to verify the precise signaling pathways involved. Despite these limitations, the robust *in vivo* phenotypic improvements observed provide strong evidence for the therapeutic utility of ELE.

In conclusion, ELE exerts protective effects against CIN through multiple pharmacological mechanisms. Therefore, oral administration of ELE represents a novel evidence-based supportive therapy with minimal burden on patients and warrants further clinical evaluation.

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### Conflict of interest

The authors declare that there is no conflict of interest.

### Data availability

The data in this study are included in the article/supplementary materials. Contact the corresponding author(s) directly to request the underlying data.

### Author contributions

Conceptualization: MG. Data curation, Formal analysis, and Investigation: MK, TY, SI, SS, YK, YS, TI, and TH. Writing – original draft: ST and MG drafted the manuscript. Supervision and Writing – review and editing: IH, TN, FA, YI-I, KK, and KI. All authors have read and approved the final manuscript.

### Ethical approval and consent to participate

The animal study protocol was approved by the Institutional Review Board of the Tokushima University Graduate School for Animal Protection (permit numbers: T2021-62 and T2024-57). All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee of the Tokushima University Graduate School.

### Patient consent for publication

Not applicable.

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