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Research Paper

Rubus sanctus protects against carbon tetrachloride-induced toxicity in rat isolated hepatocytes: isolation and characterization of its galloylated flavonoids

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Abstract

Objectives *Rubus sanctus* Schreb., known from the Bible as 'holy thorn bush', grows wild in Egypt. *Rubus sanctus* aqueous alcoholic extract (RE) contains a complicated phenolic mixture (ellagitanins, flavonoids and caffeic acid derivatives). In this study, the phytochemical investigation of the plant was re-evaluated. Herein, we report on the isolation and identification of three galloylated flavonoids, namely kaempferol-3-*O*-(6″-*O*-galloyl)-$^4C_1$-*β*-D-galactopyranoside, quercetin-3-*O*-(6″-*O*-galloyl)-$^4C_1$-*β*-D-galactopyranoside and myricetin-3-*O*-(6″-*O*-galloyl)-$^4C_1$-*β*-D-galactopyranoside for the first time from the *Rubus* genus. We further aimed at evaluating the potential protective effects of RE against carbon tetrachloride (CCl_4)-induced toxicity in isolated rat hepatocytes.

Methods Based on an initial concentration–response experiment, a concentration of 100 μg/ml was selected to investigate the hepatoprotective activity of RE.

Key findings Pretreatment with RE afforded protection as indicated by counteracting CCl_4-induced cell death, and reduced glutathione depletion. In addition, RE ameliorated CCl_4-induced enzyme leakage by 40% for lactate dehydrogenase, 30% for alanine aminotransferase and 20% for aspartate aminotransferase as compared with CCl_4-treated cells. Moreover, RE counteracted CCl_4-induced lipid peroxidation and inhibited spontaneous lipid peroxidation in the control group.

Conclusions In conclusion, RE protects against CCl_4-induced toxicity in isolated rat hepatocytes.

Keywords galloylated flavonoids; hepatoprotection; *Rubus sanctus*

Introduction

Rubus sanctus Schreb., known from the Bible as 'holy thorn bush', is the bush at which God spoke to Moses (Exodus 3). It is the only *Rubus* species that grows wild in Egypt. It has been reported to grow wild in other countries such as Turkey.[1] It is used in traditional Egyptian folk medicine to treat infectious diseases. Additionally, the plant extract was shown to possess anti-nociceptive activity in experimental animals.[2] The aqueous ethanolic extract, obtained from a homogenate of the ground dried aerial parts, was shown to contain a complicated phenolic mixture.[3] Eleven compounds were isolated from the dried aqueous alcoholic extract. Among them, four belong to the ellagitannin class of polyphenolics – sanguiin H-4, sanguiin H-6, pedunculagin and lambertianin C – exhibiting a recognizable antiviral and antibacterial activity. Others include flavonoids, such as quercetin derivatives and kaempferol derivatives,[4] and caffeic acid derivatives.[3] However, the exact content of *Rubus sanctus* extract (RE) is yet to be revealed.

The incidence of liver diseases has markedly increased. There is no commonly accepted, effective, conventional drug therapy regimen to prevent or reverse liver damage.[5] Some herbal drugs have shown a therapeutic efficacy in the treatment of liver diseases.[6] Scientific literature reports a wide use of phytomedicines in the management of liver dysfunction.[5,7] Much attention has been directed towards the potential health promoting properties of phenolic phytochemicals.[8] Plants containing phenolic compounds have been proven to possess many pharmacological effects, such as anti-inflammatory, cardioprotective, anti-cancer and hepatoprotective properties.[9] In this regard, *Rubus*

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species (family Rosaceae) have been cultivated for centuries for their fruits. These and other parts of the plants have been used traditionally for therapeutic purposes.[10] They are characterized by their capability of synthesizing and accumulating ellagitannins, a major class of phenolic compounds largely responsible for the astringent and antioxidant properties of raspberries and blackberries.[11,12] They have also been found to metabolize several phenolic carboxylic acids, such as ellagic acid, and phenyl propenoids, particularly caffeic acid. *Rubus* species have been used in traditional medicine as antimicrobials, anticonvulsants and muscle relaxants.[3]

Several animal experimental models have been in use to evaluate anti-hepatotoxicants. Liver injury induced by carbon tetrachloride (CCl_4) is one of the best characterized systems of xenobiotic-induced hepatotoxicity and is a commonly used model for the screening of anti-hepatotoxicant or hepatoprotective activity of drugs.[13] CCl_4 produces a wide array of dysfunctions in the liver, including triglyceride accumulation, centrilobular necrosis, polyribosomal desegregation and depression of protein synthesis.[14] *Silybum marianum* (milk thistle) has been shown to have clinical applications in the treatment of toxic hepatitis, fatty liver, cirrhosis, ischaemic injury, radiation toxicity and viral hepatitis via its antioxidative, anti-lipid peroxidative, anti-fibrotic, anti-inflammatory, immunomodulating and liver regenerating effects.[15] Standardized extract of the seeds of *S. marianum*, named silymarin, is a component of several commercially produced hepatoprotective remedies.[16] This compound has been considered a standard for potential hepatoprotective agents. Generally, the high polyphenolic content of RE suggests a potential antioxidant and hepatoprotective activity. This work was designed to evaluate the phytochemistry and the protective activity of RE against CCl_4-induced toxicity in isolated rat hepatocytes. The hepatoprotective activity of RE was compared with that of silymarin.

Materials and Methods

Chemicals

Bovine albumin powder (fraction V), collagenase type IV for hepatocytes isolation, dimethyl sulfoxide (DMSO), Ellman's Reagent (5,5′-dithio-bis-(2-nitrobenzoic acid)), reduced glutathione (GSH), HEPES (*N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid)), 1,1′,3′,-tetramethoxy-propane, thiobarbituric acid (TBA), Triton-X-100, alanine aminotransferase (ALT) kit, aspartate aminotransferase (AST) kit and lactate dehydrogenase (LDH) kit were purchased from Sigma-Aldrich Co. (St Louis, Mo, USA). Chloral hydrate was obtained from Riedel-de Haen Laborchemikalien GmbH and Co. (Seelze, Germany). Trichloroacetic acid (TCA) was obtained from Park Scientific Ltd (Northampton, UK). Other chemicals used were of analytical grade.

Animals

Male Sprague–Dawley rats, 200–250 g, were used in this study. The animal protocol was approved by the local animal care committee (Ain Shams University, Cairo, Egypt). Rats were obtained from The Nile Co. for Pharmaceuticals and Chemical Industries (Cairo, Egypt) and were kept in our animal facility (Faculty of Pharmacy, Ain Shams University, Cairo, Egypt) for at least one week before any experiment. Rats were kept under suitable laboratory conditions at 25 ± 2°C and were allowed free access to standard diet pellets and tap water.

Plant material

Fresh shrubs of *Rubus sanctus* Schreb. were collected from Saint Catherine City, South Sinai, Egypt, in April 2005. Specimens were authenticated by Prof. Dr Abdel Salam El Noyehy, Professor of Taxonomy, Faculty of Science, Ain Shams University, Cairo, Egypt. Voucher specimens (R-664-05) were deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

Instruments and materials for phytochemical investigation

^{1}H NMR spectra were measured on a Bruker AMX 400, relative to tetramethylsilane (TMS). ^{13}C NMR spectra were measured at 100 MHz, relative to dimethyl sulfoxide (DMSO-d_6) and converted to the TMS by adding 77. Typical conditions were 6000 Hz for ^{1}H and 22 000 Hz for ^{13}C, 32 k data points and a flip angle of 45°C. ESI/MS spectra were measured on an SSQ Finnigan MAT 4600 quadropol mass spectrometer (Institut für Chemie, Hamburg Universitat). Paper chromatography (PC) analysis was carried out on Whatman No. 1 paper, using solvent system: (1) H_2O; (2) 6% HOAc; (3) BAW (*n*-BuOH–HOAc–H_2O, 4:1:5, upper layer). Solvents (2) and (3) were used for preparative PC (PPC) on Whatman No. 3MM.

Extraction, isolation and purification of *Rubus sanctus*

Fresh leaves (3 kg) were exhaustively extracted with 20 L of aqueous alcohol (75%). The extract was dried in vacuum at low temperature (99.50 g). 2-dimensional PC (2-DPC) of the extract proved the presence of a high percentage of phenolic constituents (intense green $FeCl_3$ colour reaction and chromatographic analysis). Repeated fractionation of the extract (50 g) on Sephadex LH-20 columns, using H_2O followed by H_2O–MeOH mixtures of decreasing polarities, yielded five fractions (I–V), which were individually subjected to 2-DPC. Compounds (**1**, 26 mg; **2**, 40 mg) were obtained from fraction III (eluted with 40%) through precipitation by ether from the acetone soluble fraction (thrice), and then subsequent PPC of the precipitate, using BAW as solvent. Repeated column fractionation of fraction IV (eluted with 60%) on Sephadex LH-20 column, using *n*-BuOH saturated with H_2O, yielded pure samples of (**3**, 20 mg).

Kaempferol-3-O-*(6″*-O-*galloyl)-*$^4C_1$-*β*-D-galactopyranoside* was obtained as an amorphous dull yellow powder (100 mg); R_f values: 55 (H_2O), 45 (6% AcOH), and 72 (BAW). UV λ_{max}: MeOH (267, 319, 365). ESI/MS indicated a molecular ion at $[M\text{-}H]^-$ 599, corresponding to a molecular weight (M_r) of 600. ^{1}H NMR data: δ ppm

6.22 (1H, *d*, *J* = 2.0 Hz, H-6), 6.42 (1H, *d*, *J* = 2.0 Hz, H-8), 6.77 (2H, *d*, *J* = 8.5, H-3′, H-5′), 7.93 (2H, *d*, *J* = 8.5 Hz, H-2′, H-6′), 5.46 (1H, *d*, *J* = 7.5 Hz, H-1″), 4.25 (1H, *d*, *J* = 12.5 Hz, H_a-6″), 4.32 (1H, *dd*, *J* = 3.5, 12.5 Hz, H_b-6″), 3.2–3.8 (*m*, sugar protons), 6.93 (1H, *s*, H-2‴, H-6‴). ^{13}C NMR data: δ ppm 156.7 (C-2), 133.5 (C-3), 177.7 (C-4, [C=O]), 161.7 (C-5), 99.1 (C-6), 164.5 (C-7), 94.1 (C-8), 157.1 (C-9), 104.2 (C-10), 121.0 (C-1′), 115.4 (C-2′, C-6′), 131.1 (C-3′, C-5′), 160.3 (C-4′), 101.8 (C-1″), 74.4 (C-2″, C-3″), 69.7 (C-4″), 76.5 (C-5″), 63.1 (C-6″), 119.6 (C-1‴), 108.9 (C-2‴, C-6‴), 145.8 (C-3‴, C-5‴), 139.1 (C-4‴), 166.1 (C-7‴), ^{1}H and ^{13}C NMR data were identical to those reported in literature.[17]

Quercetin-3-O-*(6″*-O-*galloyl)-$^4C_1$-β-D-galactopyranoside* was obtained as an amorphous brown powder (75 mg); R_f values: 52 (H_2O), 49 (6% AcOH) and 59 (BAW). UV λ_{max}: MeOH (252, 319, 372). ESI/MS indicated a molecular ion at $[M\text{-}H]^-$ 615, corresponding to a M_r of 616. ^{1}H NMR data: δ ppm 6.18 (1H, *d*, *J* = 2.5 Hz, H-6), 6.37 (1H, *d*, *J* = 2.5 Hz, H-8), 6.73 (1H, *d*, *J* = 8.0 Hz, H-5′), 7.45 (1H, *d*, *J* = 3.0 Hz, H-2′), 7.59 (1H, *dd*, *J* = 3.0, 8.0 Hz, H-6′), 5.44 (1H, *d*, *J* = 7.5 Hz, H-1″), 4.25 (*d*, *J* = 12 Hz, H_a-6″), 4.32 (*dd*, *J* = 5 Hz and 12 Hz, H_b-6″), 3.3–3.8 (*m*, sugar protons), 6.90 (1H, *s*, H-2‴, H-6‴). ^{13}C NMR data: δ ppm 156.3 (C-2), 133.2 (C-3), 177.0 (C-4, [C=O]), 161.2 (C-5), 98.9 (C-6), 165.0 (C-7), 93.6 (C-8), 160.9 (C-9), 103.6 (C-10), 121.7 (C-1′), 115.6 (C-2′), 144.8 (C-3′), 148.8 (C-4′), 115.2 (C-5′), 120.7 (C-6′), 101.3 (C-1″), 73.9 (C-2″), 74.1 (C-3″), 69.3 (C-4″), 76.2 (C-5″), 62.9 (C-6″), 119.1 (C-1‴), 108.5 (C-2‴, C-6‴), 145.5 (C-3‴, C-5‴), 138.62 (C-4‴), 165.8 (C-7‴), ^{1}H and ^{13}C NMR data were identical to those reported in literature.[18]

Myricetin-3-O-*(6″*-O-*galloyl)-$^4C_1$-β-D-galactopyranoside* was obtained as an amorphous pure brown powder (108 mg); R_f values: 30 (H_2O), 28 (6% AcOH) and 35 (BAW). UV λ_{max}: MeOH (252, 319, 372). $[M\text{-}H]^-$ molecular ion in its negative ESI/MS spectrum, at 631, corresponding to a M_r of 632. ^{1}H NMR data: δ ppm 6.18 (1H, *d*, *J* = 1.5 Hz, H-6), 6.37 (1H, *d*, *J* = 1.5 Hz, H-8), 7.19 (2H, *s*, H-2′, H-6′), 5.46 (1H, *d*, *J* = 7.5 Hz, H-1″), 4.2 (*d*, *J* = 12 Hz, H_a-6″), 4.3 (*dd*, *J* = 5 Hz and 12 Hz, H_b-6″), 3.3–3.8 (*m*, sugar protons), 6.88 (1H, *s*, H-2‴, H-6‴), ^{13}C NMR data: δ ppm 155.9 (C-2, C-9), 133.5 (C-3), 177.7 (C-4, [C=O]), 161.1 (C-5), 98.6 (C-6), 164.1 (C-7), 93.3 (C-8), 103.8 (C-10), 121.8 (C-1′), 108.5 (C-2′, C-6′, C-2‴, C-6‴), 145.2 (C-3′, C-5′, C-3‴, C-5‴), 136.6 (C-4′), 101.1 (C-1″), 73.8 (C-2″), 69.7 (C-4″), 76.3 (C-5″), 63.7 (C-6″) 120.0 (C-1‴), 74.4 (C-3″), 138.4 (C-4‴), 165.7 (C-7‴). ^{1}H and ^{13}C NMR data were identical to those reported in literature.[19]

Isolation of rat hepatocytes

Rats were anaesthetized with chloral hydrate injected intraperitoneally at a dose of 360 mg/kg. Rat hepatocytes were isolated using the collagenase perfusion technique as described by Berry and Friend[20] with slight modifications as published by Figliomeni and Abdel-Rahman.[21] Briefly, the abdominal cavity was opened, the liver was exposed, and the portal vein was cannulated. The perfusion rate was started with Ca^{2+}-free perfusion buffer A (composition (g%): NaCl 0.8, KCl 0.04, $MgSO_4$ 0.01, Na_2HPO_4 0.0047, KH_2PO_4 0.006, $NaHCO_3$ 0.21 and glucose 0.1) (pH 7.4) at a flow rate of 10 ml/min and was increased slowly to reach 30 ml/min, and was maintained for 8 min to remove blood. Then the perfusion was switched to buffer B, which contained collagenase and calcium chloride (composition (g%): NaCl 0.8, KCl 0.04, $MgSO_4$ 0.01, Na_2HPO_4 0.0047, KH_2PO_4 0.006, $NaHCO_3$ 0.21, glucose 0.1, collagenase 0.067 and bovine albumin 0.5), and 200 μl of 1 M $CaCl_2$ was added. This buffer was perfused for 15–30 min at a flow rate of 15 ml/min. Hepatocytes were obtained after mechanical disruption of the liver, filtration and cold centrifugation (4°C), 600 rev/min for 2 min (twice), and suspended in Krebs–Henseleit buffer, pH 7.4 (composition (g%): NaCl 0.69, KCl 0.035, KH_2PO_4 0.016, $NaHCO_3$ 0.21, $MgSO_4$ 0.014, glucose 0.09, HEPES 0.5, bovine albumin 0.5 and 0.45 ml of 1 M $CaCl_2$). Cell viability was routinely tested with 0.4% trypan blue (TB) exclusion. A yield of 200–300 million cells per liver with viability greater than 85% was used.

Experimental protocol for evaluation of hepatoprotective activity

Optimization of the experimental conditions

Freshly prepared rat isolated hepatocytes were suspended in Krebs–Henseleit buffer (pH 7.4), at a concentration of 5×10^6 cells/ml. The suspended cells were divided into five groups (six replicates/group) in plastic containers (each containing 2 ml of the suspended cells). Isolated hepatocytes were incubated at 37°C in a shaking water bath. Three groups were incubated with different concentrations of RE (10, 100 and 1000 μg/ml), dissolved in DMSO, for 30 min. After the incubation time was over, CCl_4 (dissolved in DMSO) was added to RE groups and to a fourth group (CCl_4 group), so that the final concentration of CCl_4 was 5 mM. A fifth group, that served as a control, received only DMSO. Incubations were continued for 2 h after CCl_4 exposure. The final concentration of DMSO was 0.1%. Samples of cell suspensions from all replicates were collected at 5, 30, 60 and 120 min after CCl_4 addition. Each sample was divided into two parts, one was used for the determination of cell viability using the TB exclusion test, and the other was used for the determination of GSH level.

Potential hepatoprotective activity of aqueous ethanolic extract of Rubus sanctus

Based on the results obtained from the optimization experiment, a concentration of 100 μg/ml of RE was selected for evaluation of the hepatoprotective activity of RE using silymarin as a standard. Freshly prepared isolated rat hepatocytes were suspended in Krebs–Henseleit buffer (pH 7.4), at a concentration of 5×10^6 cells/ml. The isolated hepatocytes were incubated at 37°C in a shaking water bath. The suspended cells were divided into five groups (six replicates/group) in plastic containers (each containing 2 ml of the suspended cells). The first group received only CCl_4 (5 mM). The second group (RE100+CCl_4) was preincubated with RE at concentration 100 μg/ml for 30 min, followed by CCl_4 (5 mM). The third group (S+CCl_4) was preincubated with silymarin at concentration 100 μM for 30 min, followed by CCl_4 (5 mM). The fourth group (RE100) received RE only

at concentration 100 μg/ml and the fifth group (control group) received only DMSO. Samples of cell suspensions from all replicates were collected at 5, 30, 60 and 120 min after CCl_4 addition. The following parameters were assessed to evaluate the hepatoprotective activity of RE: TB exclusion and leakage of intracellular enzymes (LDH, ALT and AST), GSH and lipid peroxides level.

Viability testing

The viability percentage (%) of hepatocytes was assessed by the method of Baur *et al*.[22] Samples of cell suspension were collected from all replicates at different time intervals (0–5, 30, 60 and 120 min). Samples (10 μl) of hepatocytes were taken from each replicate. The same volume of 0.4% TB was added to the hepatocytes. Each sample was immediately mixed, placed on a slide, covered by cover glass and examined under a light microscope. Three fields were read from each slide for differentiation between viable and dead cells. Viability was calculated using the following equation:

$$\text{Viability } \% = (\text{No. of viable cells}/\text{No. of total cells}) \times 100 \quad (1)$$

Determination of enzyme leakage

Enzyme activity in cells, as well as in the incubation media, was assayed spectrophotometrically according to Moldeus *et al*.[23] Two samples of cell suspension (200 μl each) were collected from all replicates during the time-course studies at 5, 30, 60 and 120 min. One sample was centrifuged at 2000 rev/min (4°C) for 15 min. The supernatant was used for determination of the leakage of ALT, AST and LDH.[24,25] The other sample was centrifuged at 2000 rev/min (4°C) for 15 min, after addition of 200 μl of 1% Triton X, to obtain the lysate. The lysate was used for the determination of total enzyme activity in the cells. Enzyme leakage was expressed as a percentage of total enzyme content of hepatocytes assayed after complete cell lysis with Triton X. Enzyme leakage % was calculated using the following equation:

$$\text{Enzyme leakage } \% = (\text{supernatant absorbance}/\text{lysate absorbance}) \times 100 \quad (2)$$

Determination of reduced glutathione

Reduced glutathione (GSH) was determined spectrophotometrically according to Ellman's method.[26] A sample (0.5 ml) of cell suspension was collected from all replicates during the time-course studies at 5, 30, 60 and 120 min. Precipitation of protein thiols by TCA was carried out, and samples were centrifuged at 3000 rev/min for 10 min. The resulting supernatant was used for determination of GSH using Ellman's reagent. The absorbance was measured at 412 nm.

Assay of lipid peroxides

Lipid peroxides were determined spectrophotometrically as thiobarbituric acid-reactive substances (TBARS) according to the method of Mihara and Uchiyama.[27] The colorimetric determination of TBARS was based on the reaction of malondialdehyde (MDA) with TBA at low pH, and at high temperature. The resultant pink-coloured product was extracted by *n*-butanol, and the absorbance was determined spectrophotometrically at 535 nm.

Statistical analysis

Results are reported as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance. If the overall *P*-value was found statistically significant ($P < 0.05$), further comparisons among groups were made according to post-hoc Tukey's test. All statistical analyses were performed using GraphPad InStat 3 software (GraphPad Software, Inc., La Jolla, CA, USA). Graphs were sketched using GraphPad Prism version 4 software (GraphPad Software, Inc.).

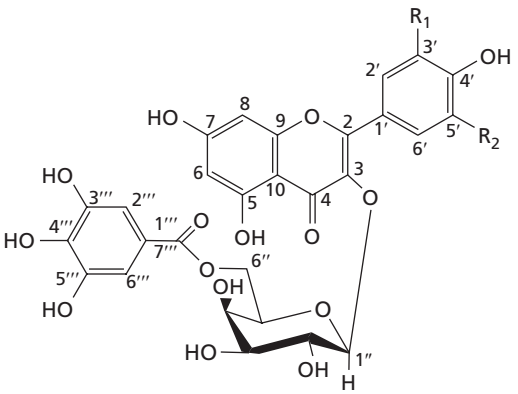
Results

Isolation of galloylated flavonoids

Our phytochemical studies indicate the presence of galloyated flavonoids in the aqueous ethanolic extract of *Rubus sanctus* (RE). We report for the first time the isolation and structure elucidation of three galloyated flavonoids, namely: kaempferol-3-*O*-(6″-*O*-galloyl)-$^4C_1$-β-D-galactopyranoside (**1**), quercetin-3-*O*-(6″-*O*-galloyl)-$^4C_1$-β-D-galactopyranoside (**2**) and myricetin-3-*O*-(6″-*O*-galloyl)-$^4C_1$-β-D-galactopyranoside (**3**) from *Rubus sanctus* (Figure 1).

Optimization of conditions for rat hepatocyte experimentation

The hepatoprotective activity of RE was examined using three different concentrations of RE (10, 100 and 1000 μg/ml). CCl_4 (5 mM) induced a significant decrease in the viability of isolated rat hepatocytes starting at 5 min, and reaching about 50% decrease at 120 min after CCl_4 exposure as compared with the corresponding control group (Table 1). In addition, CCl_4 induced a significant GSH depletion starting at 30 min, and reaching 80% at 120 min (Table 1). Pretreatment of hepatocytes with RE at concentrations of 10, 100 or 1000 μg/ml showed a significant protective effect against CCl_4-induced cytotoxicity and GSH depletion. Regarding GSH depletion, it is noteworthy that RE at a concentration of 10 μg/ml showed protection only after 120 min of CCl_4 exposure, while RE at



$R_1 = H$, $R_2 = H$ **(1)**
$R_1 = OH$, $R_2 = H$ **(2)**
$R_1 = OH$, $R_2 = OH$ **(3)**

Figure 1 Structures of isolated compounds (**1**–**3**)

Table 1 Effect of different concentrations of *Rubus sanctus* aqueous alcoholic extract on CCl_4 cytotoxicity in rat isolated hepatocytes

Time (min)	Viability (%)				
	Control	CCl_4	RE 10 μg/ml + CCl_4	RE 100 μg/ml + CCl_4	RE 1000 μg/ml + CCl_4
5	80.1 ± 1.4	53.7 ± 2.5*	74.4 ± 4.0$^{\#}$	73.1 ± 1.9$^{\#}$	70.9 ± 4.0$^{\#}$
30	78.5 ± 1.9	49.0 ± 4.3*	72.0 ± 3.6$^{\#}$	69.4 ± 2.8$^{\#\#}$	68.8 ± 1.8$^{\#}$
60	76.1 ± 3.0	44.0 ± 3.8*	69.1 ± 1.4$^{\#}$	65.7 ± 0.9$^{\#\#}$	66.7 ± 1.7$^{\#}$
120	73.0 ± 2.0	36.7 ± 2.8*	63.1 ± 0.6$^{\#}$	62.2 ± 2.8$^{\#\#}$	59.0 ± 4.7*$^{\#}$

RE, *Rubus sanctus* aqueous alcoholic extract. Values are expressed as means SEM, $n = 6$. *$P < 0.05$, compared with corresponding control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with corresponding CCl_4 group.

concentrations of 100 and 1000 μg/ml showed protection starting from 30 min after CCl_4 exposure, which was maintained thereafter. It was also found that protection conferred by RE at a concentration of 100 and 1000 μg/ml was statistically significant compared with the protection observed at a concentration of 10 μg/ml. However, protection provided by RE at concentration 100 μg/ml was not statistically different from that observed at a concentration of 1000 μg/ml. Therefore, RE at a concentration of 100 μg/ml was selected for subsequent studies (Tables 1 and 2).

Effect of aqueous ethanolic extract of *Rubus sanctus* (100 μg/ml) on CCl_4-induced cytotoxicity

Pretreatment with RE at a concentration of 100 μg/ml showed a significant protective effect against CCl_4-induced cytotoxicity, reaching about 42% at 120 min as compared with the group treated with CCl_4 alone. No statistically significant difference was observed between the protective effects of RE and silymarin. No significant difference was observed between groups treated with RE alone and the control (Table 3).

Effect of aqueous ethanolic extract of *Rubus sanctus* (100 μg/ml) on CCl_4-induced leakage of cellular enzymes in isolated rat hepatocytes

It was found that CCl_4 induced a statistically significant increase in leakage of the assessed enzymes – LDH, ALT and AST – as compared with the control group. Pretreatment with RE could ameliorate CCl_4-induced enzyme leakage. A significant difference was observed between RE and silymarin at different time intervals. No significant difference was observed between RE alone and the control group (Tables 3 and 4).

Effect of aqueous ethanolic extract of *Rubus sanctus* (100 μg/ml) on CCl_4-induced GSH depletion and TBARS formation in isolated rat hepatocytes

It was found that pretreatment with RE at a concentration of 100 μg/ml counteracted GSH depletion induced by CCl_4. A marked protection was shown starting at 30 min after incubation (40%) and maintained throughout the experiment at 60 and 120 min (58% and 83%, respectively), as compared with the CCl_4-treated group. Similarly, preincubation of hepatocytes with silymarin (100 μM) produced a significant protection against CCl_4. No statistically significant difference was observed between RE and silymarin (Figure 2a). On the other hand, CCl_4 (5 mM) induced a significant increase in lipid peroxidation (TBARS content) of isolated rat hepatocytes compared with the control group, starting at 30 min after CCl_4 exposure. Pretreatment with RE 100 μg/ml significantly counteracted CCl_4-induced TBARS formation. A marked protection was shown starting from 30 min after incubation (60%) and this was maintained thereafter, reaching about 64% at 120 min. No statistically significant difference was observed between RE and silymarin pretreated groups. A significant difference was observed between TBARS formation of RE alone and RE+CCl_4 groups and that of control group. Similar results were observed with silymarin; a significant difference was observed between TBARS formation in the silymarin-pretreated group compared with that of the control group. Both RE and silymarin could prevent the spontaneous lipid peroxidation observed in the control group (Figure 2b).

Discussion

Rubus sanctus extract (RE) has formerly been shown by our groups to contain a complicated phenolic mixture.[3,4] In

Table 2 Effect of different concentrations of *Rubus sanctus* aqueous alcoholic extract on CCl_4-induced GSH depletion in rat isolated hepatocytes

Time (min)	GSH (μmol/5 × 10^6 cells)				
	Control	CCl_4	RE 10 μg/ml + CCl_4	RE 100 μg/ml + CCl_4	RE 1000 μg/ml + CCl_4
5	0.12 ± 0.010	0.11 ± 0.007	0.12 ± 0.008	0.13 ± 0.011	0.10 ± 0.009
30	0.1 ± 0.003	0.06 ± 0.004*	0.07 ± 0.004*	0.10 ± 0.004$^{\#\dagger}$	0.10 ± 0.008$^{\#\dagger}$
60	0.07 ± 0.007	0.03 ± 0.001*	0.05 ± 0.004*	0.07 ± 0.006$^{\#\dagger}$	0.07 ± 0.007$^{\#\dagger}$
120	0.06 ± 0.006	0.01 ± 0.001*	0.04 ± 0.002*$^{\#}$	0.06 ± 0.005$^{\#\dagger}$	0.06 ± 0.003$^{\#\dagger}$

GSH, reduced glutathione; RE, *Rubus sanctus* aqueous alcoholic extract. Values are expressed as means SEM, $n = 6$. *$P < 0.05$, compared with corresponding control group; $^{\#}P < 0.05$, compared with corresponding CCl_4 group; $^{\dagger}P < 0.05$, compared with corresponding RE 10 μg/ml + CCl_4 group.

Table 3 Effect of *Rubus sanctus* aqueous alcoholic extract (100 μg/ml) on CCl_4-induced cytotoxicity and LDH leakage from rat isolated hepatocytes

Time (min)	Control		CCl_4		RE100 + CCl_4		Silymarin + CCl_4		RE100 alone	
	Viability (%)	LDH leakage (%)	Viability (%)	LDH leakage (%)	Viability (%)	LDH leakage (%)	Viability (%)	LDH leakage (%)	Viability (%)	LDH leakage (%)
5	78.4 ± 1.7	18.2 ± 0.6	55.4 ± 3.9*	30.3 ± 0.9*	72.3 ± 2.6$^{\#}$	21.8 ± 0.9*$^{\#}$	72.8 ± 0.9$^{\#}$	21.5 ± 0.7$^{\#}$	73.8 ± 2.3	17.8 ± 0.9
30	78.0 ± 2.2	21.0 ± 0.8	49.0 ± 3.8*	53.3 ± 1.4*	69.7 ± 3.0$^{\#}$	30.5 ± 0.7*$^{\#}$	66.1 ± 2.4$^{\#}$	26.8 ± 1.1*$^{\#}$	70.8 ± 3.8	20.8 ± 0.8
60	75.8 ± 3.2	24.0 ± 0.8	43.5 ± 3.6*	73.8 ± 1.3*	63.3 ± 2.0$^{\#}$	44.5 ± 0.7*$^{\#\dagger}$	62.7 ± 1.1*$^{\#}$	40.0 ± 0.4*$^{\#}$	69.7 ± 4.5	24.5 ± 0.9
120	72.7 ± 2.3	28.5 ± 0.7	36.1 ± 2.9*	77.5 ± 0.7*	61.9 ± 3.0$^{\#}$	50.0 ± 0.4*$^{\#\dagger}$	59.0 ± 1.2*$^{\#}$	44.3 ± 0.9*$^{\#}$	63.1 ± 2.8	28.8 ± 0.5

LDH, lactate dehydrogenase; RE, *Rubus sanctus* aqueous alcoholic extract. Values are expressed as means ± SEM, $n = 6$. *$P < 0.05$, compared with corresponding control group; $^{\#}P < 0.05$, compared with corresponding CCl_4 group; $^{\dagger}P < 0.05$, compared with corresponding silymarin + CCl_4 group.

Table 4 Effect of *Rubus sanctus* aqueous alcoholic extract (100 μg/ml) on CCl_4-induced AST and ALT leakage from rat isolated hepatocytes

Time (min)	Control		CCl_4		RE 100 + CCl_4		Silymarin + CCl_4		RE 100 alone	
	AST leakage (%)	ALT leakage (%)	AST leakage (%)	ALT leakage (%)	AST leakage (%)	ALT leakage (%)	AST leakage (%)	ALT leakage (%)	AST leakage (%)	ALT leakage (%)
5	28.3 ± 0.6	23.3 ± 0.9	40.8 ± 0.8*	35.0 ± 0.6*	37.3 ± 1.3*	32.0 ± 0.7*†	35.5 ± 1.9*$^{\#}$	28.5 ± 0.7*$^{\#}$	28.5 ± 0.3	23.0 ± 0.9
30	29.0 ± 0.4	24.3 ± 1.1	55.3 ± 0.9*	43.8 ± 0.5*	48.0 ± 0.9*$^{\#}$	40.3 ± 0.3*	46.3 ± 0.9*$^{\#}$	36.5 ± 1.3*$^{\#}$	29.0 ± 0.4	25.0 ± 0.7
60	30.8 ± 0.5	24.5 ± 0.7	74.5 ± 1.2*	52.5 ± 0.3*	57.0 ± 0.9*$^{\#}$	46.8 ± 0.5*$^{\#\dagger}$	55.8 ± 1.1*$^{\#}$	42.8 ± 0.6*$^{\#}$	33.5 ± 0.7	25.3 ± 0.5
120	33.5 ± 0.7	26.8 ± 0.8	94.3 ± 1.3*	79.0 ± 0.9*	74.5 ± 0.7*$^{\#\dagger}$	59.0 ± 0.9*$^{\#\dagger}$	67.0 ± 1.8*$^{\#}$	54.8 ± 0.9*$^{\#}$	34.8 ± 0.6	27.8 ± 0.5

ALT, alanine aminotransferase; AST, aspartate aminotransferase; RE, *Rubus sanctus* aqueous alcoholic extract. Values are expressed as means ± SEM, $n = 6$. *$P < 0.05$, compared with corresponding control group; $^{\#}P < 0.05$, compared with corresponding CCl_4 group; $^{\dagger}P < 0.05$, compared with corresponding silymarin + CCl_4 group.

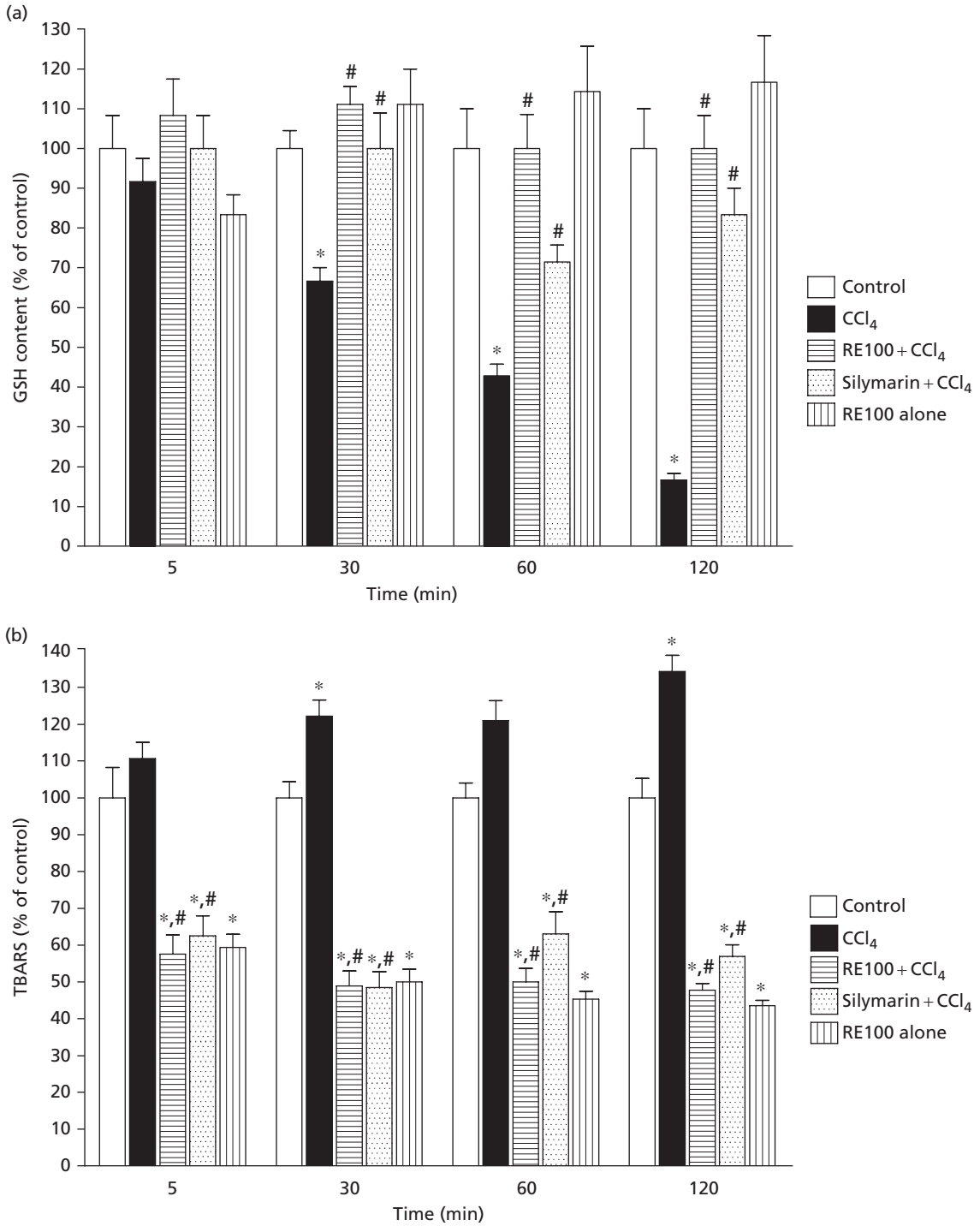


Figure 2 Effect of *Rubus sanctus* aqueous alcoholic extract (100 μg/ml) on CCl_4-induced GSH depletion (a) and CCl_4-induced TBARS formation (b) in rat isolated hepatocytes. GSH, reduced glutathione; RE, *Rubus sanctus* aqueous alcoholic extract; TBARS, thiobarbituric acid-reactive substances. Values are expressed as means ± SEM, $n = 6$. $^{*}P < 0.05$, compared with corresponding control group; $^{\#}P < 0.05$, compared with corresponding CCl_4 group.

continuation of these studies on the phenolic metabolites and biological effect of *Rubus,* this study describes the isolation and characterization of three minor galloylated flavonoids – kaempferol-3-*O*-(6″-*O*-galloyl)-$^4C_1$-*β*-D-galactopyranoside, quercetin-3-*O*-(6″-*O*-galloyl)-$^4C_1$-*β*-D-galactopyranoside and myricetin-3-*O*-(6″-*O*-galloyl)-$^4C_1$-*β*-D-galactopyranoside – from *Rubus sanctus* for the first time. Their structures were elucidated by conventional methods of analysis and spectroscopic analyses, including UV, ^{1}H NMR, ^{13}C NMR and ESI/MS.

Plants containing phenolic compounds have been proved to possess many pharmacological effects including hepatoprotective properties.[9,28] Although the use of CCl_4 has been restricted because of its distinct toxicity, it continues to provide an important service today as a model substance to induce acute liver injury.[29] Hepatocytes form the system of choice that has found ample application in the evaluation of cytotoxicity and genotoxicity of chemicals and drugs.[30,31] Thus, our study aimed at investigating the potential protective effects of the aqueous ethanolic extract of RE against CCl_4-induced toxicity in rat isolated hepatocytes. We used CCl_4 at a concentration of 5 mM to induce hepatotoxicity in rat isolated hepatocytes in accordance with several previous studies,[16,32] in addition to our preliminary results. Exposure of hepatocytes to CCl_4 adversely affected membrane integrity as assessed by decreased TB exclusion and increased enzyme (LDH, ALT and AST) leakage, starting at 5 min after CCl_4 exposure. Approximately 50% membrane damage was observed after 2 h of CCl_4 exposure. CCl_4 induced oxidative stress, as detected by depletion of GSH, and enhanced lipid peroxidation. It is generally believed that the toxicity of CCl_4 results from its reductive dehalogenation by the cytochrome P450 system into the highly reactive free radical, trichloromethyl ($CCl_3^{\cdot}$).[13] CCl_4 is also highly soluble in cell lipids and could possibly damage cells through direct solvent effects on cell membranes.[33] This would lead to leakage of intracellular enzymes and electrolytes within 3 min of exposure and could also contribute to mitochondrial toxicity.[34]

Study of the protective effects of RE was carried out in two parts. The first part aimed to optimize the experimental conditions, where cells were preincubated with various concentrations of RE (10, 100 and 1000 μg/ml) for 30 min before being subjected to CCl_4 (5 mM) for an additional 2 h. RE exhibited an observable protection at a concentration of 100 μg/ml, which was the concentration selected for subsequent in-vitro testing of the hepatoprotective activity of RE. Pretreatment with RE (100 μg/ml) showed a significant protection against CCl_4-induced cytotoxicity and diminished the enzymatic (LDH, AST and ALT) leakage. Also, RE significantly improved the oxidative status and counteracted the effect of CCl_4 on both GSH and lipid peroxides. Moreover, a significant difference was observed between the RE-pretreated group and the control group regarding lipid peroxides level. This indicates that RE could prevent the spontaneous lipid peroxidation observed in the control group.

The hepatoprotective activity of RE against CCl_4-induced toxicity may be explained in terms of the chemical composition of RE. RE contains a complicated polyphenolic mixture.[3,4] Polyphenolic compounds act as antioxidants by virtue of their chemical structure. The most important pathway is through their ability to break the free radical chain reaction, and their action as reducing agents, hydrogen atom-donors and singlet oxygen quenchers. Some polyphenols also act as antioxidants by their metal ion chelation properties, thereby reducing the metal's capacity to generate free radicals.[35] Also, it was shown that dietary plant polyphenols, namely the flavonoids, increase expression of gamma-glutamylcysteine synthetase, with a concomitant increase in the intracellular GSH concentrations.[36] GSH content in the liver plays a primary role in the protection against $CCl_3^{\cdot}$-induced liver damage. Accordingly, restoration of GSH levels by the polyphenolic content of RE might be related to its antioxidant/antihepatotoxic activity.[37] The polyphenolic content of RE includes flavonoids such as quercetin derivatives and kaempferol derivatives. Flavonoids exert a membrane-stabilizing action, thus inhibiting lipid peroxidation and cell death. These effects are in agreement with the suggested action of the flavonoids as cytoprotective agents and have been ascribed to the antioxidant properties of the flavonoids, since spontaneous or induced oxidative stress could damage cell membranes.[38] Antioxidant activity is not the only biologically important aspect of flavonoids. They are also likely to influence xenobiotic-metabolizing enzymes, as well as, antioxidant enzymes and DNA repair pathways.[39] The ubiquitous flavonoid quercetin was found to be a powerful inhibitor of iron-induced lipid peroxidation in rat liver microsomes.[38] Quercetin is one of a number of antioxidants that have been shown to reduce the high level of chromosomal aberrations found in cases of viral hepatitis.[40] Quercetin showed a strong activity on xanthine oxidase inhibition.[41] The level of xanthine oxidase is high in patients with hepatitis and mild hepatic intoxification, therefore it is suggested that selected flavonoids might be useful in treating these disorders.[42,43] Kampferol-containing extracts were also reported to show hepatoprotective activity.[44–46] In addition to flavonoids, RE contains ellagitannins and caffeic acid derivatives. Regarding ellagitannins, one of the notable effects of tannins is their antioxidant activity.[47] In addition, ellagitannins may hydrolyse to yield ellagic acid, which offers considerable promise as an antioxidant.[48,49] RE contains several ellagitannins, including sanguiin H-4 and sanguiin H-6. Sanguiin H-6 has been reported to be a major contributor to the antioxidant capacity of some *Rubus* species.[50] It has been reported that sanguiin H-6 protects against oxidative damage in mitochondria, inhibits apoptosis *in vivo*, inhibits nitric oxide production and improves cell viability.[51,52] The caffeic acid derivatives contained in RE may contribute to its hepatoprotective activity as well. A considerable amount of experimental data on the antioxidant activity of caffeic acid is available.[53] Caffeic acid has shown a potent inhibition of in-vitro lipid peroxidation and protection to hepatocytes challenged with CCl_4.[54,55] Oral administration of caffeic acids inhibited the elevation of serum lipid peroxides, as well as liver peroxides, in rats treated with CCl_4.[55]

Conclusions

In conclusion, three galloylated flavonoids were isolated for the first time from the genus *Rubus*. These were kaempferol-3-*O*-(6″-*O*-galloyl)-$^4C_1$-β-D-galactopyranoside, quercetin-3-*O*-(6″-*O*-galloyl)-$^4C_1$-β-D-galactopyranoside and myricetin-3-*O*-(6″-*O*-galloyl)-$^4C_1$-β-D-galactopyranoside. Further, aqueous ethanolic extract of *Rubus sanctus* (RE) demonstrated a hepatoprotective activity against CCl_4-induced toxicity in rat isolated hepatocytes, as demonstrated by improved cell survival and maintenance of cell membrane integrity. This is, at least partly, attributed to its antioxidant

activity shown by preservation of cellular GSH and inhibition of lipid peroxidation. The observed protection was comparable with that of silymarin. Further studies are recommended to evaluate the hepatoprotective activity of individual components of RE.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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