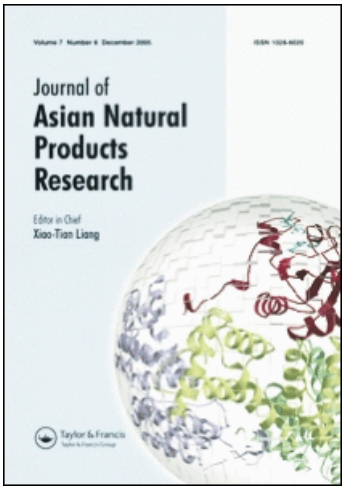

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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Online publication date: 19 January 2011

To cite this Article Sun, Zhen-Liang , Zhang, Yang , Wan, Ai-Hong , Zhang, Xue-Li and Feng, Jing(2011) 'A new active compound against kidney deficiency from the fruits of *Rubus corchorifolius*', Journal of Asian Natural Products Research, 13: 1, 68 — 74
To link to this Article: DOI: 10.1080/10286020.2010.541156
URL: http://dx.doi.org/10.1080/10286020.2010.541156

Journal of Asian Natural Products Research
Vol. 13, No. 1, January 2011, 68–74

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A new active compound against kidney deficiency from the fruits of *Rubus corchorifolius*

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(*Received 11 August 2010; final version received 15 November 2010*)

Constituents of the fruits of *Rubus corchorifolius* were investigated. A new compound, namely rubusin A (**1**), along with three known compounds, was isolated and characterized. Among them, the new compound exhibited significant activity against kidney deficiency, and quercetin and kaempferol were isolated from the fruits of *R. corchorifolius* for the first time.

Keywords: Rosaceae; *Rubus corchorifolius*; kidney deficiency; rubusin A

1. Introduction

Rubus corchorifolius (Rosaceae) is mainly distributed in the northern and southeastern parts of China. The unripe fruits of *R. corchorifolius*, commonly known as 'Ci-Hu-Lou' in Chinese, have been used as a food and a tonic agent in traditional Chinese medicine for the treatment of impotence and seminal emission for thousands of years in China [1–4]. Modern pharmacological experiments suggest that it has many biological activities such as improving sexual function [5,6], antioxidation [7], antimutagenesis [8], and immunomodulatory properties [9].

A phytochemical investigation of *R. corchorifolius* resulted in the isolation of pentacyclic triterpene acids and flavonoids [10–13]. To further understand the active components of *R. corchorifolius*, a pharmacological research and a phytochemical study of the active part were carried out. Here, we report our work about the isolation and characterization of a new compound and three known compounds from the *n*-BuOH extract of *R. corchorifolius* as a part of our ongoing research on this topic. Their structures were established with the aid of extensive NMR spectroscopic studies and chemical analysis. The protective effects of the new compound against the kidney deficiency in mice were tested as well.

2. Results and discussion

Compound **1**, obtained as yellow amorphous powder, was positive to ferric chloride solution. The molecular formula was established as $C_{12}H_8O_6$ based on the $[M + Na]^+$ ion at *m/z* 271.0218 in the positive-ion HR-FAB mass spectrum, as well as on the $[M - H]^-$ ion at *m/z* 247 in the negative-ion ESI mass spectrum. The absorption bands at 3404, 1684, 1599 cm^{-1} in the IR spectrum and the absorption maxima at 364 (4.01), 299 (4.45), 200 (4.25) nm in the UV spectrum showed the existence of a coumarin skeleton.

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ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020.2010.541156
http://www.informaworld.com

There were 12 carbon signals in ^{13}C NMR spectrum, among which two carbons at δ 160.5 and 195.5 were assigned to the carbonyl carbons, and two carbons at δ 108.0 and 140.1 to the olefinic carbons. The presence of the 3,5,8-OH groups was indicated by signals at δ 140.1, 144.2, 145.0 in the downfield region of the ^{13}C NMR spectrum. The ^{1}H NMR spectrum indicated that compound **1** contained four methylene protons at δ 2.52 and 3.51 (each 2H, m). Correspondingly, two methylene carbons at δ 33.0 and 23.8 were exhibited in the ^{13}C NMR spectrum (Table 1). A feature of the ^{1}H NMR spectrum was the olefinic proton signal at δ 8.01 (1H, s), the typical signal of H-4 for coumarin parent nucleus. In HMBC spectrum, the olefinic proton at δ 8.01 (H-4) showed long-range correlation with the carbonyl carbon at δ 160.5 (C-2), while methylene protons at δ 2.52 (H-12) and 3.51 (H-13) showed correlations with the carbonyl carbon at δ 195.5 (C-11). Complete assignment was achieved by studying the results of HMQC, HMBC, and NOESY experiment. Consequently, the structure of **1** was determined as 3,5,9-trihydroxy-7,8-dihydrocyclopenta[g]chromene-2,6-dione, named rubusin A (Figure 1).

Table 1. ^{1}H NMR, ^{13}C NMR, and HMBC spectral data of compound **1** (DMSO-d_6, δ ppm).

Position	δ_C	δ_H	HMBC (H → C)
1			
2	160.5		
3	140.1		
4	108.0	8.01 (1H, s)	2, 3, 9, 10
5	144.2		
6	115.5		
7	141.1		
8	145.0		
9	149.3		
10	113.2		
11	195.5		
12	33.0	2.52 (2H, m)	11, 13
13	23.8	3.51 (2H, m)	7, 11, 12

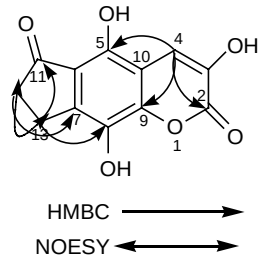

Figure 1. The main HMBC and NOESY correlations of compound **1**.

Additionally, kaempferol (**2**), quercetin (**4**), and compound **3** (Figure 2) were also identified by comparing their ^{1}H and ^{13}C NMR spectral data with those reported in literature [9,12,13].

Compound **1** was examined for its activities against kidney deficiency. The level of total testosterone in the serum was measured. The concentration of testosterone of each group was presented as mean ± SD. The concentration of testosterone decreased obviously in the model group as compared to the control group ($P < 0.01$). After administration for 15 days, the concentration of testosterone increased significantly as compared to the model group within a certain range of 10.0–40.0 mg/kg (see Table 2).

All histopathological sections were observed under light microscope (400 ×). The normal kidney histological sections showed that kidney cells were large and the cytoplasm was abundant (Figure 3(F)). Compared with the normal group, kidney tissue in the mice of model group revealed extensive kidney injuries, characterized by diminutioned kidney cells and the reduced cytoplasm (Figure 3(E)). However, the histopathological changes of kidney deficiency induced by hydrocortisone were remarkably ameliorated by compound **1** (Figure 3(A–C)).

The normal testicle histological sections showed that every developmental stage of spermatogenic cells is arranged regularly, and spermatogonium, primary

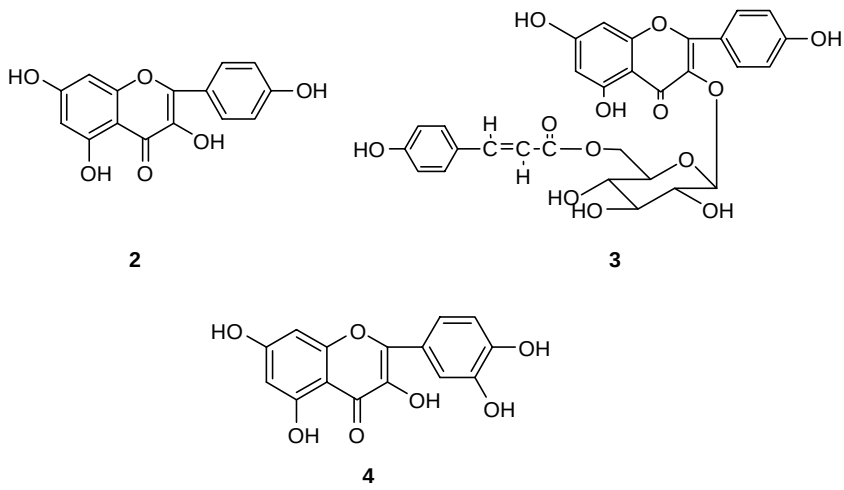

Figure 2. Structures of compounds **2**–**4** from fruits of *R. corchorifolius*.

spermatocytes, secondary spermatocytes, and sperm cells were visible (Figure 4(F)). Compared with the normal group, the testicle tissue in the model group revealed extensive testicle injuries, characterized by every developmental stage of reduced spermatogenic cells (Figure 4(E)). The tissues treated with compound **1** and Jinkui Shenqi Wan were comparable to the normal tissues, and groups II and III were more close to group IV than group I (Figure 4(A–D)). The results indicated that hydrocortisone could reduce the amount of spermatogenic cells in mice, and compound **1** could ameliorate the changes induced by hydrocortisone.

Both the kidney and testicle histopathological changes were in good agreement with the results of the concentration of testosterone in serum. In conclusion, the results indicated that the oral administration of compound **1** showed conspicuous prophylaxis of kidney deficiency induced by hydrocortisone.

3. Experimental

3.1 General experimental procedures

The melting points were measured on an X-4 digital melting point apparatus without correction. The UV spectra were measured with a Shimadzu multipurpose recording spectrometer MPS-5000 (Shanghai, China). The IR spectra were obtained on a Nicolet 470 spectrometer (San Francisco, CA, USA). ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA-600 spectrometer (San Francisco, CA, USA), with tetramethylsilane as an internal standard. ESI-MS and HR-FAB-MS were measured on the Varian MAT-212 mass spectrometer and Micromass Q-TOF mass spectrometer (San Francisco, CA, USA), respectively. Silica gel (100–200 mesh, 200–300 mesh, Qingdao Haiyang Chemical Co., Ltd., Qingdao, China), and Sephadex LH-20 (Amersham Pharmacia Biotech, Ltd., Shanghai, China) column chromatography and semipreparative HPLC (Alltech, CA, USA) were used for separation. Absorbance value was measured by a Microplate Reader 680 (Bio-Rad Laboratories, Inc., San Francisco, CA, USA) to calculate the inhibition rate. Analytical grade solvents were produced by the Beijing Chemical Factory (Beijing, China).

3.2 Plant material

Dry fruits of *R. corchorifolius* were purchased from Hangzhou, Zhejiang province of China and identified by

Table 2. The concentration of testosterone in serum.

Group	Group I	Group II	Group III	Group IV	Group V	Group VI
Concentration (ng/ml)	5.39 ± 1.41	8.32 ± 2.45*	10.33 ± 2.08**	11.11 ± 2.36**	5.56 ± 1.41	11.68 ± 2.60**

Notes: *$P < 0.05$, **$P < 0.01$, compared to group V.

Associate Professor Chang-Zheng Zhou, School of Pharmacy, Shandong University of Traditional Chinese Medicine. A voucher specimen (No. 0529) has been deposited at the herbarium of Shandong University of Traditional Chinese Medicine.

3.3 Extraction and isolation

The air-dried and powdered fruits (10 kg) were extracted under reflux with 95% EtOH for 3 h and then 50% EtOH for 1 h successively. The combined extract solution was filtered and evaporated *in vacuo* to afford an ethanol extract (2200 g). The extract was suspended in water (2000 ml) and partitioned with petroleum ether, EtOAc, and *n*-BuOH five times, successively. The *n*-BuOH extract (369 g) was subjected to column chromatography packed with D101 resin and eluted with H_2O, 30% EtOH, 60% EtOH, 95% EtOH, to give four fractions. The H_2O fraction (40 g) was subjected to column chromatography packed with silica gel and gradiently eluted with mixed CH_2Cl_2–MeOH (30:1, 20:1, 10:1, 5:1, 3:1, 1:1 and 0:1) to give seven fractions (Fractions 1–7). Fraction 2 (102 mg) and Fraction 3 (197 mg) were loaded on a Sephadex LH-20 gel column with methanol to yield **1** (15 mg) and **2** (50 mg), respectively. The 30% EtOH fraction was chromatographed on a silica gel column eluted with a gradient of CH_2Cl_2–MeOH (40:1 to 1:1) to afford five subfractions; and the subfraction 2 (206 mg) was further purified on a Sephadex LH-20 gel column using methanol to obtain **2** (60 mg). The precipitation of subfraction 3 (211 mg) was washed by methanol six times and **3** (16 mg) was obtained. Subfraction 4 (81 mg) was further purified on a Sephadex LH-20 gel column twice with methanol to yield **4** (15 mg).

*Compound **1***. Yellow powder; m.p.: 267–269°C; UV (EtOH): λ_{max} (log ε) 364 (4.01), 299 (4.45), 200 (4.25) nm; IR (KBr): ν_{max} 3404, 1684, 1599, 1305 cm^{-1}; ^1H NMR (C_5D_5N, 600 MHz) and ^{13}C

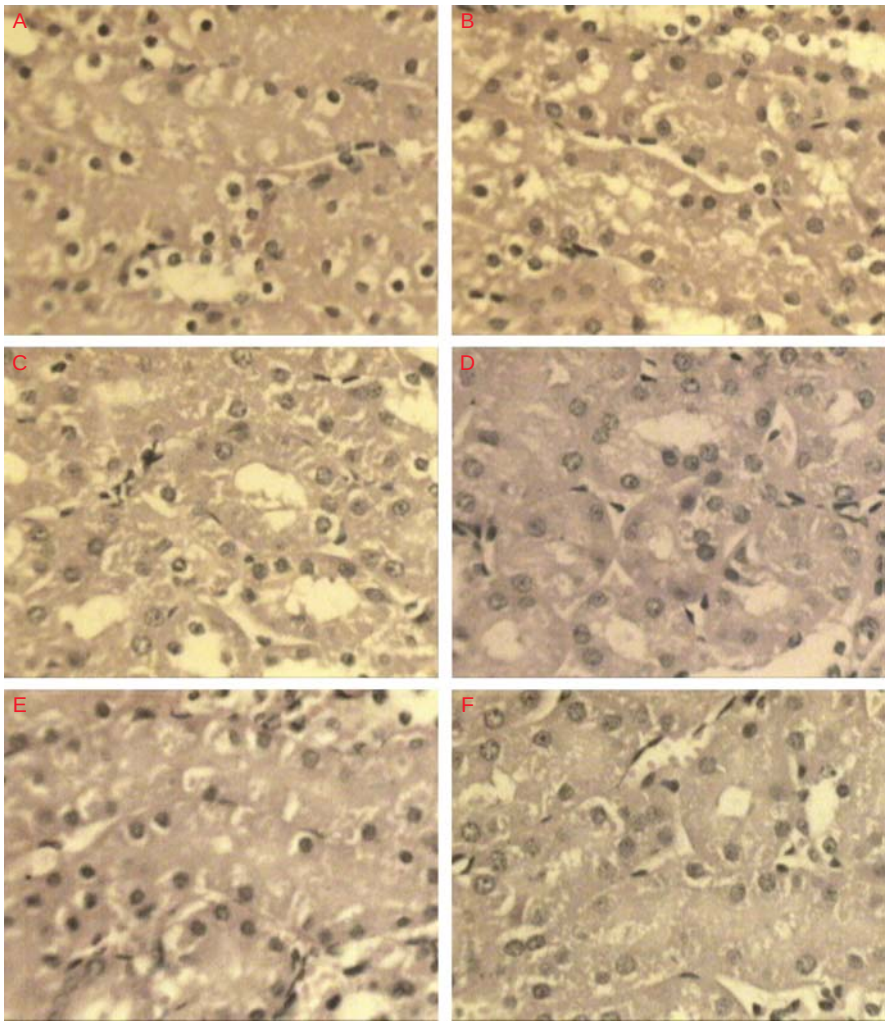

Figure 3. Kidney cells in mouse. A–F correspond to groups I–VI. (A) Treated with hydrocortisone + 10 mg/kg of compound **1**. (B) Treated with hydrocortisone + 20 mg/kg of compound **1**. (C) Treated with hydrocortisone + 40 mg/kg of compound **1**. (D) Treated with hydrocortisone + 6 g/kg of Jinkui Shenqi Wan. (E) Treated with 25 mg/kg of hydrocortisone. (F) Normal group.

NMR (C_5D_5N, 100 MHz) spectral data: see Table 1. EI-MS *m/z*: 248 $[M]^+$ (100), 164 (43). HR-ESI-MS: *m/z* 271.0218 $[M + Na]^+$ (calcd for $C_{12}H_8O_6Na$, 271.0219).

3.4 Prevention effect on hydrocortisone-induced kidney deficiency in mice

Sixty Kunming mice were randomized to six groups (10 for each), and they were given an intraperitoneal injection of 25 mg/kg of hydrocortisone for 10 days except for normal control group [14]. After this treatment, we considered the mice as a model for kidney deficiency. The normal group was injected with an equal volume of physiological saline. After 10 days, six groups were treated as follows: three test groups (groups I, II, III) of compound **1** with doses of 10.0, 20.0, and 40.0 mg/kg, respectively; Jinkui Shenqi Wan positive group (group IV) with 6 g/kg of Jinkui Shenqi Wan; model group (group V) and

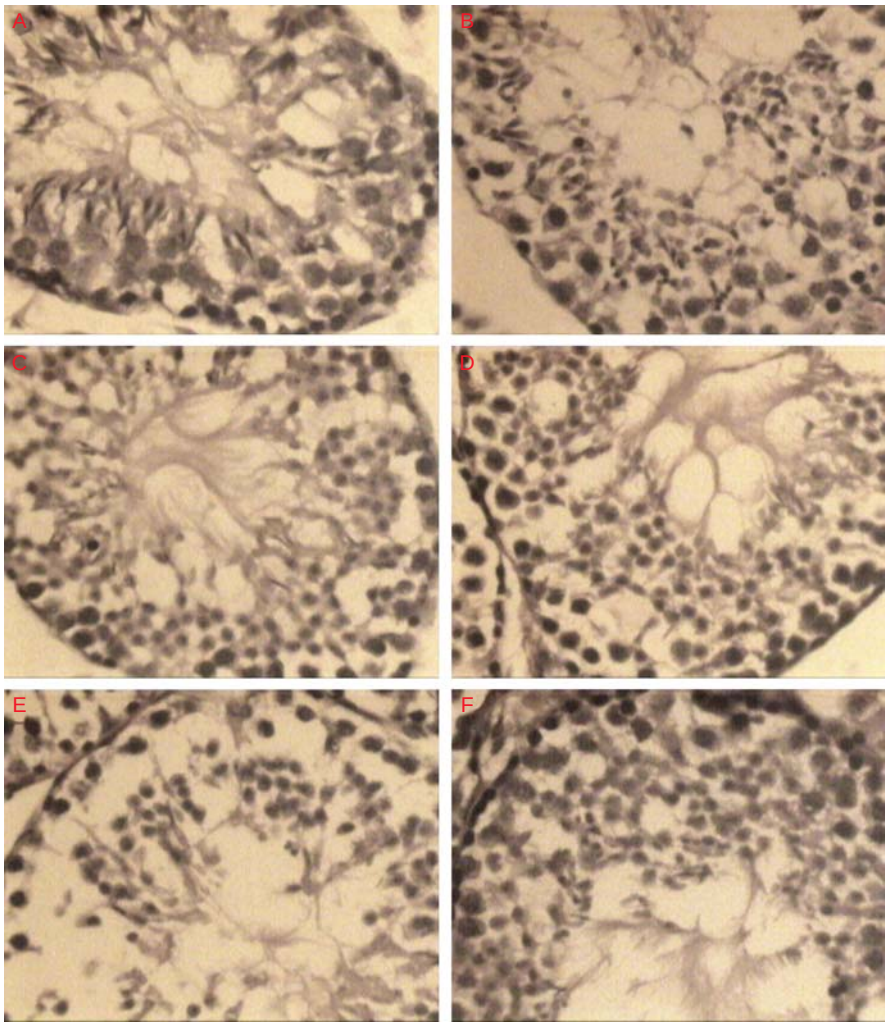

Figure 4. Testicle cells in mouse. A–F correspond to groups I–VI. (A) Treated with hydrocortisone + 10 mg/kg of compound **1**. (B) Treated with hydrocortisone + 20 mg/kg of compound **1**. (C) Treated with hydrocortisone + 40 mg/kg of compound **1**. (D) Treated with hydrocortisone + 6 g/kg of Jinkui Shenqi Wan. (E) Treated with 25 mg/kg of hydrocortisone. (F) Normal group.

normal group (group VI) with equal volume of water. Oral administration was done for 15 days. On day 15, the blood was drawn from all groups via the ophthalmic venous plexus, and centrifuged to give serum that was then stored at −20°C. The serum testosterone levels were determined by the Testosterone kit according to the manufactures' instructions. The kidney and the testicle samples were collected, and fixed in 10% formalin for the histopathological examinations. Then, they were processed routinely, embedded in paraffin, sectioned to 4 μm thickness, deparaffinized, rehydrated using standard techniques, and stained with hematoxylin and eosin (H&E, Shanghai, China). The extent of hydrocortisone-induced kidney deficiency was evaluated by assessing

morphological changes in kidney sections and testicle sections [15].

Acknowledgements

We are grateful to Prof. Chang-Zheng Zhou, College of Pharmacy, Shandong University of Traditional Chinese Medicine for his help during the performance of this work.

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