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Hepatoprotective effects of *Rubus aleaefolius* Poir. and identification of its active constituents

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ABSTRACT

The purpose of this study was to study the hepatoprotective effects of the most promising extract of the root from *Rubus aleaefolius* Poir. and to isolate and identify the active components. Various crude forms of *Rubus aleaefolius* have been evaluated for their effects on CCl_4-induced acute liver injury in mice vivo experimental model. Treatment groups contained 5 sub-groups that were ethanol crude extract; the high/low dosage ethyl acetate or n-butanol fraction; extracted with ethyl acetate or n-butanol after the residues and major constituent; intragastrically administrated with 35 mg/kg; 35, 4.6 mg/kg; 35, 5.8 mg/kg; 35 mg/kg and 3.5 mg/kg for 7 days. The serum samples were collected for biological analysis and also carried out histopathological studies.

The low-dosage ethyl acetate fraction was the most active when the fractions were compared. It was found to decrease AST, ALT; to prevent formation of hepatic MDA, NO and intensify the activity of SOD. The histopathological changes induced by CCl_4 were also significantly reduced. The separation revealed the presence of six constituents by a bioassay-guided fractionation, β-Sitosterol (1), 1β-Hydroxyeuscaphic acid (2), Oleanolic acid (3), Myrianthic acid (4), Euscaphic acid (5), and Tomentic acid (6). Among them, compounds 2, 4, 5 in *Rubus aleaefolius* root is reported here for the first time. 1β-Hydroxyeuscaphic acid (major constituent) showed a tremendous activity and the results confirm the traditional uses of *Rubus aleaefolius* in treating hepatitis.

1. Introduction

Rubus is widely distributed in Yunnan, Sichuan, Guizhou, Guangxi, Guangdong, and Fujian Provinces of China (Sun et al., 2004). *Rubus aleaefolius* Poir. (Rosaceae) is a folk medicinal herb. What the leaves and roots of *Rubus aleaefolius* can do invigorate the blood, clear heat and stop pain. It is traditionally used in treating liver and splenomegaly, mastitis, as well as stomatitis. Decoction of roots used by Anxi native who live in the mountainous regions of Fujian Province, against various types of chronic and acute hepatitis. Several compounds have been isolated from different parts of *Rubus aleaefolius*, e.g. triterpenoids (Gan et al., 1998), glycosides (Gan et al., 2000) and hydrolysable tannins (Zhao et al., 2001). Although *Rubus aleaefolius* in therapy suggests that they may indeed be effective, the lack of a scientific basis for their effectiveness remains an obstacle. No systematic study has been carried out on the plant to establish the chemical principle responsible for the hepatoprotective activities. The decoctions (Hong et al., 2005; Ye et al., 2005), ethanol extract (Zhou et al., 2007) and polysaccharide (Chen et al., 2006) of the root from *Rubus aleaefolius* improve liver functions by our previous studies support. In this report we describe the chemical composition of ethyl acetate active faction isolated from the roots of *Rubus aleaefolius* and the major constituent anti-liver damage activity.

2. Materials and methods

2.1. Analytical instruments

Optical rotations were determined by using a PerkinElmer 341 polarimeter. IR spectra were measured on a Nicolet NEXUS-670 FT-IR spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer. Chemical shifts are expressed in δ (ppm). ^1H NMR chemical shifts were referenced to the residual solvent signal [δ_H 7.26 (CD_3Cl) or δ_H 3.30 (CD_3OD)] and ^{13}C NMR to the center peak at δ_C 77.00 (CD_3Cl) or δ_C 49.00 (CD_3OD).

Abbreviations: ALT, alanine aminotransferase; ASP, aspartate aminotransferase; SOD, superoxide dismutase; MDA, malondialdehyde; NO, nitric oxide; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; HR-ESI-MS, high-resolution electrospray ionization mass spectra; NMR, nuclear magnetic resonance spectroscopy; FT-IR, fourier intelligent infrared spectroscopy; HSQC, Heteronuclear Single-Quantum Coherence Spectroscopy; HMBC, Heteronuclear Multiple-Bond Coherence Spectroscopy; CCl_4, carbon tetrachloride.

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HSQC and HMBC spectra were optimized for $^{1}J_{CH}$ = 145.0 Hz and $^{n}J_{CH}$ (n = 2 and 3) of 7.7 Hz, respectively. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were measured on a Bruker Daltonics microTOF mass spectrometer. HR-ESI-MS was carried out on a Bruker Esquire 3000plus instrument. HR-EI-MS (70 eV) was measured on a Finnigan MAT 95 mass spectrometer. Shimazu LC-8A model preparative HPLC (Japan). Column chromatography was performed using silica gel (200–300 mesh, Qingdao Ji-Yi-Da Silysia Chemical Ltd., China), MCI gel HP20P (75–120, Mitsubishi Chemical Industries, Japan), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden). Silica gel-precoated plates (GF254, 0.25 mm, Yantai Kang-Bi-Nuo Silysia Chemical Ltd., China) were used for TLC. Spots were visualized using UV light (254 and/or 366 nm) and 5% H_2SO_4–EtOH.

2.2. Plant material, extraction and isolation

Fresh *Rubus aleaefolius* were collected from Anxi mountainous regions of Fujian Province, China. The plant material was identified and authenticated as *Rubus aleaefolius* (Rosaceae), by a plant taxonomist, from the Department of Pharmacy, Fujian University of Traditional Chinese Medicine, and a voucher specimen was deposited to their herbarium with the registration number was 20070126.

The dried roots powdered (10 kg) were extracted three times with 80% ethanol under reflux for 1 h. After filtration and combination of the filtrates, they were concentrated to dryness in the rotary evaporator to provide a crude EtOH extract (1500 g). The crude ethanolic extract was re-dissolved in water and partitioned successively with four organic solvents to provide a petroleum ether fraction (5 g), $CHCl_3$ fraction (5 g), EtOAc fraction (50 g), and n-BuOH fraction (250 g). All the fractions were subjected to bioactive evaluation using the CCl_4-induced acute liver injury mice model. The ethyl acetate fraction was found to be the most active when the other fractions were compared.

The dried ethyl acetate extract (30 g) was fractionated by silica gel column chromatography (200–300 mesh) eluting with chloroform–methanol (100:1–0:100). Elution was started with 100% chloroform, and the polarity was sequentially increased in a step wise fashion of 0.5% increments of chloroform–methanol. A total of 126 test tube fractions were collected, similar fractions were combined according to the TLC characteristics to afford four different Sub-fractions which were coded SF-1–SF-4. SF-1 was separated using a silica gel column chromatography (100–200 mesh) eluting with chloroform–methanol (100:1.5) and recrystallized with ethyl acetate to give compound 1 (8 mg). SF-2 was further purified on a silica gel column eluting with chloroform–methanol (25:1). Further purification was necessary using preparative HPLC, with a Shimazu PRC-ODS, 10 × 250 mm column, 80% MeOH as the solvent, flow rate of 2 ml/min, to furnish compounds 2 (705 mg), 4 (18 mg), and 6 (15 mg). SF-3 was further purified on a silica gel column and elute with chloroform–methanol (50:1). Further purification was proceeded using preparative HPLC under similar conditions described above for SF-2 but with a flow rate of 3 ml/min, to afford compound 5 (31 mg). SF-4 was subsequently chromatographed on a silica gel (chloroform–methanol, 20:1). Further purification was achieved using a Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) to yield compound 3 (13 mg).

2.3. Animal

Male ICR mice (20–22 g) were used for the animal model experiment, purchased from Laboratory Animal Center of the Fujian University of Traditional Chinese Medicine (Fuzhou, China), were housed in an environmentally-controlled room at a temperature of 22 ± 1 °C, relative humidity 65–70%. Air ventilation 12–18 times/h, and a 12-h light/dark cycle of 150–300 lux, with feed and water ad libitum. The animal studies were approved by the Fujian Institute of Traditional Chinese Medicine Animal Ethics Committee (Fuzhou, China). The experimental procedures were carried out in accordance with the Guidelines for Animal Experimentation of Fujian University of Traditional Chinese Medicine (Fuzhou, China).

2.4. Reagents

CCl_4 was purchased from Changjiang Chemical Co. Ltd. (Shanghai, China). Assay kits for SOD, NO and MDA activity were obtained from the Jiancheng Institute of Biotechnology (Nanjing, China). Assay kits for ALT, AST, were purchased from Fosun Long March

Table 1
Effect of ethanol extract, ethyl acetate and n-butanol factions and compound 2 of *Rubus aleaefolius* on serum ALT, AST, SOD, NO, and MDA in CCl_4-intoxicated mice.

Treatment	ALT (IU/L)	AST (IU/L)	SOD (NU/ml)	NO (μmol/L)	MDA (nmol/mg)
Normal control	21.7750 ± 1.5496	20.4667 ± 9.3237	310.9383 ± 40.8472	5.3033 ± 1.3304	1.27 ± 0.09
CCl_4 model	28.2980 ± 4.0200▲	37.1550 ± 19.2809▲	229.3967 ± 20.6634▲	8.7300 ± 1.9354▲	3.38 ± 0.55▲
Ethanol crude extract (35 mg/kg) + CCl_4	24.7143 ± 3.8763**	46.4917 ± 15.6362**	279.2817 ± 19.7712**	5.3517 ± 0.8533**	2.70 ± 0.50**
Low-dosage n-BAF (5.8 mg/kg) + CCl_4	28.8233 ± 4.1430**	32.4867 ± 10.8611**	261.6400 ± 53.3337**	7.9300 ± 1.9446**	3.20 ± 0.63**
High-dosage n-BAF (35 mg/kg) + CCl_4	22.4417 ± 4.3114**	29.2967 ± 7.6474**	289.9467 ± 55.4449**	7.3417 ± 1.5689**	3.12 ± 0.65**
Extracted with n-butanol after the residues (35 mg/kg) + CCl_4	24.7900 ± 6.4674**	36.1700 ± 7.9940**	276.2883 ± 36.4202**	4.2933 ± 1.0802**	3.28 ± 1.08**
Low-dosage EtOAcAF (4.6 mg/kg) + CCl_4	21.3667 ± 7.3272**	19.7617 ± 6.3655**	308.3133 ± 20.4429**	4.7150 ± 0.8816**	1.87 ± 0.14**
High-dosage EtOAcAF (35 mg/kg) + CCl_4	24.8850 ± 4.3446**	41.8600 ± 12.5598**	298.4500 ± 41.8027**	4.4667 ± 0.8639**	2.74 ± 0.32**
Extracted with ethyl acetate after the residues (35 mg/kg) + CCl_4	25.9367 ± 2.9398**	45.6967 ± 15.3154**	259.7333 ± 26.4009**	6.5200 ± 1.9847**	2.82 ± 0.63**
Compound 2 (3.5 mg/kg) + CCl_4	22.4576 ± 6.6361**	22.8534 ± 6.4743**	303.4249 ± 19.5129**	5.7280 ± 1.9635**	2.18 ± 0.86**

Values are expressed as mean ± standard error, n = 10.
▲ $P \leq 0.05$ versus normal control.
** $P \leq 0.05$ versus CCl_4 control.