Acta Pharmacologica Sinica (2012) 33: 1260–1270
 1671-4083/12 $32.00
www.nature.com/aps

Original Article

Differential hepatoprotective mechanisms of rutin and quercetin in CCl_4-intoxicated BALB/cN mice

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Aim: To investigate the mechanisms underlying the protective effects of quercetin-rutinoside (rutin) and its aglycone quercetin against CCl_4-induced liver damage in mice.
Methods: BALB/cN mice were intraperitoneally administered rutin (10, 50, and 150 mg/kg) or quercetin (50 mg/kg) once daily for 5 consecutive days, followed by the intraperitoneal injection of CCl_4 in olive oil (2 mL/kg, 10% *v/v*). The animals were sacrificed 24 h later. Blood was collected for measuring the activities of ALT and AST, and the liver was excised for assessing Cu/Zn superoxide dismutase (SOD) activity, GSH and protein concentrations and also for immunoblotting. Portions of the livers were used for histology and immunohistochemistry.
Results: Pretreatment with rutin and, to a lesser extent, with quercetin significantly reduced the activity of plasma transaminases and improved the histological signs of acute liver damage in CCl_4-intoxicated mice. Quercetin prevented the decrease in Cu/Zn SOD activity in CCl_4-intoxicated mice more potently than rutin. However, it was less effective in the suppression of nitrotyrosine formation. Quercetin and, to a lesser extent, rutin attenuated the inflammation in the liver by down-regulating the CCl_4-induced activation of nuclear factor-kappa B (NF-κB), tumor necrosis factor-α (TNF-α) and cyclooxygenase (COX-2). The expression of inducible nitric oxide synthase (iNOS) was more potently suppressed by rutin than by quercetin. Treatment with both flavonoids significantly increased NF-E2-related factor 2 (Nrf2) and heme oxygenase (HO-1) expression in injured livers, although quercetin was less effective than rutin at an equivalent dose. Quercetin more potently suppressed the expression of transforming growth factor-β1 (TGF-β1) than rutin.
Conclusion: Rutin exerts stronger protection against nitrosative stress and hepatocellular damage but has weaker antioxidant and anti-inflammatory activities and antifibrotic potential than quercetin, which may be attributed to the presence of a rutinoside moiety in position 3 of the C ring.

Keywords: hepatotoxicity; carbon tetrachloride (CCl_4) poisoning; rutin; quercetin; nuclear factor-kappa B; tumor necrosis factor-α; cyclooxygenase-2; nitric oxide synthase; NF-E2-related factor 2; heme oxygenase-1; transforming growth factor-β1

Acta Pharmacologica Sinica (2012) 33: 1260–1270; doi: 10.1038/aps.2012.62; published online 20 Aug 2012

Introduction

Toxic liver injury may lead to acute liver failure, resulting in organ dysfunction. Numerous drugs and toxic substances could cause hepatic damage, with the severity of the changes proportional to the duration of the toxic exposure[1]. Carbon tetrachloride (CCl_4) poisoning is one of the most commonly used models of acute liver damage. The hepatotoxic effects of CCl_4 were attributed to the excessive production of free radicals[2]. Previous studies have shown that natural compounds with antioxidant activity could ameliorate CCl_4-induced liver damage, thus preventing acute liver failure[3, 4].

Rutin is a naturally occurring flavonol consisting of aglycone quercetin and a rutinoside moiety in position 3 of the C ring (Figure 1). These widespread flavonoids, commonly found in various foods[5], exert numerous biochemical and pharmacological activities, such as antioxidant[6], anti-inflammatory[7] and antitumor activities[8]. However, the pharmacological effects of rutin and its aglycone may differ, suggesting

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Received 2012-01-17 Accepted 2012-05-07

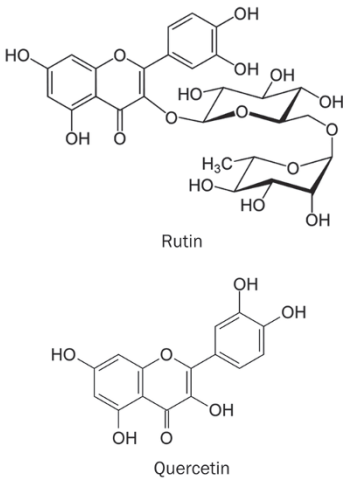

Figure 1. The chemical structures of rutin and quercetin.

that the presence of the rutinoside moiety is crucial for some of the protective effects of rutin. In several studies, rutin exerted anti-inflammatory activity, whereas quercetin was either not effective against or actually aggravated the inflammatory response both *in vivo* and *in vitro*[9, 10]. However, other authors demonstrated that quercetin acted as a strong inhibitor of inflammation in an experimental model of rat colitis[11]. Interestingly, rutin was an effective anti-inflammatory agent in chronic inflammatory conditions, such as adjuvant arthritis, whereas quercetin, but not rutin, potently suppressed acute inflammation and reduced carrageenan-induced paw edema[7]. Similarly, the cytochromes CYP1A1 and CYP1B1 were strongly inhibited by quercetin, while rutin exerted no inhibition or only weak inhibitory potential[12]. Thus, the pharmacological activities of rutin and quercetin may differ substantially, which could be attributed to the presence of a sugar moiety in position 3 of the C ring.

Previous investigations showed that rutin and quercetin could ameliorate chemically induced liver damage in rodents[13, 14]. The objective of this study was to elucidate the molecular mechanisms of the hepatoprotective activity of rutin against acute toxic liver damage in mice and compare this activity to its aglycone quercetin.

Materials and methods

Chemicals and antibodies

Rutin, quercetin, olive oil, bovine superoxide dismutase (SOD), xanthine, xanthine oxidase, cytochrome *c*, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), bovine Cu/Zn superoxide dismutase (Cu/Zn SOD), glutathione (GSH), glutathione reductase (GR), reduced nicotinamide adenine dinucleotide phosphate (NADPH), metaphosphoric acid, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), dimethyl sulfoxide (DMSO), ammonium molybdate, 2,2-diphenyl-1-picryl-hydrazyl (DPPH$^{\cdot}$), hydrogen peroxide, potassium ferricyanide, Trolox, sodium acetate, Tris(hydroxymethyl) aminomethane (Tris), Tween 20 and Entelan were purchased from Sigma-Aldrich (Taufkirchen, Germany). Trichloroacetic acid was purchased from Acros Organics (Geel, Belgium). Iron (III) chloride was obtained from Riedel-de Haën (Seelze, Germany). The radioimmunoprecipitation assay (RIPA) buffer (sc-24948) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CCl_4, ethanol, sodium phosphate and sulfuric acid were purchased from Kemika (Zagreb, Croatia). The diagnostic kits for the determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were from Dijagnostika (Sisak, Croatia). Mouse monoclonal antibodies to tumor necrosis factor-alpha (TNF-α) (ab1793) and 3-nitrotyrosine (3-NT) (ab78163) and rabbit polyclonal antibodies to nuclear factor-kappa B (NF-κB) p65 (ab7970), cyclooxygenase (COX)-2 (ab15191), inducible nitric oxide synthase (iNOS) (ab3523), heme oxygenase (HO)-1 (ab13243), NF-E2-related factor 2 (Nrf2) (ab31163) and TGF-β1 (ab92486) were purchased from Abcam (Cambridge, UK). The DAKO EnVision+ System was from DAKO Corporation (Carpinteria, CA, USA).

Reducing power assay

The reducing power of the samples was determined by the method of Oyaizu[15], as described previously[16]. Briefly, an aliquot of the sample (1.0 mL) at various concentrations (1.25–100 μg/mL) was mixed with phosphate buffer (0.2 mol/L, pH 6.6, 2.5 mL) and 1% potassium ferricyanide (2.5 mL). The mixture was incubated at 50 °C for 20 min. After the addition of 10% trichloroacetic acid (2.5 mL), the mixture was centrifuged at 1000×*g* for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% iron (III) chloride (0.5 mL), and the absorbance was measured at 700 nm using an appropriate blank. All experiments were performed in triplicate. Trolox was used as a reference.

DPPH radical scavenging assay

The free radical scavenging activity of the samples was measured using the stable DPPH$^{\cdot}$ radical according to the method of Blois[17], as described previously[18]. Briefly, a 0.1 mmol/L solution of DPPH$^{\cdot}$ in ethanol was prepared, and this solution (0.5 mL) was added to sample solution in ethanol (1.5 mL) at different concentrations (0.39–50 μg/mL). After the reaction was performed in the dark at room temperature for 30 min, the absorbance was measured at 517 nm. The capability to scavenge the DPPH$^{\cdot}$ radical was calculated using the following equation: (%)=$[(A_0-A_1)/A_0]\times100$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample, corrected for the absorbance of the sample itself. All experiments were performed in triplicate. Trolox was used as a reference.

Total antioxidant capacity assay

The total antioxidant capacity of rutin and quercetin was evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al*[19], as described previously[16]. Briefly, the sample was dissolved in ethanol, and an aliquot of the solution (0.3 mL) was combined in a vial with the reagent

solution (0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate, 2.7 mL). The vials were capped and incubated in a water bath at 95 °C for 90 min. After the mixture was cooled to room temperature, the absorbance was measured at 695 nm against a blank. All experiments were performed in triplicate. The antioxidant capacity of the sample was expressed as Trolox equivalents, utilizing a calibration curve of Trolox in the concentration range from 0.78 to 100 μg/mL.

Nitric oxide radical scavenging assay

The nitric oxide ($NO^{\cdot}$) scavenging activity of the samples was determined according to the method described by Rai *et al*[20] with a slight modification. The $NO^{\cdot}$ generated from sodium nitroprusside in an aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction. Equal volumes of 10 mmol/L sodium nitroprusside in phosphate buffered-saline (pH 7.4) were mixed with different concentrations of the sample (0.39–50 μg/mL) and incubated at 25 °C for 150 min. After the incubation, 1.0 mL of the reaction mixture was mixed with 1% sulfanilamide (0.5 mL). After 5 min, 0.1% naphthylethylenediamine dihydrochloride (0.5 mL) was added, the solution was mixed, and the absorbance of a pink-colored chromophore was measured at 540 nm against the corresponding blank solution. Trolox was used as a standard. All experiments were performed in triplicate. The $NO^{\cdot}$ scavenging activity was expressed as the percentage of inhibition according to the following equation: $(\%)=[(A_0-A_1)/A_0]\times100$, where A_0 is the absorbance of the control without a sample and A_1 is the absorbance in the presence of the sample.

Animals

Male BALB/cN mice, 10–12 weeks old and weighing 23–25 g, were obtained from our breeding colony. The animals were housed under standard environmental conditions and had free access to tap water and a standard rodent diet (pellet, type 4RF21 GLP, Mucedola, Italy). All experimental procedures were approved by the Ethical Committee of the Medical Faculty, University of Rijeka.

Experimental design

The mice were divided into six groups, each containing five animals. The normal control (group I) received saline, and group II received CCl_4 dissolved in olive oil (2 mL/kg, 10% *v/v*) intraperitoneally (ip). Rutin or quercetin, dissolved in 5% (*v/v*) DMSO, was administered ip at 10, 50, and 150 mg/kg (groups III, IV, and V, respectively) and 50 mg/kg (group VI), respectively, once daily for five consecutive days. Immediately after the last dose, the mice were given CCl_4. The doses of rutin were selected on the basis of our preliminary studies (data not shown), whereas the middle dose of quercetin (50 mg/kg) was used for the comparison with rutin. The mice were sacrificed 24 h after the injection of CCl_4. Blood was collected by cardiac puncture, and heparinized plasma was separated for the determination of the ALT and AST activities. The gall bladder was removed, and the liver was carefully excised, washed with saline, blotted dry and divided into samples. The tissue specimens were snap frozen in liquid nitrogen and stored at -80 °C if not used on the same day. The liver samples were used to assess the Cu/Zn SOD activity, GSH and protein concentration and also for immunoblotting. Portions of the livers were immersed in 4% paraformaldehyde for histology and immunohistochemistry.

Determination of hepatotoxicity

The activity of transaminases (ALT and AST) in plasma was measured using a Bio-Tek EL808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT, USA) according to the manufacturer's instructions.

Measurement of oxidative stress

Mouse livers were homogenized in 50 mmol/L phosphate buffer saline (PBS), pH 7.4, using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The supernatants were separated by centrifugation at 15000×*g* for 20 min at 4 °C (Beckman L7-65 Ultracentrifuge, Beckman, Fullerton, CA, USA) and used to measure the Cu/Zn SOD activity and GSH content. The Cu/Zn SOD activity was determined as described previously[3]. The GSH content was evaluated according to Anderson[21], with modifications. Briefly, the supernatants were deproteinized with 1.25 mol/L metaphosphoric acid and centrifuged at 5000×*g* for 10 min at room temperature (Rotina 420R, Andreas Hettich GmbH, Tuttlingen, Germany). Then, 25 μL of the deproteinized sample was mixed in a cuvette with 700 μL of 0.3 mmol/L NADPH in PBS, 100 μL of 6 mmol/L DTNB and water to give a final volume of 1.0 mL. The reaction was started by the addition of 10 μL of GR (50 units/mL), and the absorbance was monitored at 405 nm for 25 min. The GSH concentration in the samples was determined using the standard curve generated with different GSH solutions under the same condition. The protein content in the liver homogenates was estimated by the Bradford method[22].

Histopathology

Paraformaldehyde-fixed tissues were processed routinely, embedded in paraffin, sectioned, deparaffinized and rehydrated using standard techniques[23]. Hepatocellular necrosis was evaluated by measuring the size of the necrotic area in hematoxylin and eosin (H&E) stained liver sections. The necrotic areas were manually selected, and their size was determined using Cell F v3.1 software (Olympus Soft Imaging Solutions, Münster, Germany).

Immunohistochemistry

For immunohistochemistry, 4-μm thick liver tissue sections were used, as described elsewhere[3]. Briefly, the slides were deparaffinized using xylene and washed with ethanol. The liver slices were incubated overnight with antibodies against TNF-α (diluted 1:50), NF-κB (diluted 1:1000) and iNOS, 3-NT, Nrf2, and TGF-β1 (diluted 1:100). The detection of antibodies was performed using DAKO EnVision+ System, Peroxidase/

DAB kit (DAKO Corporation, Carpinteria, CA, USA). Later, the slides were counterstained with hematoxylin, dehydrated using graded ethanol and xylene and mounted with Entelan. The expression profile and cellular localization of NF-κB, iNOS, Nrf2, and 3-NT were analyzed by light microscopy (Olympus BX51, Tokyo, Japan). The relative staining intensity was analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA)[23].

Immunoblotting

The liver samples were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mmol/L PMSF, 1 mmol/L sodium orthovanadate and 2 μg/mL each of aprotinin, leupeptin and pepstatin. Volumes equivalent to 50 μg of proteins were loaded onto a 12% polyacrylamide gel. After electrophoresis, the gels were blotted onto a polyvinylidene fluoride membrane (Roche Diagnostics GmbH, Mannheim, Germany). After the protein transfer, the membranes were blocked overnight with a milk blocking reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C. TNF-α, COX-2, and HO-1 were visualized by the addition of their respective antibodies, followed by peroxidase-labeled goat anti-mouse or anti-rat antibodies (Amersham Pharmacia Biotech, Uppsala, Sweden). β-Actin was used as a control for protein loading. The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBST), pH 7.6, incubated with Amersham ECL Prime (GE Healthcare, Uppsala, Sweden) for 5 min and exposed to X-ray film (Ortho CP-G Plus film, Agfa-Gevaert NV, Belgium) for 1 min.

Statistical analysis

The data were analyzed using StatSoft STATISTICA version 7.1 and Microsoft Excel 2000 software. Differences between the groups were assessed by a one-way ANOVA and Dunnett's *post hoc* test. The values in the text are mean±standard deviation (SD). For the *in vitro* studies, the concentration of samples that provide 50% inhibition (IC_{50}) was obtained by interpolation from a linear regression analysis. Differences from $P<0.05$ were considered statistically significant.

Results

In vitro antioxidant activity

The ability of the tested samples to reduce iron (III) and scavenge $DPPH^{\cdot}$ and $NO^{\cdot}$ was assessed on the basis of their IC_{50} values. Figure 2A shows the plot of the reducing power of rutin and quercetin compared with Trolox as a reference antioxidant. Quercetin exhibited the most powerful effect (IC_{50}=2.42 μg/mL) compared with rutin (IC_{50}=7.18 μg/mL) and Trolox (IC_{50}=6.66 μg/mL) ($P<0.05$). Quercetin also showed the most effective $DPPH^{\cdot}$ and $NO^{\cdot}$ radical scavenging activity (IC_{50}=0.56 μg/mL and 4.04±1.03 μg/mL, respectively), which was significantly stronger ($P<0.05$) than that of both rutin (IC_{50}=2.07 μg/mL and 9.32±1.24 μg/mL, respectively) and Trolox (IC_{50}=1.11 μg/mL and 8.51±1.03 μg/mL, respectively) (Figure 2B and 2C). Similarly, the total antioxidant capacity of quercetin (Figure 2D) was higher than that of rutin ($P<0.05$).

Liver weight and plasma activity of transaminases

The relative liver weight in CCl_4-intoxicated mice decreased compared with that in the controls. Rutin dose-dependently prevented liver weight loss significantly more than quercetin at the equivalent dose. The plasma AST and ALT activities significantly increased 24 h after CCl_4-intoxication ($P<0.05$). Treatment with rutin decreased the activity of these transaminases in a dose-dependent manner (Table 1). However, quercetin showed a less protective effect against hepatocellular damage than rutin at the equivalent dose.

Effect of rutin and quercetin on hepatic oxidative stress

Our results showed that CCl_4 administration induced oxidative stress in mouse livers. The Cu/Zn SOD activity and GSH concentration were significantly lower compared with the control group (Table 1) ($P<0.05$). Treatment with rutin elevated the Cu/Zn SOD activity and GSH concentration in a dose-dependent manner. Quercetin at 50 mg/kg significantly attenuated the decrease in the oxidative stress markers in CCl_4-intoxicated mice, restoring Cu/Zn SOD activity more potently than rutin at 50 mg/kg ($P<0.05$).

Table 1. Effect of CCl_4, rutin and quercetin on relative liver weight, plasma transaminase activity and oxidative stress markers in mice liver.

Group		Relative liver weight (g/100 g)	AST (U/L)	ALT (U/L)	Cu/Zn SOD (U/g protein)	GSH (μmol/g liver)
I	Control	5.7±0.4	41.4±8.6	16.1±1.2	14.4±0.7	7.5±0.5
II	CCl_4	4.7±0.6[b]	3261±365[b]	5208±466[b]	8.1±0.5[b]	3.7±0.3[b]
III	CCl_4+rutin 10 mg/kg	4.9±0.5	3241±319	5066±205	8.5±0.5	4.2±0.6
IV	CCl_4+rutin 50 mg/kg	5.3±0.6	990±143[e]	2190±56[e]	10.8±1.1[e]	5.5±0.8[e]
V	CCl_4+rutin 150 mg/kg	5.8±0.7[e]	226±74[e]	662±98[e]	13.6±0.9[e]	5.8±0.6[e]
VI	CCl_4+quercetin 50 mg/kg	5.1±0.4	1621±74[eh]	3892±294[eh]	14.3±0.7[eh]	6.3±0.7[e]

Mice were treated with rutin or quercetin intraperitoneally (ip) once daily for five consecutive days. Immediately after the last dose, mice received CCl_4 dissolved in olive oil (10% *v/v*, 2 mg/kg), ip, except the control group that received saline. Relative liver weights were expressed as liver weight/100 g of body weight. Each value represents the mean±SD for five mice. [b]$P<0.05$, CCl_4-intoxication *vs* control. [e]$P<0.05$, rutin and quercetin treatment *vs* CCl_4-intoxication. [h]$P<0.05$, rutin 50 mg/kg *vs* quercetin 50 mg/kg.

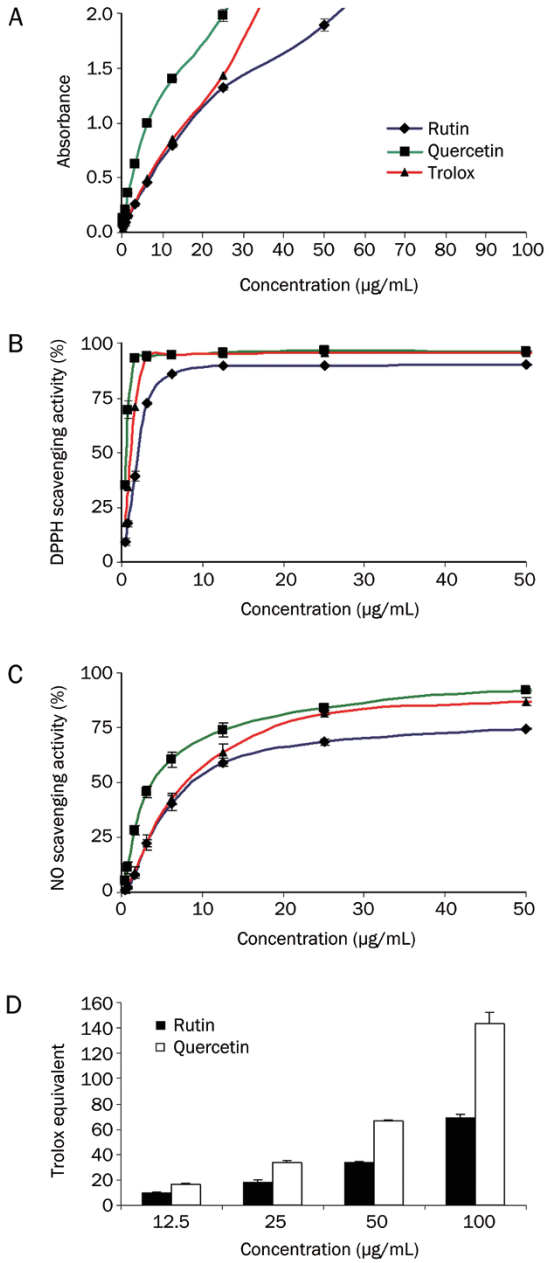

Figure 2. Evaluation of the *in vitro* antioxidant properties of rutin and quercetin. (A) Reducing power, (B) DPPH˙ radical scavenging effect, (C) NO˙ radical scavenging effect and (D) total antioxidant activity of rutin and quercetin. Each value represents the mean±SD from three independent measurements.

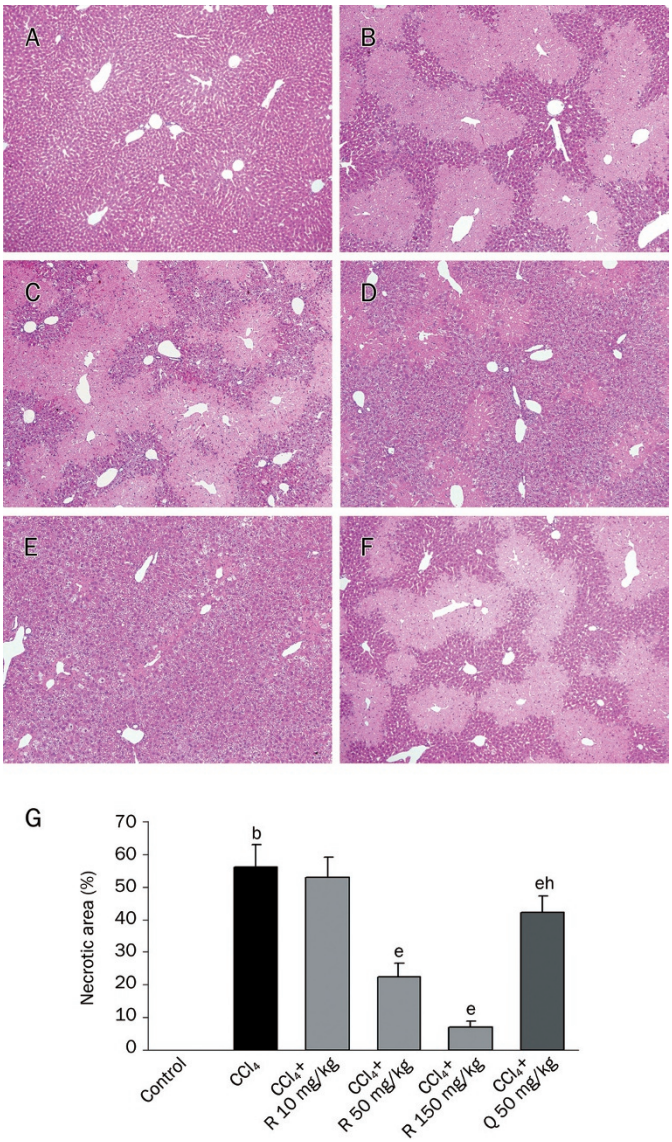

Figure 3. Representative hematoxylin and eosin stained sections of livers from experimental mice. (A) Control group. (B) CCl_4-intoxicated mice. (C–F) Mice treated with CCl_4 and protected with rutin (R) at 10 mg/kg, 50 mg/kg and 150 mg/kg and with quercetin (Q) at 50 mg/kg. Original magnification ×100, insets ×400. (G) Measurement of the size of the necrotic areas (n=5). ^{b}P<0.05, CCl_4-intoxication *vs* control. ^{e}P<0.05, rutin and quercetin treatment *vs* CCl_4-intoxication. ^{h}P<0.05, 50 mg/kg rutin *vs* 50 mg/kg quercetin.

Amelioration of histopathological changes by rutin and quercetin in the liver

Representative images of histological sections from the experimental groups are shown in Figure 3. The livers of the control mice showed a normal morphology and architecture (Figure 3A). In the CCl_4-intoxicated mice, severe hepatic damage with a massive centrilobular necrosis was detected (Figure 3B). In the livers of mice treated with rutin at 10 mg/kg, no histological improvement was found (Figure 3C). Rutin at 50 mg/kg markedly reduced the size of the hepatic necrotic areas (Figure 3D), whereas the high-dose rutin (150 mg/kg) almost completely prevented hepatocellular damage (Figure 3E). In mice treated with quercetin at 50 mg/kg, larger necrotic areas were present than in the livers of mice treated with rutin at the equivalent dose (Figure 3F and 3G).

Amelioration of hepatic inflammation by rutin and quercetin

To determine whether rutin or quercetin could reverse the acute liver inflammation induced by CCl_4, we analyzed pro-

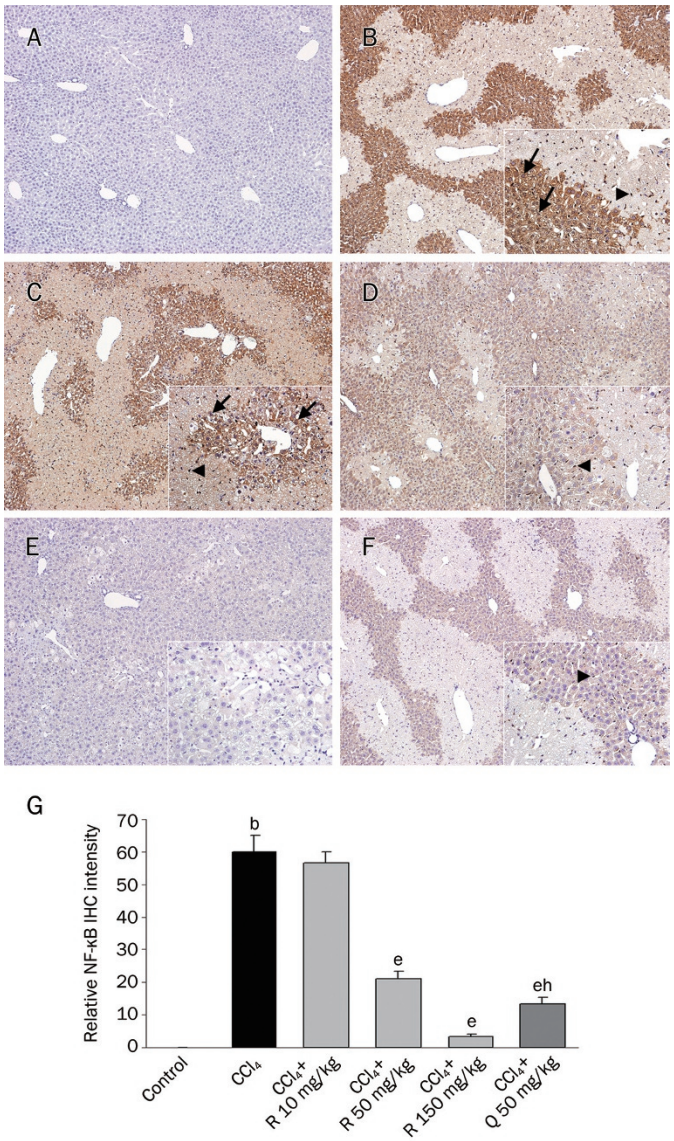

Figure 4. NF-κB immunostaining in liver sections. (A) Control group. (B) CCl_4-intoxicated mice. (C–F) Mice treated with CCl_4 and protected with rutin (R) at 10, 50, and 150 mg/kg and with quercetin (Q) at 50 mg/kg. The arrows show NF-κB p65-positive hepatocyte nuclei, and the arrowheads show NF-κB p65-positive Kupffer cells. Original magnification ×100, insets ×400. Representative results from five mice. (G) Measurement of NF-κB immunostaining intensity (n=5). ^{b}P<0.05, CCl_4-intoxication *vs* control; ^{e}P<0.05, rutin and quercetin treatment *vs* CCl_4-intoxication; ^{h}P<0.05, 50 mg/kg rutin *vs* 50 mg/kg quercetin.

teins involved in the proinflammatory response, such as NF-κB, TNF-α, and COX-2. The livers of control mice were NF-κB immunonegative (Figure 4A). In contrast, strong NF-κB immunoreactivity was detected in the CCl_4-treated mice (Figure 4B). Immunohistochemical analysis revealed NF-κB p65 localization in both the cytoplasm and the nucleus of hepatocytes and in Kupffer cells. The low-dose rutin treatment (10 mg/kg) did not substantially affect NF-κB immunopositivity (Figure 4C). However, the higher doses of rutin, 50 and 150 mg/kg, gradually decreased NF-κB expression and prevented the accumulation of NF-κB p65 in the nuclei (Figure 4D and 4E). Quercetin had a more pronounced effect on NF-κB suppression than rutin at the equivalent dose (Figure 4F and 4G). CCl_4 administration also increased the hepatic levels of TNF-α and COX-2 (Figure 5). Rutin dose-dependently reduced both TNF-α and COX-2 expression compared with the CCl_4-treated mice. Similarly to NF-κB, quercetin more effectively suppressed TNF-α and COX-2 expression than rutin at the equivalent dose.

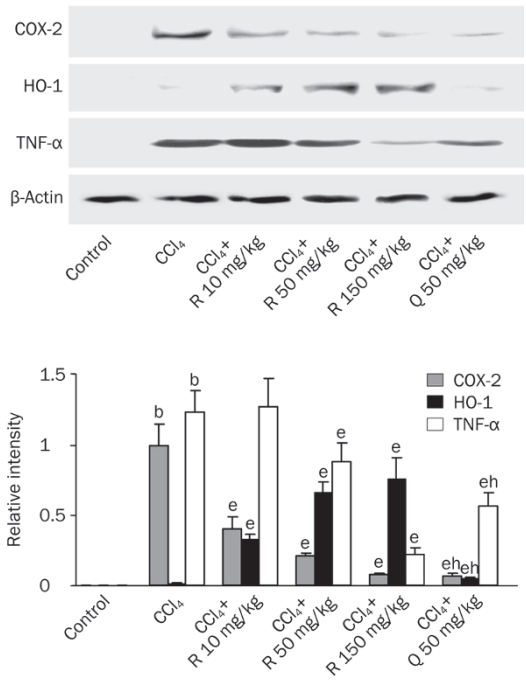

Figure 5. Western blot analysis of TNF-α, COX-2, and HO-1 in mouse livers. Representative results from five mice. Quantification of band intensity relative to β-actin intensity is shown below the Western blot. ^{b}P<0.05, CCl_4-intoxication *vs* control. ^{e}P<0.05, rutin and quercetin treatment *vs* CCl_4-intoxication. ^{h}P<0.05, 50 mg/kg rutin *vs* 50 mg/kg quercetin. R, rutin; Q, quercetin.

Effect of rutin and quercetin on hepatic nitrosative stress

The analysis of the hepatic expression of iNOS and the formation of the $NO^{\cdot}$-dependent product, 3-NT, revealed no immunopositivity in the control mice (Figure 6A and 7A). CCl_4 administration induced a strong expression of iNOS and 3-NT (Figure 6B and 7B). Rutin dose-dependently reduced the iNOS (Figure 6C, 6D, and 6E) and 3-NT (Figure 7C, 7D, and 7E) immunopositivity compared with the CCl_4-treated mice. However, quercetin was less effective than rutin in the reduction of both iNOS (Figure 6F and 6G) and 3-NT (Figure 7F and 7G) immunoreactivity.

Induction of the Nrf2/HO-1 pathway in the liver by rutin and quercetin

The hepatic expression of HO-1 (Figure 5), the cytoprotective

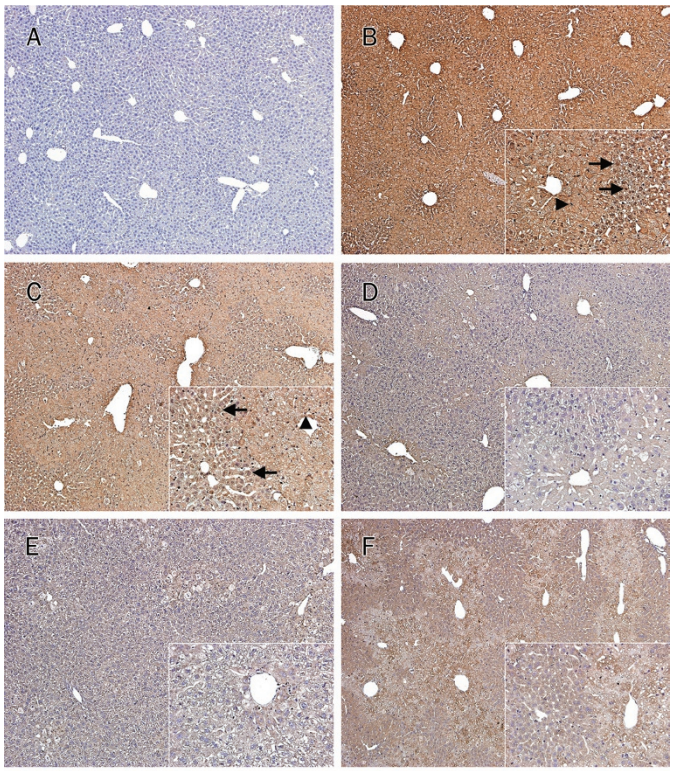

Figure 6. Immunohistochemical analysis of iNOS expression in mouse livers. (A) Control group. (B) CCl_4-intoxicated mice. (C–F) Mice treated with CCl_4 and protected with rutin (R) at 10, 50, and 150 mg/kg and with quercetin (Q) at 50 mg/kg. The arrows show iNOS-positive hepatocyte nuclei, and the arrowheads show iNOS-positive Kupffer cells. Original magnification ×100, insets ×400. Representative results from five mice. (G) Measurement of iNOS immunostaining intensity (n=5). ^{b}P<0.05, CCl_4-intoxication *vs* control. ^{e}P<0.05, rutin and quercetin treatment *vs* CCl_4-intoxication. ^{h}P<0.05, 50 mg/kg rutin *vs* 50 mg/kg quercetin.

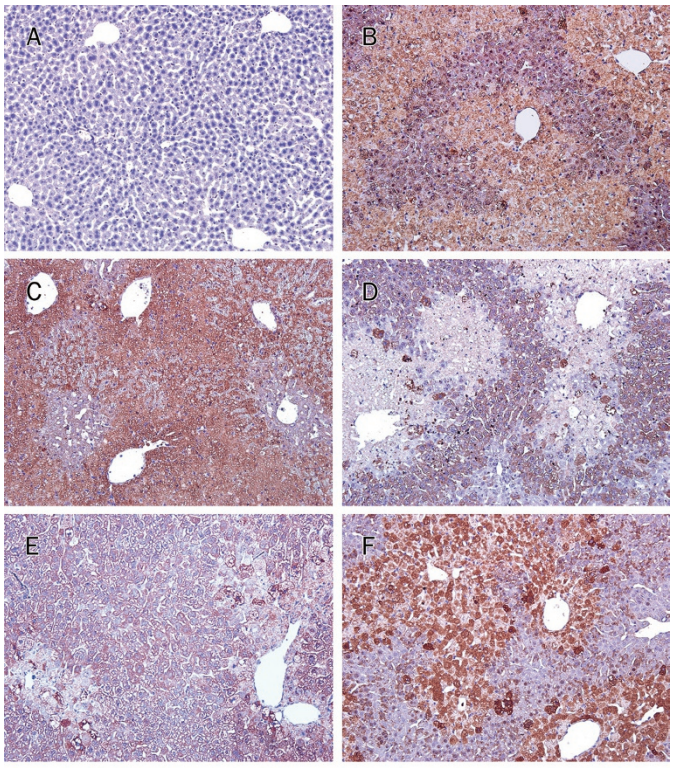

Figure 7. Immunohistochemical detection of 3-NT production in mouse livers. (A) Control group. (B) CCl_4-intoxicated mice. (C–F) Mice treated with CCl_4 and protected with rutin (R) at 10, 50, and 150 mg/kg and with quercetin (Q) at 50 mg/kg. Original magnification ×200. Representative results from 5 mice. (G) Measurement of 3-NT immunostaining intensity (n=5). ^{b}P<0.05, CCl_4-intoxication vs control. ^{e}P<0.05, rutin and quercetin treatment *vs* CCl_4-intoxication. ^{h}P<0.05, 50 mg/kg rutin *vs* 50 mg/kg quercetin.

enzyme that plays a critical role in the response to stressful conditions, and Nrf2, its upstream inducer[24], is shown in Figure 8. The livers of control mice were Nrf2 immunonegative (Figure 8A). The livers of CCl_4-intoxicated mice were weakly Nrf2 immunopositive (Figure 8B). However, the treatment with rutin resulted in a marked dose-dependent induction of Nrf2 (Figure 8C, 8D, and 8E) in the cytoplasm and nucleus of hepatocytes and Kupffer cells. Nrf2 immunoreactivity in hepatocytes of mice treated with quercetin (Figure 8F) markedly increased compared with the CCl_4 group but was less expressed compared with mice receiving rutin at the equivalent dose (Figure 8G). Similarly, rutin dose-dependently increased the hepatic HO-1 levels compared with CCl_4-treated mice, whereas quercetin was a less potent up-regulator of HO-1 than rutin at the equivalent dose (Figure 5).

Antifibrotic potential of rutin and quercetin in the liver

The livers of control mice did not show substantial TGF-β1 immunopositivity (Figure 9A). In contrast, TGF-β1 was over-

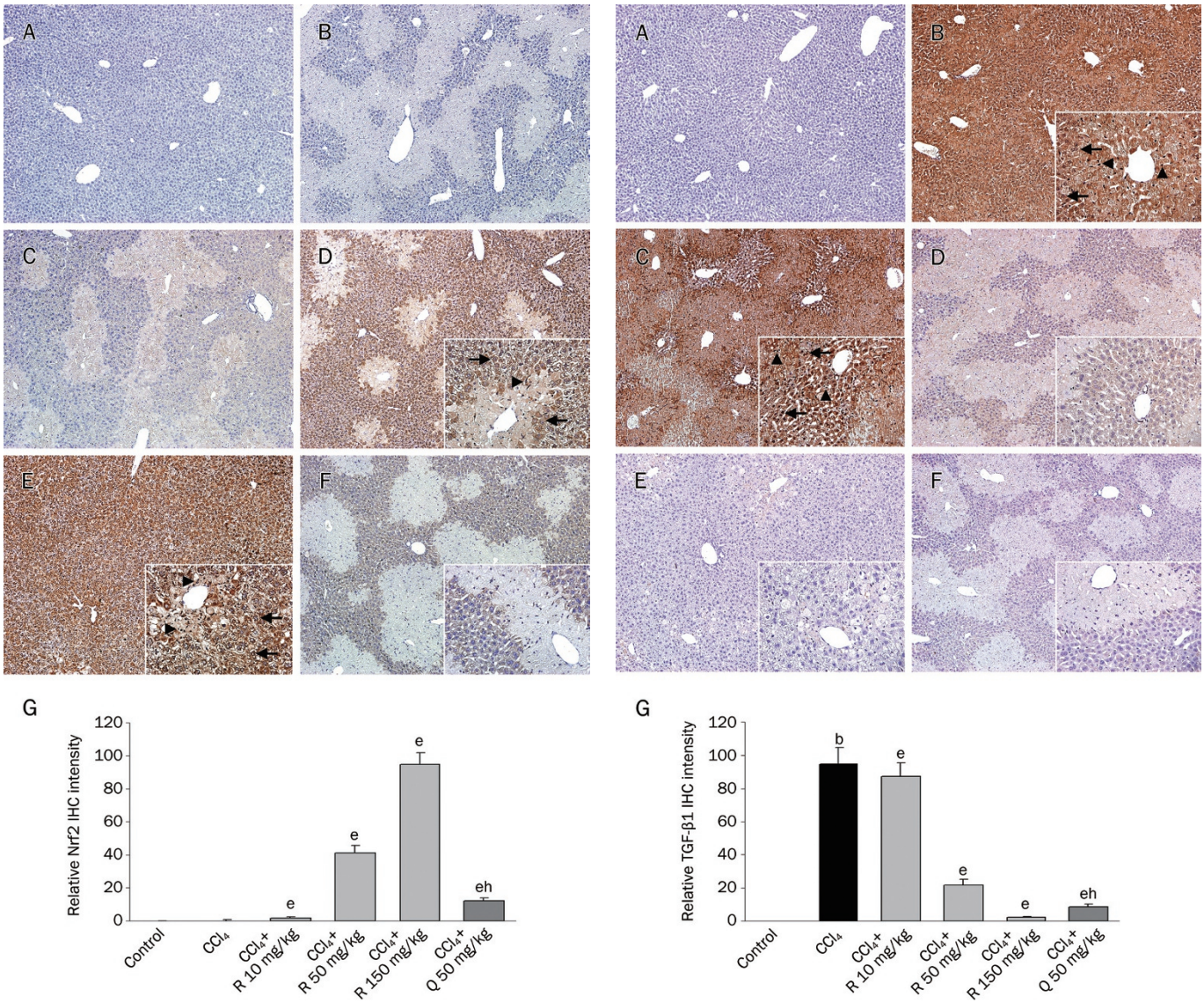

Figure 8. Immunohistochemical detection of Nrf2 expression in mouse livers. (A) Control group. (B) CCl_4-intoxicated mice. (C–F) Mice treated with CCl_4 and protected with rutin (R) at 10, 50, and 150 mg/kg and with quercetin (Q) at 50 mg/kg. The arrows show Nrf2-positive hepatocyte nuclei, and the arrowheads show Nrf2-positive Kupffer cells. Original magnification ×100, insets ×400. Representative results from five mice. (G) Measurement of Nrf2 immunostaining intensity (n=5). ^{b}P<0.05, CCl_4-intoxication *vs* control. ^{e}P<0.05, rutin and quercetin treatment *vs* CCl_4-intoxication. ^{h}P<0.05, 50 mg/kg rutin *vs* 50 mg/kg quercetin.

Figure 9. Immunohistochemistry of TGF-β1 expression in mouse livers. (A) Control group. (B) CCl_4-intoxicated mice. (C–F) Mice treated with CCl_4 and protected with rutin (R) at 10, 50, and 150 mg/kg and with quercetin (Q) at 50 mg/kg. The arrows show TGF-β1-positive hepatocyte nuclei, and the arrowheads show TGF-β1-positive Kupffer cells. Original magnification ×100, insets ×400. Representative results from five mice. (G) Measurement of TGF-β1 immunostaining intensity (n=5). ^{b}P<0.05, CCl_4-intoxication *vs* control. ^{e}P<0.05, rutin and quercetin treatment *vs* CCl_4-intoxication. ^{h}P<0.05, 50 mg/kg rutin *vs* 50 mg/kg quercetin.

expressed in the livers of CCl_4-intoxicated mice (Figure 9B). The administration of rutin dose-dependently ameliorated TGF-β1 expression (Figure 9C, 9D, and 9E). However, the hepatic TGF-β1 immunoreactivity in mice treated with quercetin (Figure 9F) was significantly lower than that in mice receiving rutin at the equivalent dose (Figure 9G) (P<0.05).

Discussion

The results of the present study suggest the importance of the sugar moiety in position 3 of the C ring for the specific pharmacological activities of rutin and quercetin. The glycosylation of flavonoids reduces their antioxidant activity compared with their corresponding aglycones[25]. Our results showed that quercetin possessed higher reducing power and DPPH$^{\cdot}$ and NO$^{\cdot}$ free radical scavenging abilities *in vitro* compared with rutin. Quercetin more potently prevented the decrease in Cu/Zn SOD activity in mouse livers. However, it was less effective in the amelioration of protein nitrosylation, suggesting

that the *in vitro* free radical scavenging ability of compounds does not necessary correlate with their *in vivo* efficacy, which indicates that absorption and/or metabolism are key modulators of *in vivo* activity[26]. Additionally, the hepatic lesions and necrosis induced by CCl_4 were markedly reduced in mice treated with rutin, whereas quercetin was less effective. The results of the current study showed that the amelioration of hepatic necrosis was closely related to the reduction of nitrosative but not oxidative stress.

Reactive oxygen species (ROS) generated during CCl_4 intoxication may induce NF-κB activation and consequently stimulate the production of cytotoxic and proinflammatory cytokines, such as TNF-α[27]. Our results showed that rutin, and more potently quercetin, suppressed both NF-κB and TNF-α expression in injured livers. NF-κB is also involved in the regulation of COX-2 and iNOS gene expression by binding to their promoter regions[28]. COX-2 and iNOS exert a prominent role under inflammatory conditions by producing prostaglandins and $NO^{\cdot}$, respectively[29, 30]. Thus, the anti-inflammatory effect of quercetin and rutin in CCl_4-injured liver could be attributed to the inhibition of the NF-κB pathway, which is in agreement with previous findings[31]. Additionally, the increased $NO^{\cdot}$ synthesis and superoxide generation could result in the formation of peroxynitrite[32] and nitration of protein tyrosine residues[33], which actively contribute to the development of hepatic necrosis[34]. In this study, the activation and nuclear accumulation of NF-κB p65 in CCl_4-intoxicated mice coincided with COX-2 and iNOS overexpression, which was prevented by both flavonoids. The nuclear presence of COX-2 and iNOS suggests their involvement in the regulation of some nuclear functions. However, rutin exhibited lower potency in reducing COX-2 expression compared with quercetin, which is in agreement with previous findings[35]. Interestingly, rutin more effectively suppressed the expression of both iNOS and 3-NT compared with quercetin. The latter coincided with the amelioration of hepatic necrosis. These findings agree with Raghav *et al*[36], who showed a stronger inhibition of iNOS than COX-2 gene expression by rutin in murine macrophages.

TGF-β, a pleiotrophic growth factor, has been shown to stimulate fibroblast proliferation and increase the synthesis of ECM proteins[37]. Previously, we showed that the fibrogenic potential of the CCl_4-injured liver is closely related to TGF-β1 expression[38]. In the current study, TGF-β1 expression markedly increased in both hepatocytes and non-parenchymal cells of the CCl_4-intoxicated liver. Although Kupffer cells and HSCs are considered the main sources of TGF-β[39], hepatocytes may also produce TGF-β. In cultured hepatocytes, latent TGF-β was rapidly detectable during culture due to demasking of the mature TGF-β by calpains[40]. The nuclear presence of TGF-β1 could be attributed to increased nuclear membrane permeability during the activation of apoptosis[41]. Our results showed that treatment with both flavonoids markedly ameliorated the overexpression of TGF-β1, suggesting the reduction of the fibrogenic potential in the liver.

HO-1, the inducible isoform of heme oxygenase, plays a critical role in cell protection against acute and chronic liver injury[42]. The transcription factor Nrf2 is considered a key regulator of HO-1 expression[24]. In the current study, hepatic injury was associated with low expression of HO-1 and its upstream inducer Nrf2 but with high expression of NF-κB. The treatments with rutin and, to a lesser extent, quercetin were associated with increased Nrf2 and HO-1 expression in the liver, which coincided with the suppression of NF-κB activation. Most recently, Liu *et al*[43] showed that quercetin acts as an inducer of HO-1 in primary rat hepatocytes. Interestingly, the induction of HO-1 was found in quercetin-treated but not in rutin-treated rat glioma C6 cells[44] and in the hydrogen peroxide-induced apoptosis of RAW264.7 macrophages[45]. However, rutin acted as a strong HO-1 inducer in a rat model of liver ischemia-reperfusion injury[46], suggesting a cell type-dependent activation of this enzyme. Additionally, the nuclear translocation of HO-1 could be involved in the regulation of genes responsible for cytoprotection against oxidative stress[47].

In conclusion, the results of this study demonstrate that rutin and quercetin can ameliorate acute liver damage by at least four mechanisms: acting as scavengers of free radicals, inhibiting NF-κB activation and the inflammatory response, exerting antifibrotic potential and inducing the Nrf2/HO-1 pathway. The rutinoside moiety in position 3 of the C ring could be responsible for more pronounced protective effects against iNOS induction, nitrosative stress and hepatocellular necrosis. The aglycone quercetin exerted higher antioxidant and anti-inflammatory activities and antifibrotic potential than rutin. The antioxidant actions of rutin and quercetin were partially responsible for their beneficial effects in injured liver tissue. However, the antioxidant properties of these flavonoids cannot solely explain the stronger protective activity of rutin against hepatocellular damage. Thus, the modulation of signaling pathways emerges as an important mode of action of these flavonoids, which should be considered as a specific therapeutic strategy. The application of these flavonoids in medical practice should be further confirmed by conveying preliminary placebo-controlled clinical studies.

Acknowledgements

This research was supported by the Ministry of Science, Education and Sport, Republic of Croatia (projects 062-0000000-3554 and 006-0061117-1238).

Author contribution

Robert DOMITROVIĆ designed the research and wrote the paper; Hrvoje JAKOVAC, Vanja VASILJEV MARCHESI, Sanda VLADIMIR-KNEŽEVIĆ, Olga CVIJANOVIĆ, and Žarko TADIĆ performed the research; Željko ROMIĆ contributed new reagents and analytic tools; and Dario RAHELIĆ analyzed the data.

References

1 Gimson AE. Fulminant and late onset hepatic failure. Br J Anaesth 1996; 77: 90–8.

2 Brattin WJ, Glende EA Jr, Recknagel RO. Pathological mechanisms in carbon tetrachloride hepatotoxicity. J Free Radic Biol Med 1985; 1: 27–38.
3 Domitrović R, Jakovac H, Blagojević G. Hepatoprotective activity of berberine is mediated by inhibition of TNF-α, COX-2, and iNOS expression in CCl_4-intoxicated mice. Toxicology 2011; 280: 33–43.
4 Tipoe GL, Leung TM, Liong EC, Lau TY, Fung ML, Nanji AA. Epigallocatechin-3-gallate (EGCG) reduces liver inflammation, oxidative stress and fibrosis in carbon tetrachloride (CCl_4)-induced liver injury in mice. Toxicology 2010; 273: 45–52.
5 Erlund I, Kosonen T, Alfthan G, Maenpaa J, Perttunen K, Kenraali J, *et al*. Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. Eur J Clin Pharmacol 2000; 56: 545–53.
6 Kandaswami C, Middleton E. Free radical scavenging and antioxidant activity of plant flavonoids. Adv Exp Med Biol 1994; 366: 351–76.
7 Rotelli AE, Guardia T, Juárez AO, de la Rocha NE, Pelzer LE. Comparative study of flavonoids in experimental models of inflammation. Pharmacol Res 2003; 48: 601–6.
8 Araújo JR, Gonçalves P, Martel F. Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines. Nutr Res 2011; 31: 77–87.
9 Kwon KH, Murakami A, Tanaka T, Ohigashi H. Dietary rutin, but not its aglycone quercetin, ameliorates dextran sulfate sodium-induced experimental colitis in mice: attenuation of proinflammatory gene expression. Biochem Pharmacol 2005; 69: 395–406.
10 Kwon KH, Murakami A, Ohigashi H. Suppressive effects of natural and synthetic agents on dextran sulfate sodium-induced interleukin-1β release from murine peritoneal macrophages. Biosci Biotechnol Biochem 2004; 68: 436–9.
11 Comalada M, Camuesco D, Sierra S, Ballester I, Xaus J, Gálvez J, *et al*. *In vivo* quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-κB pathway. Eur J Immunol 2005; 35: 584–92.
12 Chaudhary A, Willett KL. Inhibition of human cytochrome CYP 1 enzymes by flavonoids of St. John's wort. Toxicology 2006; 217: 194–205.
13 Janbaz KH, Saeed SA, Gilani AH. Studies on the protective effects of caffeic acid and quercetin on chemical-induced hepatotoxicity in rodents. Phytomedicine 2004; 11: 424–30.
14 Janbaz KH, Saeed SA, Gilani AH. Protective effect of rutin on paracetamol- and CCl_4-induced hepatotoxicity in rodents. Fitoterapia 2002; 73: 557–63.
15 Oyaizu M. Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr 1986; 44: 307–15.
16 Vladimir-Knežević S, Blažeković B, Štefan MB, Alegro A, Koszegi T, Petrik J. Antioxidant activities and polyphenolic contents of three selected *Micromeria* species from Croatia. Molecules 2011; 16: 1454–70.
17 Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958; 181: 1199–200.
18 Domitrović R, Jakovac H, Romić Ž, Rahelić D, Tadić Ž. Antifibrotic activity of *Taraxacum officinale* root in carbon tetrachloride-induced liver damage in mice. J Ethnopharmacol 2010; 130: 569–77.
19 Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Anal Biochem 1999; 269: 337–41.
20 Rai S, Wahile A, Mukherjee K, Saha BP, Mukherjee PK. Antioxidant activity of *Nelumbo nucifera* (sacred lotus) seeds. J Ethnopharmacol 2006; 104: 322–7.
21 Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 1985; 113: 548–55.
22 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248–54.
23 Domitrović R, Jakovac H, Marchesi VV, Šain I, Romić Z, Rahelić D. Preventive and therapeutic effects of oleuropein against carbon tetrachloride-induced liver damage in mice. Pharmacol Res 2012; 65: 451–64.
24 Yang YC, Lii CK, Lin AH, Yeh YW, Yao HT, Li CC, *et al*. Induction of glutathione synthesis and heme oxygenase 1 by the flavonoids butein and phloretin is mediated through the ERK/Nrf2 pathway and protects against oxidative stress. Free Radic Biol Med 2011; 51: 2073–81.
25 Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 1996; 20: 933–56.
26 Reyes-Gordillo K, Segovia J, Shibayama M, Vergara P, Moreno MG, Muriel P. Curcumin protects against acute liver damage in the rat by inhibiting NF-κB, proinflammatory cytokines production and oxidative stress. Biochim Biophys Acta 2007; 1770: 989–96.
27 Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, *et al*. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. Mutat Res 2001; 480-481: 243–68.
28 Vane JR, Botting RM. Anti-inflammatory drugs and their mechanism of action. Inflamm Res 1998; 47 Suppl 2: 78–87.
29 Vodovotz Y, Kim PK, Bagci EZ, Ermentrout GB, Chow CC, Bahar I, *et al*. Inflammatory modulation of hepatocyte apoptosis by nitric oxide: *in vivo*, *in vitro*, and *in silico* studies. Curr Mol Med 2004; 4: 753–62.
30 Wu CH, Wu CF, Huang HW, Jao YC, Yen GC. Naturally occurring flavonoids attenuate high glucose-induced expression of proinflammatory cytokines in human monocytic THP-1 cells. Mol Nutr Food Res 2009; 53: 984–95.
31 Park HH, Lee S, Son HY, Park SB, Kim MS, Choi EJ, *et al*. Flavonoids inhibit histamine release and expression of proinflammatory cytokines in mast cells. Arch Pharm Res 2008; 31: 1303–11.
32 Pryor WA, Squadrito GL. The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. Am J Physiol 1995; 268: L699–722.
33 Beckman JS. Oxidative damage and tyrosine nitration from peroxynitrite. Chem Res Toxicol 1996; 9: 836–44.
34 Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. Toxicol Sci 2002; 65: 166–76.
35 Jones DJL, Lamb JH, Verschoyle RD, Howells LM, Butterworth M, Lim CK, *et al*. Characterisation of metabolites of the putative cancer chemopreventive agent quercetin and their effect on cyclo-oxygenase activity. Br J Cancer 2004; 91: 1213–9.
36 Raghav SK, Gupta B, Agrawal C, Goswami K, Das HR. Anti-inflammatory effect of *Ruta graveolens* L in murine macrophage cells. J Ethnopharmacol 2006; 104: 234–9.
37 Cutroneo KR. TGF-beta-induced fibrosis and SMAD signaling: oligo decoys as natural therapeutics for inhibition of tissue fibrosis and scarring. Wound Repair Regen 2007; 15: S54–60.
38 Domitrović R, Jakovac H. Antifibrotic activity of anthocyanidin delphinidin in carbon tetrachloride-induced hepatotoxicity in mice. Toxicology 2010; 272:1–10.
39 Reeves HL, Friedman SL. Activation of hepatic stellate cells-a key issue in liver fibrosis. Front Biosci 2002; 7: d808–26.
40 Gressner OA, Lahme B, Siluschek M, Rehbein K, Herrmann J,

Weiskirchen R, *et al*. Activation of TGF-β within cultured hepatocytes and in liver injury leads to intracrine signaling with expression of connective tissue growth factor. J Cell Mol Med 2008; 12: 2717–30.

41 Ferrando-May E, Cordes V, Biller-Ckovrić I, Mirković J, Görlich D, Nicotera P. Caspases mediate nucleoporin cleavage, but not early redistribution of nuclear transport factors and modulation of nuclear permeability in apoptosis. Cell Death Differ 2001; 8: 495–505.

42 Sass G, Barikbin R, Tiegs G. The multiple functions of heme oxygenase-1 in the liver. Z Gastroenterol 2012; 50: 34–40.

43 Liu S, Hou W, Yao P, Li N, Zhang B, Hao L, *et al*. Heme oxygenase-1 mediates the protective role of quercetin against ethanol-induced rat hepatocytes oxidative damage. Toxicol *In Vitro* 2012; 26: 74–80.

44 Chen TJ, Jeng JY, Lin CW, Wu CY, Chen YC. Quercetin inhibition of ROS-dependent and -independent apoptosis in rat glioma C6 cells. Toxicology 2006; 223: 113–26.

45 Chow JM, Shen SC, Huan SK, Lin HY, Chen YC. Quercetin, but not rutin and quercitrin, prevention of H_2O_2-induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages. Biochem Pharmacol 2005; 69: 1839–51.

46 Acquaviva R, Lanteri R, Li Destri G, Caltabiano R, Vanella L, Lanzafame S, *et al*. Beneficial effects of rutin and *L*-arginine coadministration in a rat model of liver ischemia-reperfusion injury. Am J Physiol Gastrointest Liver Physiol 2009; 296: G664–70.

47 Lin Q, Weis S, Yang G, Weng YH, Helston R, Rish K, *et al*. Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. J Biol Chem 2007; 282: 20621–33.