

Renoprotective effect of *Chrysanthemum coronarium* L. extract on adenine-induced chronic kidney disease in mice

Yiseul Kim

Food Functionality Research, Korea Food Research Institute

Ae Sin Lee

Food Functionality Research, Korea Food Research Institute

Haeng Jeon Hur

Food Functionality Research, Korea Food Research Institute

Sang Hee Lee

Food Functionality Research, Korea Food Research Institute

Hyun Jin Na

Food Functionality Research, Korea Food Research Institute

Mi Jeong Sung (✉ dulle5@kfri.re.kr)

Food Functionality Research, Korea Food Research Institute

Article

Keywords: *Chrysanthemum coronarium* L. extract, inflammation, fibrosis, chronic kidney disease

Posted Date: May 11th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2884236/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Chronic kidney disease (CKD) is a progressive loss of kidney function associated with inflammation and fibrosis. *Chrysanthemum coronarium* L. (CC), a leafy vegetable, possesses various beneficial properties, including anti-oxidative, anti-inflammatory, and antiproliferative effects. This study aimed to determine the renoprotective effects of CC on adenine-induced CKD in mice. CKD was induced by feeding mice an adenine diet (0.25% w/w) for 4 weeks. Changes in renal function, histopathology, inflammation, and renal interstitial fibrosis were analyzed. The adenine-fed mice were characterized by increased blood urea nitrogen, serum creatinine, and histological changes, including inflammation and fibrosis; however, these changes were significantly restored on treatment with CC. Additionally, CC inhibited the expression of inflammatory markers, monocyte chemoattractant protein-1, interleukins-6 and -1 β , intercellular adhesion molecule-1, and cyclooxygenase 2. Moreover, CC suppressed the expression of the fibrotic markers, type IV collagen, and fibronectin. Furthermore, CC attenuated the expression of profibrotic genes (tumor growth factor- β and α -smooth muscle actin) in adenine-induced renal injury mice. These results suggest that CC has the potential to attenuate adenine-induced renal injury and might offer a new option as a renoprotective agent or functional food supplement for moderate CKD.

Introduction

Chronic kidney disease (CKD) is a global health challenge in most countries ¹. CKD is characterized by a progressive and irreversible loss of kidney function, leading to glomerular injury and tubulointerstitial fibrosis, resulting in end-stage renal disease through a common pathological process ^{2,3}. Although accumulating evidence has focused on developing new and efficient treatments, there are no effective agents for preventing or delaying the development of CKD ⁴. Therefore, identifying an effective agent for satisfactorily treating CKD is crucial.

Several studies have documented the beneficial effects of herbs, edible medicinal products, and traditional foods on CKD. Several edible medicinal foods reportedly have renoprotective effects on adenine (AD)-induced CKD, such as Erhuang Formula ³, *Garcinia lucida* Vesque (Clusiaceae) ⁵, *Laminaria japonica* ⁶, *Lindera aggregate* ⁷, *Salvia miltiorrhiza* ⁸, and Chinese chive (*Allium tuberosum* Rottl. Ex Spreng). In addition, edible natural products contain many bioactive components that can act on multiple targets and exert various protective effects against CKD. Therefore, an in-depth study of edible natural products to protect against or delay CKD is essential.

Glebionis coronaria (*Chrysanthemum coronarium* L.; CC) is widely distributed in the Mediterranean region. It is considered a healthy food in Asia. CC, also known as Ssukgat in Korea, is a precious vegetable and medicinal plant ^{9,10}. Studies have shown that CC exerts potent antioxidant effects ^{11–13}. Similarly, we previously found the preventive effects of CC on bone metabolism in vitro and in vivo ⁹. In addition, we recently showed that CC protects against premature senescence in vitro ¹⁰. Although CC has

been shown to possess various activities, its role in CKD is unknown. Hence, in the present study, we aimed to determine whether CC exerted renoprotective effects against AD-induced renal injury.

Material and Methods

Preparation of CC Extracts

CC extract was purchased from a local market in Korea. The dried CC (1 kg) was crushed and extracted twice with 18 mL of 50% ethanol for 24 h. After filtration, the extracts were evaporated by freeze-drying at -70°C . The extraction yield was 8% (w/w). All experiments on plant including the collection of plant material, were performed in accordance with the institutional, national, and international guidelines and legislation.

Animals

Seven-week-old male C57BL/6 mice were purchased from Oriental Biotechnology (Daejeon, South Korea). The mice were housed under controlled laboratory conditions (the temperature at $22 \pm 2^{\circ}\text{C}$, humidity at $50 \pm 60\%$, and with a light-dark cycle of 12 h) and had free access to food and tap water. The experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Korea Food Research Institute (KFRI-M-22001); the experiments were conducted per the institutional guidelines established by the Committee. The animal studies are reported with the recommendations in the ARRIVE guidelines. All the relevant institutional guidelines, along with the ARRIVE guidelines were followed during the study.

Experimental procedure

The mice were randomly divided into three experimental groups: control (Cont, $n = 10$), AD (Ade, $n = 10$), and AD with CC (Ade + CC, $n = 10$). The control group was fed normal saline diet. CKD was induced by feeding mice with an AD diet (0.25% w/w). The Ade group was fed an AD diet supplemented with saline. The Ade + CC group was fed an AD diet supplemented with CC. CC was orally administrated once daily at 100 mg/kg/day. Body weights were measured weekly. After 4 weeks of feeding, all the mice were euthanized with 1.5% isoflurane (BKPharma Corp., Kyonggi-Do, Korea). Serum and kidney samples were collected from each mouse.

Biochemical parameters

Common serum biochemical parameters, including blood urea nitrogen (BUN), Serum creatinine (SCr), calcium, and phosphorous, were measured using a biochemical kit (Chema Diagnostic Di Marco Flore, Monsano, Italy), following the manufacturer's instructions.

Histopathological analysis

The kidneys were harvested, fixed in 10% formalin, dehydrated, and embedded in paraffin wax. The tissues were cut into 4 µm-sections from the paraffin blocks, dewaxed, and rehydrated. Sections were stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Masson's trichrome (MT) to analyze inflammation, fibrosis, atrophy, and dilation.¹⁴ The slides were analyzed under a panoramic microscope (3D HISTECH Panoramic 250, Hungary).

Immunohistochemistry (IHC)

The expression of α -smooth muscle actin (SMA) was analyzed with immunohistochemistry (IHC) staining. After blocking at room temperature for 10 min, the slides were incubated with the anti- α -SMA (1:500) at 4 °C overnight. After incubation with secondary antibodies, the sections were developed using 3,3'-Diaminobenzidine (Dako, CA, USA). The stained slides were observed under a panoramic microscope (3D HISTECH Panoramic 250, Hungary). Five randomly selected microscopic fields (200× magnification) were analyzed quantitatively in a blinded manner.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the kidney tissues (100 mg) using an RNeasy RNA isolation kit (GeneAll). Subsequently, the RNA was reverse-transcribed to cDNA using an iScript cDNA synthesis kit with random primers (Bio-Rad). Finally, the synthesized cDNA was used for qRT-PCR using the SYBR Green PCR Master Mix Kit (Invitrogen). The expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase served as the endogenous control. The actual primer sequences are listed in Table 1.

Table 1
List of primers used in the study

Gene	Primer sequence (5'-3')	Tm (°C)
MCP-1	<i>F: GCCTGCTGTTACAGTTGC</i>	59.1
	<i>R: CAGGTGAGTGGGGCGTTA</i>	58.0
IL-6	<i>F: TGAGAGTAGTGAGGAACAAG</i>	52.8
	<i>R: CGCAGAATGAGATGAGTTG</i>	53.0
IL-1 β	<i>F: TGAGCTCGCCAGTGAAATGAT</i>	59.1
	<i>R: TCCATGGCCACAACAACCTGA</i>	58.8
F4/80	<i>F: CCTGGACGAATCCTGTGAAG</i>	57.0
	<i>R: GGTGGGACCACAGAGAGTTG</i>	59.0
Type IV Collagen	<i>F: TTAAAGGACTCCAGGGACCAC</i>	58.0
	<i>R: CCCACTGAGCCTGTCACAC</i>	59.3
Fibronectin	<i>F: CCCTATCTCTGATACCGTTGTCC</i>	58.8
	<i>R: TGCCGCAACTACTGTGATTCCG</i>	62.4
TGF β -1	<i>F: TCAGACATTCGGGAAGCAGT</i>	58.0
	<i>R: ACGCCAGGAATTGTTGCTAT</i>	56.9
α -SMA	<i>F: GCCCAGAGCAAGAGAGG</i>	55.6
	<i>R: TGTCAGCAGTGTCGGATG</i>	56.1
GAPDH	<i>F: AAATGGTGAAGGTCGGTGTG</i>	60.0
	<i>R: TGAAGGGGTCGTTGATGG</i>	60.0

Western blotting analysis

Western blotting analysis

The Kidney tissues were homogenized in a radioimmunoprecipitation assay buffer (Beyotime, Los Altos, CA, USA). Similarly, 20 μ g of tissue lysates were loaded onto SDS-PAGE gels, separated, and transferred onto polyvinylidene fluoride membranes. Subsequently, the membranes were blocked with 5% skimmed milk and incubated the intercellular adhesion molecule (ICAM)-1, cyclooxygenase (COX)-2, and β -actin (Santa Cruz Biotechnology, Dallas, TX, USA). Next, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Cell Signaling Technology). Finally, signals were detected using an image analyzer (ChemiDoc[™] XRS + System; Bio-Rad Laboratories). Finally, densitometry was performed with ImageJ (NIH).

Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD) and analyzed using GraphPad Prism software version 9.0 (Inc., La Jolla, CA, USA). One-way analysis of variance, followed by Tukey's multiple comparison test, were used to detect statistical differences. Differences were considered statistically significant at $p < 0.05$.

Results

CC attenuates AD-induced renal injury

Mice were fed an AD diet for 4 weeks to establish a CKD model. The body weight in the Cont group increased; however, it decreased in the Ade group. Although the body weight of mice in the Ade + CC group was decreased, it was higher than that of those in the Ade group (Figure. 1A). BUN and SCr levels are known biomarkers of kidney function. Next, the levels of BUN and SCr in the Ade group were significantly increased compared with those in the Cont group ($p < 0.0001$ for both). However, compared with the Ade group, BUN and SCr levels in the Ade + CC group were reduced ($p < 0.01$, $p < 0.05$, respectively). CKD is associated with an imbalance in calcium and phosphorus homeostasis (Figure. 1B and 1C). The levels of calcium and phosphorus in the Ade group were significantly increased compared with those in the Cont group ($p < 0.0001$, for both). However, compared with the Ade group, the levels of calcium and phosphorus in the Ade + CC group were decreased (ns and $p < 0.01$, respectively) (Figure. 1D and 1E). H&E, PAS, and MT stainings were performed on the kidney sections from each group to investigate the effects of CC on histopathological changes (Figure. 2). Extensive renal damages, including evident renal tubular dilation, inflammatory cell infiltration, and fibrosis, were observed in the Ade group compared with the Cont group. However, renal damage was significantly reduced in the Ade + CC group ($p < 0.05$). These results suggest that CC could improve renal function and suppress renal damage in CKD mice.

CC suppress inflammatory responses in AD-induced renal injury mice

It is well-established that chronic inflammation accelerates the progression of fibrosis. To define the effect of CC in regulating inflammation, we analyzed the expression of inflammatory cytokines, such as monocyte chemoattractant protein (MCP)-1, interleukins (IL)-6 and -1β , and ICAM-1, COX-2, and macrophage activation markers F4/80. The qRT-PCR results showed that MCP-1, IL-6, and IL-1 β levels were significantly increased in the Ade group compared with those in the Cont group ($p < 0.0001$, for all), which were decreased in the Ade + CC group ($p < 0.01$, $p < 0.001$, and $p < 0.05$, respectively; Figure. 3A–C). In addition, western blotting analysis showed that the expression levels of ICAM-1 and COX-2 were increased in the Ade group compared with those in the Cont group (Figure. 3D–F, $p < 0.0001$) and were decreased in the Ade + CC group ($p < 0.05$). Moreover, qRT-PCR results showed that the expression level of F4/80 was increased in the Ade group compared with that in the Cont group (Figure. 3G, $p < 0.0001$),

which was decreased on CC treatment ($p < 0.05$). These data indicate that CC attenuated inflammation in AD-induced renal injury by suppressing inflammatory cytokine production and macrophage infiltration.

CC suppress extracellular matrix (ECM) deposit and tubulointerstitial fibrosis in AD-induced renal injury mice

To determine the effect of CC on AD-induced fibrotic responses, we assessed two of the ECM proteins, including type IV collagen and fibronectin (FN), using RT-PCR. Compared with the Cont group, the mRNA expression levels of type IV collagen and FN were significantly increased in the Ade group ($p < 0.01$ and $p < 0.001$, respectively). However, the mRNA expression levels were decreased in the Ade + CC group compared with the Ade group (Figure. 4A and B, $p < 0.05$, $p < 0.001$, respectively). Our results suggest that CC attenuated extracellular matrix overproduction and renal fibrosis in CKD mice.

CC attenuates the expression of profibrotic genes in AD-induced renal injury mice

To define the effect of CC in regulating fibrosis, we assessed the expression levels of fibrosis-related genes, including TGF- β and α -SMA, using qRT-PCR. A significant increase in TGF- β and α -SMA expression levels in the Ade group compared with the Cont group was observed (Figure. 5A and B, $p < 0.001$ and $p < 0.01$, respectively). In contrast, the Ade + CC group exhibited a reduced those of all parameters related with renal fibrosis ($p < 0.001$ for both). Furthermore, we assessed the α -SMA using IHC staining. Tubulointerstitial fibrosis in the Ade group mice was significantly higher than that in the Cont group ($p < 0.0001$). However, compared with the Ade group, tubulointerstitial fibrosis in mice in the Ade + CC group mice was also decreased (Figure. 5C and D, $p < 0.0001$). These results suggest that CC attenuated profibrotic gene expression in CKD mice.

Discussion

CKD is a progressive and irreversible disease, increasingly becoming more common and a critical health challenge with no effective therapy. Thus, many researchers have investigated the pathogenesis of CKD, its underlying mechanisms, and the development of effective and new treatments. In humans, AD causes metabolic abnormalities that mimic CKD. Accumulating evidence has shown that CKD is induced in mice fed an AD diet, a well-established model for exploring renal injury^{15,16}. After feeding AD, the base is metabolized to 2,8-dihydroxyadenine, deposited and crystallized in the proximal renal tubules owing to renal injury, leading to accumulation of urine and inflammation of the tubule, and causing tubulointerstitial fibrosis¹⁷. This study's results indicate that mice fed with AD exhibited significant weight loss and renal dysfunction owing to markedly increased levels of BUN and SCr. In addition, our findings showed that the kidneys of AD-fed mice exhibited significant tubular and interstitial injuries, including tubular atrophy, dilation, interstitial inflammation, and fibrosis. Consistent with previous studies

^{16–18}, these findings provide sufficient evidence of renal damage in AD-fed mice. In the present study, we observed that CC treatment effectively reduced the increase in BUN and SCr levels and histological renal morphology in AD-induced CKD mice. These findings suggest that CC is a potential inhibitor of renal injury.

Furthermore, evidence indicates that the progression of CKD involves inflammation and fibrosis. Many studies have reported that the increased levels of inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, facilitate the progression of CKD ^{19,20}. Similarly, AD feeding increased the expression of renal inflammatory factors, including IL-6 and IL-1 β ²¹. In addition, MCP-1 expression was associated with increased COX-2 ²². MCP-1, an inflammatory cytokine, increases the proliferation, infiltration, and production of several cytokines and chemokines in inflammatory cells ^{21,23}. ICAM-1 is a chemotactic factor that enables macrophages to maintain an inflammatory response. Activating ICAM-1 recruits macrophage. Thus, ICAM-1 is an essential factor for macrophage infiltration into the renal tissue. Moreover, F4/80 is commonly recognized as a marker of macrophage infiltration. Thus, increased expression of F4/80 in the kidneys indicates active inflammatory responses and has been well-documented in kidney injuries ²⁴. In this study, CC inhibited the increased expression of MCP-1, IL-6, IL-1 β , COX-2, ICAM-1, and F4/80 in AD-induced kidney injury. These findings suggest that CC exerts anti-inflammatory effects on AD-induced renal injury.

Furthermore, the development of CKD is associated with tubulointerstitial fibrosis ²⁵. TGF- β 1 has been established as a key factor of the renal fibrosis process, ²⁶ which induces the development of epithelial-to-mesenchymal transition (EMT) ^{27,28}. Myofibroblasts generate inflammatory cytokines and collagenous matrix, leading to renal fibrosis. The renal tubules with EMT show upregulated expression of α -SMA and ECM proteins (FN and type IV collagen) ¹⁷. TGF- β 1 promotes increased ECM production, including FN and type IV collagen ²⁹. Several studies reported that -feeding mice with an AD diet increases the expression of TGF- β 1 and ECM proteins such as type 4 collagen and FN, resulting in renal fibrosis ^{4,29}. In this study, AD treatment significantly increased the expression levels of TGF- β 1, type IV collagen, FN, and α -SMA in kidneys, which were significantly suppressed by CC treatment. Our findings suggest that CC exerts antifibrotic effects in patients with CKD.

Epidemiological evidence indicates that consuming a diet rich in vegetables, medicinal plants, or their derived flavonoids can delay the progression of CKD, possibly owing to the bioactivity of these compounds and other phytochemicals that act synergistically. Hence, we investigated whether CC can be used as a potential target to alleviate CKD in AD-fed mice. Several studies have reported that CC and CC-derived compounds have beneficial effects ⁹. Similar to our findings, previous studies have demonstrated that CC contains caffeic acid, chlorogenic acid, rutin, cynarin, 3,4-dicaffeoylquinic acid, and 3,5-dicaffeoyl-4-succinoyl quinic acid. Rutin has numerous beneficial health effects, including anti-inflammatory and anti-fibrosis in CKD. Caffeic acid exhibits pharmacological activities, including antioxidant and antifibrotic effects, and reduces renal tubulointerstitial fibrosis. Chlorogenic acid attenuates renal fibrosis. Furthermore, 3,4-dicaffeoylquinic acid possesses antioxidant and anti-

inflammatory properties. Therefore, we hypothesized that the CC extract, containing several bioactive compounds, would exert an inhibitory effect on AD-induced renal injury. Herein, the CC extract showed protective effects in improving renal function in AD-induced CKD by suppressing inflammatory factors and fibrosis, suggesting that the extract might be developed as a new renoprotective agent or functional food supplement to moderate CKD.

The present study had some limitations. First, we focused on inflammation and fibrosis in the kidneys. Hence, we assessed only the associated parameters. Second, we did not determine the therapeutic effects of the various CC-derived compounds. Therefore, further studies are needed to understand the mechanistic signaling pathways involved in the effects of CC and CC-derived components.

Conclusions

We confirmed that CC could improve renal function and protect against AD-induced chronic renal injury. In addition, the mechanism of renoprotection may be related to its anti-inflammatory and antifibrotic effects. Thus, CC may offer a new option for moderate CKD as a renoprotective agent or functional food supplement.

Declarations

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments

We thank Editage (www.editage.co.kr) for English language editing.

Author contributions

K.Y. and S.M.J designed the study; K.Y. and S.M.J. performed the measurements. S.M.J., H.H.J., L.S.H., N.H., and L.A.S. analyzed the data, interpreted the results, and prepared the figures. K.Y. and S.M.J. wrote and edited the manuscript. All authors have read and agreed to the final version of the manuscript.

Funding

This study was supported by a grant from the Korea Food Research Institute (grant number E0210102-03).

Competing interests:

The authors declare that they have no competing interests.

References

1. Webster, A. C., Nagler, E. V., Morton, R. L. & Masson, P. Chronic kidney disease. *The lancet* 389, 1238–1252 (2017).
2. Sohel, B. M. *et al.* Renal function trajectory over time and adverse clinical outcomes. *Clin. Exp. Nephrol.* 20, 379–393 (2016).
3. Zhang, C.-y. *et al.* Erhuang Formula ameliorates renal damage in adenine-induced chronic renal failure rats via inhibiting inflammatory and fibrotic responses. *Biomed. Pharmacother.* 95, 520–528 (2017).
4. Yi, H. *et al.* Metformin attenuates renal fibrosis in a mouse model of adenine-induced renal injury through inhibiting TGF- β 1 signaling pathways. *Front. Cell Dev. Biol.* 9, 603802 (2021).
5. Sonfack, C. S. *et al.* The aqueous extract from the stem bark of *Garcinia lucida* Vesque (Clusiaceae) exhibits cardioprotective and nephroprotective effects in adenine-induced chronic kidney disease in rats. *Evid. Based Complement. Alternat. Med.* 2021, 1–11 (2021).
6. Long, M. *et al.* Renoprotective effect of *Laminaria japonica* polysaccharide in adenine-induced chronic renal failure. *Molecules* 24, 1491 (2019).
7. Cai, H. *et al.* *Lindera aggregata* intervenes adenine-induced chronic kidney disease by mediating metabolism and TGF- β /Smad signaling pathway. *Biomed. Pharmacother.* 134, 111098 (2021).
8. Cai, H. *et al.* Protective effects of *Salvia miltiorrhiza* on adenine-induced chronic renal failure by regulating the metabolic profiling and modulating the NADPH oxidase/ROS/ERK and TGF- β /Smad signaling pathways. *J. Ethnopharmacol.* 212, 153–165 (2018).
9. Kim, S. A., Lee, A. S., Hur, H. J., Lee, S. H. & Sung, M. J. Preventive effects of chrysanthemum coronarium L. Extract on bone metabolism in vitro and in vivo. *Evid. Based Complement. Alternat. Med.* 2020, 6975646 (2020).
10. Sung, M. J. & Lee, A. S. Chrysanthemum coronarium L. Protects against Premature Senescence in Human Endothelial Cells. *Curr. Issues Mol. Biol.* 44, 5839–5847 (2022).
11. Kim, S.-Y. *et al.* Comparison of physicochemical properties and antioxidant activity between raw and heat-treated vegetables. *Korean J Community Living. Sci.* 25, 5–18 (2014).
12. Abd El Raheim, M. D. Biological activity of Chrysanthemum coronarium L. extracts. *Annu. Res. Rev. Biol.* 4, 2617–2627 (2014).
13. Abd-Alla, H. I., Albalawy, M. A., Aly, H. F., Shalaby, N. M. M. & Shaker, K. H. Flavone composition and antihypercholesterolemic and antihyperglycemic activities of Chrysanthemum coronarium L. *Z. Naturforsch. C.* 69, 199–208 (2014).
14. Ali, B. H. *et al.* Ameliorative effect of chrysin on adenine-induced chronic kidney disease in rats. *PLoS One* 10, e0125285 (2015).
15. Jia, T. *et al.* A novel model of adenine-induced tubulointerstitial nephropathy in mice. *BMC Nephrol.* 14, 1–8 (2013).

16. Santana, A. C. *et al.* Thalidomide suppresses inflammation in adenine-induced CKD with uraemia in mice. *Nephrol. Dial. Transplant.* 28, 1140–1149 (2013).
17. Yuan, H. *et al.* Contribution of TFEB-mediated autophagy to tubulointerstitial fibrosis in mice with adenine-induced chronic kidney disease. *Biomed. Pharmacother.* 133, 110949 (2021).
18. Wang, J., Zhang, Q., Jin, W., Niu, X. & Zhang, H. Effects and mechanism of low molecular weight fucoidan in mitigating the peroxidative and renal damage induced by adenine. *Carbohydr. Polym.* 84, 417–423 (2011).
19. Tbahrity, H. F. *et al.* Inflammatory status in chronic renal failure: The role of homocysteinemia and pro-inflammatory cytokines. *World J. Nephrol.* 2, 31–37 (2013).
20. Hamdy, M. M., Abdel-Rahman, M. S., Badary, D. M. & Sabra, M. S. Effects of furosemide and tadalafil in both conventional and nanoforms against adenine-induced chronic renal failure in rats. *Eur. J. Med. Res.* 27, 1–17 (2022).
21. Gong, Q. *et al.* Anemoside B4 protects rat kidney from adenine-induced injury by attenuating inflammation and fibrosis and enhancing podocin and nephrin expression. *Evid. Based Complement. Alternat. Med.* 2019, 8031039 (2019).
22. Singh, M. P., Sharma, C. & Kang, S. C. Morin hydrate attenuates adenine-induced renal fibrosis via targeting cathepsin D signaling. *Int. Immunopharmacol.* 90, 107234 (2021).
23. Kondo, M. *et al.* Renoprotective effects of novel interleukin-1 receptor-associated kinase 4 inhibitor AS2444697 through anti-inflammatory action in 5/6 nephrectomized rats. *Naunyn-Schmiedeb. Arch. Pharmacol.* 387, 909–919 (2014).
24. Wang, Y.-Y. *et al.* Macrophage-to-myofibroblast transition contributes to interstitial fibrosis in chronic renal allograft injury. *J. Am. Soc. Nephrol.* 28, 2053–2067 (2017).
25. Lv, W., Booz, G. W., Wang, Y., Fan, F. & Roman, R. J. Inflammation and renal fibrosis: recent developments on key signaling molecules as potential therapeutic targets. *Eur. J. Pharmacol.* 820, 65–76 (2018).
26. Jung, G.-S., Hwang, Y. J., Choi, J.-H. & Lee, K.-M. Lin28a attenuates TGF- β -induced renal fibrosis. *BMB reports* 53, 594–599 (2020).
27. Fan, J.-M. *et al.* Transforming growth factor- β regulates tubular epithelial-myofibroblast transdifferentiation in vitro. *Kidney Int.* 56, 1455–1467 (1999).
28. Ito, S., Manabe, E., Dai, Y., Ishihara, M. & Tsujino, T. Juzentaihoto improves adenine-induced chronic renal failure in BALB/c mice via suppression of renal fibrosis and inflammation. *J. Pharmacol. Sci.* 148, 172–178 (2022).
29. Mulsow, J. J. W., Watson, R. W. G., Fitzpatrick, J. M. & O'Connell, P. R. Transforming growth factor- β promotes pro-fibrotic behavior by serosal fibroblasts via PKC and ERK1/2 mitogen activated protein kinase cell signaling. *Ann. Surg.* 242, 880–889 (2005).

Figures

Figure 1.

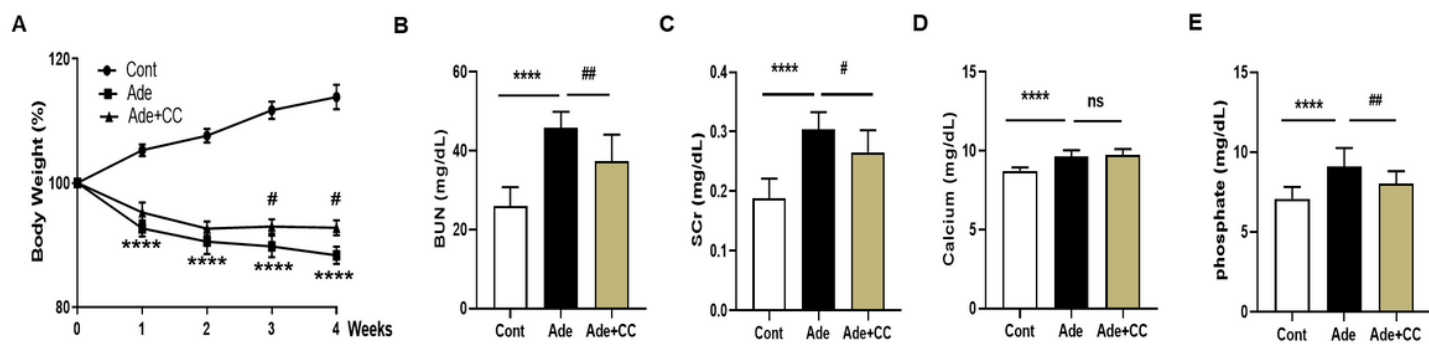


Figure 1

CC attenuates adenine (AD)-induced renal injury. Chronic kidney disease (CKD) was induced by adenine (0.25% w/w) for 28 days, and CC (100 mg/kg b.w) was given orally by gavage either alone or additionally with adenine. **(A)**. Bodyweight. **(B)**. BUN level. **(C)**. SCr level. **(D)**. Calcium level. **(E)**. Phosphate level. All data are represented as mean±standard deviation (SD). N=8. ****p<0.0001 vs. Cont group. #p<0.05, and ##p<0.01, ns: not significant, vs. Ade group.

Figure 2.

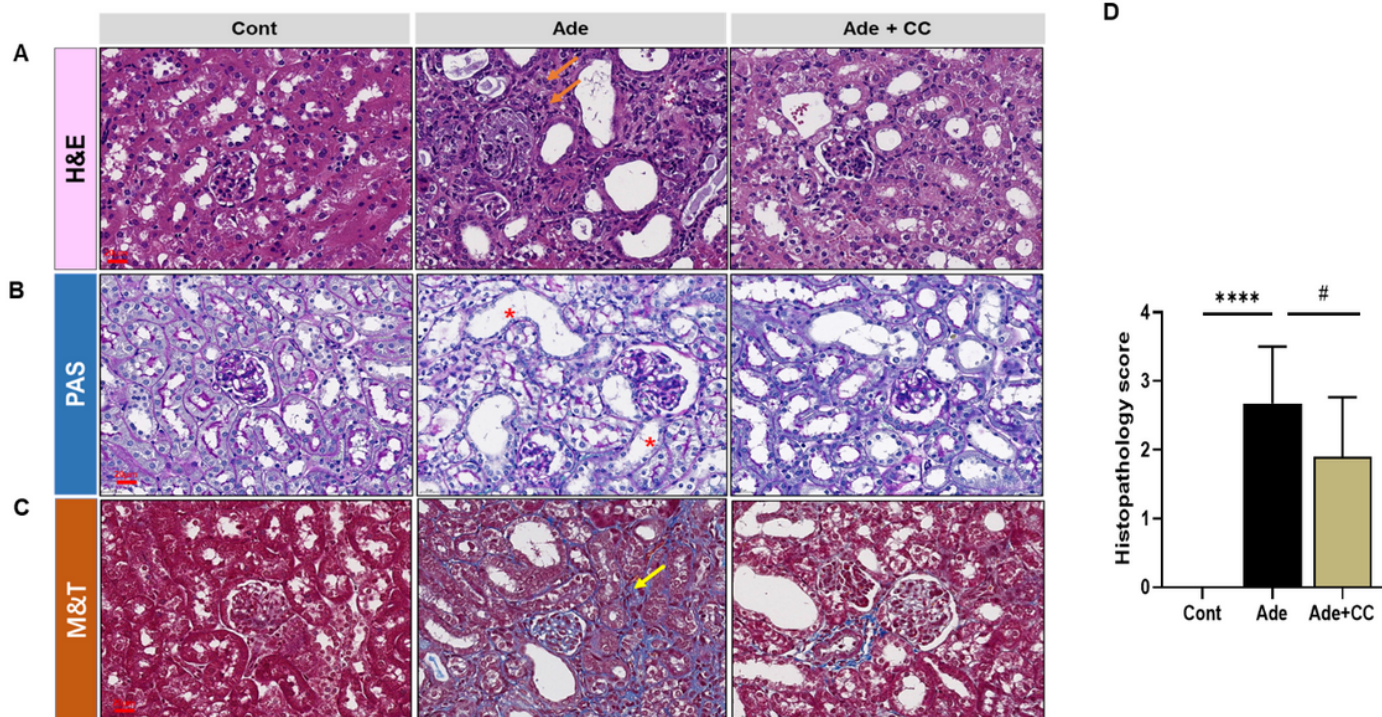


Figure 2

CC attenuates AD-induced morphological changes. **(A)**. Hematoxylin and eosin (H&E) stainings were used for identification and semiquantitative scoring of inflammation. The orange arrows indicate inflammatory cell infiltration. **(B)**. Periodic acid-Schiff (PAS) staining indicates the identification and semiquantitative scoring of basal membrane atrophy and dilatation. White arrows indicate examples of atrophic basal membranes, and red stars indicate tubule dilation. **(C)**. Masson's trichrome (MT) staining was used for identification and semiquantitative fibrosis scoring. The yellow arrows indicate interstitial fibrosis. **(D)**. Histopathology was evaluated from the kidney stained with HE, PAS, and MT. All data are represented as mean±SD. N=8. ****p<0.0001 vs. control group. #p<0.05, vs. Ade group.

Figure 3.

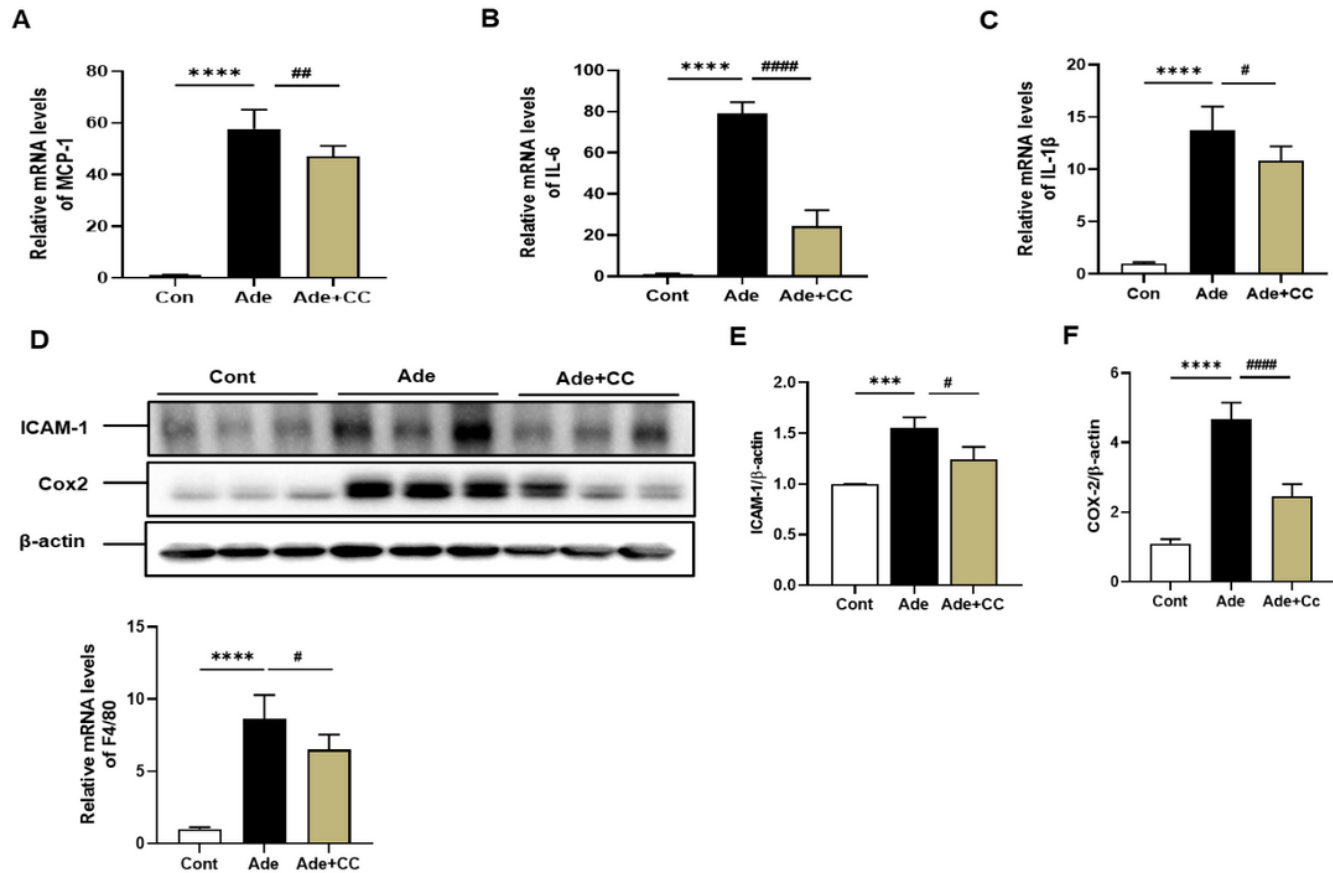


Figure 3

CC suppress inflammatory responses in AD-induced renal injury mice. qRT-PCR analysis of MCP-1 **(A)**, IL-6 **(B)**, and IL-1β **(C)** mRNA expression. Western blotting analysis showing protein expression of ICAM-1 **(D and E)** and COX-2**(D and F)**. qRT-PCR analysis showing the mRNA expression of F4/80 **(G)**. All data are presented as mean±SD. N=8. ***p<0.001 and ****p<0.0001 vs. Cont group. #p<0.05, and ##p<0.01, ####p<0.0001, vs. Ade group.

Figure 4.

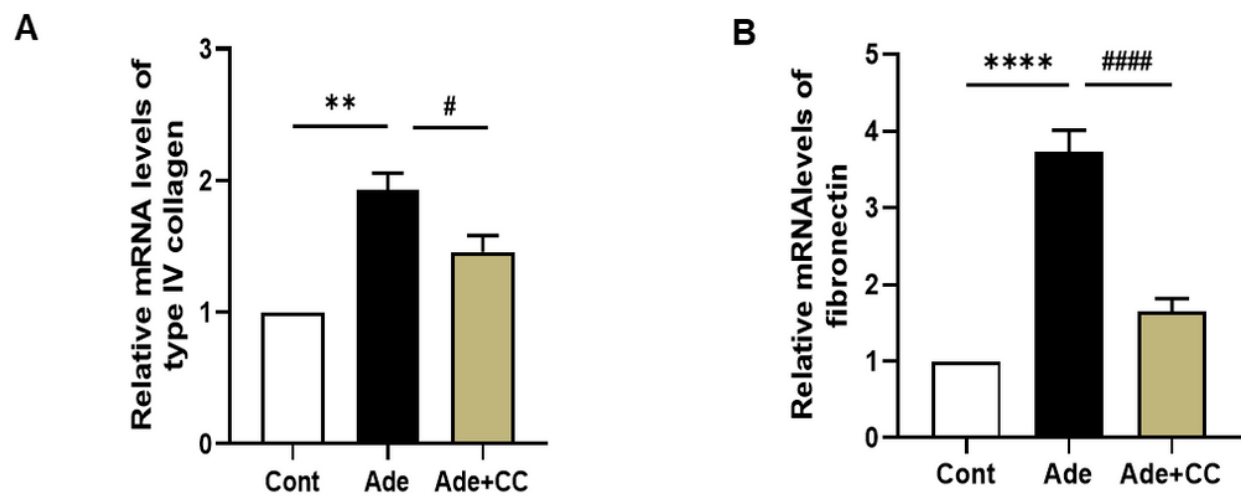


Figure 4

CC reduces extracellular matrix deposition and tubulointerstitial fibrosis in a mouse model of AD-induced renal injury. qRT-PCR results showing the mRNA level of type IV collagen (A) and fibronectin (B). All data are presented as mean±SD. N=8. **p<0.01 and ****p<0.0001 vs. Cont group. #p<0.05, and ###p<0.001, vs. Ade group.

Figure 5.

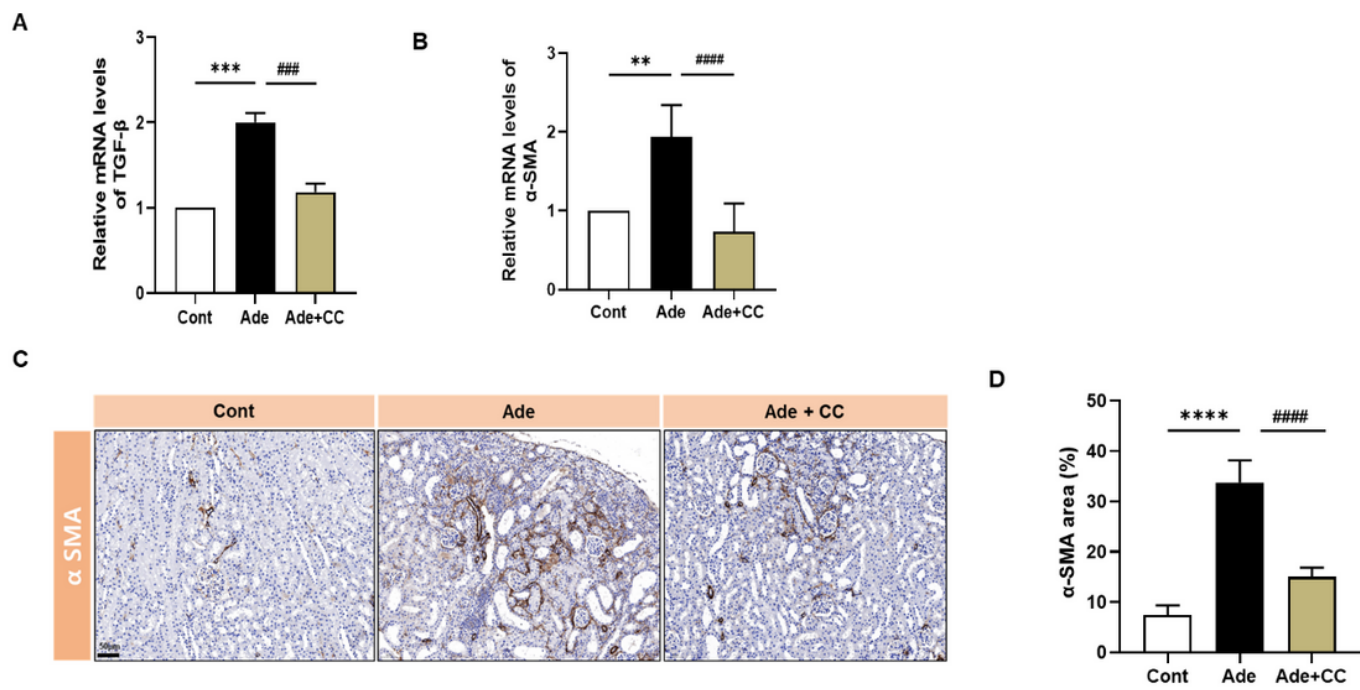


Figure 5

CC suppresses the upregulation of TGF- β 1 and α -SMA expression in the mouse model of AD-induced renal injury. The qRT-PCR results represent the mRNA levels of TGF- β 1 (**A**) and α -SMA (**B**). Immunohistochemical staining showed the expression of α -SMA (**C** and **D**). All data are presented as mean $\pm$ SD. N=8. **p<0.01, ***p<0.001, and ****P<0.0001, vs. Cont group. ###p<0.001, and ####p<0.0001 vs. Ade group.