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Ferulic Acid Is Nephrodamaging While Gallic Acid Is Renal Protective In Long Term Treatment of Chronic Kidney Disease

Chiung-Chi Peng[a,b,c,g], **Chiu-Lan Hsieh**[d], **Hui-Er Wang**[e], **Jin-Yuan Chung**[a], **Kuan-Chou Chen**[f,g*], **Robert Y. Peng**[h]

[a]Department of Physical Therapy, [b]Graduate Institute of Rehabilitation Science, [c]Department of Nutrition, College of Health Care, China Medical University, 91 Hsueh-Shih Rd., Taichung, Taiwan 40202

[d]Graduate Institute of Biotechnology, National Changhua University of Education, 1, Jin-De Rd., Changhua, Taiwan 500

[e]Department of Food and Applied Biotechnology, [h]Research Institute of Biotechnology, Hungkuang University, 34 Chung-Chi Rd., Shalu County, Taichung Hsien, Taiwan 43302

[f]Department of Urology, Taipei Medical University-Shuang Ho Hospital, [g]Taipei Medical University, 250, Wu-Xin St., Xin-Yi District, Taipei, Taiwan 110

[*]Corresponding authors: Dr. Kuan-Chou Chen

Department of Urology, Taipei Medical University-Shuang Ho Hospital, Taipei Medical University, 250, Wu-Xin St., Xin-Yi District, Taipei, Taiwan 110

E-mail: kc.chen416@msa.hinet.net; Mobile: +886-958-828-839; Tel: +886-2-27299723.

***Running title*: Ferulic Acid Worsened Chronic Kidney Disease**

Abstract

Backgrounds & aims: The long term therapeutic effect of ferulic (FA) and gallic (GA) in treatment of chronic kidney disease (CKD) has been lacking.

Methods: Doxorubicin (DR, Adriamycin)-induced CKD rat model was established for this study.

Results: DR significantly reduced levels of serum albumin, GOT, GPT, RBC, TNF-α, and urinary creatinine and elevated serum cholesterol, TG, BUN, creatinine, uric acid, WBC, platelet count, and IL-6. In DRCKD rats, FA and GA significantly increased kidney weight and glomerular volume. FA reduced glomerular filtration rate but GA did not. FA enhanced more collagen deposition than GA in renal cortex and glomeruli. Both FA and GA showed crucial hyperlipidemic activity. The inhibitory effects of FA and GA on MMP-2 were very comparable. GA suppressed MMP-2 more effectively than FA in DRCKD rats. Both FA and GA induced SOD elevation and MDA elimination. In DRCKD rats, Western blot analysis indicated that FA further up-regulated CD34, α-SMA, tissue pDGFR, p-PDGFR, and TGF-β; and down-regulated p-PI3K, and p-Akt. Since both PDGF-BB and TGF-β are considered to induce kidney prefibrosis stage, GA was proved to be more beneficial in this regard.

Conclusions: GA tends to protect the CKD while FA is not recommended for the long term CKD therapy.

***Keywords*:** gallic acid; ferulic acid; chronic kidney disease; PDGF; α-SMA

1. Introduction

Flavonoids comprise the most common group of plant polyphenols and provide much of the flavor and color to fruits and vegetables[1]. Most of the flavonoids present in plants occur in glycosidic forms, although occasionally as aglycones. Interest in the role of flavonoids to act as health benefits is emerging owing to their potential antioxidative and free-radical scavenging activities. However, up to present, epidemiologic studies exploring the role of flavonoids in human health have been inconclusive[1,2]. Some studies support a protective effect of flavonoid consumption in cardiovascular disease and cancer, others demonstrate no effect, and conversely a few suggest potential harm[1].

Gallic acid (3,4,5-trihydroxybenzoic acid) (GA) widely occurs in plants including gallnuts, grapes, tea, hops and oak bark[3]. GA yields numerous esters and salts including digallic acid. GA seems to have anti-fungal and anti-viral properties. More recently, GA was found to show cytotoxicity against cancer cells, without harming healthy cells[4]. GA is also used to treat albuminuria and diabetes. Some ointments for treatment of psoriasis and external haemorrhoids contain mainly gallic acid[3].

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (FA), an effective component of Chinese medicine herbs such as *Angelica sinensis, Cimicifuga heracleifolia* and *Lignsticum chuangxiong*, is a ubiquitous phenolic acid in the plant kingdom[5]. FA exhibits many physiological functions, including antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis, and anti-cancer activities. It protects against coronary disease by lowering serum cholesterol. Moreover, it enhances the viability of spermatozoa[5].

Doxorubicin (DR, commercial name Adriamycin) has been used as an anticancer (antineoplastic) medication. It interferes with cancer cell growth and slows their

migration in body[6]. DR had been used to induce nephropathy as a model of chronic progressive glomerular disease[7], generally named “The Chronic Kidney Disease (CKD)”. DR produced chronic, progressive glomerular changes in rats, which led to terminal renal failure. The segmental glomerular sclerosis and IgM-dominant glomerular deposition in these animals are similar to the pathological characteristics of focal and segmental glomerular sclerosis seen clinically[7]. Referring to the recent report[2], we suspect that some flavonoid antioxidants may be safe for use while some may damage the kidney in a CKD status. In this work, we adopted DR to create the CKD model in rats and investigated whether the potentially used phytoantioxidants (PAO) like gallic and ferulic acids can improve CKD to some extent.

2. Materials and methods

2.1 Animals

Thirty six male Sprague Dawley (SD) rats, age 4 weeks, having mean body weight 155 g (range of 150–164 g), were purchased from the Biolasco Animal Centre, Taiwan. Rats were individually housed in animal room maintained at 22±1^{o}C and a relative humidity of 65% on a 12h/12 h light-dark cycle. The access of distilled water was ad libitum, but the maximum amount of feed was restricted at 10% of body weight per day. The body weight change and the amount of food intake were recorded daily. All the protocols had been previously approved before experimentation by the Institutional Animal Care and Use Committee of the China Medical University.

2.2 CKD induction and animal grouping

The DR-CKD rat modeling was performed according to Okuda et al.[7]. Briefly, in the first week, CKD was induced by subcutaneous injection of 8.5 mg/kg of DR (Pfizer, Milano, Italia) under ether anesthesia. The DR-induced rats were divided into six groups, 6 rats in each. Group 1 served as the diet control was fed normal diet only (Normal group). Group 2 was DR-induced and fed normal diet (DRCKD group). Commercially, the pharmaceutical preparation for human use is fabricated into tablets, containing 182 mg FA per tablet[8]. When prescribed with the order 2-3 tablets tid, a total of 1092-1638 mg/day will be administered. Assuming a 60 kg male is to receive this dosage, a single

dosage will correspond to 18.2-27.3mg FA/kg-day. Alternatively, pure GA at 50 mg/dose had been tried for testing its bioavailability in human[9], while the dosage of GA reported to be safe for rats in doses ranging within 119-128 mg/kg/day (Niho et al., 2001)[10].Obviously rats are able to endure at least 2-3 folds human dosage. Consequently, Group 3 received FA (Sigma-Aldrich, USA)-containing diet at FA 70mg/kg-day (FA group)[11]. Group 4, the DR-induced rats, received FA-containing diet at FA 70mg/kg-day (DRCKD+FA group)[11]. Group 5 rats were fed GA (Sigma-Aldrich, MO, USA)-diet containing only GA 70mg/kg/day (GA group). Group 6, the DR-induced rats, was given GA-containing diet at GA 70mg/kg/day (DRCKD+GA group). The two compositions were correctly weighed and thoroughly blended with normal diet before feeding.

2.3 Glomerular volume

The glomerular volume was determined by the formula[12].

$$\mathbf{GV} = (\beta/k)\,(G_A)^{3/2} \quad \ldots\ldots 1$$

Where **GV** = glomerular volume (mm^3)

G_A =cross-sectional tuft area (mm^2)

β is the shape coefficient (1.38 in this case, for a sphere β= 1.38)

k is the size distribution coefficient (= 1.1 in this case)

2.4 Glomerular filtration rate (GFR)

The glomerular clearance rate (GRF) is defined by Eq. 2[13]

$$GFR = (CR_c \times BN_c)^{1/2} \quad \ldots\ldots 2$$

Where

CR_c is the creatinine clearance, and BN_c is BUN clearance.

And

$CR_c = 1000C_{Cr,u}/C_{Cr,s}$ ……3

Here $C_{Cr,u}$ is the volume concentration of creatinine in urine, and $C_{Cr,s}$ is the volume concentration of creatinine in serum. And

$BN_c = C_{BN,u}/CB_{N,s}$ ……4

Here $C_{BN,u}$ denotes the volume concentration of BUN in urine; and $CB_{N,s}$ means the volume concentration of BUN in serum.

2.5 Histopathological examination

The animals were ether-euthanized at the end of week 12, the kidneys were immediately picked up and fixed in 10% formalin and embedded in paraffin. The embedded tissues were stained with hematoxylin and eosin reagent (H&E stain). Renal histology was examined with Olympus- CKX41 Microscope. Glomerular areas were measured using an image analyzer. The collagen content was estimated by Sirius Red stain. For Sirius Red staining, the paraffin embedded sections were first dewaxed, hydrateds, and sliced. The nuclei in the tissue slices were stained with Weigert's Haematoxylin and then stained in Saturated aqueous picric acid with Sirius Red (Sigma-Aldrich, USA) for one hour. The treated slides were rapidly dehydrated by a concentration gradient alcohols (starting with 70% to absolute alcohol), then to xylene and finally the slices were covered in Permount.

2.6 Biochemical analysis

The blood was collected for the measurement of serum albumin, blood urea nitrogen (BUN), creatinine, cholesterol, triglyceride, calcium, phosphorus, uric acid. At week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11, the blood was collected via the tail artery and was collected by arteria coeliaca at week 12. It was measured by reagent (Siemens, Bakersfield, CA, USA) and automatic analyzer (Ciba-Corning Express Plus) (Ciba-Corning, USA). All the

157 rats were weighed and placed in metabolic cages week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11,
158 to determine BUN, creatinine and protein excretion in 12 h urine. Urine BUN and
159 creatinine were measured by reagent (Siemens, Bakersfield, CA, USA) and automatic
160 analyzer (Ciba-Corning Express Plus) (Ciba-Corning, USA). Urine protein was measured
161 by ELISA reader.

162 ***2.7 ELISA of superoxide dismutase and malondialdehyde***

163 All ELISA protocols were performed by following the manufacturer's instruction. The
164 serum superoxide dismutase (SOD) and malondialdehyde (MDA, or generally termed the
165 thiobarbituric acid reactive substance, TBARs) were assayed with the commercial ELISA
166 kits provided by Cayman Chemical Co. (MI, USA). The optical density was read using
167 the SYSMEX K-1000 Reader (San-Tong Instrument Co., Taipei, Taiwan).

168 ***2.8 Western blot analysis***

169 One hundred mg of frozen renal cortex was homogenized with 1 mL of protein extraction
170 solution (EDTA free) (Intron Biotechnology, Korea). After incubated on ice for 40 min,
171 the homogenate was centrifuged at 12000×g for 20 min at 4^{o}C. The supernatant (tissue
172 lysate) was collected. Lysates containing approximately an amount of protein (50 μg)
173 were boiled for 10 min in PBS. The boiled sample solutions were loaded onto a 7.5 %
174 polyacrylamide SDS gel. Proteins were transferred to a PVDF membrane and rinsed with
175 TBS-Tween buffer (TBST), and blocked at 4^{o}C overnight in TBST containing 5% w/v
176 non-fat powdered milk. The PVDF membranes were incubated with the primary
177 antibodies, which contains Akt (1:1000), phospho-Akt (1:1000) , PDGF receptor β
178 (1:1000), phospho-PDGF receptor β (1:1000), and PI3-kinase (1:1000) (Cell Signaling,
179 USA) ; phospho-PI3K (1:500), CD34 (Santa Cruz, USA); and α- smooth muscle actin

(1:1000) (Sigma-Aldrich, USA) etc. in TBST at 4^{o}C overnight. The PVDF membrane was then rinsed three times with TBST and incubated with the secondary antibodies containing anti-mouse, anti-rabbit and anti-goat (each at 1:5000 dilution in TBST with 5% w/v non-fat powdered milk). After incubated at room temperature for 1 h, the PVDF membranes were rinsed three times with TBST. The secondary antibodies bound were detected using the chemiluminescent HRP substrate (Minipore, USA).

2.9 Statistical analyses

Data obtained in the same group were analyzed by Student's *t* test with computer statistical software SPSS 10.0 (SPSS, Chicago, IL). ANOVA statistical analysis system software with Tukey test was used to analyze the variances and significances of difference between paired means. Significance level was judged by a confidence level $p < 0.05$.

3. Results

3.1. Both ferulic and gallic acids did not harm normal kidney, but FA aggravated CKD

DR caused significant body weight loss, the body weight was significantly decreased from 516 g of the normal to 304 g. Although FA control group showed normal body as the control, GA alone seemed to have a moderate body weight reducing effect (Table 1). Conversely, in DRCKD+FA rats, FA reduced body weight more significantly than DRCKD+GA, giving rise to 239 g and 357 g, respectively (Table 1). Anatomically, DR caused renal tubular and glomerular damages with formation of a number of vacuoles, glomerular sclerosis and tubulointerstitial degeneration at week 28 (Figure 1), but slighter extent with CKD+GA group, although all DR-induced rats revealed renal inflammation accompanied with apparent swelling and edema. Figure 1 exhibits the status of nephroedema (Figure 1B: DRCKD; D: DRCKD+FA; F: DRCKD+GA). Nonetheless, GA more efficiently secured the DR injury (Figure 1F) in this regard. The average kidney weight of the DRCKD, DRCKD+FA and DRCKD+GA groups was 5.0, 4.7, and 3.9 g respectively, comparing to 3.1 g of the normal, a significantly larger extent of recovery was seen in DRCKD+GA rats (Table 1). Similar results were found in the ratio kidney/body weight, glomerular volume (GV) and the glomerular filtration rate (GFR) (Table 1). Surprisingly, in DRCKD rats FA reduced the GFR to 120 mL/h. Contrary to this, GA increased GFR to a value 515 mL/h (Table 1).

3.2 Histopathological examination

In the renal cortex of DRCKD rats, a huge amount of collagen deposition wa observed occurred (Figure 1B; Figure 2B). Contrast with this, a much larger amount of collagen deposit was found in the DRCKD+FA group (Figure 2D), less deposit in DRCKD+GA group (Figure 2F).

3.3 Biochemical parameters affected in doxorubicin induced CKD

DR severely down-regulated the serum albumin, GOT, GPT, and RBC clearance, but up-regulated levels of serum cholesterol, triglyceride (TG), BUN, creatinine, calcium, phosphate, uric acid, WBC, platelets, and levels of urinary BUN and protein (Table 2).

3.3.1. Serum creatinine level was significantly raised

DRCKD rats exhibited higher serum creatinine levels (1.4 mg/dL) comparing with the normal 0.7 mg/dL, and FA further increased the level to 2.9 mg/dL (Table 2). Apparently, the DRCKD+FA rats were suffering from a moderate renal failure (2-4 mg/dL).

3.3.2. Effect of FA and GA on serum uric acid level

Interestingly, FA alone activated, conversely GA alone inhibited uric acid synthesis (Table 2). Controversially in CKD victims, FA completely recovered the uric acid level to 1.5 mg/dL (in DRCKD+FA), comparing to the control (1.7 mg/dL) and the DRCKD+GA (3.0 mg/dL) (Table 2). Otherwise, the activity of SOD was activated in FA and GA groups, which may be correlated with the enhancement of TNF-α expression in these groups (Table 2), a phenomenon consistent with Wong and Goeddel[14].

3.3.3. Effect on SOD induction

The activity of superoxide anion dismutase (SOD) was elevated in all groups comparing to the normal control (Figure 3a). DR activated SOD, FA failed to suppress such

activation of SOD. As contrast, GA promisingly inhibited the elevation of SOD (Fig. 3a).

3.3.4. Effect on MDA suppression

Comparing with the MDA data, the serum MDA level in DRCKD group once having reached 86 μM was totally abolished by FA and GA (Figure 3b). Results indicate FA to be a better antioxidant than GA with respect to MDA suppression.

3.3.5. Effect on the hyperlipidemic status in CKD

DR induced hypercholesterolemia and triglyceridemia in CKD victims (Table 2). By comparison of the pharmacological action between the FA-alone or GA-alone diet, some amazing phenomena were observed. FA-alone and GA-alone diets did not show any apparent different effect on serum cholesterol and triglyceride levels. However in DRCKD rats, both the serum levels were significantly suppressed by FA and GA (Table 2).

3.3.6. Hepatoprotective effect

Similar trend was seen for serum BUN, GOT, and GPT, implicating the hepatoprotective effect of both FA and GA, consistent with Balasubashini et al.[15]. The anatomical and histological examination also confirmed such a result (unpublished).

3.3.7. Effect on leukopoiesis and eryhtrocytopenia induced by DRCKD

FA and GA exhibited moderate leukopoiesis effect (Table 2). FA even enhanced it to a greater extent in the DRCKD rats (1.8×10^4 count/μL). Whereas GA suppressed it to the normal level (8×10^3 count/μL). As a contrast, DR destroyed RBC in CKD rats to a level of 5.8×10^6 counts/μL. FA further reduced it to 4.5×10^6 counts/μL. In this regard, GA did not improve the CKD to any extent (Table 2).

3.3.8. Effect on the platelet count

Based on the platelet count of normal control (6.3×10^5 count/μL), FA-alone diet seemed to be a platelet proliferation inhibitor though the effect is not statistically significant. FA-alone diet suppressed the platelets to a number of 4.9×10^5 count/μL, but GA totally did not show any effect. Astonishingly, FA conversely increased the platelet count in DRCKD+FA rats to 1.52×10^6 count/μL, comparing to the DRCKD control (Table 2).

3.3.9. Effect on IL-6 and TNF-α in DRCKD victims

After 28 weeks, DR had moderately up-regulated the inflammatory cytokine IL-6 but significantly down-regulated TNF-α in DRCKD group. FA or GA when used alone was able to up-regulate IL-6 to a level 4.5 or 3.5 ng/mL and TNF-α to 1476.1 or 1099.65 pg/mL, pointing to the moderate inflammatory effect of FA and GA (Table 2). However, in DRCKD rats level of TNF-α was greatly suppressed to 370.2, 97.6, and 563.1 pg/mL respectively, comparing to the normal 936.1 pg/mL (Table 2). Whereas IL-6 still remained at a level higher than the normal.

3.3.10. Effect on level of urinary BUN in DRCKD victims

The urinary BUN level was significantly raised by DR to 547 mg/dL, comparing with the normal control level 108 mg/dL, which was cured by FA and GA to only 385 and 457 mg/dL, respectively. And interestingly, FA-alone and GA-alone-diets respectively raised the levels to 484 and 188 mg/dL (Table 2).

3.3.11. Doxorubicin upregulated PDGF-BB, and TGF-β□caused prefibrosis in kidney

DR up-regulated the platelet derived growth factor-BB (PDGF-BB) in the renal tissue of DRCKD rats to 4739.3 *p*g/mL (normal value, 693.9 pg/mL) and simultaneously the

294 TGF-β□to 2118 pg/mL (normal level, 1788 pg/mL) (Figure 4a,b). When administered
295 with FA or GA in DRCKD rats, FA showed a lower level of PDGF-BB than GA in the
296 DRCKD rat renal tissues (2747.8 pg/mL vs. 4238.2 pg/mL in Figure 4a), indicating FA
297 could be more damaging to the renal cells than GA (Figure 1D and 1F), which was
298 evidenced by the up-regulation of PDGFR, p-PDGFR and tissue TGF-β□in DRCKD+FA
299 and DRCKD+GA rats (Fig. 4b). While the slightly increased level of FA- (842.8 *p*g/mL)
300 and GA-controls (1240.2 *p*g/mL) may be crucially the concentration of FA or GA
301 required for cell growth promotion (Figure 4a).

302 **3.3.12. DR downregulated p-PI3K and p-Akt, but upregulated levels of CD34 and**
303 **α-SMA in DRCKD**

304 Western blotting revealed that FA when used alone did not affect the levels of PI3K,
305 p-Akt, CD34, and α-SMA. Likewise, GA alone did not show any effect on PI3K, p-Akt,
306 and α-SMA (Figure 5). On application of DR, DR down-regulated PI3K, p-PI3K and
307 p-Akt, but up-regulated levels of, Akt, CD34 and α-SMA. In DRCKD rats, FA
308 down-regulated levels of PI3K, p-PI3K, p-Akt and up-regulated Akt, CD34, and □
309 α-SMA. Conversely, GA slightly had restored levels of PI3K and p-Akt to normal levels,
310 and to lesser extent, the down-regulation of Akt and up-regulation of α-SMA (Figure 5).

311 **3.4. Zymography of MMP-2 and MMP-9**

312 Zymography of whole-kidney extracts showed very prominent two bands, 72 and 92-kDa
313 bands for MMP-2 and MMP-9, respectively (Figure 6), consistent with Rankin et al.[16].
314 High levels of MMP-2 seemed to result from increased expression by DR treatment. GA

315 down-regulated MMP-2 in DRCKD+GA rats, but FA did not show any recovery effect
316 (Figure 6). A similar but less intense response was found for MMP-9.
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322 ## 4. Discussion

323 ### *4.1. Why FA tends to aggravate CKD?*

324 As evidenced by the body weight gain, the glomerular volume, the glomerular filtration
325 rate (GFR), the ratio kidney/body weight (Table 1), and pathological changes (Figure 1),
326 FA at dosage 70mg/kg was detrimental to kidneys in CKD patients, conversely GA can be
327 protective. Histological examination revealing much more severe pre-fibrotic collagen
328 deposition in the renal cortex of DRCKD rats treated with FA (Figure 2D) than GA
329 (Figure 2F) has strongly supported this result. Both FA and GA are potentially potent
330 prooxidants[17,18]. Much of the literature has indicated FA is more potent in view of
331 pro-oxidative bioactivity. Overproduction of superoxide anions and prooxidants can elicit
332 cytotoxicity and induce apoptosis[18]. Pascale et al.[19] reported the order for scavenging
333 superoxide anions ($\bullet O_2^-$) is GA (76×10^{-4} M) > FA (10×10^{-4} M); and for scavenging
334 hydroxyl free radicals ($\bullet$OH) is FA (29×10^{-4} M) > GA (7×10^{-4} M)[19], indicating GA to be a
335 better superoxide anion scavenger; conversely, FA a better hydroxyl free radical
336 scavenger. With respect to prevention of the upstream overproduction of superoxide
337 anion, GA would be a better protective agent, an elucidation well supports our

speculation. Comparing with the MDA data, the serum MDA level in DRCKD group once having reached 86 μM was totally abolished by FA and GA (Figure 3b). Results indicates FA is a better antioxidant than GA with respect to MDA suppression.

4.2. Increased serum creatinine level can be ascribed to the inhibition of creatine phosphate kinase, and decreased urinary level of creatinine can be caused by energy deficiency

Since an increase in serum creatinine from 0.6 to 1.2 mg/dL represents a 50% decline in renal function, accompanied with the GFR 120 mL/h (normal 435 mL/h)[20]. In the early stages of renal failure, major decreases in GFR are often associated with what appear to be minor changes in serum creatinine[20]. However, worth noting, serum creatinine levels correlate with GFR only in the steady state. Therefore, significant errors in the estimation of GFR may occur if the serum creatinine level is rapidly changing[20]. Alternatively, the feature of creatinine clearance was severely impaired in groups DRCKD, DRCKD+FA, and DRCKD+GA (Table 2), similar to the findings of Yokozawa et al.[21]. The effects of doxorubicin on the energy metabolism had been reported by Bachmann et al.[22]. DR not only reduced oxygen consumption in heart mitochondria ex vivo, but also uncoupled oxidative phosphorylation, inhibited creatinine phosphate kinase (CPK), and damaged the semipermeability of the inner mitochondrial membrane (measured as creatine influx) [22]. Until recently, the only site of the transamidinating enzyme in mammals has been thought to be the kidney[23]. The reduced activity of CPK would lead to the accumulation of serum creatine, which in the absence of further phosphorylation to create CP will be spontaneously converted to creatinine by nonenzymatic reaction[23]. As a consequence, the serum creatinine level was increased (Table 2). In addition, renal clearance is responsible

for 80% of kidney's total energy requirement[24]. Under malnutrition status (Table 1) and lacking high energy creatine phosphate formation, the renal clearance may be retarded, resulting in reduced urinary creatinine excretion (Table 2).

4.3. FA and GA acted differently on serum uric acid level

The reason why FA alone activated, conversely GA alone inhibited, uric acid synthesis (Table 2) can be explained by their effect on xanthine oxidase[25] and the status of glomerular clearance[26]. FA might inhibit, but GA might activate, the enzyme xanthine oxidase in CKD victims. Literature elsewhere indicated accumulation of a broad spectrum of toxins due to failure of the kidney to eliminate these substances. Under normal conditions, the glomerular filter clears molecules with a molecular weight up to 58,000 Da. All these substances are supposed to be retained in renal failure and are candidate uremic toxins. Ninety compounds are known as uremic toxins; 68 of them have a molecular weight <500 Da (small water-soluble compounds), 22 have a molecular weight >500 Da (middle molecules), and 25 solutes (27.8%) are protein bound[26].

4.4. FA may enhance platelet aggregation

As mentioned, FA increased the platelet count in DRCKD+FA rats to 1.52×10^6 count/μL, comparing to the DRCKD control (Table 2). In patients on hemodialysis (HD), platelet aggregation was impaired before as well as after the HD session[27], an implication in the possible feature of FA to affect the cell growth, proliferation and blood coagulation.

4.5. Effect on IL-6 and TNF-α in DRCKD victims

In DRCKD rats, level of TNF-α was greatly suppressed, whereas IL-6 still remained at a level higher than the normal (Table 2), a phenomena being very similar to polymyalgia rheumatica (PMR). Active PMR is characterized by increased serum levels of IL-6, but

not those of other pro-inflammatory cytokines. Worth noting, all the DR, FA alone, DRCKD+FA, and DRCKD+GA groups exhibited significantly elevated WBC counts, indicating in parallel significantly increased monocytes. As circulating monocytes do not show increased production of proinflammatory cytokines, IL-6 might be mainly produced in the inflamed tissue[28].

Tumor necrosis factor TNF-α and TNF-α are soluble ligands binding to TNF receptors with similar activities. TNF is a multifunctional cytokine that plays important roles in diverse cellular events. In regard to cancer, TNF is a double dealer, acting as either a promoter or a killer[29]. Soluble TNF receptor in inflammatory bowel disease (IBD) mucosa inhibited TNF activity. Type 2 soluble receptor release from IBD mucosa was increased in active inflammation; release from lamina cells was not increased[30]. Mucosal TNF-α production correlated with severity of disease. In some diseases, soluble TNF-α receptors neutralize TNF-α activity by acting as inhibitors[30]. Conversely, DR suppressed level of TNF-α. To enhance TNF-α level to induce MnSOD has been reported to be a possible protective mechanism of ghrelin for DR-induced cardiomyopathy and heart failure[14, 31]. In our case, both FA and GA when used alone increased the levels of TNF-α to 1476.1 pg/mL and 1099.7 pg/mL respectively, comparing to the normal 936.1 pg/mL (Table 2). On induction with DR, the DRCKD rats might first down-regulated TNF-α, which was further suppressed by FA to a much lower level (97.6 pg/mL). Conversely, level of TNF-α in DRCKD+GA was enhanced by GA to a higher level (563.1 pg/mL) (Table 2), evidencing the possible imbalance between the TNF-α and the TNF-α□ receptor in the DRCKD+FA as mentioned by Noguchi et al.[30].

4.6. Why level of urinary BUN was intensely increased by FA and GA?

FA-alone and GA-alone-diets respectively raised the levels to 484 and 188 mg/dL (Table 2), indicating that FA and GA were not sufficiently effective for suppressing the level of urinary protein, consistent with Okuda et al.[7]. On administration of DR, massive proteinuria, hypoalbuminemia, and hyperlipidemia were observed (Table 2). Both BUN and serum creatinine increased at week 16 and reached the uremic level at week 28[7]. Comparing with the serum BUN levels, the high level of urinary BUN can be ascribed to more rapid renal excretion of urea when affected by DR, FA, and GA (Table 2).

4.7. Doxorubicin up-regulated PDGF-BB, and TGF-β□caused prefibrosis, and FA may potentiate the pathological status due its pro-oxidant bioactivity

As indicated in Figure 1, prefibrosis of kidney occurred as a consequence of DR therapy. (Figure 1), and similarly the level of PDGF-BB (Figure 4a). Okuda et al. reported that IgM with a small amount of IgG and C_3 appeared in the sclerosing glomeruli from week 16 on treatment with DR[7]. As mentioned, FA acts as a strong pro-oxidant, and previously, we also had found a certain degree of cardiac injury in rats when treated with DR (data not shown). FA thus may enhanced the severity of fibrotic status. As well cited, PDGF-BB activates all combinations of PDGF receptor subunits[32], serving to potentiate autocrine stimulation of growth[33]. PDGF-BB is associated with excessive cell migration, proliferation and many growth-related diseases[34]. PDGF-BB plays an important role in the cellular metabolism of vascular wall by regulating the rate of macrophage-colony stimulating factor (MCSF) production in vascular smooth muscle cells[35]. PDGF-BB is also a potent wound-healing hormone accelerating incisional repair[36].

Transforming growth factor (TGF)-β is strongly implicated in the progression of renal fibrosis. TGF-β1 is reported to cause epithelial-mesenchymal transition, inhibition of

epithelial cell proliferation, increased apoptosis, auto-induction of TGF-β1 production and induction of secondary mediators of tissue fibrosis such as connective tissue growth factor (CTGF, CCN2)[37]. Ras/MAP kinase pathway, specifically through N-Ras, mediates TGF-β1 auto-induction and TGF-β1 induced CTGF expression in human renal tubule epithelial cells[37].

Rats received N-nitro-L-arginine methyl ester (L-NAME) developed severe hypertensive nephrosclerosis. Levels of TGF-β1 mRNA in the renal tissue was also significantly increased compared with control spontaneously hypertensive rats[38]. By inhibiting both TGF-β1 production and apoptosis induction, glomerular and arteriolar damages can be prevented and renal functions can be secured[38].

In its normal state, the TGF-β pathway restricts cell growth, differentiation and cell death[39]. When a normal cell becomes cancerous, various components of the TGF-β□ signaling pathway become mutated, which makes the newly cancerous cell resistant to the effects of normally functioning TGF-β. These resistant cells then grow without regulation[39].

4.8. DR down-regulated p-PI3K and p-Akt, but up-regulated levels of CD34 and □-SMA in DRCKD

As mentioned, in DRCKD rats, FA down-regulated levels of PI3K, p-PI3K, p-Akt and up-regulated Akt, CD34, and α-SMA. Conversely, GA had slightly restored levels of PI3K and p-Akt to normal levels, lesser extent in down-regulation of Akt and up-regulation of α-SMA (Figure 5).

Among 30 patients with glomeronephritis (GN), CD34 is present in the extraglomerular mesangium in 50% (15 patients) of the GN patients. 73% (11 patients) of the latter may

453 show concomitant intraglomerular and extraglomerular mesangial CD34 immunostaining,
454 while 26.7% (four patients) show only extraglomerular mesangial immunostaining, and
455 in 20% (3 patients) of patients, CD34 immunostaining is present only in the
456 intraglomerular mesangium[40]. In fact there is a fair degree of relationship, which did not
457 reach statistical significance between CD34 in the extraglomerular mesangium and CD34
458 in the intraglomerular mesangium. In the intraglomerular mesangium, CD34 does not
459 significantly correlate with mesangial α-SMA and activity or chronicity index. In the
460 extraglomerular mesangium, CD34 does not show a significant correlation with α-SMA[40].
461 Instead, the activity index and the chronicity index may show a good correlation with
462 serum creatinine (Table 2). Mesangial cell proliferation correlates well with the
463 mesangial matrix increase, while interstitial vimentin shows a good correlation with
464 interstitial α-SMA[41]. In addition, the immunoreactivity of α-SMA is closely correlated
465 with and necroinflammatory activity (p = 0.022). The degree of α-SMA expression and
466 the scores of fibrosis (in periportal, perisinusoidal and pericentral areas) were highly
467 correlated[41]. Neglecting the role of CD34, we suspect that DR up-regulated TGF-β,
468 p-PDGFR, and PDGFR to trigger the signal cascade PDGF→ PDGFR→ (CD34??)
469 →α-SMA signaling pathway, and simultaneously down-regulated the pathway PI3K
470 (p-PI3K) → Akt (p-Akt), resulting in severe kidney damages that FA and GA are unable
471 to inhibit.

472 ***4.9. Zymography of MMP-2 and MMP-9***

473 Levels of MMP-2 was up-regulated by DR treatment. Under normal physiological
474 conditions, much of the increased MMP was present in the inactive zymogen form. In
475 pathological renal cysts, MMP-2 is abnormally localized to the interstitium and to foci

476 between cysts, suggesting that MMP-2 may regulate collagen accumulation at those sites,
477 thus allowing cyst enlargement and limiting the severity of interstitial fibrosis[16]. GA was
478 found effective for down-regulation of MMP-2, but FA did not show any recovery effect
479 (Figure 6). The whole experiment had been observed for a period of 28 weeks,
480 corresponding to 47 year-human life of 60 years, which seemingly was equivalent to
481 approximately 3 year-life of rats, i.e. such an experiment could be considered as a long
482 term observation. A similar result had been previously reported in our laboratory[11]. The
483 relationship between food and disease is indeed extremely complex. It is generally
484 accepted that diet is a contributory factor in the aetiology of a large proportion of
485 diseases[42]. Furthermore, polyphenols may interact with certain pharmaceutical agents like
486 DR and enhance their biologic effects (refer to Figure 1). Considering the outcome may
487 deviate between a short term and a long term therapy with FA and GA, the possible
488 mechanism may involve i) the unique prooxidant effect of some antioxidants, ii) the
489 pathological changes altering the signaling peptides and signaling pathways, iii) the
490 optimum dosage required may vary depending on the stage of pathological event, and
491 finally iv) the individual variation in biochemical response[18]. As mentioned[43], it is
492 important to consider the doses at which these effects occur, in relation to the
493 concentrations that naturally occur in the human body. Future studies evaluating either
494 beneficial or adverse effects should therefore include relevant forms and doses of
495 polyphenols and, before the development of fortified foods or supplements with
496 pharmacologic doses, safety assessments of the applied doses should be performed[43].

497 ***4.10. Hypertension is another risk***

498 In DRCKD victims, severe hypertensive status is always observed. Blood pressure of

DRCKD, DRCKD+FA, and DRCKD+GA groups reached 160, 145, and 144 mmHg in DRCKD, DRCKD+FA, and DRCKD+GA groups respectively, comparing to the normal value 99 mmHg (Table 2). As often cited, hypertension is a risk factor to induce renal disease, neural degeneration and a diversity of cardiovascular diseases[44].

To conclude, both FA and GA failed to retard the down-regulation of *p*-PI3K and *p*-Akt; and the up-regulation of PI3K, Akt, CD34 and α-SMA caused by DRCKD. Although both FA and GA were able to down-regulate serum PDGF-BB and up-regulate tissue PDGFR, long term treatment of CKD with ferulic acid has revealed that ferulic acid tends to aggravate, on the contrary, GA tends to protect the damages caused by CKD. Thus, FA is not recommended to be used as a long term therapy for patients with CKD.

Statement of Authorship

We state that **Chiung-Chi Peng** is responsible for study design, monitor the progress of the experiments, and data interpretation. **Chiu-Lan Hsieh** and **Jin-Yuan Chung** are responsible for performing experiments. **Kuan-Chou Chen** and **Robert Y. Peng** are responsible for trouble shooting and article writing.

Conflict of Interest

The authors do not have any conflict of interest.

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673 ## Figure Legends

674 **Figure 1. The histopathological findings of renal tubules and glomeruli in all groups**.
675 A: normal control. B: DRCKD control. C: ferulic acid control (FA). D:
676 DRCKD+ ferulic acid (DRCKD+FA). E: GA control (GA). F: DRCKD+ gallic
677 acid (DRCKD+GA) (magnification□ 400).

678 **Figure 2. The Sirius Red Staining of collagen deposition in kidney tissues of**
679 **different groups.** Ferulic acid accelerates the collagen deposition (stained red)
680 in DRCKD tissue (figure D) when compared with the gallic acid treated
681 DRCKD tissue (figure F). [A: normal control. B: DRCKD control. C: FA
682 control. D: DRCKD+ FA. E: GA control. F: DRCKD+ GA (magnification×
683 400).

684 **Figure 3. Serum superoxide dismutas (SOD) (3A), and MDA levels (3B) in different**
685 **experimental groups.** Values in each bar with different superscripts (a to b)
686 indicate significantly different with each other at confidence level of $p<0.05$. .

687 **Figure 4.The serum PDGF-BB level (4A) and protein expression of**
688 **PDGFR ,p-PDGFR, TGF-β□(4B) in different experimental groups.** Values
689 in each bar with different superscripts (a to d) indicate significantly different

690 with each other at confidence level of $p<0.05$(4A). The amount of protein
691 expressed is expressed in fold(s) of control (β-actin) (4B).

692 **Figure 5. The expression of signaling proteins in kidney tissues of different**
693 **experimental group.**

694 The experimental groups comprise normal (normal control), DRCKD
695 (DR-induced CKD), FA (ferulic acid control), DRCKD+FA (DRCKD+ferulic
696 acid), GA (gallic acid control), and DR-GA (DRCKD + gallic acid). The amount
697 of protein expressed is expressed in fold of control (β-actin).

698 **Figure 6. The expression of matrix metalloproteinases MMP-2 and MMP-9 in**
699 **kidney tissues of different experimental groups.** Data are expressed in
700 inhibition percent of MMP-2 when comparing to the normal group.

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Table Caption

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Table 1. Effect of gallic and ferulic acids on the physiological parameters of rats having chronic kidney disease.[§]

Table 2. Effect of gallic and ferulic acids on the serum and urinary biochemical parameters in rats having chronic kidney disease.[§]

728

*Manuscript

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Ferulic Acid Is Nephrodamaging While Gallic Acid Is Renal Protective In Long Term Treatment of Chronic Kidney Disease

Chiung-Chi Peng[a,b,c,g], Chiu-Lan Hsieh[d], Hui-Er Wang[e], Jin-Yuan Chung[a], Kuan-Chou Chen[f,g*], Robert Y. Peng[h]

[a]Department of Physical Therapy, [b]Graduate Institute of Rehabilitation Science, [c]Department of Nutrition, College of Health Care, China Medical University, 91 Hsueh-Shih Rd., Taichung, Taiwan 40202

[d]Graduate Institute of Biotechnology, National Changhua University of Education, 1, Jin-De Rd., Changhua, Taiwan 500

[e]Department of Food and Applied Biotechnology, [h]Research Institute of Biotechnology, Hungkuang University, 34 Chung-Chi Rd., Shalu County, Taichung Hsien, Taiwan 43302

[f]Department of Urology, Taipei Medical University-Shuang Ho Hospital, [g]Taipei Medical University, 250, Wu-Xin St., Xin-Yi District, Taipei, Taiwan 110

[*]Corresponding authors: Dr. Kuan-Chou Chen

Department of Urology, Taipei Medical University-Shuang Ho Hospital, Taipei Medical University, 250, Wu-Xin St., Xin-Yi District, Taipei, Taiwan 110

E-mail: kc.chen416@msa.hinet.net; Mobile: +886-958-828-839; Tel: +886-2-27299723.

***Running title*: Ferulic Acid Worsened Chronic Kidney Disease**

Abstract

Backgrounds & aims: The long term therapeutic effect of ferulic (FA) and gallic (GA) in treatment of chronic kidney disease (CKD) has been lacking.

Methods: Doxorubicin (DR, Adriamycin)-induced CKD rat model was established for this study.

Results: DR significantly reduced levels of serum albumin, GOT, GPT, RBC, TNF-α, and urinary creatinine and elevated serum cholesterol, TG, BUN, creatinine, uric acid, WBC, platelet count, and IL-6. In DRCKD rats, FA and GA significantly increased kidney weight and glomerular volume. FA reduced glomerular filtration rate but GA did not. FA enhanced more collagen deposition than GA in renal cortex and glomeruli. Both FA and GA showed crucial hyperlipidemic activity. The inhibitory effects of FA and GA on MMP-2 were very comparable. GA suppressed MMP-2 more effectively than FA in DRCKD rats. Both FA and GA induced SOD elevation and MDA elimination. In DRCKD rats, Western blot analysis indicated that FA further up-regulated CD34, α-SMA, tissue pDGFR, p-PDGFR, and TGF-β; and down-regulated p-PI3K, and p-Akt. Since both PDGF-BB and TGF-β are considered to induce kidney prefibrosis stage, GA was proved to be more beneficial in this regard.

Conclusions: GA tends to protect the CKD while FA is not recommended for the long term CKD therapy.

***Keywords*:** gallic acid; ferulic acid; chronic kidney disease; PDGF; α-SMA

1. Introduction

Flavonoids comprise the most common group of plant polyphenols and provide much of the flavor and color to fruits and vegetables[1]. Most of the flavonoids present in plants occur in glycosidic forms, although occasionally as aglycones. Interest in the role of flavonoids to act as health benefits is emerging owing to their potential antioxidative and free-radical scavenging activities. However, up to present, epidemiologic studies exploring the role of flavonoids in human health have been inconclusive[1,2]. Some studies support a protective effect of flavonoid consumption in cardiovascular disease and cancer, others demonstrate no effect, and conversely a few suggest potential harm[1].

Gallic acid (3,4,5-trihydroxybenzoic acid) (GA) widely occurs in plants including gallnuts, grapes, tea, hops and oak bark[3]. GA yields numerous esters and salts including digallic acid. GA seems to have anti-fungal and anti-viral properties. More recently, GA was found to show cytotoxicity against cancer cells, without harming healthy cells[4]. GA is also used to treat albuminuria and diabetes. Some ointments for treatment of psoriasis and external haemorrhoids contain mainly gallic acid[3].

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (FA), an effective component of Chinese medicine herbs such as *Angelica sinensis, Cimicifuga heracleifolia* and *Lignsticum chuangxiong*, is a ubiquitous phenolic acid in the plant kingdom[5]. FA exhibits many physiological functions, including antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis, and anti-cancer activities. It protects against coronary disease by lowering serum cholesterol. Moreover, it enhances the viability of spermatozoa[5].

Doxorubicin (DR, commercial name Adriamycin) has been used as an anticancer (antineoplastic) medication. It interferes with cancer cell growth and slows their

migration in body[6]. DR had been used to induce nephropathy as a model of chronic progressive glomerular disease[7], generally named “The Chronic Kidney Disease (CKD)”. DR produced chronic, progressive glomerular changes in rats, which led to terminal renal failure. The segmental glomerular sclerosis and IgM-dominant glomerular deposition in these animals are similar to the pathological characteristics of focal and segmental glomerular sclerosis seen clinically[7]. Referring to the recent report[2], we suspect that some flavonoid antioxidants may be safe for use while some may damage the kidney in a CKD status. In this work, we adopted DR to create the CKD model in rats and investigated whether the potentially used phytoantioxidants (PAO) like gallic and ferulic acids can improve CKD to some extent.

2. Materials and methods

2.1 Animals

Thirty six male Sprague Dawley (SD) rats, age 4 weeks, having mean body weight 155 g (range of 150–164 g), were purchased from the Biolasco Animal Centre, Taiwan. Rats were individually housed in animal room maintained at 22±1^{o}C and a relative humidity of 65% on a 12h/12 h light-dark cycle. The access of distilled water was ad libitum, but the maximum amount of feed was restricted at 10% of body weight per day. The body weight change and the amount of food intake were recorded daily. All the protocols had been previously approved before experimentation by the Institutional Animal Care and Use Committee of the China Medical University.

2.2 CKD induction and animal grouping

The DR-CKD rat modeling was performed according to Okuda et al.[7]. Briefly, in the first week, CKD was induced by subcutaneous injection of 8.5 mg/kg of DR (Pfizer, Milano, Italia) under ether anesthesia. The DR-induced rats were divided into six groups, 6 rats in each. Group 1 served as the diet control was fed normal diet only (Normal group). Group 2 was DR-induced and fed normal diet (DRCKD group). Commercially, the pharmaceutical preparation for human use is fabricated into tablets, containing 182 mg FA per tablet[8]. When prescribed with the order 2-3 tablets tid, a total of 1092-1638 mg/day will be administered. Assuming a 60 kg male is to receive this dosage, a single

dosage will correspond to 18.2-27.3mg FA/kg-day. Alternatively, pure GA at 50 mg/dose had been tried for testing its bioavailability in human[9], while the dosage of GA reported to be safe for rats in doses ranging within 119-128 mg/kg/day (Niho et al., 2001)[10].Obviously rats are able to endure at least 2-3 folds human dosage. Consequently, Group 3 received FA (Sigma-Aldrich, USA)-containing diet at FA 70mg/kg-day (FA group)[11]. Group 4, the DR-induced rats, received FA-containing diet at FA 70mg/kg-day (DRCKD+FA group)[11]. Group 5 rats were fed GA (Sigma-Aldrich, MO, USA)-diet containing only GA 70mg/kg/day (GA group). Group 6, the DR-induced rats, was given GA-containing diet at GA 70mg/kg/day (DRCKD+GA group). The two compositions were correctly weighed and thoroughly blended with normal diet before feeding.

2.3 Glomerular volume

The glomerular volume was determined by the formula[12].

$$\mathbf{GV} = (\beta/k)\,(G_A)^{3/2} \quad \ldots\ldots 1$$

Where **GV** = glomerular volume (mm^3)

$\mathbf{G_A}$ =cross-sectional tuft area (mm^2)

β is the shape coefficient (1.38 in this case, for a sphere β= 1.38)

k is the size distribution coefficient (= 1.1 in this case)

2.4 Glomerular filtration rate (GFR)

The glomerular clearance rate (GRF) is defined by Eq. 2[13]

$$GFR = (CR_c \times BN_c)^{1/2} \quad \ldots\ldots 2$$

Where

CR_c is the creatinine clearance, and BN_c is BUN clearance.

And

$CR_c = 1000C_{Cr,u}/C_{Cr,s}$ ……3

Here $C_{Cr,u}$ is the volume concentration of creatinine in urine, and $C_{Cr,s}$ is the volume concentration of creatinine in serum. And

$BN_c = C_{BN,u}/CB_{N,s}$ ……4

Here $C_{BN,u}$ denotes the volume concentration of BUN in urine; and $CB_{N,s}$ means the volume concentration of BUN in serum.

2.5 Histopathological examination

The animals were ether-euthanized at the end of week 12, the kidneys were immediately picked up and fixed in 10% formalin and embedded in paraffin. The embedded tissues were stained with hematoxylin and eosin reagent (H&E stain). Renal histology was examined with Olympus- CKX41 Microscope. Glomerular areas were measured using an image analyzer. The collagen content was estimated by Sirius Red stain. For Sirius Red staining, the paraffin embedded sections were first dewaxed, hydrateds, and sliced. The nuclei in the tissue slices were stained with Weigert's Haematoxylin and then stained in Saturated aqueous picric acid with Sirius Red (Sigma-Aldrich, USA) for one hour. The treated slides were rapidly dehydrated by a concentration gradient alcohols (starting with 70% to absolute alcohol), then to xylene and finally the slices were covered in Permount.

2.6 Biochemical analysis

The blood was collected for the measurement of serum albumin, blood urea nitrogen (BUN), creatinine, cholesterol, triglyceride, calcium, phosphorus, uric acid. At week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11, the blood was collected via the tail artery and was collected by arteria coeliaca at week 12. It was measured by reagent (Siemens, Bakersfield, CA, USA) and automatic analyzer (Ciba-Corning Express Plus) (Ciba-Corning, USA). All the

157 rats were weighed and placed in metabolic cages week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11,
158 to determine BUN, creatinine and protein excretion in 12 h urine. Urine BUN and
159 creatinine were measured by reagent (Siemens, Bakersfield, CA, USA) and automatic
160 analyzer (Ciba-Corning Express Plus) (Ciba-Corning, USA). Urine protein was measured
161 by ELISA reader.

162 ***2.7 ELISA of superoxide dismutase and malondialdehyde***

163 All ELISA protocols were performed by following the manufacturer's instruction. The
164 serum superoxide dismutase (SOD) and malondialdehyde (MDA, or generally termed the
165 thiobarbituric acid reactive substance, TBARs) were assayed with the commercial ELISA
166 kits provided by Cayman Chemical Co. (MI, USA). The optical density was read using
167 the SYSMEX K-1000 Reader (San-Tong Instrument Co., Taipei, Taiwan).

168 ***2.8 Western blot analysis***

169 One hundred mg of frozen renal cortex was homogenized with 1 mL of protein extraction
170 solution (EDTA free) (Intron Biotechnology, Korea). After incubated on ice for 40 min,
171 the homogenate was centrifuged at 12000×g for 20 min at 4^{o}C. The supernatant (tissue
172 lysate) was collected. Lysates containing approximately an amount of protein (50 μg)
173 were boiled for 10 min in PBS. The boiled sample solutions were loaded onto a 7.5 %
174 polyacrylamide SDS gel. Proteins were transferred to a PVDF membrane and rinsed with
175 TBS-Tween buffer (TBST), and blocked at 4^{o}C overnight in TBST containing 5% w/v
176 non-fat powdered milk. The PVDF membranes were incubated with the primary
177 antibodies, which contains Akt (1:1000), phospho-Akt (1:1000) , PDGF receptor β
178 (1:1000), phospho-PDGF receptor β (1:1000), and PI3-kinase (1:1000) (Cell Signaling,
179 USA) ; phospho-PI3K (1:500), CD34 (Santa Cruz, USA); and α- smooth muscle actin

(1:1000) (Sigma-Aldrich, USA) etc. in TBST at 4^{o}C overnight. The PVDF membrane was then rinsed three times with TBST and incubated with the secondary antibodies containing anti-mouse, anti-rabbit and anti-goat (each at 1:5000 dilution in TBST with 5% w/v non-fat powdered milk). After incubated at room temperature for 1 h, the PVDF membranes were rinsed three times with TBST. The secondary antibodies bound were detected using the chemiluminescent HRP substrate (Minipore, USA).

2.9 Statistical analyses

Data obtained in the same group were analyzed by Student's *t* test with computer statistical software SPSS 10.0 (SPSS, Chicago, IL). ANOVA statistical analysis system software with Tukey test was used to analyze the variances and significances of difference between paired means. Significance level was judged by a confidence level $p < 0.05$.

3. Results

3.1. Both ferulic and gallic acids did not harm normal kidney, but FA aggravated CKD

DR caused significant body weight loss, the body weight was significantly decreased from 516 g of the normal to 304 g. Although FA control group showed normal body as the control, GA alone seemed to have a moderate body weight reducing effect (Table 1). Conversely, in DRCKD+FA rats, FA reduced body weight more significantly than DRCKD+GA, giving rise to 239 g and 357 g, respectively (Table 1). Anatomically, DR caused renal tubular and glomerular damages with formation of a number of vacuoles, glomerular sclerosis and tubulointerstitial degeneration at week 28 (Figure 1), but slighter extent with CKD+GA group, although all DR-induced rats revealed renal inflammation accompanied with apparent swelling and edema. Figure 1 exhibits the status of nephroedema (Figure 1B: DRCKD; D: DRCKD+FA; F: DRCKD+GA). Nonetheless, GA more efficiently secured the DR injury (Figure 1F) in this regard. The average kidney weight of the DRCKD, DRCKD+FA and DRCKD+GA groups was 5.0, 4.7, and 3.9 g respectively, comparing to 3.1 g of the normal, a significantly larger extent of recovery was seen in DRCKD+GA rats (Table 1). Similar results were found in the ratio kidney/body weight, glomerular volume (GV) and the glomerular filtration rate (GFR) (Table 1). Surprisingly, in DRCKD rats FA reduced the GFR to 120 mL/h. Contrary to this, GA increased GFR to a value 515 mL/h (Table 1).

3.2 Histopathological examination

In the renal cortex of DRCKD rats, a huge amount of collagen deposition wa observed occurred (Figure 1B; Figure 2B). Contrast with this, a much larger amount of collagen deposit was found in the DRCKD+FA group (Figure 2D), less deposit in DRCKD+GA group (Figure 2F).

3.3 Biochemical parameters affected in doxorubicin induced CKD

DR severely down-regulated the serum albumin, GOT, GPT, and RBC clearance, but up-regulated levels of serum cholesterol, triglyceride (TG), BUN, creatinine, calcium, phosphate, uric acid, WBC, platelets, and levels of urinary BUN and protein (Table 2).

3.3.1. Serum creatinine level was significantly raised

DRCKD rats exhibited higher serum creatinine levels (1.4 mg/dL) comparing with the normal 0.7 mg/dL, and FA further increased the level to 2.9 mg/dL (Table 2). Apparently, the DRCKD+FA rats were suffering from a moderate renal failure (2-4 mg/dL).

3.3.2. Effect of FA and GA on serum uric acid level

Interestingly, FA alone activated, conversely GA alone inhibited uric acid synthesis (Table 2). Controversially in CKD victims, FA completely recovered the uric acid level to 1.5 mg/dL (in DRCKD+FA), comparing to the control (1.7 mg/dL) and the DRCKD+GA (3.0 mg/dL) (Table 2). Otherwise, the activity of SOD was activated in FA and GA groups, which may be correlated with the enhancement of TNF-α expression in these groups (Table 2), a phenomenon consistent with Wong and Goeddel[14].

3.3.3. Effect on SOD induction

The activity of superoxide anion dismutase (SOD) was elevated in all groups comparing to the normal control (Figure 3a). DR activated SOD, FA failed to suppress such

activation of SOD. As contrast, GA promisingly inhibited the elevation of SOD (Fig. 3a).

3.3.4. Effect on MDA suppression

Comparing with the MDA data, the serum MDA level in DRCKD group once having reached 86 μM was totally abolished by FA and GA (Figure 3b). Results indicate FA to be a better antioxidant than GA with respect to MDA suppression.

3.3.5. Effect on the hyperlipidemic status in CKD

DR induced hypercholesterolemia and triglyceridemia in CKD victims (Table 2). By comparison of the pharmacological action between the FA-alone or GA-alone diet, some amazing phenomena were observed. FA-alone and GA-alone diets did not show any apparent different effect on serum cholesterol and triglyceride levels. However in DRCKD rats, both the serum levels were significantly suppressed by FA and GA (Table 2).

3.3.6. Hepatoprotective effect

Similar trend was seen for serum BUN, GOT, and GPT, implicating the hepatoprotective effect of both FA and GA, consistent with Balasubashini et al.[15]. The anatomical and histological examination also confirmed such a result (unpublished).

3.3.7. Effect on leukopoiesis and eryhtrocytopenia induced by DRCKD

FA and GA exhibited moderate leukopoiesis effect (Table 2). FA even enhanced it to a greater extent in the DRCKD rats (1.8×10^4 count/μL). Whereas GA suppressed it to the normal level (8×10^3 count/μL). As a contrast, DR destroyed RBC in CKD rats to a level of 5.8×10^6 counts/μL. FA further reduced it to 4.5×10^6 counts/μL. In this regard, GA did not improve the CKD to any extent (Table 2).

3.3.8. Effect on the platelet count

Based on the platelet count of normal control (6.3×10^5 count/μL), FA-alone diet seemed to be a platelet proliferation inhibitor though the effect is not statistically significant. FA-alone diet suppressed the platelets to a number of 4.9×10^5 count/μL, but GA totally did not show any effect. Astonishingly, FA conversely increased the platelet count in DRCKD+FA rats to 1.52×10^6 count/μL, comparing to the DRCKD control (Table 2).

3.3.9. Effect on IL-6 and TNF-α in DRCKD victims

After 28 weeks, DR had moderately up-regulated the inflammatory cytokine IL-6 but significantly down-regulated TNF-α in DRCKD group. FA or GA when used alone was able to up-regulate IL-6 to a level 4.5 or 3.5 ng/mL and TNF-α to 1476.1 or 1099.65 pg/mL, pointing to the moderate inflammatory effect of FA and GA (Table 2). However, in DRCKD rats level of TNF-α was greatly suppressed to 370.2, 97.6, and 563.1 pg/mL respectively, comparing to the normal 936.1 pg/mL (Table 2). Whereas IL-6 still remained at a level higher than the normal.

3.3.10. Effect on level of urinary BUN in DRCKD victims

The urinary BUN level was significantly raised by DR to 547 mg/dL, comparing with the normal control level 108 mg/dL, which was cured by FA and GA to only 385 and 457 mg/dL, respectively. And interestingly, FA-alone and GA-alone-diets respectively raised the levels to 484 and 188 mg/dL (Table 2).

3.3.11. Doxorubicin upregulated PDGF-BB, and TGF-β□caused prefibrosis in kidney

DR up-regulated the platelet derived growth factor-BB (PDGF-BB) in the renal tissue of DRCKD rats to 4739.3 *p*g/mL (normal value, 693.9 pg/mL) and simultaneously the

294 TGF-β□to 2118 pg/mL (normal level, 1788 pg/mL) (Figure 4a,b). When administered
295 with FA or GA in DRCKD rats, FA showed a lower level of PDGF-BB than GA in the
296 DRCKD rat renal tissues (2747.8 pg/mL vs. 4238.2 pg/mL in Figure 4a), indicating FA
297 could be more damaging to the renal cells than GA (Figure 1D and 1F), which was
298 evidenced by the up-regulation of PDGFR, p-PDGFR and tissue TGF-β□in DRCKD+FA
299 and DRCKD+GA rats (Fig. 4b). While the slightly increased level of FA- (842.8 *p*g/mL)
300 and GA-controls (1240.2 *p*g/mL) may be crucially the concentration of FA or GA
301 required for cell growth promotion (Figure 4a).

302 **3.3.12. DR downregulated p-PI3K and p-Akt, but upregulated levels of CD34 and**
303 **α-SMA in DRCKD**

304 Western blotting revealed that FA when used alone did not affect the levels of PI3K,
305 p-Akt, CD34, and α-SMA. Likewise, GA alone did not show any effect on PI3K, p-Akt,
306 and α-SMA (Figure 5). On application of DR, DR down-regulated PI3K, p-PI3K and
307 p-Akt, but up-regulated levels of, Akt, CD34 and α-SMA. In DRCKD rats, FA
308 down-regulated levels of PI3K, p-PI3K, p-Akt and up-regulated Akt, CD34, and □
309 α-SMA. Conversely, GA slightly had restored levels of PI3K and p-Akt to normal levels,
310 and to lesser extent, the down-regulation of Akt and up-regulation of α-SMA (Figure 5).

311 **3.4. Zymography of MMP-2 and MMP-9**

312 Zymography of whole-kidney extracts showed very prominent two bands, 72 and 92-kDa
313 bands for MMP-2 and MMP-9, respectively (Figure 6), consistent with Rankin et al.[16].
314 High levels of MMP-2 seemed to result from increased expression by DR treatment. GA

315 down-regulated MMP-2 in DRCKD+GA rats, but FA did not show any recovery effect
316 (Figure 6). A similar but less intense response was found for MMP-9.
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322 ## 4. Discussion

323 ### *4.1. Why FA tends to aggravate CKD?*

324 As evidenced by the body weight gain, the glomerular volume, the glomerular filtration
325 rate (GFR), the ratio kidney/body weight (Table 1), and pathological changes (Figure 1),
326 FA at dosage 70mg/kg was detrimental to kidneys in CKD patients, conversely GA can be
327 protective. Histological examination revealing much more severe pre-fibrotic collagen
328 deposition in the renal cortex of DRCKD rats treated with FA (Figure 2D) than GA
329 (Figure 2F) has strongly supported this result. Both FA and GA are potentially potent
330 prooxidants[17,18]. Much of the literature has indicated FA is more potent in view of
331 pro-oxidative bioactivity. Overproduction of superoxide anions and prooxidants can elicit
332 cytotoxicity and induce apoptosis[18]. Pascale et al.[19] reported the order for scavenging
333 superoxide anions ($\bullet O_2^-$) is GA (76×10^{-4} M) > FA (10×10^{-4} M); and for scavenging
334 hydroxyl free radicals ($\bullet$OH) is FA (29×10^{-4} M) > GA (7×10^{-4} M)[19], indicating GA to be a
335 better superoxide anion scavenger; conversely, FA a better hydroxyl free radical
336 scavenger. With respect to prevention of the upstream overproduction of superoxide
337 anion, GA would be a better protective agent, an elucidation well supports our

speculation. Comparing with the MDA data, the serum MDA level in DRCKD group once having reached 86 μM was totally abolished by FA and GA (Figure 3b). Results indicates FA is a better antioxidant than GA with respect to MDA suppression.

4.2. Increased serum creatinine level can be ascribed to the inhibition of creatine phosphate kinase, and decreased urinary level of creatinine can be caused by energy deficiency

Since an increase in serum creatinine from 0.6 to 1.2 mg/dL represents a 50% decline in renal function, accompanied with the GFR 120 mL/h (normal 435 mL/h)[20]. In the early stages of renal failure, major decreases in GFR are often associated with what appear to be minor changes in serum creatinine[20]. However, worth noting, serum creatinine levels correlate with GFR only in the steady state. Therefore, significant errors in the estimation of GFR may occur if the serum creatinine level is rapidly changing[20]. Alternatively, the feature of creatinine clearance was severely impaired in groups DRCKD, DRCKD+FA, and DRCKD+GA (Table 2), similar to the findings of Yokozawa et al.[21]. The effects of doxorubicin on the energy metabolism had been reported by Bachmann et al.[22]. DR not only reduced oxygen consumption in heart mitochondria ex vivo, but also uncoupled oxidative phosphorylation, inhibited creatinine phosphate kinase (CPK), and damaged the semipermeability of the inner mitochondrial membrane (measured as creatine influx) [22]. Until recently, the only site of the transamidinating enzyme in mammals has been thought to be the kidney[23]. The reduced activity of CPK would lead to the accumulation of serum creatine, which in the absence of further phosphorylation to create CP will be spontaneously converted to creatinine by nonenzymatic reaction[23]. As a consequence, the serum creatinine level was increased (Table 2). In addition, renal clearance is responsible

for 80% of kidney's total energy requirement[24]. Under malnutrition status (Table 1) and lacking high energy creatine phosphate formation, the renal clearance may be retarded, resulting in reduced urinary creatinine excretion (Table 2).

4.3. FA and GA acted differently on serum uric acid level

The reason why FA alone activated, conversely GA alone inhibited, uric acid synthesis (Table 2) can be explained by their effect on xanthine oxidase[25] and the status of glomerular clearance[26]. FA might inhibit, but GA might activate, the enzyme xanthine oxidase in CKD victims. Literature elsewhere indicated accumulation of a broad spectrum of toxins due to failure of the kidney to eliminate these substances. Under normal conditions, the glomerular filter clears molecules with a molecular weight up to 58,000 Da. All these substances are supposed to be retained in renal failure and are candidate uremic toxins. Ninety compounds are known as uremic toxins; 68 of them have a molecular weight <500 Da (small water-soluble compounds), 22 have a molecular weight >500 Da (middle molecules), and 25 solutes (27.8%) are protein bound[26].

4.4. FA may enhance platelet aggregation

As mentioned, FA increased the platelet count in DRCKD+FA rats to 1.52×10^6 count/μL, comparing to the DRCKD control (Table 2). In patients on hemodialysis (HD), platelet aggregation was impaired before as well as after the HD session[27], an implication in the possible feature of FA to affect the cell growth, proliferation and blood coagulation.

4.5. Effect on IL-6 and TNF-α in DRCKD victims

In DRCKD rats, level of TNF-α was greatly suppressed, whereas IL-6 still remained at a level higher than the normal (Table 2), a phenomena being very similar to polymyalgia rheumatica (PMR). Active PMR is characterized by increased serum levels of IL-6, but

not those of other pro-inflammatory cytokines. Worth noting, all the DR, FA alone, DRCKD+FA, and DRCKD+GA groups exhibited significantly elevated WBC counts, indicating in parallel significantly increased monocytes. As circulating monocytes do not show increased production of proinflammatory cytokines, IL-6 might be mainly produced in the inflamed tissue[28].

Tumor necrosis factor TNF-α and TNF-α are soluble ligands binding to TNF receptors with similar activities. TNF is a multifunctional cytokine that plays important roles in diverse cellular events. In regard to cancer, TNF is a double dealer, acting as either a promoter or a killer[29]. Soluble TNF receptor in inflammatory bowel disease (IBD) mucosa inhibited TNF activity. Type 2 soluble receptor release from IBD mucosa was increased in active inflammation; release from lamina cells was not increased[30]. Mucosal TNF-α production correlated with severity of disease. In some diseases, soluble TNF-α receptors neutralize TNF-α activity by acting as inhibitors[30]. Conversely, DR suppressed level of TNF-α. To enhance TNF-α level to induce MnSOD has been reported to be a possible protective mechanism of ghrelin for DR-induced cardiomyopathy and heart failure[14, 31]. In our case, both FA and GA when used alone increased the levels of TNF-α to 1476.1 pg/mL and 1099.7 pg/mL respectively, comparing to the normal 936.1 pg/mL (Table 2). On induction with DR, the DRCKD rats might first down-regulated TNF-α, which was further suppressed by FA to a much lower level (97.6 pg/mL). Conversely, level of TNF-α in DRCKD+GA was enhanced by GA to a higher level (563.1 pg/mL) (Table 2), evidencing the possible imbalance between the TNF-α and the TNF-α□ receptor in the DRCKD+FA as mentioned by Noguchi et al.[30].

4.6. Why level of urinary BUN was intensely increased by FA and GA?

FA-alone and GA-alone-diets respectively raised the levels to 484 and 188 mg/dL (Table 2), indicating that FA and GA were not sufficiently effective for suppressing the level of urinary protein, consistent with Okuda et al.[7]. On administration of DR, massive proteinuria, hypoalbuminemia, and hyperlipidemia were observed (Table 2). Both BUN and serum creatinine increased at week 16 and reached the uremic level at week 28[7]. Comparing with the serum BUN levels, the high level of urinary BUN can be ascribed to more rapid renal excretion of urea when affected by DR, FA, and GA (Table 2).

4.7. Doxorubicin up-regulated PDGF-BB, and TGF-β□caused prefibrosis, and FA may potentiate the pathological status due its pro-oxidant bioactivity

As indicated in Figure 1, prefibrosis of kidney occurred as a consequence of DR therapy. (Figure 1), and similarly the level of PDGF-BB (Figure 4a). Okuda et al. reported that IgM with a small amount of IgG and C_3 appeared in the sclerosing glomeruli from week 16 on treatment with DR[7]. As mentioned, FA acts as a strong pro-oxidant, and previously, we also had found a certain degree of cardiac injury in rats when treated with DR (data not shown). FA thus may enhanced the severity of fibrotic status. As well cited, PDGF-BB activates all combinations of PDGF receptor subunits[32], serving to potentiate autocrine stimulation of growth[33]. PDGF-BB is associated with excessive cell migration, proliferation and many growth-related diseases[34]. PDGF-BB plays an important role in the cellular metabolism of vascular wall by regulating the rate of macrophage-colony stimulating factor (MCSF) production in vascular smooth muscle cells[35]. PDGF-BB is also a potent wound-healing hormone accelerating incisional repair[36].

Transforming growth factor (TGF)-β is strongly implicated in the progression of renal fibrosis. TGF-β1 is reported to cause epithelial-mesenchymal transition, inhibition of

epithelial cell proliferation, increased apoptosis, auto-induction of TGF-β1 production and induction of secondary mediators of tissue fibrosis such as connective tissue growth factor (CTGF, CCN2)[37]. Ras/MAP kinase pathway, specifically through N-Ras, mediates TGF-β1 auto-induction and TGF-β1 induced CTGF expression in human renal tubule epithelial cells[37].

Rats received N-nitro-L-arginine methyl ester (L-NAME) developed severe hypertensive nephrosclerosis. Levels of TGF-β1 mRNA in the renal tissue was also significantly increased compared with control spontaneously hypertensive rats[38]. By inhibiting both TGF-β1 production and apoptosis induction, glomerular and arteriolar damages can be prevented and renal functions can be secured[38].

In its normal state, the TGF-β pathway restricts cell growth, differentiation and cell death[39]. When a normal cell becomes cancerous, various components of the TGF-β□ signaling pathway become mutated, which makes the newly cancerous cell resistant to the effects of normally functioning TGF-β. These resistant cells then grow without regulation[39].

4.8. DR down-regulated p-PI3K and p-Akt, but up-regulated levels of CD34 and □-SMA in DRCKD

As mentioned, in DRCKD rats, FA down-regulated levels of PI3K, p-PI3K, p-Akt and up-regulated Akt, CD34, and α-SMA. Conversely, GA had slightly restored levels of PI3K and p-Akt to normal levels, lesser extent in down-regulation of Akt and up-regulation of α-SMA (Figure 5).

Among 30 patients with glomeronephritis (GN), CD34 is present in the extraglomerular mesangium in 50% (15 patients) of the GN patients. 73% (11 patients) of the latter may

453 show concomitant intraglomerular and extraglomerular mesangial CD34 immunostaining,
454 while 26.7% (four patients) show only extraglomerular mesangial immunostaining, and
455 in 20% (3 patients) of patients, CD34 immunostaining is present only in the
456 intraglomerular mesangium[40]. In fact there is a fair degree of relationship, which did not
457 reach statistical significance between CD34 in the extraglomerular mesangium and CD34
458 in the intraglomerular mesangium. In the intraglomerular mesangium, CD34 does not
459 significantly correlate with mesangial α-SMA and activity or chronicity index. In the
460 extraglomerular mesangium, CD34 does not show a significant correlation with α-SMA[40].
461 Instead, the activity index and the chronicity index may show a good correlation with
462 serum creatinine (Table 2). Mesangial cell proliferation correlates well with the
463 mesangial matrix increase, while interstitial vimentin shows a good correlation with
464 interstitial α-SMA[41]. In addition, the immunoreactivity of α-SMA is closely correlated
465 with and necroinflammatory activity (p = 0.022). The degree of α-SMA expression and
466 the scores of fibrosis (in periportal, perisinusoidal and pericentral areas) were highly
467 correlated[41]. Neglecting the role of CD34, we suspect that DR up-regulated TGF-β,
468 p-PDGFR, and PDGFR to trigger the signal cascade PDGF→ PDGFR→ (CD34??)
469 →α-SMA signaling pathway, and simultaneously down-regulated the pathway PI3K
470 (p-PI3K) → Akt (p-Akt), resulting in severe kidney damages that FA and GA are unable
471 to inhibit.

472 ***4.9. Zymography of MMP-2 and MMP-9***

473 Levels of MMP-2 was up-regulated by DR treatment. Under normal physiological
474 conditions, much of the increased MMP was present in the inactive zymogen form. In
475 pathological renal cysts, MMP-2 is abnormally localized to the interstitium and to foci

476 between cysts, suggesting that MMP-2 may regulate collagen accumulation at those sites,
477 thus allowing cyst enlargement and limiting the severity of interstitial fibrosis[16]. GA was
478 found effective for down-regulation of MMP-2, but FA did not show any recovery effect
479 (Figure 6). The whole experiment had been observed for a period of 28 weeks,
480 corresponding to 47 year-human life of 60 years, which seemingly was equivalent to
481 approximately 3 year-life of rats, i.e. such an experiment could be considered as a long
482 term observation. A similar result had been previously reported in our laboratory[11]. The
483 relationship between food and disease is indeed extremely complex. It is generally
484 accepted that diet is a contributory factor in the aetiology of a large proportion of
485 diseases[42]. Furthermore, polyphenols may interact with certain pharmaceutical agents like
486 DR and enhance their biologic effects (refer to Figure 1). Considering the outcome may
487 deviate between a short term and a long term therapy with FA and GA, the possible
488 mechanism may involve i) the unique prooxidant effect of some antioxidants, ii) the
489 pathological changes altering the signaling peptides and signaling pathways, iii) the
490 optimum dosage required may vary depending on the stage of pathological event, and
491 finally iv) the individual variation in biochemical response[18]. As mentioned[43], it is
492 important to consider the doses at which these effects occur, in relation to the
493 concentrations that naturally occur in the human body. Future studies evaluating either
494 beneficial or adverse effects should therefore include relevant forms and doses of
495 polyphenols and, before the development of fortified foods or supplements with
496 pharmacologic doses, safety assessments of the applied doses should be performed[43].

497 ***4.10. Hypertension is another risk***

498 In DRCKD victims, severe hypertensive status is always observed. Blood pressure of

DRCKD, DRCKD+FA, and DRCKD+GA groups reached 160, 145, and 144 mmHg in DRCKD, DRCKD+FA, and DRCKD+GA groups respectively, comparing to the normal value 99 mmHg (Table 2). As often cited, hypertension is a risk factor to induce renal disease, neural degeneration and a diversity of cardiovascular diseases[44].

To conclude, both FA and GA failed to retard the down-regulation of *p*-PI3K and *p*-Akt; and the up-regulation of PI3K, Akt, CD34 and α-SMA caused by DRCKD. Although both FA and GA were able to down-regulate serum PDGF-BB and up-regulate tissue PDGFR, long term treatment of CKD with ferulic acid has revealed that ferulic acid tends to aggravate, on the contrary, GA tends to protect the damages caused by CKD. Thus, FA is not recommended to be used as a long term therapy for patients with CKD.

Statement of Authorship

We state that **Chiung-Chi Peng** is responsible for study design, monitor the progress of the experiments, and data interpretation. **Chiu-Lan Hsieh** and **Jin-Yuan Chung** are responsible for performing experiments. **Kuan-Chou Chen** and **Robert Y. Peng** are responsible for trouble shooting and article writing.

Conflict of Interest

The authors do not have any conflict of interest.

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673 ## Figure Legends

674 **Figure 1. The histopathological findings of renal tubules and glomeruli in all groups**.
675 A: normal control. B: DRCKD control. C: ferulic acid control (FA). D:
676 DRCKD+ ferulic acid (DRCKD+FA). E: GA control (GA). F: DRCKD+ gallic
677 acid (DRCKD+GA) (magnification□ 400).

678 **Figure 2. The Sirius Red Staining of collagen deposition in kidney tissues of**
679 **different groups.** Ferulic acid accelerates the collagen deposition (stained red)
680 in DRCKD tissue (figure D) when compared with the gallic acid treated
681 DRCKD tissue (figure F). [A: normal control. B: DRCKD control. C: FA
682 control. D: DRCKD+ FA. E: GA control. F: DRCKD+ GA (magnification×
683 400).

684 **Figure 3. Serum superoxide dismutas (SOD) (3A), and MDA levels (3B) in different**
685 **experimental groups.** Values in each bar with different superscripts (a to b)
686 indicate significantly different with each other at confidence level of $p<0.05$. .

687 **Figure 4.The serum PDGF-BB level (4A) and protein expression of**
688 **PDGFR ,p-PDGFR, TGF-β□(4B) in different experimental groups.** Values
689 in each bar with different superscripts (a to d) indicate significantly different

690 with each other at confidence level of $p<0.05$(4A). The amount of protein
691 expressed is expressed in fold(s) of control (β-actin) (4B).

692 **Figure 5. The expression of signaling proteins in kidney tissues of different**
693 **experimental group.**

694 The experimental groups comprise normal (normal control), DRCKD
695 (DR-induced CKD), FA (ferulic acid control), DRCKD+FA (DRCKD+ferulic
696 acid), GA (gallic acid control), and DR-GA (DRCKD + gallic acid). The amount
697 of protein expressed is expressed in fold of control (β-actin).

698 **Figure 6. The expression of matrix metalloproteinases MMP-2 and MMP-9 in**
699 **kidney tissues of different experimental groups.** Data are expressed in
700 inhibition percent of MMP-2 when comparing to the normal group.

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Table Caption

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Table 1. Effect of gallic and ferulic acids on the physiological parameters of rats having chronic kidney disease.[§]

Table 2. Effect of gallic and ferulic acids on the serum and urinary biochemical parameters in rats having chronic kidney disease.[§]

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Figure 1

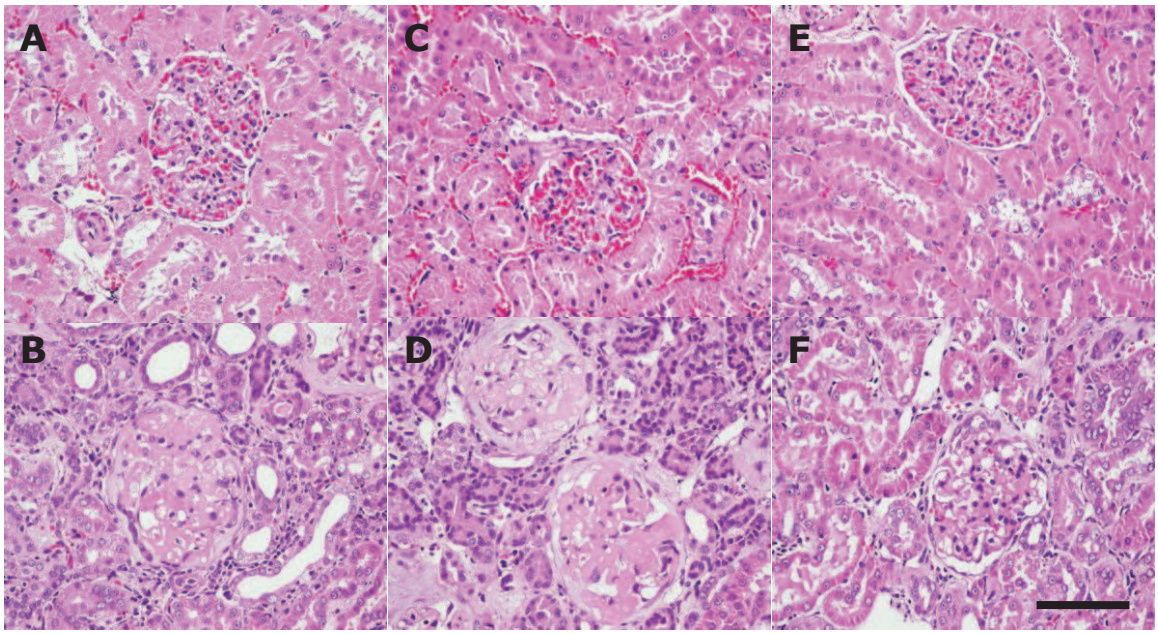


Figure 2

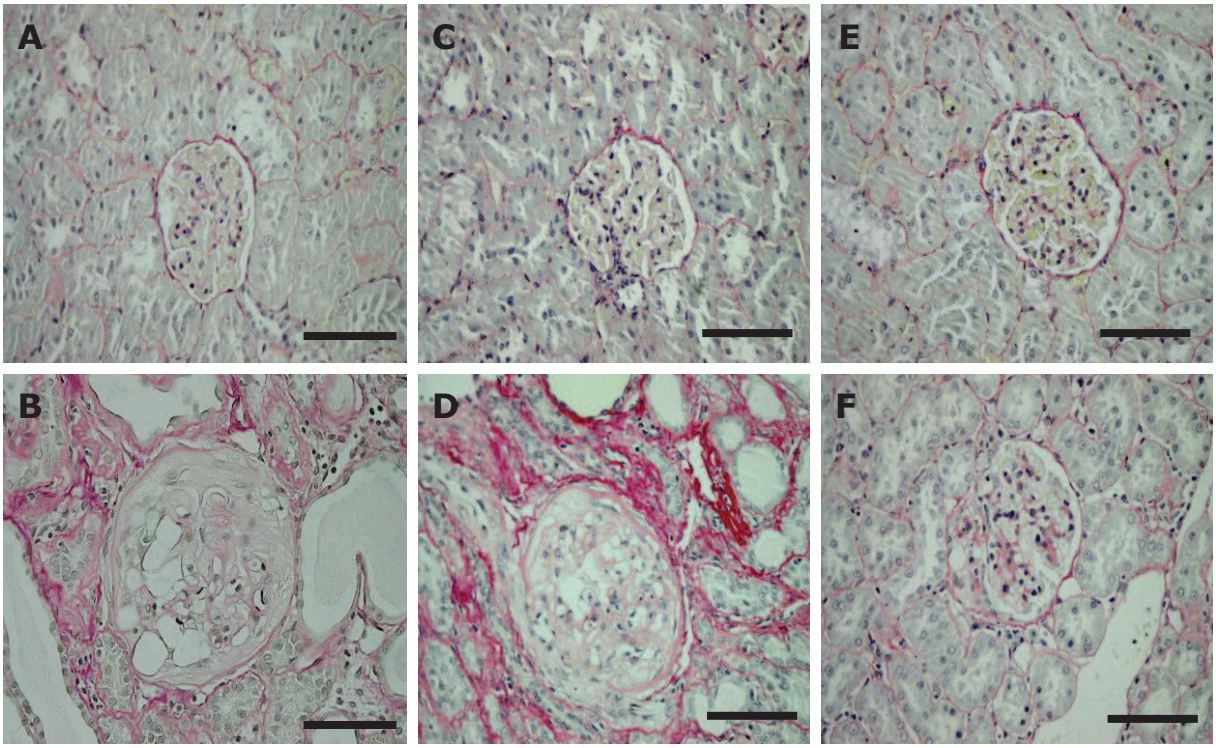


Figure 3 A

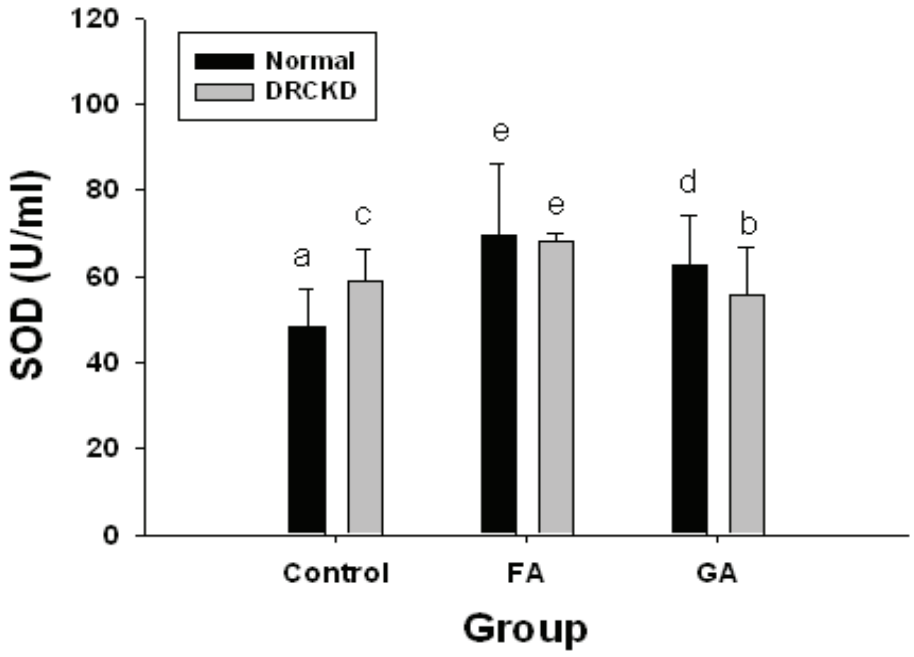


Figure 3B

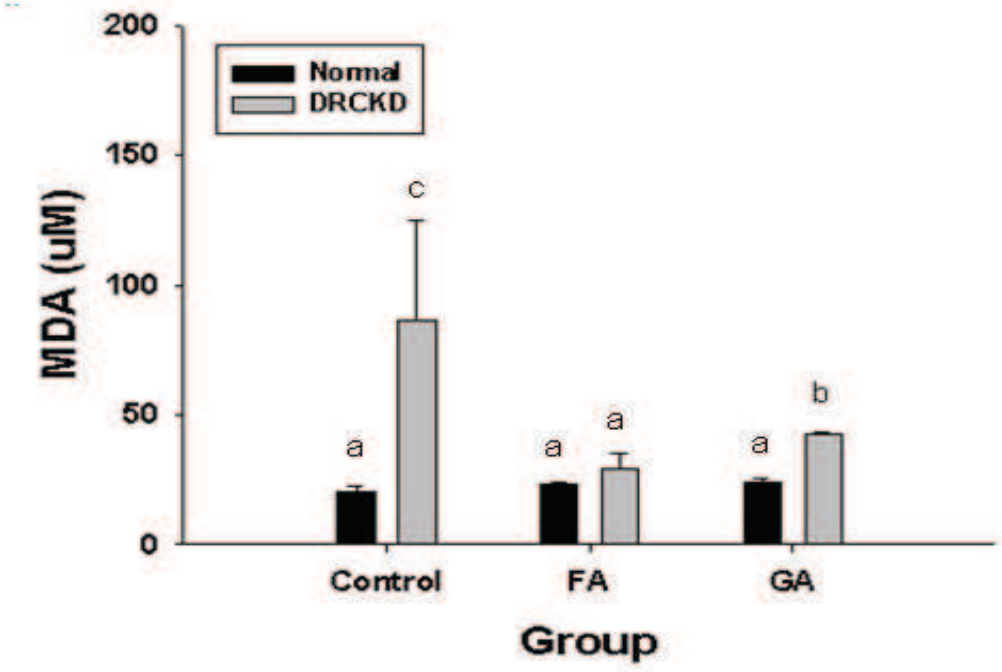
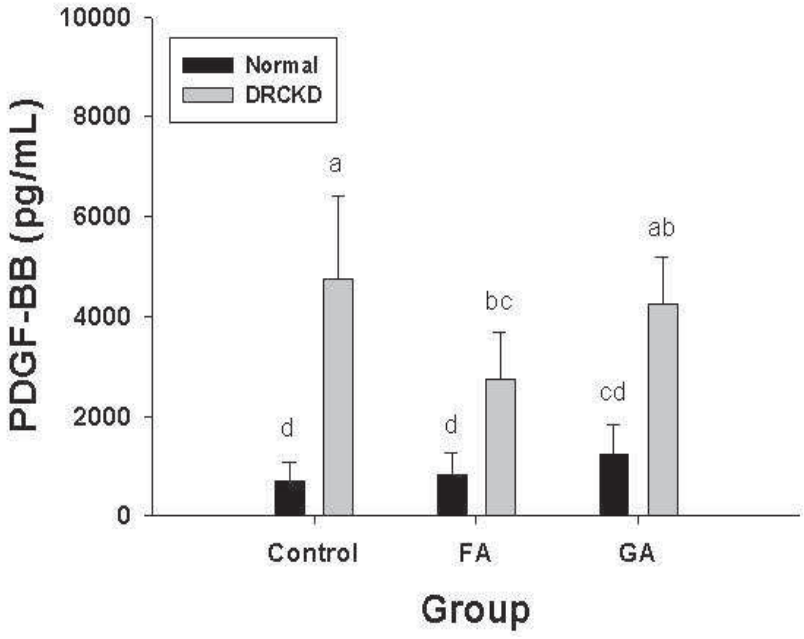
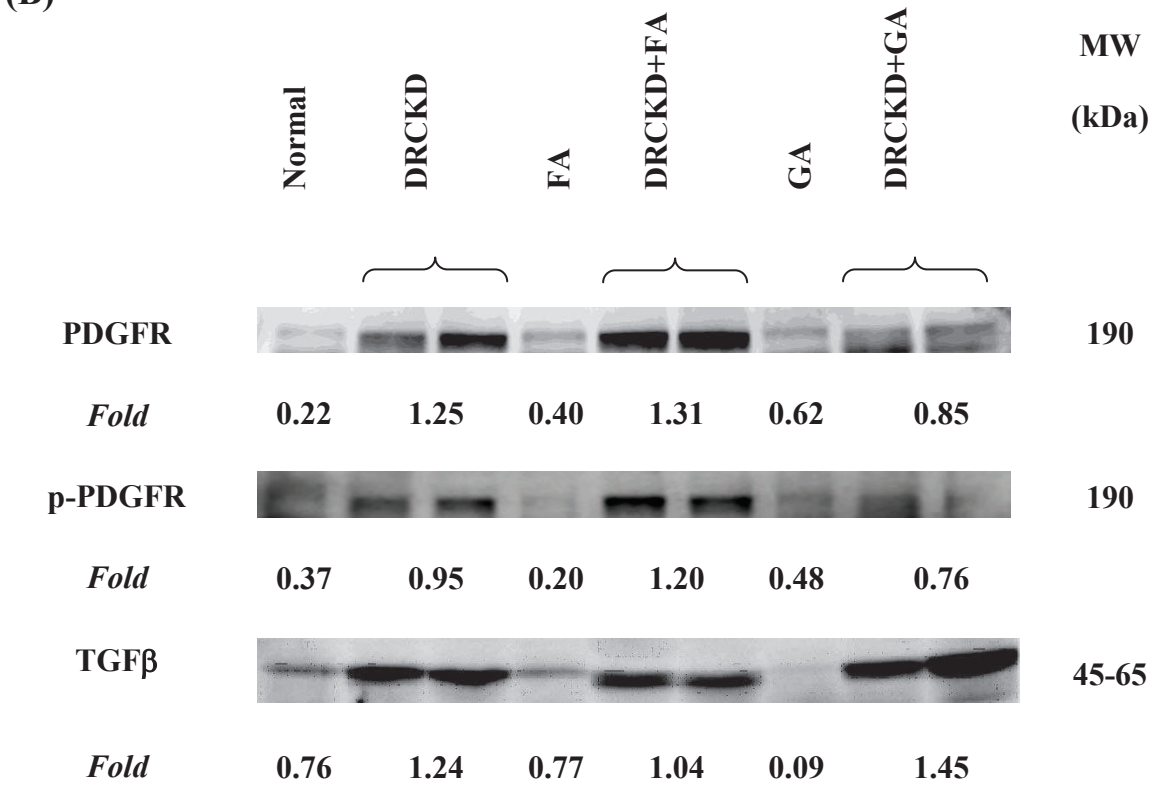


Figure 4

(A)


10000
8000
6000
4000
2000
0
PDGF-BB (pg/mL)
Normal
DRCKD
a
ab
bc
cd
d
d
Control
FA
GA
Group

(B)


Normal
DRCKD
FA
DRCKD+FA
GA
DRCKD+GA
MW
(kDa)
PDGFR
190
Fold
0.22
1.25
0.40
1.31
0.62
0.85
p-PDGFR
190
Fold
0.37
0.95
0.20
1.20
0.48
0.76
TGFβ
45-65
Fold
0.76
1.24
0.77
1.04
0.09
1.45

β-actin  42

Figure 5

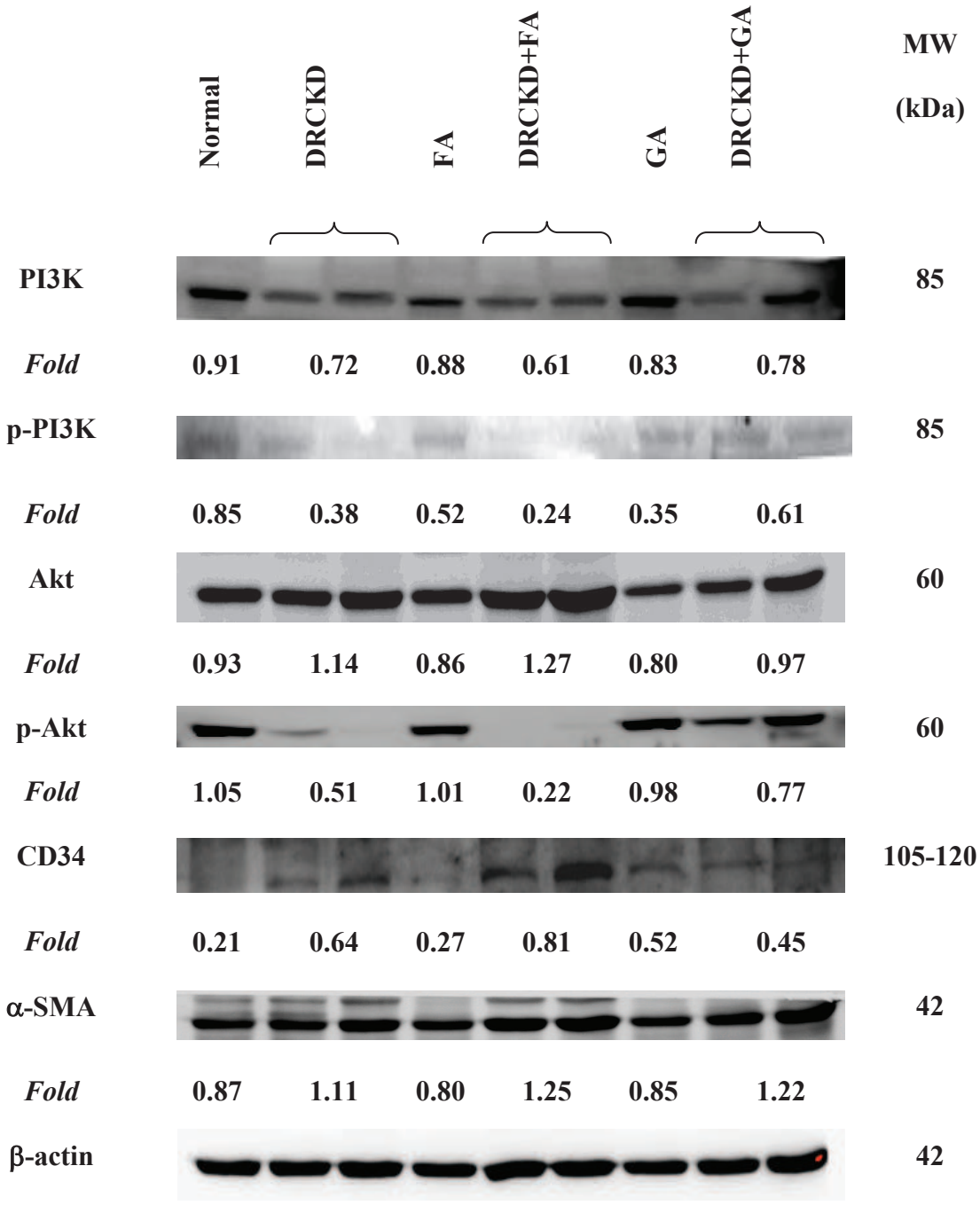

Normal
DRCKD
FA
DRCKD+FA
GA
DRCKD+GA
MW (kDa)
PI3K
85
Fold 0.91 0.72 0.88 0.61 0.83 0.78
p-PI3K
85
Fold 0.85 0.38 0.52 0.24 0.35 0.61
Akt
60
Fold 0.93 1.14 0.86 1.27 0.80 0.97
p-Akt
60
Fold 1.05 0.51 1.01 0.22 0.98 0.77
CD34
105-120
Fold 0.21 0.64 0.27 0.81 0.52 0.45
α-SMA
42
Fold 0.87 1.11 0.80 1.25 0.85 1.22
β-actin
42

Figure 6

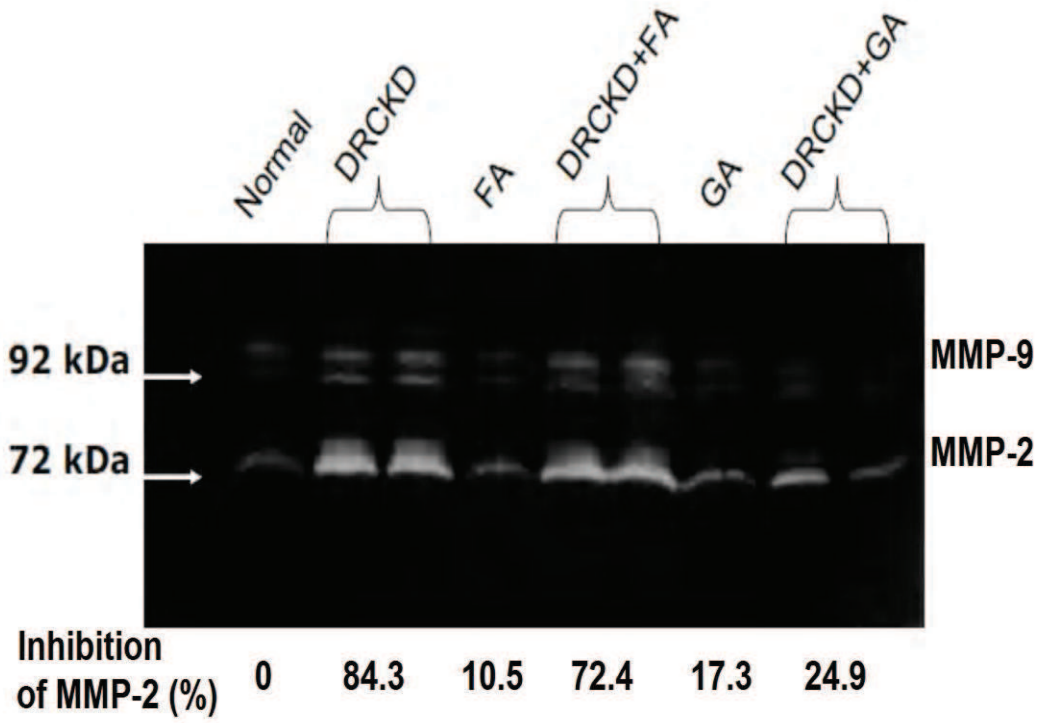

Normal
DRCKD
FA
DRCKD+FA
GA
DRCKD+GA
92 kDa
72 kDa
MMP-9
MMP-2
Inhibition of MMP-2 (%)
0
84.3
10.5
72.4
17.3
24.9

Table 1. Effect of gallic and ferulic acids on the physiological parameters of rats having chronic kidney disease.[§]

Items	Normal	DRCKD	FA	DRCKD+FA	GA	DRCKD+GA
Bw (g)	516 ± 12^{e}	$304\pm31^{b,B}$	$523\pm9^{e,E}$	$239\pm34^{a,A}$	$450\pm19^{d,D}$	$357\pm48^{c,C}$
Kw (g)	3.1 ± 0.3^{b}	$5.0\pm1.0^{f,E}$	$3.0\pm0.1^{a,A}$	$4.7\pm0.6^{e,D}$	$3.3\pm0.3^{c,B}$	$3.9\pm0.5^{d,C}$
Kw/Bw(%)	0.6 ± 0.0^{a}	$1.7\pm0.4^{c,C}$	$0.6\pm0.1^{a,A}$	$2.0\pm0.2^{d,D}$	$0.7\pm0^{a,A}$	$1.1\pm0.1^{b,B}$
GV (mm^3)	1.3 ± 0.2^{b}	$1.9\pm0.4^{d,C}$	$1.1\pm0.1^{a,A}$	$2.4\pm0.5^{e,D}$	$1.1\pm0.1^{a,A}$	$1.8\pm0.3^{c,B}$
GFR, mL/h	435±26	260±15	435±25	120±12	400±24	515±25

[a]Bw: body weight; Kw: kidney weight. GV: glomerular volume. GFR: glomerular filtration rate. The superscripts in lower case in each row indicate significant difference with level of confidence $p<0.05$ between the normal and the other tested groups (n=6). The superscripts in upper case in each row indicate significant difference with level of confidence $p<0.05$ between the DR and the other tested groups (n=6). Data are expressed in mean± S.D. from triplicate experiments.

Table 2. Effect of gallic and ferulic acids on the serum and urinary biochemical parameters in rats having chronic kidney disease. [§]

	Normal	DRCKD	FA	DRCKD+FA	GA	DRCKD+GA
Serum (mg/dL)						
Albumin (g/dL)	4.8±0.3^{a}	2.8±0.4^{c}	4.6±0.2^{a}	3.3±0.3^{b,c}	4.7±0.3^{a}	3.4±0.4^{b}
Cholesterol	74±11^{c}	831±39^{a}	72±11^{c}	554±61^{b}	68±18^{c}	539±31^{b}
TG	47±2^{b}	548±29^{a}	71±8^{b}	409±16^{a}	49±10^{b}	493±20^{a}
BUN	15±2^{b}	70±12^{a}	17±2^{b}	57±10^{a}	18±2^{b}	37±9^{a,b}
Creatinine	0.7±0.0^{b}	1.4±0.6^{b}	0.7±0.1^{b}	2.9 ±1.4^{a}	0.7±0.1^{b}	0.7±0.1^{b}
Calcium	9.8±2.3^{a}	11.9±1.6^{a}	11.3±1.9^{a}	12.9±2.7^{a}	10.9±2.3^{a}	12.0±1.5^{a}
Phosphate	6.3±0.4^{b}	8.2±4.2^{a,b}	5.7±0.3^{b}	11.5±2.6^{a}	6.8±0.0^{b}	8.3±1.5^{a,b}
Uric Acid	1.7±0.5^{b}	4.1±2.3^{a}	2.0±0.7^{b}	1.5±0.2^{b}	1.2±0.2^{b}	3.0±1.3^{a,b}
GOT (U/L)	70±13^{a}	42±2^{b}	57±4^{a}	22±2^{b}	71±11^{a}	42±2^{b}
GPT (U/L)	38±14^{b}	30±3^{b,c}	26±2^{b,c}	25±2^{c}	41±3^{a}	28±3^{b,c}
Cell count						
WBC (10^3/μL)	8±4^{b}	10±2^{b}	10±3^{b}	18±3^{a}	10±3^{b}	8±3^{b}
RBC (10^6/μL)	7.5±2.0^{a,b}	5.8±2.0^{a,b}	7.8±1.9^{a}	4.5±3^{c}	8.0±2^{a}	5.8±3^{b,c}
Platelet (10^5/μL)	6.3±4.0^{b}	14.5±5.0^{a}	4.9±3.4^{b}	15.2±3.1^{a}	6.0±4.0^{b}	14.9±2.0^{a}
Cytokines						
IL-6 (ng/mL)	2.2±0.1^{b}	5.0±0.2^{a}	4.5±0.1^{a}	3.9±0.1^{a}	3.5±0.1^{a,b}	4.2±0.2^{a}
TNF α (pg/mL)	936.1±174.3^{a,b}	370.2±100.6^{b}	1476.1±210.3^{a}	97.6±6.1^{b}	1099.7±127.6^{a,b}	563.1±84.3 ^{a,b}
Urinary (mg/dL)						
BUN	108±5^{b}	547±5^{a}	484±28^{a}	385±49^{a}	188±47^{b}	457±37^{a}
Creatinine	172±48^{a}	57±37^{b}	178±20^{a}	31±23^{b}	177±18^{a}	68±15^{b}
Protein	52±5^{d}	811±23^{a}	41±3^{d}	676±12^{c}	49±4^{d}	735±21^{b}
Creatinine clearance	1.85±0.2^{a}	0.21±0.1^{b}	2.00±0.9^{a}	0.09±0.0^{b}	2.14±0.7^{a}	1.2±0.1^{a,b}

Blood pressure (mmHg)	99±1[b]	160±2[a]	104±2[b]	145±2[a]	104±2[b]	144±9[a]

[§]Different letters indicate significant difference ($p<0.05$). (n=6). Data are expressed in mean± S. D. from triplicate experiments.

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