RESEARCH ARTICLE

Selected nutraceutic screening by therapeutic effects on doxorubicin-induced chronic kidney disease

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Scope: The number of patients with chronic kidney disease (CKD) are increasing. Interventions such as controlling hypertension and specific pharmacologic options are recommended. Some nutraceutics may have benefits in this regard.

Methods and results: Naringenin (a flavanon), catechin (a flavanol), and quercetin (a flavonol) and rutin (a flavonol rutinoside) were tried on CKD in a Sprague Dawley rat model. Results indicated quercetin to be the most effective therapeutic candidate with respect to renal edema, hypertension, serum creatinine, hematocrit, cardiopathy, aorta calcification, glomerular amyloidosis, erythrocyte depletion in bone marrow, collagen deposition, expressions of TNF-α, cleaved caspase-3, IκBα, PPARα, and serum insulin. But quercetin was only partially effective in restoring glomerular filtration rate, albuminuria, serum cholesterol, triglyceride, blood urea nitrogen (BUN), uric acid, malondialdehyde, superoxide dismutase; urinary BUN and urinary creatinine. As for signaling, quercetin was completely effective in alleviating the cleaved caspase-3, being only partially effective in suppressing Bax and Bad, restoring Bcl-2, and rescuing DNA damage.

Conclusion: The CKD status cannot to be ameliorated by naringenin, rutin, and catechin. Comparatively, quercetin may be a better therapeutic candidate.

Keywords:
Apoptosis / Chronic kidney disease / Cytokines / Hyperlipidemia / Quercetin

1 Introduction

Kidney is the major site for elimination of many cytokines. The delicate equilibrium of proinflammatory cytokines, and their inhibitors are clearly dysregulated in chronic kidney disease (CKD) patients [1]. Pathophysiologically, glomerular filtration rate (GFR) has been used to differentiate five stages of human kidney disease. GFR indexing allows correlation of severity of kidney function loss and the prevalence of comorbidities associated with the kidney disease [2]. CKD and cardiovascular disease share two major risk factors—diabetes and high blood pressure, both can damage the blood vessels in the kidney [3] (Tremblay, L., Heart attack and chronic kidney disease. http://ABOUT.COM, Heart Health Center. Updated November 04, 2008. (http://heartdisease.about.com/lw/Health-Medicine/Conditions-and-diseases/Heart-Attack-and-Chronic-Kidney-Disease.htm) 2008.). The main pathological symptoms of CKD involve overt proteinuria, a focal

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Abbreviations: BUN, blood urea nitrogen; **CKD**, chronic kidney disease; **DRCKD**, Doxorubicin-induced CKD; **GFR**, glomerular filtration rate; **MDA**, malondialdehyde; **SOD**, superoxide dismutase; **TUNEL**, Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling

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increase in reabsorption droplets in tubular cells, numerous intraluminal casts, glomerular vacuolation, mild focal glomerular sclerosis, and even further to become extensive focal and even global glomerular sclerosis, associated with moderate interstitial expansion and severe inflammation [4]. In recent years, glomerular and tubular epithelial cell apoptosis and cellular transdifferentiation have been proposed as putative primary pathomechanisms that may underlie progression of renal disease [5].

There is also a high burden of cardiac disease in the CKD population [6]. Interventions such as controlling hypertension, specific pharmacologic options, and lifestyle modification are recommended when appropriate [2].

Since the past few decades, the potential bioactivities of profound nutraceutics have attracted much attention of clinical physicians, including their hypotensive, hypolipidemic, antioxidative, and anticancer bioactivities. Naringenin, a naturally occurring flavonone, has shown cytotoxicity in various human cancer cell lines as inhibitory effects on tumor growth and there is increasing interest in its therapeutic applications. Naringenin itself does not have cytotoxic effect in lung cancer cells [7]. Naringenin protects against cadmium-induced oxidative renal dysfunction in rats, and this nephroprotective potential of naringenin is due to its antioxidant and metal chelating properties [8]. Rutin exhibits a beneficial effect on the kidney of streptozotocin (STZ)-induced diabetic rats via a modulation of metalloproteinase levels in the kidney and a reduction of plasma glucose levels [9]. Rutin helps inflammation recovery [9], arthritis [10], and cancers [11]. In addition, rutin shows heart protective, cholesterol lowering and blood pressure lowering effects [12]. Recent data indicated that tea catechins suppressed proliferative changes in glomeruli and inhibited the development of glomerulosclerosis in partially nephrectomized rats [13]. Catechin combined with vitamins C and E was shown effective in ameliorating insulin resistance (IR) and atherosclerotic changes in aged rats with chronic renal failure (CRF) [14]. Chronic green tea extract supplementation (catechin) reduced hemodialysis-stimulated production of hydrogen peroxide, hypochlorous acid, atherosclerotic factors, and proinflammatory cytokines [15]. Quercetin, a member of the flavonoids family, is one of the most prominent dietary antioxidants. It is ubiquitously present in foods including vegetables, fruit, tea, and wine as well as countless food supplements and is claimed to exert beneficial health effects [16]. Greater consumption of the flavonoid quercetin may reduce the risk of renal cell cancer among male smokers, according to data obtained from a study of men in southwestern Finland [17]. Quercetin is used for treating atherosclerosis, hypercholesterolemia, and vascular cardiac diseases. It is also used for diabetes, cataracts, hay fever, peptic ulcer, inflammation, asthma, preventing cancer, and for treating chronic infections of the prostate. Quercetin reduces systolic blood pressure and plasma-oxidized low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype [18]. The ability of quercetin to scavenge highly reactive species such as peroxynitrite and the hydroxyl radical is suggested to be involved in these possible beneficial health effects [16]. Recently, we pointed out the nephrocarcinoma could be induced by a long-term administration of quercetin or ferulic acid. This adverse effect was attributed to (i) the prooxidant effect, (ii) the insulin-secretagogue bioactivity, and (iii) the competitive and non-competitive inhibition on the O-methyltransferase to enhance the estradiol-induced tumorigenesis [19].

In 2002, the National Kidney Foundation sponsored Kidney Disease Outcomes Quality Initiative published evidence-based guidelines targeting earlier evaluation and intervention in patients who have CKD [20].

We hypothesize that these nutraceutics extensively used as the complementary adjuvant medicine (CAM) may be beneficial to CKD patients if appropriately administered in short term therapy. Naringenin (a flavanon), catechin (a flavanol), and quercetin (a flavonol) and rutin (a flavonol rutinoside) were selected and administered to doxorubicin-induced CKD rats. The relevant histological examination, biochemical tests, and immunoblotting of some cytokines and signals were carried out. A comparison of their therapeutic effect on CKD was established.

2 Materials and methods

2.1 Chemicals and kits

Doxorubicin was a product of Pfizer (Milano, Italia), Pro-PREP lysis buffer was purchased from iNTRON Biotechnology (Seongnam, Korea). Antibodies Bax (1:1000), BcL-2 (1:1000), Caspase 3 (1:1000), IκBα (1:500), and β-actin (1:1000) were purchased from Cell Signaling (Danvers, MA, USA). MMP-2 (1:1000) and Bad (1:1000), was provided by Epitomics (Burlingame, CA, USA). PPARα was a product of Abcam (Cambridge, MA, USA). The ELISA was performed using rat TNF-α ELISA kit of PeproTech (Rocky Hill, NJ, USA). SOD and TBARS were manufactured by Cayman Chemical Company (MI, USA). Chemiluminescent HRP Substrate was the product of Millipore (MA, USA). Rat Insulin EIA KIT was a product of Mercodia (Uppsala, Sweden). Naringenin, rutin, catechin, and quercetin were supplied by Sigma-Aldrich (Saint Louis, MO, USA).

2.2 Preparation of the animal diet

The basic rat chow was purchased from Fu-So Feed Stock Company (Taichung-Hsien, Taiwan), the basic diet was macerated and pulverized by milling machine. The required amounts of nutraceutics as indicated were blended thoroughly. To the blended powder, double-distilled water was added to raise the moisture content to 18% w/w, the wet moisturized powder was subjected to the granulator. The obtained granules were stored at −20°C for regular rat feeding. The nutraceutics were administered to rats at a doe approximately 70 mg/kg body weight per day.

2.3 Animal CKD model

This experimental protocol was approved by the China Medical University Ethic Committee of Experimental Animals (100–71-N) (Taichung, Taiwan). Principles of laboratory animal care (NIH publication) were followed. Thirty-six 4-week old Sprague-Dawley adult male rats (BioLASCO Taiwan, Ltd. Resources) weighing 225–250 g were used in the study. These rats were randomly assigned to six groups: the normal, the doxorubicin induced CKD (DRCKD, DR), the DRCKD + Naringenin (DR + N), the DRCKD + Rutin (DR + R), the DRCKD + Catechin (DR + C) groups and the DRCKD + Quercetin (DR + Q). These rats were acclimated and fed ordinary laboratory chow during the first week. The rats were housed in animal room in 12 colony cages, 3 rats in each. The animal room was maintained at a relative humidity 65–75% within 23 ± 1°C with a 12-h/12-h light/dark cycle. The animals were allowed free access to water. In the very beginning of second week, CKD was induced by a single subcutaneous injection of 7.5 mg/kg of doxorubicin [21] and at the same time, the nutraceutics containing diet was administered ad libitum. The entire period of experiment lasted for 12 weeks.

2.4 Blood, urine, and tissue sample collection

2.4.1 Urine collection

On finishing the treatments at week 11, rats were moved to the metabolic cage two days before the end of week 11. Urine was collected from 8.00 am to 8.00 am of the next day. The total volume of urine per day for each rat was taken. The urine samples were freshly analyzed for its urinary creatinine and blood urea nitrogen (BUN) or immediately stored in the freezer at 0–4°C when not in use. Urine BUN and creatinine were measured by reagent (Siemens, Bakersfield, CA, USA) and automatic analyzer (Ciba-Corning Express Plus, Ciba-Corning, USA).

2.4.2 Blood collection

After urine collection, the blood samples were immediately withdrawn from the abdominal aorta under ether intraperitoneal ketamine and xylasine anesthesia. The sample blood was centrifuged at 3000 × *g* to separate the serum. The serum obtained were used for measurement of parameters including serum albumin, uric acid, cholesterol, triglyceride, BUN, and creatinine, which were measured by reagent (Siemens, Bakersfield, CA, USA) and automatic analyzer (Ciba-Corning Express Plus).

2.4.3 Tissue collection

After euthanized, the organs were inspected by vision for their outer look morphological changes. The kidneys were exsiccated and immediately frozen with liquid nitrogen and stored in −80°C.

2.5 Glomerular filtration rate (GFR)

The GFR is typically recorded in units of volume per time, e.g. milliliters per minute mL/min by the expression [22]:

$$\mathrm{GFR} = (\text{urine concentration} \times \text{urine flow})/\text{plasma concentration} \quad (1)$$

GFR was measured by method of creatinine clearance, C_{Cr}. Briefly, 24-h urine was collected to determine the amount of creatinine that was removed from the blood over 24-h interval.

Creatinine clearance (C_{cr}) is calculated from the creatinine concentration in the collected urine sample (U_{cr}), urine flow rate (V), and the plasma concentration (P_{cr}). Since the product of urine concentration and urine flow rate yields creatinine excretion rate, which is the rate of removal from the blood, creatinine clearance is calculated as removal rate per min ($U_{cr} \times V$) divided by the plasma creatinine concentration (KDOQI CKD Guidelines. Retrieved 2010-08-25. http://www.kidney.org/professionals/kdoqi/guidelines_ckd/p5_lab_g4.htm.). This is commonly represented mathematically as:

$$C_{cr} = (U_{cr} \times V)/P_{cr} \quad (2)$$

2.6 Histochemical examinations

Organs excised were fixed by immersion with 10% formalin in PBS (pH 7.4) at 4°C for 24 h and processed for paraffin embedding. Paraffin sections were dewaxed in xylene and rehydrated in a series of ethanol washes. The nuclei of these specimens were subjected to Weigert's Haematoxylin-Eosin stain. Otherwise, the collagen content was stained with Sirius Red (Sigma-Aldrich).

2.7 Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay

TUNEL reaction was carried out according to the protocol given by the manufacturer. Paraffin-embedded tissue sections were stained with the in situ Cell Death Detection Kit (Roche Applied Science, IN, USA). Briefly, the whole procedure was as follows: The paraffin-embed sections were deparaffinized in two changes of xylene for 5 min each, and hydrate with two changes of 100% ethanol for 3 min each, and 95% ethanol for 1 min. The sections were rinsed in distilled water. For frozen sections on slides, samples should be pretreated with 0.2% Triton X-100 in PBS-Tween for 30 min before proteinase K digestion treatment. These sections were rinsed in two changes of PBS-Tween 20, 2 min each. The rinsed sections were preincubated in TdT Reaction Buffer

for 10 min, then followed by TdT Reaction, i.e. incubated in TdT Reaction Mixture for 1–2 h at 37–40°C in humidified chamber. To stop the reaction, the sections were rinsed in stop wash buffer for 10 min, then rinsed in PBS-Tween 20 for 6 min. For detection, the sections were incubated in reaction mixture (34 mU/mL terminal transferase, 280 pmol of dATP, 90 pmole of flourescein-11 dUTP, 30 mM Tris-HCl, 140 mM sodium cacodylate, 1 mM $CoCl_2$, pH 7.2) for 1 h at 37°C in the dark. Cells were subsequently washed with PBS and examined under a fluorescence microscope. Positive controls were carried out by incubating sections with DNase I (3000 U/mL in 50 mM Tris-HCl, pH 7.5, 1 mg/mL BSA) for 10 min at 15–25°C to induce DNA strand breaks, prior to labeling procedure. Negative controls were conducted by incubating sections with label solution only (without terminal transferase) instead of TUNEL reaction mixture.

2.8 ELISA for serum TNF-α, malondialdehyde (MDA), superoxide dismutase (SOD), and insulin

Serum levels of TNF-α, MDA, SOD, and insulin were measured using ELISA Kits. All ELISA protocols were performed by following the manufacturer's instruction. The SYSMEX K-1000 Reader used was a product of San-Tong Instrument Co. (Taipei, Taiwan).

2.9 Western blotting

Frozen renal cortex tissue samples (approximately 100 mg) were homogenized with the homogenizer (T10 basic, The IKA Company, Germany) in 1 mL of Pro-PREP lysis buffer (pH 7.2). The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C, and the supernatant was collected as tissue sample lysate. The sample protein lysates were heated at 100°C for 10 min before loading and separated on precasted 7.5% SDS-PAGE. The proteins content was analyzed before loading according to the manufacturer's instruction. Aliquots of the treated lysates containing 50 μg/μL of protein were electrotransferred onto the PVDF membrane in transfer buffer for 1 h. The nonspecific binding to the membrane was blocked for 1 h at room temperature with 5% nonfat milk in tris-buffered saline (TBS) buffer. The membranes were then incubated for 16 h at 4°C with various primary antibodies. After extensive washing in TBS buffer, the membranes were the incubated with secondary antibody in blocking buffer containing 5% nonfat milk for 1 h at room temperature. Membranes were then washed with TBS buffer and the signals were visualized using the Luminescent Image Analyzer LAS-4000 (Fujifilm, Tokyo, Japan). Levels of Bad, Bax, Bcl-2, IκBα, cleaved caspase-3, PPARα, MMP2, and β-actin were analyzed, respectively by immunoassay according to the manufacturers' instruction as mentioned in the section "Chemicals And Kits." β-Actin was used as the reference protein.

2.10 Statistical analysis

Data obtained in the same group were analyzed by Duncan's multiple range test with computer statistical software SAS 9.0 (SAS Institute, Cary, NC, USA). Significance of difference was judged by a confidence level of $p < 0.05$.

3 Results

3.1 Body weight of the rats

DR induced body weight loss, changing from 600 g/rat to 291.5 g/rat ($p < 0.05$) at the end of week 12 (Fig. 1A). All nutraceutics showed poor alleviation effect. Only quercetin had raised the body weight to 422.5 g/rat ($p < 0.05$), better than any of the other nutraceutics (Fig. 1A).

3.2 Effect of nutraceutics on the morphology of DR damaged kidneys

The doxorubicin induced CKD. DR-treated groups evidenced the slightly pale colored kidney with edema (DR). Rutin and catechin seemed to have more severely damaged the kidneys. Rutin caused kidneys look pale colored with whitish spots in appearance (DR + R), while catechin induced the severest swelling of kidneys (DR + C), narigenin (DR + N) caused swelling but not very enlarged, quercetin (DR + Q) showed shrinking of renal outer membrane with uneven blood color (Fig. 1B).

3.3 Effect of nutraceutics on DR-induced macroscopic renal symptoms

The percent ratios kidney weight to body weight (%KW/BW) in DR and catechin groups were rather comparables (0.81 ± 0.08, compared with 0.33 ± 0.04 of the control) ($p < 0.001$), lesser yet significant extent was seen for naringenin- and rutin-treated victims, showing ratios of only 0.65 ± 0.03 and 0.64 ± 0.02 (DR + N and DR + R) ($p < 0.05$). Only quercetin was able to completely ameliorate the kidney swelling induced by DR (DR + Q) (Fig. 1C). DR greatly reduced the GFR to 25 mL/h comparing to 191 mL/h of the control ($p < 0.001$). All nutraceutics failed to ameliorate the GFR ($p < 0.001$). Only quercetin effectively recovered the GFR to 125 mL/h, approximately 66.1% of the normal ($p < 0.001$) (Fig. 1D). DR highly raised the blood pressure to 153 ± 5 mmHg, a status of hypertentsion ($p < 0.001$). Rutin and quercetin were totally ineffective to suppress such a hypertensive status. Catechin showed moderate effect ($p < 0.05$). Astonishingly, only naringenin restored completely the hypertensive status (109 ± 9 mmHg) (Fig. 1E).

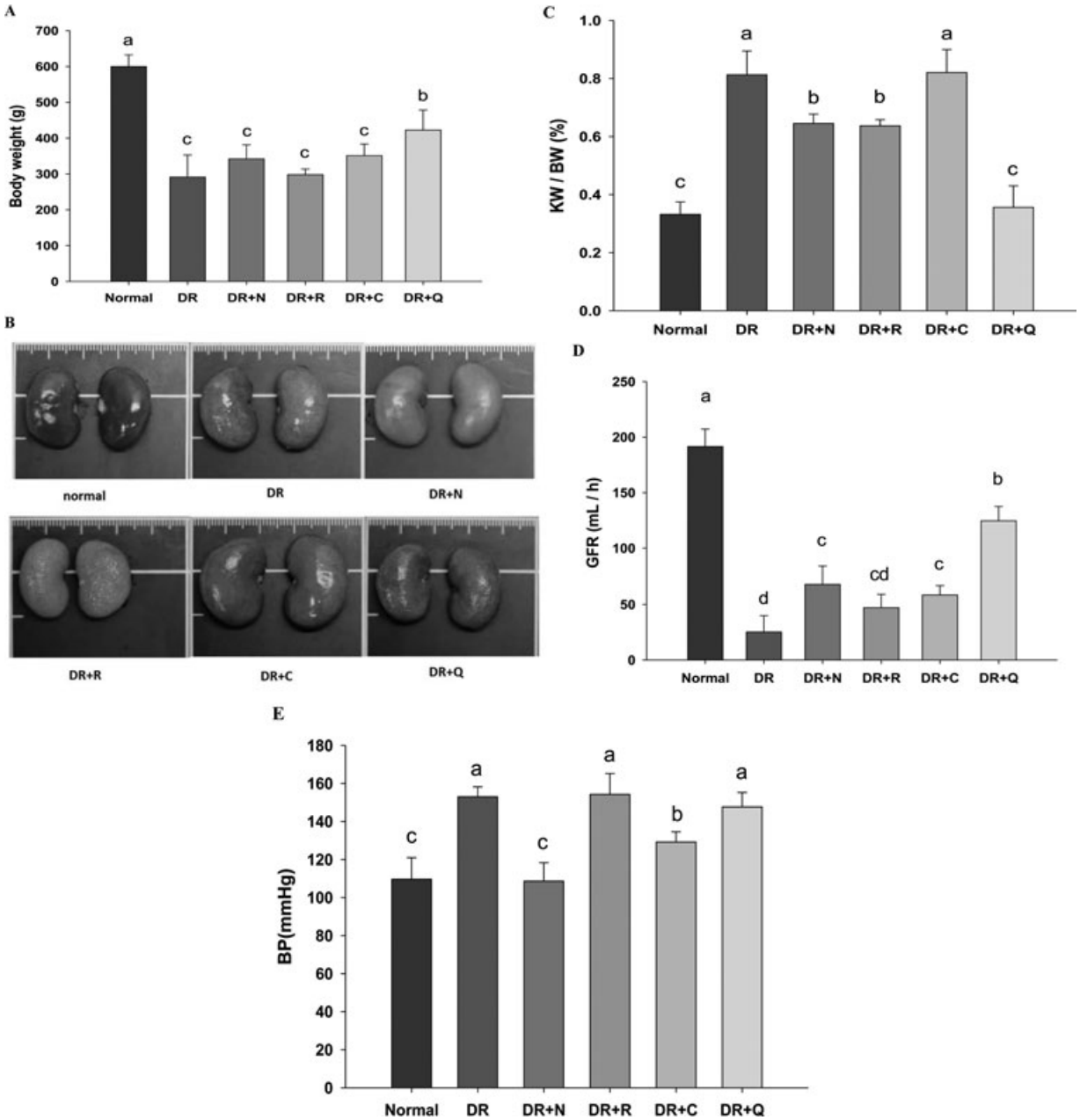

Figure 1. Effect of nutraceutics on the morphology of DR-damaged kidneys and related macroscopic renal symptoms. Body weight (A). Appearance of kidneys (B). The ratio of kidney to body weight (C). The glomerular filtration rates (D). Blood pressure (E). DR: DRCKD. DR + N: DRCKD + naringenin. DR + R: DRCKD + rutin. DR + C: DRCKD + catechin, and DR + Q: DRCKD + quercetin. Normal: normal control. Values in each bar with different superscripts indicate significant differences between each other ($p < 0.05$, or $p < 0.01$, or $p < 0.001$). Normal BP range: 84–134/60 mmHg. KW, kidney weight; BW, body weight.

3.4 Effect of nutraceutics on DR-induced microscopic biochemical changes

3.4.1 Hypoalbuminemia

DR highly significantly reduced the serum albumin content to 2.0 ± 0.1 g/dL ($p < 0.01$). All nurtraceutics only partially recovered the level, and quercetin was the most effective in retaining the albumin level at 2.7 ± 0.08 g/dL ($p < 0.05$) (Fig. 2A).

3.4.2 Hypercholesterolemia

Comparing to the normal level 65 ± 11 mg/dL ($p < 0.001$), DR was extremely hypercholesterolemic (342 ± 14 mg/dL)

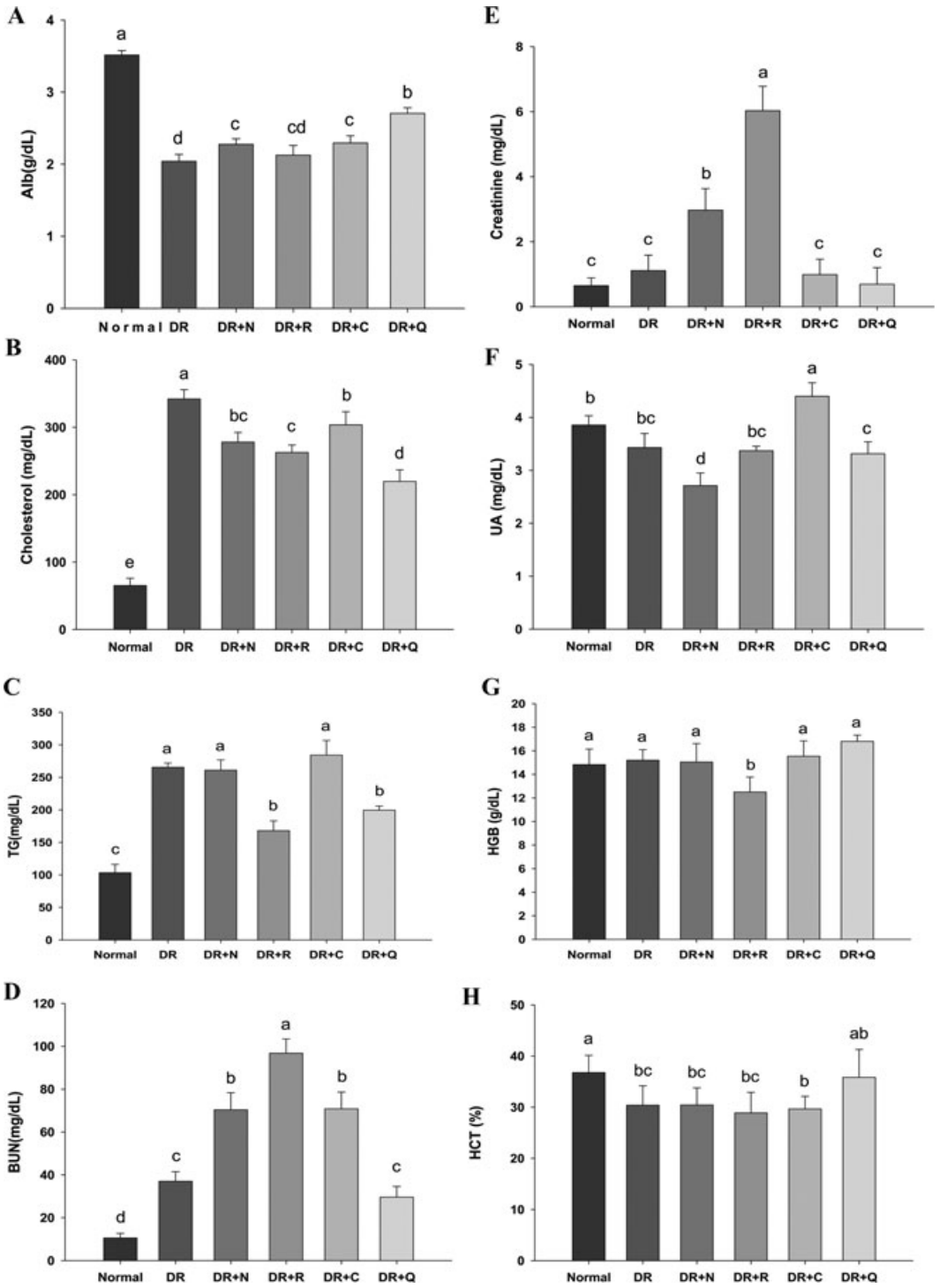

Figure 2. Effect of nutraceutics on DR-induced macroscopic renal symptoms. Serum albumin levels (A), serum cholesterol levels (B), serum triglyceride levels (C), blood urea nitrogen levels (D), serum creatinine levels (E), serum uric acid levels (F), serum hemoglobin levels (G), and hematocrit values (H). DR: DRCKD. DR + N: DRCKD + naringenin. DR + R: DRCKD + rutin. DR + C: DRCKD + catechin, and DR + Q: DRCKD + quercetin. Normal: normal control. Values in each bar with different superscripts in lower case (a–c) indicate significant differences between each other ($p < 0.05$, or $p < 0.01$, or $p < 0.001$). Normal ranges: serum albumin, 3.8–4.8 g/dL. Cholesterol, 40–130 mg/dL. Triglycerides, 26–145 mg/dL. BUN, 15–21 mg/dL. Serum creatinine, 0.2–0.8 mg/dL. Serum hemoglobin, 11.0–18.0 g/dL. Hematocrit value, 36–48%.

($p < 0.001$), which could be only suppressed to a slight degree by the nutraceutics. Although quercetin showed the most prominent reducing effect, yet the level still remained high at 220 ± 17 mg/dL ($p < 0.001$) (Fig. 2B).

3.4.3 Hypertriglyceridemia

The normal serum triglyceride (TG) level of rats ranges within 103 ± 13 mg/dL. Similar to cholesterol, DR was hypertriglyceridemic (TG level, 266 ± 6 mg/dL) ($p < 0.001$) and could not be suppressed by any of the nutraceutics regarding TG levels. Interestingly, catechin even was more hypertriglyceridemic (284 ± 22 mg/dL) ($p < 0.001$). As contrast, rutin and quercetin were only moderately effective, their levels still retained at 168 ± 15 and 200 ± 6 mg/dL, respectively ($p < 0.001$) (Fig. 2C).

3.4.4 Upregulated serum BUN level

Naringenin, rutin, and catechin all prominently increased the BUN production in CKD groups. Their levels all exceeded 70 mg/dL compared to the DR (37 ± 4 mg/dL) ($p < 0.001$) and the control (11 ± 2 mg/dL) ($p < 0.001$). Quercetin effectively alleviated it to 30 ± 5 mg/dL ($p < 0.05$) (Fig. 2D).

3.4.5 Effect on serum creatinine level

Normal serum creatinine level is 0.6 ± 0.2 mg/dL. DR, catechin, and quercetin did not show any effect on its level. Instead naringenin and rutin exhibited strong stimulating effect on creatinine production. The levels were raised to 3.0 ± 0.7 mg/dL and 6.0 ± 0.7 mg/dL, respectively ($p < 0.01$) (Fig. 2E).

3.4.6 Effect on serum uric acid level

Naringenin, rutin, and quercetin significantly suppressed the serum uric acid level. On the contrary, catechin significantly raised its level to 4.4 ± 0.3 mg/dL, contrasting with the control level 3.9 ± 0.2 mg/dL ($p < 0.05$) (Fig. 2F).

3.4.7 Effect on plasma hemoglobin level

The hemoglobin level was almost unaffected by all nutraceutics except rutin, comparing to 14.8 ± 1.3 g/dL of the control ($p < 0.05$). Rutin significantly induced hypohemoglobinemia to 12.5 ± 1.3 g/dL ($p < 0.05$) (Fig. 2G).

3.4.8 Effect on hematocrit

The hematocrit (HCT) was significantly reduced by DR ($p < 0.05$), a parameter indicating anemia. All nutraceutics except quercetin were shown ineffective to alleviate the change. Quercetin completely restored HCT level to normal range (36 ± 5%) (Fig. 2H).

3.4.9 Effect on urinary BUN and creatinine levels

The normal urinary BUN and creatinine levels are 2196 ± 180 mg/dL and 154 ± 13 mg/dL. DR severely reduced these two levels to 899 ± 162 mg/dL and 75 ± 16 mg/dL, respectively. The nutraceutics all showed only slight restoring effect for BUN (Fig. 3A), but totally ineffective for urinary creatinine (Fig. 3B), evidencing the severely damaged GFR was unalleviated by these nutraceutics (Figs. 1C, 3A and 3B).

3.4.10 Effect on serum oxidative stress (MDA) and anti-oxidative stress (SOD) levels

DR stimulated MDA production up to 25 ± 3 μM ($p < 0.05$). Naringenin and rutin only showed moderately effective, quercetin totally ineffective, in anti-MDA formation (Fig. 3C), and amazingly, catechin potently simulated MDA production to yield a peak value 58.2 ± 3 μM (Fig. 3C), which might be due to the potent prooxidant effect of catechin [23]. Hemoglobin-H_2O_2-induced protein carbonyl formation was significantly enhanced by (+)-catechin at lower concentrations, while it was efficiently inhibited when higher concentrations were used [23].

The antioxidative defensive weapon SOD was severely downregulated in DRKD (31 ± 4 U/mL) contrasting with the control (54 ± 2 U/mL) (Fig. 3D). While all nutraceutics were unable to alleviate the decreasing effect of DR (retaining within 30–45 U/mL). Quercetin was shown to be moderately effective for amelioration of SOD, which was only able to recover SOD activity to 46 ± 2 U/mL (Fig. 3D).

3.5 Histochemical examinations

Hematoxylin-Eosin staining showed all heart tissues obtained from DR-injured victims more or less revealed moderate to severe cardiopathy (as indicated by arrows), the severest with rutin, and naringenin and catechin the next. Quercetin seemed to be the most potent protective (Fig. 4A). In aorta only rutin severely elicited focal calcification in aorta tissues (Fig. 4B). Alternatively, DR and all nutraceutics seemed not having had any effect on liver (Fig. 4C). In contrast, DR severely damaged the glomerules and interstitial tissues (Fig. 4D). Furthermore, in all DR-treated groups, moderate to severe amyloidosis (indicated by arrows) was seen. These nutraceutics seemed ineffective in ameliorating these changes (Fig. 4D). Bone marrows were not affected at all (Fig. 4E). More advanced pathological changes were observed with collagen deposition in interstitial tissues as revealed by Sirus Red stain (Fig. 4F, top panel). With respect to collagen deposition, only quercetin showed the most

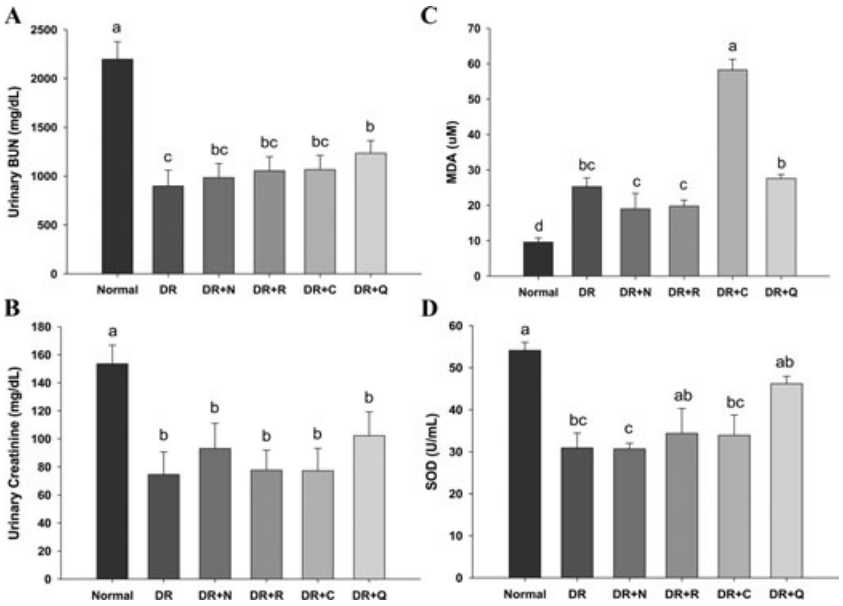

Figure 3. Variation of urinary parameters. Urinary urea nitrogen (A), and creatinine levels (B).Values in each bar with different superscripts in lower case (a–b) indicate significantly different between each other ($p < 0.05$, or $p < 0.01$, or $p < 0.001$). Normal: normal control. DR: DRCKD. DR + N: DRCKD + naringenin. DR + R: DRCKD + rutin. DR + C: DRCKD + catechin, and DR + Q: DRCKD + quercetin.Serum MDA levels (3C), Serum SOD levels (3D).

effective suppressing effect (Fig. 4F, middle panel), as evidenced by the restoration of MMP-2 by quercetin (Fig. 4F, bottom panel).

3.6 Effect on serum inflammatory cytokines TNF-α

The change of TNF-α level (Fig. 5) was seen very similar to the finding for body weight (Fig. 1A). Naringenin, rutin, and catechin were entirely ineffective in restoring the TNF-α level. As contrast, quercetin uniquely exhibited complete ameliorating effect (Fig. 5).

3.7 Western blot analysis

3.7.1 Bax level

Bax level was highly upregulated by DR, no any combination with naringenin, rutin, or catechin was effective to rescue the overproduction of Bax (Fig. 6A). Conversely, quercetin exhibited the most beneficial effect, which completely suppressed the overproduction of Bax (Fig. 6A).

3.7.2 Bad level

Similar phenomena were seen for Bad level. All nutraceutic combinations failed to suppress the upregulation of Bad. Rutin even further upregulated to a peak Bad/β-actin ratio 0.92 (Fig. 6B). While quercetin showed better suppressing effect in this regard (Fig. 6B).

3.7.3 Bcl-2 level

DR significantly downregulated Bcl-2. All nutraceutics were ineffective in rescuing such changes (Fig. 6C).

3.7.4 Cleaved caspase-3 level

The significant overexpression of cleaved caspase-3 (19 kDa) by DR was seen effectively suppressed by all nutraceutics (Fig. 7A).

3.7.5 Cytosolic IκBα level

In DR victims, the level of IκBα was highly upregulated. Rutin was totally ineffective, naringenin and catechin were moderately effective to ameliorate such an effect. While only quercetin exhibited complete alleviative effect (Fig. 7B).

3.7.6 PPARα level

DR reduced PPAR level significantly. Naringenin, rutin and catechin all failed to retard this change, implicating ineffective for the hypercholesterolemic status (Fig. 2B). Only quercetin was potentially ameliorated the reduced PPARα level (Fig. 8A and B).

3.7.7 Insulin level

The insulin level was only slightly affected in all groups (Fig. 8C). Normal serum insulin level ranged within 1.7 ± 0.5 μg/L ($= 1.7 \pm 0.5$ ng/mL).

3.8 TUNEL assay

TUNEL assay revealed that DR seriously damaged the cell nuclei. The repairing effect of naringenin, rutin, and catechin

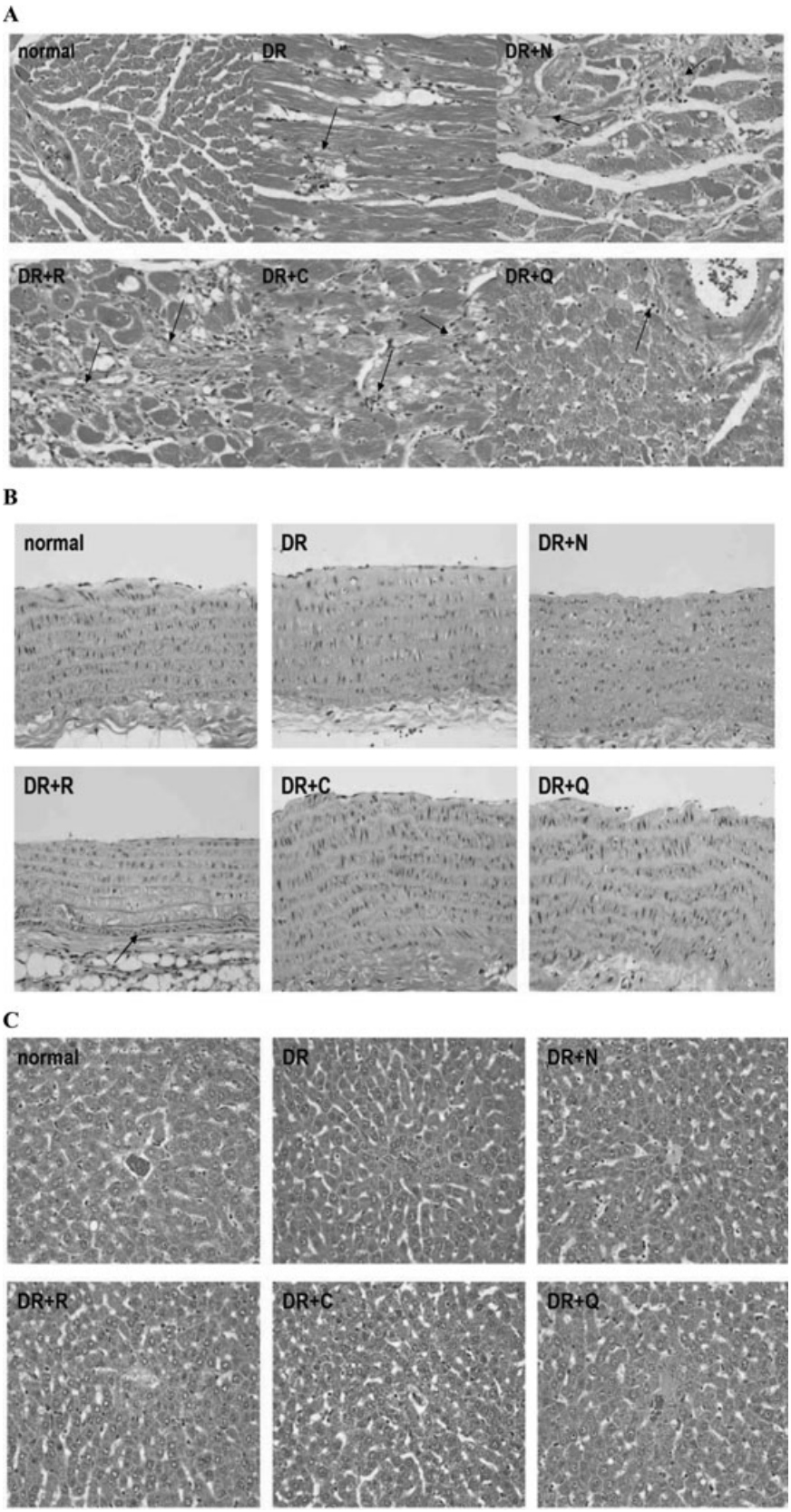

Figure 4. Histopathological findings in various organs by Hematoxylin-Eosin staining and Sirus Red staining. Heart by Hematoxylin-Eosin staining (A). Aorta by Hematoxylin-Eosin staining (B). Medium calcification was found. Histopathological findings of liver by Hematoxylin-Eosin staining in all rat groups (C). Histopathological findings of kidney by Hematoxylin-Eosin staining in all rat groups, glomerular amyloidosis was observed (D). Bone marrow by Hematoxylin-Eosin staining (E). Erythrocytes depletion was found. The collagen deposition in kidney by Sirus Red staining in all rat groups (F, top panel) and its quantification (F, bottom panel), Magnification ×400. Western blot for MMP2 expression (F-c). Normal: normal control. DR: DRCKD. DR + N: DRCKD + naringenin. DR + R: DRCKD + rutin. DR + C: DRCKD + catechin, and DR + Q: DRCKD + quercetin.

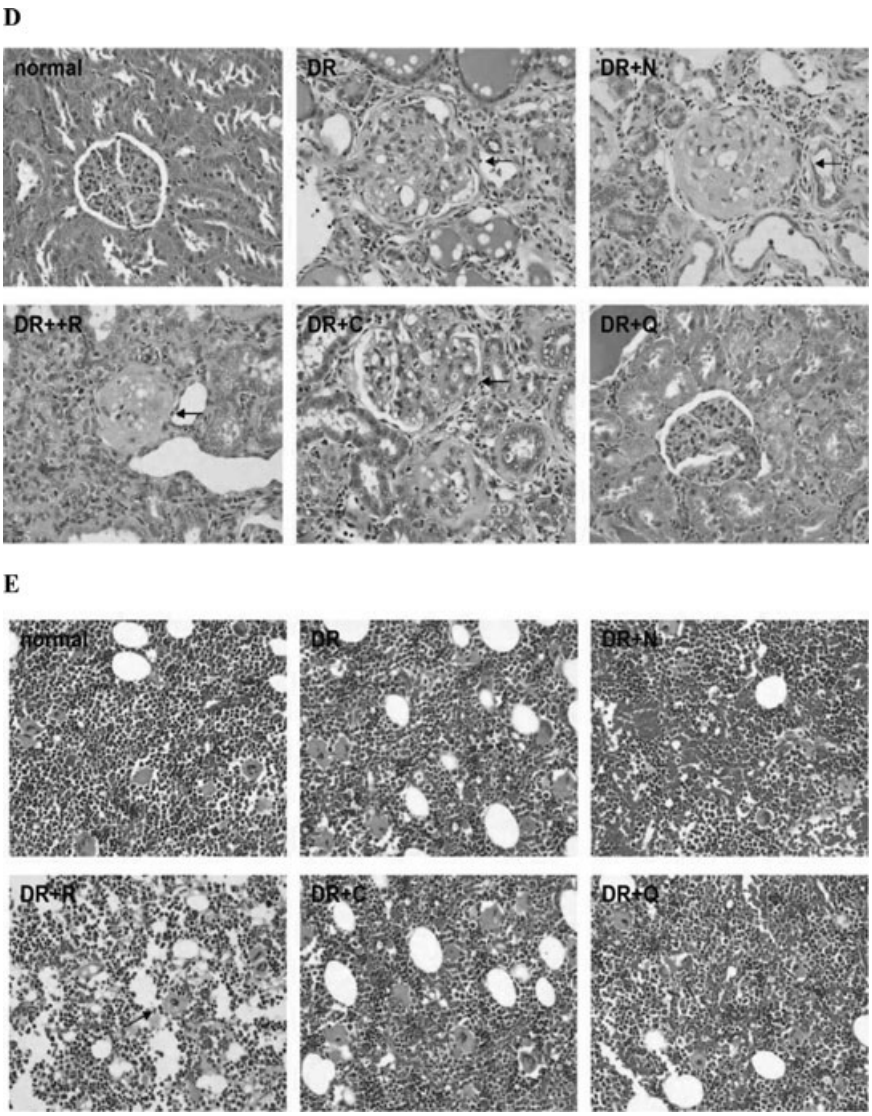

Figure 4. Continued.

was very limited, while quercetin was able to ameliorate most of the damage (Fig. 9).

4 Discussion

4.1 Reduced body weight might be due to malnutritional status

In DRCKD rats, the body weight was severely reduced to 290 $\pm$ 70 g compared to the control 600 $\pm$ 50 g ($p < 0.01$) (Fig. 1A). Only quercetin was moderately effective to restore it to 420 $\pm$ 60 g ($p < 0.05$). All other nutraceutics seemed to be entirely ineffective in body weight restoration. The incidence of malnutrition disorders in CKD appears unchanged overtime [24, 25]. Thus, this body loss effect can be attributed to the outcome of inadequate food intake and exhaustive energy expenditure [25].

4.2 Effect of nutraceutics on kidney morphology in DRCKD victims

The typical symptoms of doxorubicin induced CKD are renal swelling due to edema and elevation of percent ratio of kidney to body weight (%KW/BW), consistent with our previously report [21]. Rutin and catechin seemed to have damaged the kidneys more severely (Fig. 1A, B), which might be ascribed to the simultaneous hypertensive status (Fig. 1D) and reduced glomerular filtration rates (Fig. 1C).

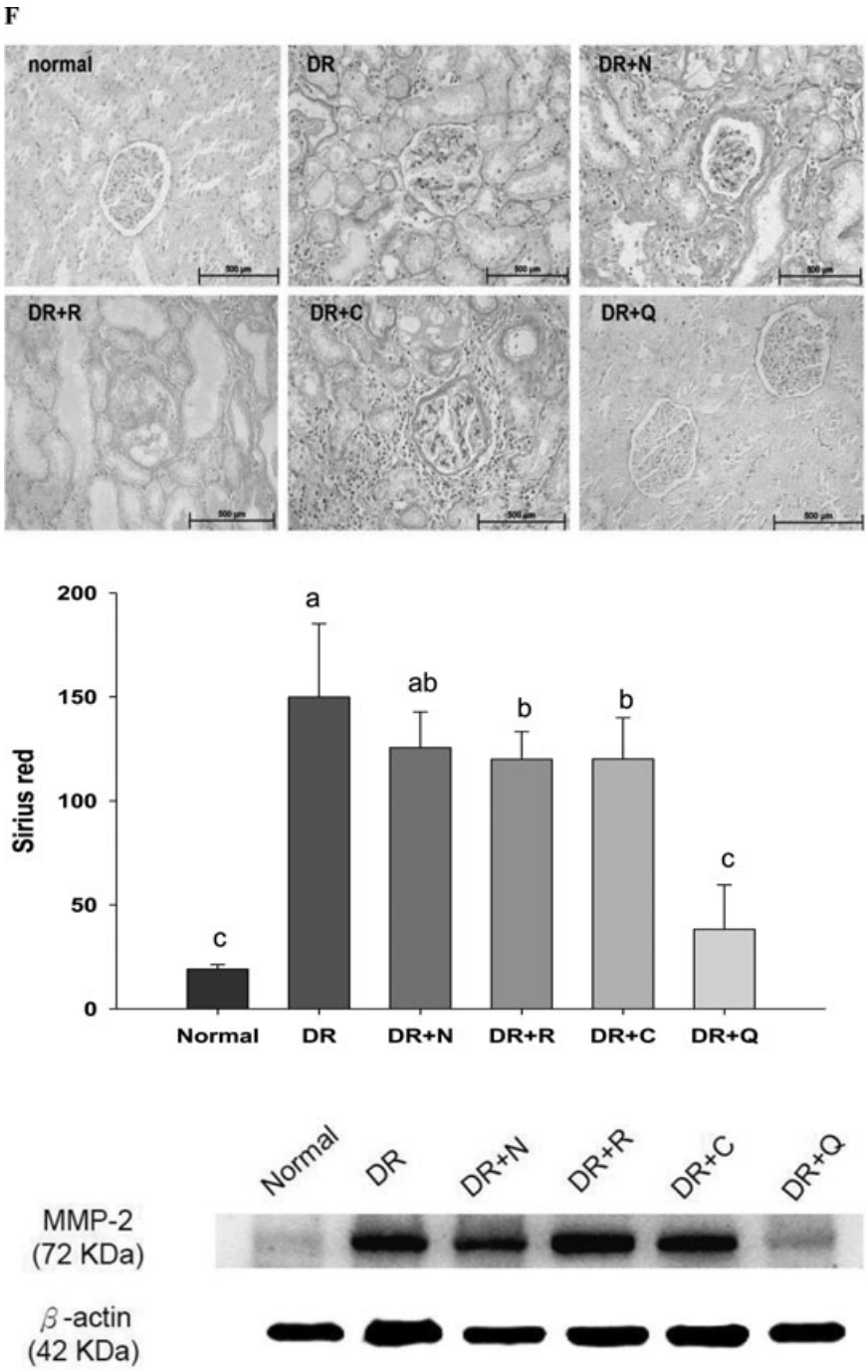

Figure 4. Continued.

4.3 Antihypertensive effect of nutraceutics in DRCKD victims

Much of literature has cited the antihypertensive effect of rutin [26], quercetin, and catechin [27]. Angiotensin II (AT_2) induced redox-dependent signaling pathways and cell behavior. A mixture of quercetin plus catechin reduced AT_2-stimulated NAD(P)H oxidase activation and p47phox translocation to the cell membrane, without affecting NOX2 expression [27]. Obviously, the DR-induced renal type hypertension (CKD type hypertension) could not be alleviated by rutin and quercetin, alternatively suppressed by catechin and

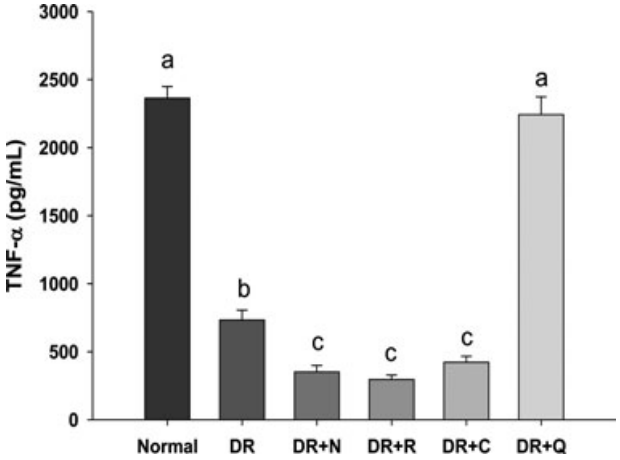

Figure 5. Level of serum TNF-α.Serum tumor necrosis factor–alpha (TNF-α) levels. Values in each bar with different superscripts in lower case (a–b) indicate significantly different between each other ($p < 0.05$, or $p < 0.001$, or $p < 0.001$). Normal: normal control. DR: DRCKD. DR + N: DRCKD + naringenin. DR + R: DR-CKD + rutin. DR + C: DRCKD + catechin, and DR + Q: DRCKD + quercetin.

naringenin, indicating the hypertensive mechanism evoked by CKD would be entirely different from the conventional cardiovascular type hypertension. Literature elsewhere indicated mono-7-O-(beta-hydroxyethyl)-rutosides (HR) to be a potent skin capillary resistance enhancer [28]. Whether rutin could similarly have acted in the same manner to raise the blood pressure remains unclear.

4.4 Antialbuminuria and antihyperlipidemia by nutraceutics in DRCKD victims

Proteinuria or albuminuria usually is a sign of CKD, which can result from diabetes, high blood pressure, and diseases that cause inflammation in the kidneys. Hase et al. reported catechin (5 mg/day) significantly reduced albuminuria in streptozotocin-treated diabetic rats (the urinary albumin excretion rate, AER) ($p = 0.017$). The interstitial fibrosis in the kidney was completely normalized with catechin treatment and the serum levels of TBARS and blood pressure were comparable among all groups [13]. However, our results were rather contradicting.

The hypertension induced by DR (Fig. 1D) actually was reflecting the status of albuminuria (Fig. 2A). Interestingly, the experimental nutraceutics frequently cited to practically possess hypolipidemic effect [29] were all ineffective in ameliorating hypercholesterolemia (Fig. 2B) and hypertriglyceridemia (Fig. 2C), evidencing the hyperlipidemic conditions of CKD unlike the common hepatic or metabolic type.

4.5 Anti-BUN and antihypercreatininemia by nutraceutics in DRCKD victims

The liver produces urea in the urea cycle as a waste product of the digestion of protein. The BUN test is a measure of the amount of nitrogen in the blood in the form of urea, and a measurement of renal function. Urea is a byproduct from metabolism of proteins by the liver and is removed from the blood by the kidneys. Normal human adult blood should contain between 7–21 mg/dL [30]. Comparable magnitude was seen for serum BUN (11 ± 2 mg/dL) in our result (Fig. 2D).

Amidinotransferase (transamidinase, L-arginine: glycine amidinotransferase, EC 2.1.4.1) is an enzyme that catalyses the first step in creatine synthesis primarily in the kidney and pancreas [31]. Kidney transamidinase activity was decreased in mercury intoxication ($p < 0.001$) [31]. Supposedly, rutin and naringenin might have stimulated the enzyme transamidinase (Fig. 2E) and simultaneously induced uremic syndrome (elevation of serum BUN) as evidenced by the highly raised levels of serum creatinine (Fig. 2E) and serum BUN (Fig. 2D). Similar phenomena were observed in mercury chloride induced acute uremic syndrome, i.e. urea and creatinine levels in blood plasma were significantly elevated 48 h after the induction of acute uremic syndrome ($p < 0.001$) [31].

To explain the role of uric acid, Cos et al. [32] classified the flavonoids into six categories according to their xanthine oxidase inhibition (XOI) and superoxide scavenging activities (SSA) [32]. Rutin and quercetin belonged to category C (XOI+, SSA+), hence rutin and quercetin showed comparable serum uric acid levels (Fig. 2F). Catechin was involved in category A (XOI 0, SSA+) (0 denotes no effect) [32], hence yielded higher level of uric acid (Fig. 2F). Naringenin was classified as category F (XOI 0, SAA 0) [32]. Uric acid itself is an in vivo antioxidant, part of the uric acid produced may be consumed while acting as an antioxidant, leading to lowered serum level of uric acid (Fig. 2F).

Literature indicated that the increase in antioxidant capacity of blood after the consumption of flavonoid-rich foods may not be caused directly by the flavonoids themselves, but is probably due to increased production of uric acid resulting from excretion of flavonoids from the body (Stauth, D., (Ed.), "Studies force new view on biology of flavonoids", EurekAlert!. Adapted from a news release issued by Oregon State University. http://www.eurekalert.org/pub_releases/2007–03/osu-sfn030507.php).

Although the serum BUN level was highly raised by naringenin, rutin, and catechin (Fig. 2D), yet their urinary BUN levels were only comparable to DR group (Fig. 3A), implicating the severely damaged glomerular filtration rates in these groups (Fig. 1C).

Similar phenomenon was found for the urinary creatinine level. Naringenin and rutin had significantly elevated the serum creatinine (Fig. 2E), while the levels of urinary

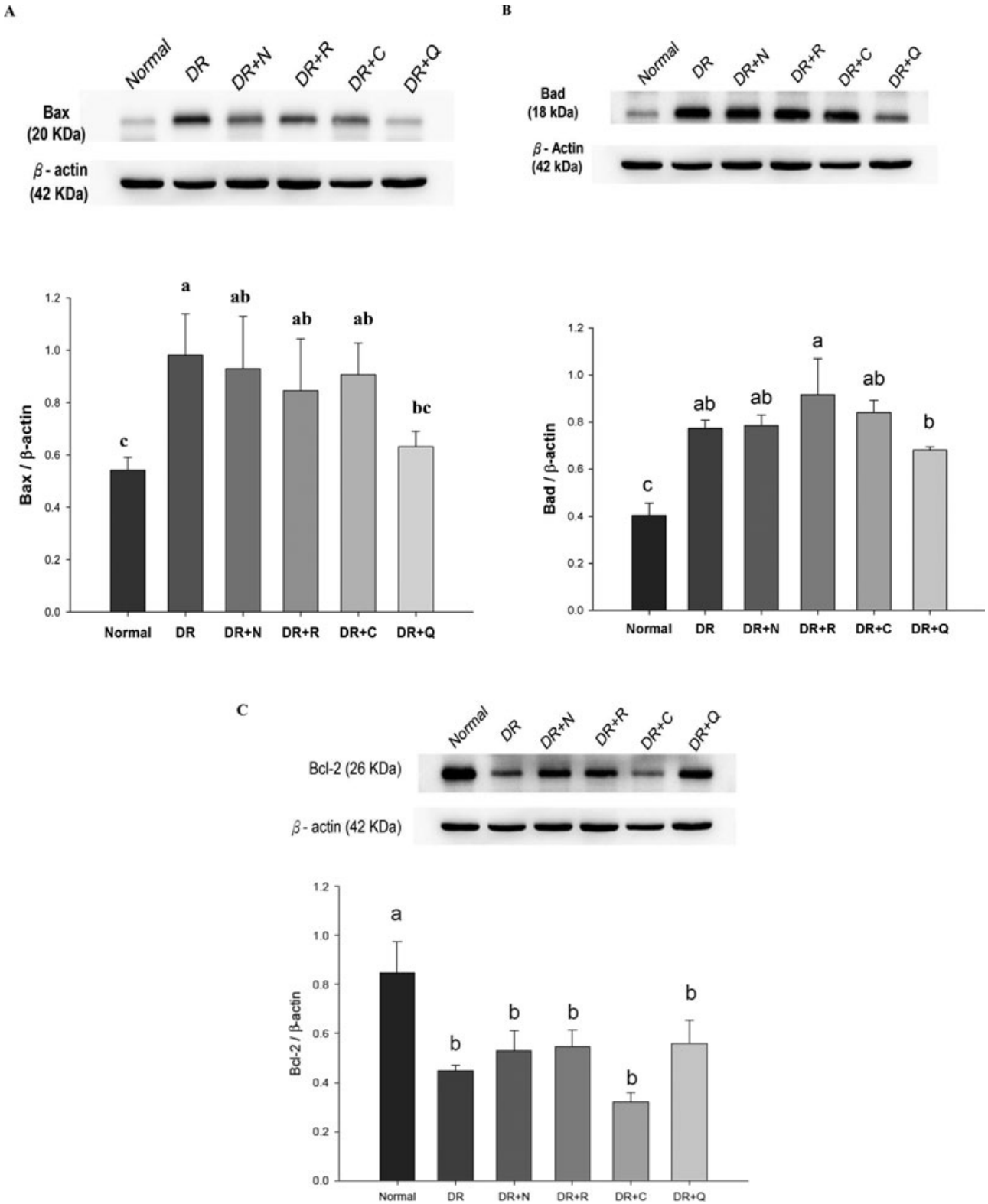

Figure 6. Western blot analysis of Bax (A, top and bottom panel); Bad (B, top and bottom panel), and Bcl-2 (C, top and bottom panel). The protein loading was 50 μg/μL.

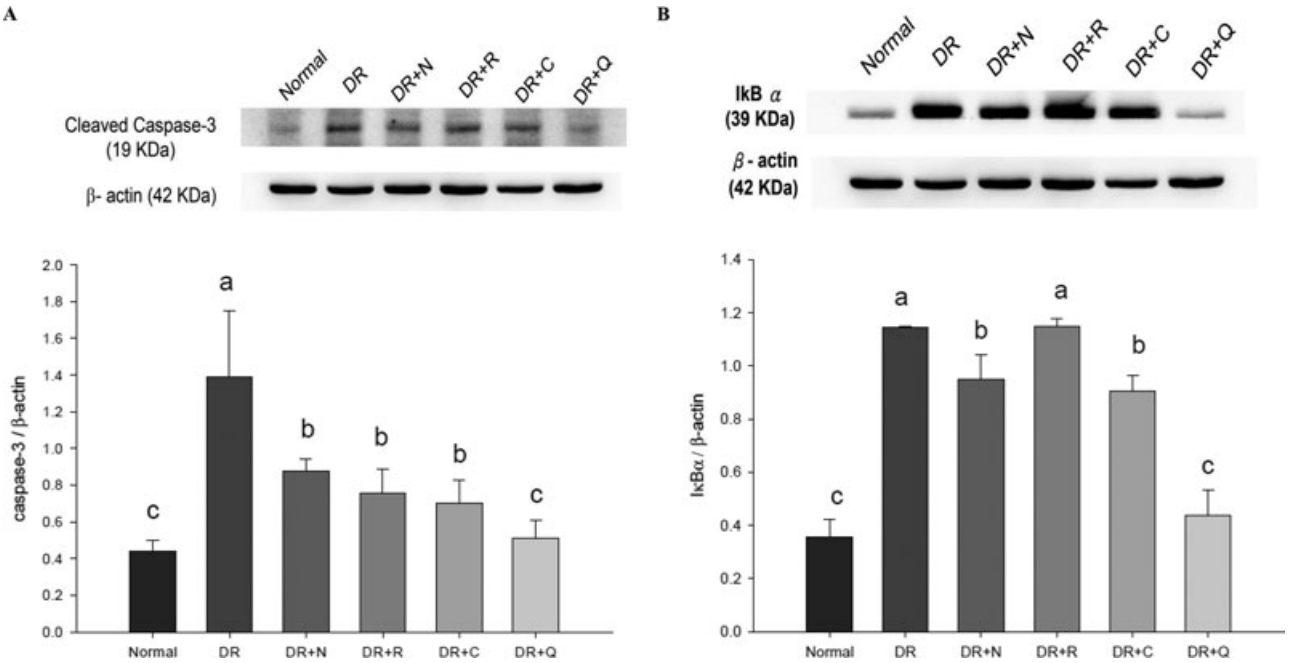

Figure 7. Western blot analysis of cleaved caspase-3 (A, top and bottom panel) and IkBα (B, top and bottom panel). The protein loading was 50 μg/μL.

creatinine were rather comparable among the nutraceutic-treated groups (Fig. 3B).

4.6 The highly prooxidant bioactivity of catechin in DRCKD victims

Green tea aqueous and ethanolic extracts exhibit prooxidant activity at lower concentrations [33]. While at high concentrations it reveals antioxidant activity as verified by oxygen radical absorbance capacity (ORAC) and deoxyribose assays using generated hydroxyl radicals [33]. Suggestively, the peak level of MDA produced by catechin could be attributed to its prooxidant bioactivity (Fig. 3C).

4.7 All nutraceutics except quercetin aggravated the CKD status

DR damaged kidneys and induced CKD (Figs. 1A, 4D). Interestingly, all nutraceutics except quercetin were shown not only ineffective to have ameliorated, but also aggravated the CKD status (Fig. 4A and F). Tubular cell epithelial-mesenchymal transition (EMT) is a fundamental contributor to renal fibrosis. In moderate and severe tubulo-interstitial damage, increased expression of MMP-2 had been noted [21]. MMP-2 may regulate collagen accumulation at those sites. During severe tubulo-interstitial damage, highly upregulated MMP-2 may contribute the pathological basement membrane splitting and disruption of type-IV collagen. Similar yet lesser expression was seen with MMP-9 [21].

4.8 Quercetin completely restored tissue TNF-α level

CKD victims were characteristic of highly suppressed tissue TNF-α levels. Narigenin, rutin, and catechin all reduced further the corresponding TNF-α levels. Only quercetin was shown to have completely restored its level (Fig. 5). Olszanecka-Gliniaowiczet et al. reported body loss induced decreased TNF-α levels [34]. Protein energy malnutrition decreased the production of TNF-α [35]. Otherwise, the level of TNF-α could be significantly reduced in parallel to body weight loss if the initial TNF-α level was >10 pg/mL [36]. We show DRCKD induced severe body weight loss (Fig. 1A), thus the decreased level of TNF-α can be ascribed to body weight loss or malnutrition (Fig. 5).

4.9 Western blot showed only quercetin successfully retarded the cell apoptosis

DR significantly upregulated the apoptotic biomarkers Bax, Bad, and suppressed the antiapoptotic Bcl-2. All experimental nutraceutics except quercetin were moderately effective in retarding these changes (Fig. 6A–C), similarly seen for the level of IκBα (Fig. 7B). The raised level of cytosolic IκBα indicated that DR induced cell apoptosis [37], while all nutraceutics except quercetin were only partially effective in rescuing such

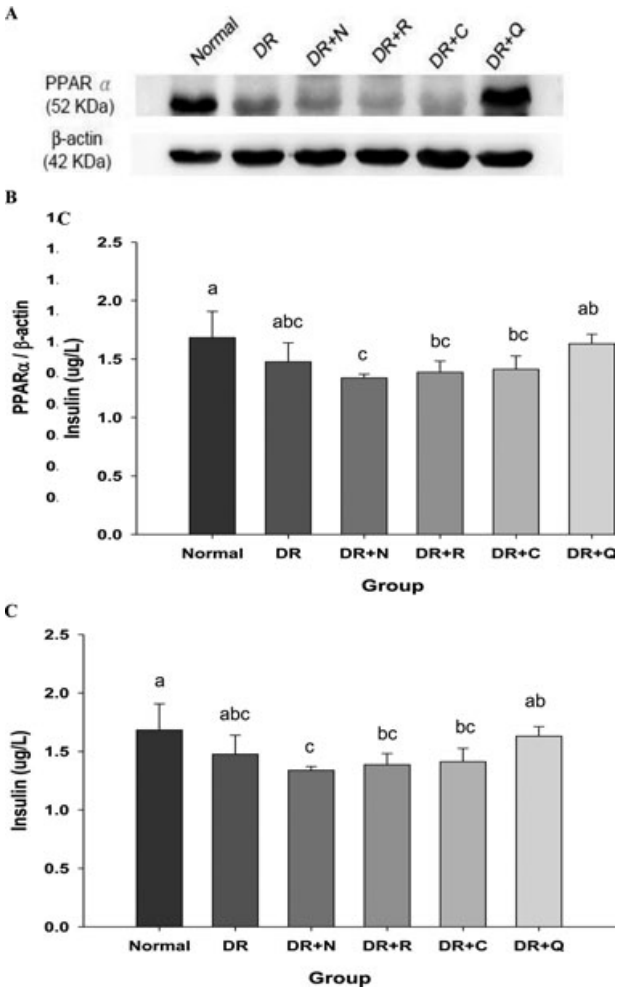

Figure 8. Western blot analysis of PPARα and the insulin level affected by the four nutraceutics. Western blot (A), quantification (B), and the insulin level (C). The protein loading was 50 μg/μL.

a tendency. Conversely, the level of cleaved caspase-3 overexpressed by DR was seen effectively downregulated to normal level (Fig. 7A).

4.10 PPARα downregulated by DR was ameliorated by quercetin only

PPARα is expressed in liver, kidney, heart, muscle adipose tissue, and others [38]. PPARα was able to improve insulin sensitivity [39]. DR and all nuratraceutic groups except quercetin were characterized with suppressed PPARα (Fig. 8A and B). Insulin resistance is often associated with increased levels of intracellular triglycerides (Fig. 2C), diacylglycerol and decreased fat β-oxidation [40].

Conversely, quercetin was shown to have retained full strength PPARα (Fig. 8B) and to be most effective in acting as antihypercholesterolemic (Fig. 2B), a fact pointing to the complicate hypercholesterolemia of CKD not merely controlled by mitochondrial fat oxidation.

Moreover, the insulin level was slightly suppressed by nutraceutics except quercetin (Fig. 8C). Normal insulin value ranges within 1.7 ± 0.5 ng/mL (42.5 ± 12.5 μIU/mL) (Fig. 8C). Translated to human, the normal range would be 0.076–1.184 ng/mL (1.9–29.6 μIU/mL) with a median 0.632 ng/mL (15.8 μIU/mL), or 0.58 ± 0.28 ng/mL (14.5 ± 6.96 μIU/mL) [41]. The parallel modulation pattern of PPARα and insulin (Fig. 8) implicated the close link between these both parameters.

Hotamisligil demonstrated that insulin receptor signaling is an important target for TNF-α [42]. There seemed to have a twofold increase in insulin-stimulated tyrosine phosphorylation of insulin receptor in the muscle and adipose tissue of TNF-α knockout mice. Other possible mediators of TNF-α-induced insulin resistance include circulating free fatty acids and leptin [42]. Contrasting to the suppressed levels of PPARα (Fig. 8B), the free fatty acid level might be elevated to synergize the status of insulin resistance.

4.11 TUNEL assay indicated quercetin was the most effective protective agent for DR-CKD

TUNEL assay indicated DR extensively induced apoptosis in situ in kidney cells. Comparatively, quercetin was the most effective antiapoptotic agent (Fig. 9). Previously, we indicated that quercetin tends to induce renal carcinoma under condition of prolonged administration [19]. However, contrasting with the above mentioned, much of the data have revealed quercetion to be the most effective protective nutraceutics against the DRCKD, at least in the short-term medication. CKD is a renopathy accompanied with renal inflammation [21], glomerular amyloidosis (Fig. 4D), and collagen deposition (Fig. 4F), hence fibrosis and necrosis could accompany the occurrence of apoptosis [21]. Suggestively, to ameliorate these symptoms using nutraceutic therapy, some conditions have to be taken into consideration: the pathophysiological specificity of nutraceutics, the pharmacological action of nutraceutics, the dose and duration of administration.

A summary of the biochemical effects exerted by these four selected nutraceutics on CKD rats is listed in Table 1. In conclusion, among the selected nutraceutics tried, quercetin showed the most effective alleviation effect. Most nutraceutics, commonly reported to have rather prominent bioactivities, are ineffective to ameliorate CKD. Only quercetin has shown the most effective alleviation effect. In a future study, quercetin will be used in combination with other medicines clinically applicable to treat CKD.

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The authors have declared no conflicts of interest.

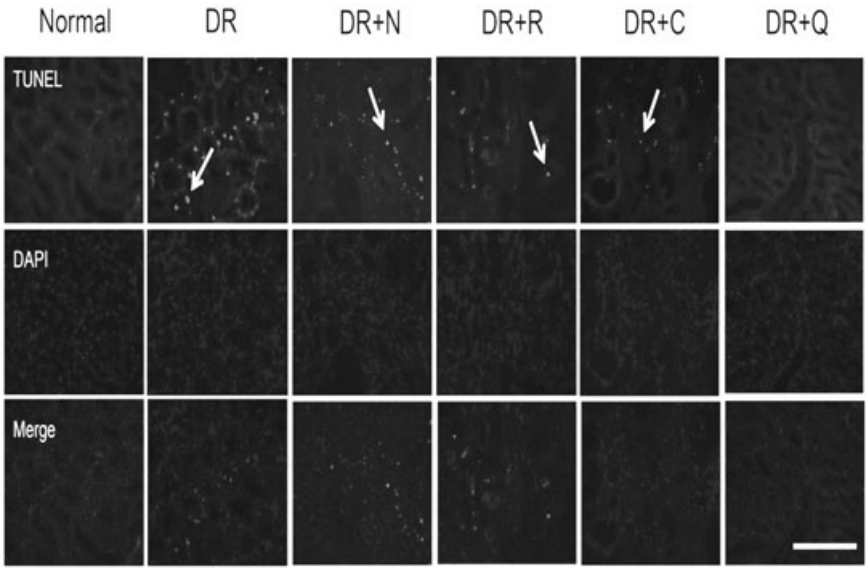

Figure 9. TUNEL assays: Normal: normal control. DR: DRCKD. DR + N: DRCKD + naringenin. DR + R: DRCKD + rutin. DR + C: DRCKD + catechin, and DR + Q: DRCKD + quercetin.

Table 1. Summary of biochemical effects of the selected nutraceutics on the chronic kidney diseased rats

Groups	Normal	DR	DR + N	DR + R	DR + C	DR + Q
Body weight (g/rat)	600^{a}	291.5^{c}	342.2^{c}	294.8^{c}	347.7^{c}	422.5^{b}
KW/BW (%)	0.33 ± 0.04^{c}	0.81 ± 0.08^{a}	0.65 ± 0.03^{b}	0.64 ± 0.02^{b}	0.82 ± 0.07^{a}	0.36 ± 0.06^{c}
GFR (mL/h)	191^{a}	25^{d}	69^{c}	$47^{c,d}$	59^{c}	125^{b}
Blood pressure (mmHg)	110 ± 10^{c}	153 ± 5^{a}	109 ± 9^{c}	154 ± 11^{a}	129 ± 5^{b}	147 ± 8^{a}
Serum albumin (g/dL)	3.5 ± 0.05^{a}	2.0 ± 0.1^{d}	2.3 ± 0.06^{c}	$2.1 \pm 0.1^{c,d}$	2.3 ± 0.08^{c}	2.7 ± 0.08^{b}
Serum cholesterol (mg/dL)	65 ± 11^{e}	342 ± 14^{a}	$277 \pm 14^{b,c}$	264 ± 10^{c}	305 ± 18^{b}	220 ± 17^{d}
Serum triglyceride (mg/dL)	103 ± 13^{c}	266 ± 6^{a}	261 ± 16^{a}	168 ± 15^{b}	284 ± 22^{a}	200 ± 6^{b}
Blood urea nitrogen (mg/dL)	11 ± 2^{d}	37 ± 4^{c}	71 ± 7^{b}	97 ± 6^{a}	71 ± 7^{b}	30 ± 5^{c}
Serum creatinine (mg/dL)	0.6 ± 0.2^{c}	1.1 ± 0.5^{c}	3.0 ± 0.7^{b}	6.0 ± 0.7^{a}	1.0 ± 0.4^{c}	0.7 ± 0.5^{c}
Serum uric acid (mg/dL)	3.9 ± 0.2^{b}	$3.4 \pm 0.3^{b,c}$	2.7 ± 0.2^{d}	$3.4 \pm 0.1^{b,c}$	4.4 ± 0.3^{a}	3.3 ± 0.2^{c}
Serum hemoglobin (g/dL)	14.8 ± 1.3^{a}	15.3 ± 0.8^{a}	15.2 ± 1.4^{a}	12.5 ± 1.3^{b}	15.5 ± 1.2^{a}	16.7 ± 0.5^{a}
Hematocrit (%)	37 ± 3^{a}	$30 \pm 4^{b,c}$	$30 \pm 4^{b,c}$	$29 \pm 4^{b,c}$	30 ± 2^{b}	$36 \pm 5^{a,b}$
Urinary urea nitrogen (mg/dL)	2196 ± 180^{a}	899 ± 162^{c}	$994 \pm 133^{b,c}$	$1065 \pm 135^{b,c}$	$1074 \pm 134^{b,c}$	1234 ± 125^{b}
Urinary creatinine (mg/dL)	154 ± 13^{a}	75 ± 16^{b}	93 ± 18^{b}	78 ± 14^{b}	77 ± 16^{b}	102 ± 16^{b}
Serum MDA (μM)	9.7 ± 1.0^{d}	$25.4 \pm 3.2^{b,c}$	19.3 ± 4.1^{c}	20.0 ± 1.5^{c}	58.5 ± 2.7^{a}	27.4 ± 1.4^{b}
Serum SOD (U/mL)	54 ± 2^{a}	$31 \pm 4^{b,c}$	31 ± 1^{c}	$34 \pm 6^{a,b}$	$34 \pm 4^{b,c}$	$46 \pm 2^{a,b}$
Serum TNF-α (pg/mL)	2370.8 ± 76.2^{a}	730.7 ± 65.2^{b}	362.4 ± 38.1^{c}	305.7 ± 19.4^{c}	419.1 ± 47.4^{c}	2239.6 ± 131.3^{a}

Values in each row with different superscripts indicate significantly different between each other ($p < 0.05$, or $p < 0.01$, or $p < 0.001$). Data are expressed in mean $\pm$ SD from triplicate experiments. Bw, body weight; Kw, kidney weight. GV, glomerular volume; GFR, glomerular filtration rate. MDA: malondialdehyde. SOD: superoxide anion dismutase.

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