Journal of Ethnopharmacology 157 (2014) 201–205

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Research Paper

Pallidifloside D, a saponin glycoside constituent from *Smilax riparia*, resist to hyperuricemia based on URAT1 and GLUT9 in hyperuricemic mice

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ARTICLE INFO

Article history:
Received 21 January 2014
Received in revised form
10 April 2014
Accepted 15 September 2014
Available online 28 September 2014

Keywords:
Saponin glycoside
Smilax riparia
Pallidifloside D
Hyperuricemia
URAT1
GLUT9

ABSTRACT

Ethnopharmacological relevance: The roots and rhizomes of *Smilax riparia* (SR), called "Niu-Wei-Cai" in traditional Chinese medicine (TCM), are believed to be effective in treating hyperuricemia and gout symptoms. This study was designed to isolate a saponin glycoside named pallidifloside D from the total saponins of *Smilax riparia* and to examine its effect in reducing serum uric acid levels in a hyperuricemic mouse model induced by potassium oxonate.

Materials and methods: We examined the effects of pallidifloside D treated with 5, 10 and 20 mg/kg on serum uric acid levels (S_{UA}), Serum creatinine (S_{Cr}) and blood urea nitrogen (BUN) levels in a hyperuricemic mouse. A colorimetric method was used to evaluate the effects of pallidifloside D on the XOD activities, and Western Blotting analysis were carried out to observe protein levels of mURAT1, mGLUT9 and mOTA1 in hyperuricemic mice after treatment with pallidifloside D.

Results: The levels of serum uric acid levels (S_{UA}) were suppressed significantly with dose-dependence by pallidifloside D treated with 5, 10 and 20 mg/kg ($p<0.05$, $p<0.01$ and $p<0.01$ respectively). Pallidifloside D could down-regulate the expression levels of renal mURAT1 protein in hyperuricemic mice in a dose-dependent manner ($p<0.05$, $p<0.01$, and $p<0.001$ respectively), and the *p*rotein levels of mGLUT9 could be down-regulated with dose-dependence ($p<0.05$ and $p<0.01$ respectively) by pallidifloside D at the dose of 10 and 20 mg/kg.

Conclusion: These results suggest that pallidifloside D possesses a potent uricosuric effect in hyperuricemic mice through decreasing renal mURAT1 and GLUT9, which contribute to the enhancement of uric acid excretion and attenuate hyperuricemia-induced renal dysfunction.

1. Introduction

Hyperuricemia is one of the most extensive metabolic diseases. It is characterized by high uric acid level in the blood, causing deposition of urate crystals in the joints and kidneys, and is well known as important risk factor for gout, hyperlipidemia, hypertension and diabetes (Boffetta et al., 2009; Tong et al., 2012). Underexcretion of urate has been proved to result in hyperuricemia (Boffetta et al., 2009). Urate transporter in kidney such as URAT1, GLUT9 and OAT1 may have important roles in the impaired urate excretion and hyperuricemia (Habu et al., 2003; Enomoto and Endou, 2005; Eraly et al., 2008; Preitner et al., 2009) and they constitute an important target for drugs to treat hyperuricemia. Anti-hyperuricemic drugs such as allopurinol and probenecid are demonstrated to produce adverse effects such as hypersensitivity and agranulocytosis (Harris et al., 1999; Bardin, 2004). Therefore, it underlines much impetus for urgent need of safer and more effective anti-hyperuricemic agents, especially herbal medicine (Ahmad et al., 2008; An et al., 2010).

Smilax riparia A. DC. (SR) which belongs to genus Smilax and family Liliaceae is distributed in the south and midland of China.

Abbreviations: mURAT1, mouse Urate Transporter 1; mGLUT9, mouse Glucose Transporter 9; mOAT1, mouse Organic Anion Transporter 1; mGAPDH, mouse Glyceraldehyde-3-phosphate dehydrogenase; EtOH, Ethyl alcohol; BuOH, n-Butyl alcohol; EtOAc, Ethyl acetate; HR-ESI-MS, High-Resolution Mass Spectrometer; NMR, Nuclear Magnetic Resonance; XOD, xanthine oxidase; UA, uric acid; Cr, creatinine; BUN, blood urea nitrogen; FEUA, fraction excretion of uric acid

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http://dx.doi.org/10.1016/j.jep.2014.09.034
0378-8741/

The roots and rhizomes of *Smilax riparia* in traditional Chinese medicine (TCM) are a source of Chinese folk drug "Niu-Wei-Cai", and are supposed to be effective as gout, hyperuricemia diuretic, anti-inflammatory, and antitumor agents in folklore medicine (Zhang and Han, 2012), and as edible wild herbs in some regions of China (Wang et al., 2000). Although some constituents such as phenylpropanoid glycosides, steroidal saponins, and aromatic compounds have been isolated from the roots and rhizomes of *Smilax riparia* in the past (Sashida et al., 1992; Li et al., 2006; Sun et al., 2012), it is not clear that the active constituents and their pharmacological mechanisms of *Smilax riparia* support for its therapeutic action such as gout and hyperuricemia diuretic etc.

In this study, we have isolated a saponin glycoside named pallidifloside D from total saponins of *Smilax riparia* (SR), and then examined it in reducing serum uric acid levels in mice model of hyperuricemia induced by potassium oxonate. We found that this saponin glycoside possesses a potent uricosuric effect in hyperuricemic mice through decreasing renal mURAT1 and GLUT9, which are attributable to the enhancement of uric acid excretion and attenuate hyperuricemia-induced renal dysfunction.

2. Materials and methods

2.1. Plant material

The roots and rhizomes of *Smilax riparia* were collected from Tieling, Liaoning Province of China, in September 2011, and were authenticated by Prof. Ye Zhou, Tianjin Medical University, China. A voucher specimen (SR-2011-12) was deposited at School of Pharmacy, Tianjin Medical University, Tianjin, People's Republic of China.

2.2. Extraction and isolation

Dried roots and rhizomes of *Smilax riparia* powder were ground and extracted with 90% EtOH. The 90% EtOH extract was suspended in water and extracted with petroleum ether, chloroform, EtOAc and BuOH, respectively. The BuOH layer was concentrated under reduced pressure to give the brown extract. The BuOH fraction was passed through a D101 macropore resin (0.25–0.84 mm) and eluted successively with 30% EtOH, 50% EtOH, 70% EtOH and 90% EtOH, respectively, and the total saponins of *Smilax riparia* were obtained from the 70% EtOH fraction. Then the total saponin fraction was passed through a Sephadex LH-20 column with MeOH; the fraction eluted with MeOH was passed through silica gel column chromatography with a $CHCl_3$/MeOH/H_2O solvent system (10:1:0.5–5:1:0.5). Finally, the fractions eluted by $CHCl_3$/MeOH/H_2O (9:1:0.5) were subjected to semi-preparative HPLC (10–100 mm) to afford pallidifloside D. HPLC showed the purity of this compound was 97.8%.

2.3. Characterization of pallidifloside D

An amorphous power, $[\alpha]_D^{20}$ −50.9 (MeOH), had a molecular formula of $C_{34}H_{50}O_{12}$ determined by the HR-ESI-MS: (m/z 651.3389, $[M+H]^+$) as well as its ^{13}C and NMR data. By UV, IR, MS and extensive ^1H and ^{13}C NMR spectra analysis and comparison with literature data (Shen et al., 2012), the structure of this compound was identified as (3β,16β)-3,16-dihydroxypregna-5,20-diene-20-carboxylic acid γ-lactone 3-O-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranoside, and named pallidifloside D (Fig. 1).

2.4. Preparation of hyperuricemia model and drug administration

Male Kunming mice (20 ± 2.0 g) were purchased from the China BK Experimental Animal Center (Beijing, China). Experiments reported in this study were carried out in accordance with guidelines for the care of laboratory animals at Tianjin Medical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Tianjin Medical University, Tianjin, China (Permit Number: 2011-X8-21). All surgery was performed under chloral hydrate anesthesia, and all efforts were made to minimize suffering.

The uricase inhibitor potassium oxonate was used to induce hyperuricemia in mice according to previous reports (Wang et al., 2010; Li et al., 2011). Except for the normal control (NC) mice, Potassium oxonate (250 mg/kg) dissolved in 0.9% saline solution was administrated intraperitoneally to each animal once daily for 7 consecutive days in order to induce hyperuricemic mice. The normal control mice and hyperuricemic mice were divided into 6 subgroups, NC (normal control group, orally receiving water only), MC (model group, potassium oxonate only), allopurinol (10 mg/kg, as a positive control drug), pallidifloside D (5 mg/kg, 10 mg/kg and 20 mg/kg respectively). Dosages of allopurinol and pallidifloside D were determined based on the conversions from clinical adult dosages (Chinese Pharmacopoeia Committee, 2005) and our preliminary studies. The test compounds (pallidifloside D and allopurinol) were dispersed in 0.3% CMC–Na aqueous solution and were orally administered once daily from day 1 to day 7, while the NC mice were treated with a solvent vehicle. After 7 days of treatment, diets were removed from the cages 12 h before the animals were sacrificed. The blood was allowed to clot for approximately 1 h at room temperature and centrifuged at 3000 rpm for 10 min to obtain serum. The serum and urine were stored at −20 °C until assayed. The levels of XOD activities, uric acid (UA), creatinine (Cr), blood urea nitrogen (BUN) in liver and kidney were determined by a colorimetric method using commercially available kits (purchased from Beijing Aoboseng Bioengineering Institute, China) according to the manufacturers' instructions. The index of fractional excretion of urate (FEUA) was calculated as follows (Kong et al., 2004):

$$FEUA = \frac{S_{Cr} \times U_{UA}}{U_{Cr} \times S_{UA}} \times 100$$

- S_{Cr}: Serum Creatinine, S_{UA}: Serum Uric Acid.
- U_{UA}: Urine Uric Acid, U_{Cr}: Urine Creatinine.

Simultaneously the kidney cortex was rapidly and carefully separated on ice-plate and stored at −80 °C for protein assays of mURAT1, mGLUT9 and mOAT1.

2.5. Western Blotting analysis of mURAT1, mGLUT9 and mOAT1 in kidney tissues

Mice renal cortical brush-border membrane vesicles for analysis of mURAT1, mGLUT9, mOAT1 and mNa^+–K^+ ATPase were prepared by a modified procedure (Hosoyamada et al., 2004). Immunoblotting was assayed using anti-URAT1 (1: 200), mGLUT9 (1: 200), mOAT1 (1: 200) as well as mGAPDH (1: 400) antibodies (Santa Cruz Biotech, USA). The contents of target proteins were analyzed via densitometry using Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA) and normalized by the respective blotting from mNa^+–K^+ ATPase or mGAPDH.

2.6. Statistical analysis

All data were expressed as the mean ± standard error of the mean (± SD) and statistical analysis was performed using a one-way analysis of variance (ANOVA) to determine the level of significance. A value of $p < 0.05$ was considered statistically