Hepatoprotective Activity of the Constituents in *Swertia pseudochinensis*

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A new xanthone derivative was isolated together with other 13 known constituents from a Chinese natural medicine, *Swertia pseudochinensis* HARA. Their structures were determined based on the spectral and chemical evidences. Furthermore, respective hexane, ethyl acetate, 1-BuOH, MeOH and water extracts of *S. pseudochinensis* and purified compounds were respectively evaluated for their hepatoprotective activities against hepatocyte injury induced by CCl4. All the extracts and isolated compounds exhibited significant hepatoprotective activities at a dose showing no hepatotoxicity.

Key words  
*Swertia pseudochinensis*; hepatoprotective activity; hepatotoxicity

*Swertia pseudochinensis* HARA belonging to Gentianaceae widely distributes in northern China. The whole plant is used to treat digest dysfunction, acute and chronic dysentery, toothache, and canker as a folk medicine in China. In recent years, it has been used very often as an important anti-hepatitis drug. Especially, it has collected much attention since this herb was listed in *Chinese Pharmacopoeia* at 1977. However, the composition and bioactivity of this crude drug have not yet been clearly reported. In our study, its constituents and their hepatoprotective activities against cytotoxicity of cultured liver cells induced by CCl4 have been examined. As a result, the respective hexane, ethyl acetate, 1-BuOH, MeOH and water extracts, and the purified components exhibited significant hepatoprotective activities. Meanwhile, we have also examined their cytotoxicity.

By various column chromatography of the methanolic extract prepared from the dried whole plant of the crude drug, we have obtained 5 iridoids compounds as swertiamarin (1), sweroside (2), gentiopicroside (3), amarogentin (8), amaroswerin (9) and 8 xanthenes compounds, a new compound (5), which was named pseudonolin, together with the following known compounds, tetrahydrosvertianolin (4), bellidifoline (6), swertianolin (7), metlyswertianin (10), 1-hydroxy-3,7-dimethoxyxanthone (11), methylbellelidifolin (12), desmethylbellelidifolin (13). Among them, 5 was a new compound and 11 was isolated for the first time from *Swertia* genus.5,6)

**MATERIALS AND METHODS**

**Spectral Instruments**  
Optical rotations were performed using a JASCO DIP-1000 KUYY polarimeter (l=5 cm). NMR spectra was measured in pyridine-d5 using a JEOL α-500 spectrometer and chemical shifts (ppm) were referenced to tetramethylsilane (TMS). The UV spectrum was measured with a V-550 spectrometer. FAB-MS, HR-FAB-MS were recorded on JEOL JMS-DX 300 and JEOL HX-110, respectively. The following reagents were used in the experiments: GOT assay kit (transaminase CII-test Wako); CCl4 (Kanto chemical); DMSO (Wako); fetal bovine serum (FBS) (GIBCO); dexamethasone (Wako); insulin (Sigma); kanamycin (Wako); collagenase (Wako) and Williams E medium (GIBCO). All of the other reagents were of special grade.

**Plant Material**  
The plant of *S. pseudochinensis* was purchased from the Anguo medicinal material market in China. A voucher specimen was deposited in the herbarium of the Hebei Province Pharmaceutical Examining Institute.

**Extraction and Isolation**  
The dried whole plant (750 g) of *S. pseudochinensis* was extracted with refluxing MeOH twice. The MeOH extract (71 g) was defatted by refluxing with hexane, then the hexane fraction (16 g) and the hexane-insoluble fraction were obtained. The latter was partitioned into chloroform–water system, giving a chloroform layer (11 g) and H2O layer (38 g). The H2O layer (8.2 g) was subjected to a silica gel column chromatography and eluted with CHCl3–MeOH–H2O (10: 1: 0.05→9: 1: 0.1→8: 2: 0.2) to give Fr. 1→8. Fr. 4 (409 mg) was subjected ODS column chromatography eluted with MeOH–H2O (10: 90→15: 85) to give Fr. 4-1 (52 mg) and Fr. 4-2 (98 mg). Further, Fr. 4-1 was chromatoraphed on a silica gel column eluted with CHCl3–MeOH–H2O (11: 1: 0.1→9: 1: 0.1) to give 1 (35 mg). Compounds 2 (20 mg) and 3 (12 mg) were obtained from the Fr. 4-2 by silica gel column chromatography eluted with CHCl3–MeOH (15: 1: 1→10: 1). Fr. 6 (882 mg) was subjected to ODS column chromatography eluted with MeOH–H2O (40: 60→50: 50→60: 40) to give Fr. 6-1→10. Furthermore, 4 (15 mg) from Fr. 6-3 and 5 (15 mg) from Fr. 6-7 were obtained. Fr. 6-10 (22 mg) was further separated by Sephadex LH-20 column chromatography with 80% MeOH to afford 6 (3 mg). The Fr. 6-5 (71 mg) was subjected to a silica gel column chromatography eluted with CHCl3–MeOH (12: 1: 1→10: 1) to provide 7 (6 mg). Fr. 4 (206 mg) was subjected to ODS column with MeOH–H2O (30: 70→40: 60) to give 8 (68 mg). Fr. 5 (592 mg) was sub-
jected to Sephadex LH-20 column chromatography with MeOH–H2O (25 : 75 → 70) to yield 9 (35 mg). Meanwhile, a part (9 g) of the hexane-soluble portion was chromatographed on a silica gel eluted with hexane–EtOAc solvent system (20: 1 → 10: 1) to give Fr. 9—21 and Fr. 13 was refined by washing with a small amount of hexane to provide 10 (20 mg). Fr. 14 (84 mg) was further purified by HPLC with MeOH–H2O (85 : 15) to give 11 (28 mg). The chloroform layer (11 g) was subjected to silica gel column chromatography with hexane–EtOAc (4 : 1 → 2 : 1 : 1) system to give Fr. 22—30. Fr. 23 (416 mg) was subjected to ODS (Calcd 552.4878). UV \( \lambda_{max} \) (in CH3CN–H2O) 270, 323 nm. 13C-NMR (pyridine-\( D_5 \)), 1.82 (3H, d, rha H-3), 4.39 (1H, o, rha H-4), 5.07 (1H, dd, \( J = 7 , 3 \), 9.2 Hz, xyl H-2), 4.39 (1H, o, xyl H-5), 4.32 (1H, dd, \( J = 9.2 , 9.2 \) Hz, xyl H-3), 4.26 (1H, m, xyl H-4), 3.79 (1H, o, xyl H-5). Sugar Analysis of 5: Solution of 5 (4 mg) in 1 \( \times \) HCl was heated at 80 °C for 3 h. The reaction mixture was diluted with H2O and the solution was neutralized with Amberlite IR-120. The fraction was concentrated in \( \text{vacuo} \) to give a residue, which was analyzed by liquid chromatography with a JASCO OR-20 rotation detector, and column: YMC-Pack R&D Polyamine (III) 4.6 mm i.d.×250 mm, elute solvent: 80% acetonitrile; flow rate: 0.8 ml/min. As a result, the retention time (min) of sugars was 7.19 min (−) and 9.86 min (+) respectively. [standard: \( \alpha \)-rhamnose 7.19 min; \( \beta \)-xylose 9.95 min].

Preparation of Hepatocytes: Male Wistar rats (6 weeks old, body weight 140—170 g, purchased from SLC, Japan) were used. The isolation of rat liver cells was performed according to the Berry and Friend’s method8 with a slight modifying. Culture medium was composed of Williams E medium, supplemented with 15% fetal bovine serum (FBS), 10−4 M dexamethasone, 10−4 M insulin and 30 μg/ml kanamycin. The isolated cells were diluted to 5×107/ml using the culture medium, and every 1×105 cells (0.2 ml) were seeded into a 24-well plate. Then, the cells were incubated at 37 °C in a humidified 5% CO2 and 95% air atmosphere. After 3 h incubation, the culture medium was changed and cells were incubated continuously for another 24 h.9—12)

Hepatocyte Injury by CCl4: In order to decide suitable CCl4 concentration for GOT measure, 20 μl CCl4/DMSO solution (50 mM) and 5, 7.5, 10, 15, 20 μl CCl4/DMSO solution (500 mM) were respectively added, into 1 ml of the culture medium for liver cells preincubated for 1 d. After incubating for 1 h, each GOT value was measured. Analyzing the results of GOT measure, we finally selected 500 mM CCl4/DMSO solution 7.5 μl (final concentration of CCl4 was 3.75 mM) to use in the following hepatocyte injury experiment.

Measurement of Hepatoprotective Effects: After preincubation for 24 h, the cells were exposed to the above prepared medium containing CCl4 and sample (final concentrations: 0; 0.04; 0.2; 1; 10; 50 μg/ml). One hour later, the
medium was taken to measure GOT.

The similar examination was also applied to evaluate the each S. pseudochinensis extract, MeOH, hexane, acetic ether, 1-BuOH and H₂O extracts, at concentrations of 0; 0.05; 0.25; 1; 10; 50; 100; 200; 500 μg/ml, respectively.

Cytotoxicity Assay (without CCl₄) One hour after addition of 5 μl each test sample solution (compound concentration: 50 μg/ml; extract concentration: 500 μg/ml, in DMSO) into 1 ml hepatocytes culture medium, its cytotoxicity was evaluated by the above method.

The reference was determined by addition of DMSO to cells, without exposure with CCl₄ or samples. The control of the GOT value was determined by challenging cells with CCl₄ but no treatment with samples.

Statistical Analysis The data are shown as means±S.D. (n=3). After analysis of variance, Bonferroni/Dunn’s method was employed to determine the significance of differences between reference and experimental samples.

RESULTS AND DISCUSSION

Chemical Constituents S. pseudochinenses was used to treat acute or chronic hepatitis frequently and was listed into China Pharmacopoeia in 1977. However, its chemical and pharmacological studies have not been performed. We have obtained 13 compounds from S. pseudochinenses and identified their structures for the first time. Among them, 5 was a new compound. The UV spectra of 5 showed maximum absorptions at 248, 270 and 323 nm, which indicated the presence of aromatic structure. In the 13C-NMR spectrum, besides signals of tow sugars, the remain moiety exhibited presence of a xanthone skeleton by the carbon numbers and their chemical shifts. A signal at δ 56.1 could be assigned to a methoxy, which is attached to the xanthone skeleton. In the acid hydrolysate, the presence of l-rhamnose and d-xylene were detected, and their structures were confirmed by the 1H-NMR and a specific LC analysis method. The NMR data were assigned with the aid of 1H–1H COSY, HMQC and HMBC experiments. The sugar’s anomeric proton signals at δ 5.62 (xyl H-1) and 6.50 (rham H-1), exhibited the HMBC between δ 140.3 (C-4) and δ 79.0 (xyl C-2), respectively (Fig. 1). Moreover, by comparison of its 13C-NMR data with those of reported related compounds,5,11) the above data indicated that the structure of 5 was represented as 4-O-α-L-rhamnopyranosyl-(1→2)-β-D-xlypyranosyl-1,4,5-trihydroxy-7-methoxystanthon, designated as pseudonolin.

Similarly, other compounds, 1—4, 6—13, were also identified by comparison of their spectrum with those of previously reported compounds (Fig. 2).

Hepatoprotective Effects The constituents and hepatoprotective effect of Swertia japonica were previously reported.4,13,14) Hikino et al. examined the antihepatotoxic activities for its 11 pure compounds and hot water, 50% EtOH and 95% EtOH extracts, by CCl₄-injury primary culture rat hepatocytes.4) As a result, each extract exhibited significant GPT inhibiting activity, and five same compounds also isolated in our experiment, which are amarogentin, amaroswerin, swertiamarin, bellidifolin and methylswertianin, similarly revealed some extent of activity, indicating a high similarity with our present results. However, as for a compound, tetrahydroswertianolin, Hase’s report claimed it having extreme antihepatotoxic activity even than the medicine glycyrhrizin.5) In contrast, we gained a different result that it has only normal activity when compared to the other compounds, such as 5 and 7.

In our results, the 1-BuOH extract began to exhibiting an inhibit activity for cultured hepatocyte GOT from an initial concentration of 0.25 μg/ml, and showed the strongest activity at 1 μg/ml. Both methanol and ethyl acetate extracts showed effects at 1 μg/ml, without further enhancement by increasing concentration. The hexane extract began to demonstrate activity from the concentration of 1 μg/ml and reached to its strongest effect at 10 μg/ml. The water extract exhibited effect at concentration as low as 0.05 μg/ml, and the activity-concentration relation was observed in a range of concentration up to 100 μg/ml (Fig. 3A). So in a conclusion, all extracts have good hepatoprotective activity.

As for the results of the pure compounds, the respective compounds also showed evident antihepatotoxic activity. This is consistent with the data of above extracts tightly. At the lowest concentration of 0.04 μg/ml, activities of 5 and 12 were stronger than the others. On the other hand, after measuring the GOT indexes at the lower three grade concentrations 0.04, 0.2 and 1 μg/ml, we calculated the EC₅₀ value by the Probit’s method. The results indicated that the GOT inhibiting activity of 9 was the strongest (0.42 μg/ml), and 11

![Fig. 3. Hepatoprotective Effect and Toxicity of Respective Extract from S. pseudochinensis against Cultured Hepatocyte Injury by CCl₄](image-url)
was the weakest (2.33 \( \mu g/ml \)) (Fig. 4A).

In this study, we have surveyed their hepatoprotective activity for four secoiridoids and seven xanthones isolated from \textit{S. peudochinensis}. From the results, the following facts are discovered: in the secoiridoids, the presence of 5-hydroxyl enhanced the hepatoprotective activity; and in xanthone group, the activity of glycoside was stronger than that of aglycone, indicating that the sugar section contributes to the activity.

In addition, the toxicity test showed that all the samples did not demonstrate significant cytotoxicity at concentration of 50 \( \mu g/ml \) for the respective compounds (Fig. 4B) and 500 \( \mu g/ml \) for the respective extracts (Fig. 3B).

The new compound, pseudonolin (5) exhibited stronger hepatoprotective activity (Fig. 5).

In order to investigate whether the samples have inhibitory effects on GOT caused by normal hepatocytes, we also measured GOT value of hepatocyte culture solution with samples at a high concentrations (extracts: 500 \( \mu g/ml \); compounds: 50 \( \mu g/ml \)) in the absence of CCl\(_4\). As a result, each sample did not exhibit significant difference when compared to the control (DMSO), suggesting that the samples have no inhibitory activity on normal GOT.

Based on the experimental data, we can conclude that \textit{S. pseudochinensis} shows remarkable and trustworthy hepatoprotective effect, without evident toxicity. Furthermore, the high similarity in components and GOT inhibiting activity between \textit{S. pseudochinensis} and \textit{S. japonica}, indicate that two plants could probably be used for same application. Therefore \textit{S. pseudochinensis} is supposed of much wider application for a hepatitis treatment drug.

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REFERENCES