

# Study on the structures and anti-hepatic fibrosis activity of stilbenoids from *Arundina graminifolia* (D. Don) Hochr.

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**Abstract.** A phytochemical study was performed on *Arundina graminifolia* (D. Don) Hochr. by silica gel column and semi-preparative HPLC, and ten stilbenoids were obtained. Their structures were elucidated by NMR and MS spectra and identified as 7-hydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene (1), 4,7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene (2), 2,7-dihydroxy-4-methoxy-9,10-dihydrophenanthrene (3), 3,3'-dihydroxy-5-methoxy-bibenzyl (4), 7-hydroxy-2,8-dimethoxy-phenanthrene-1,4-dione (5), 7-hydroxy-2,10-dimethoxy-phenanthrene-1,4-dione (6), 7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene-1,4-dione (7), 7-hydroxy-2-methoxy-phenanthrene-1,4-dione (8), 7-hydroxy-1-(p-hydroxybenzyl)-2,4-dimethoxy-9,10-dihydroxy-phenanthrene (9), 2,7-dihydroxy-1-(p-hydroxybenzyl)-4-methoxy-9,10-dihydroxy-phenanthrene (10). Compounds 5 and 6 were isolated from this plant for the first time. The isolated compounds were examined for their anti-hepatic fibrosis activity against HSC-T6 cells in vitro. The results showed that compounds 4 and 5 exhibited moderate growth inhibitory effects with IC<sub>50</sub> 61.9 µg/mL and 52.7 µg/mL, respectively.

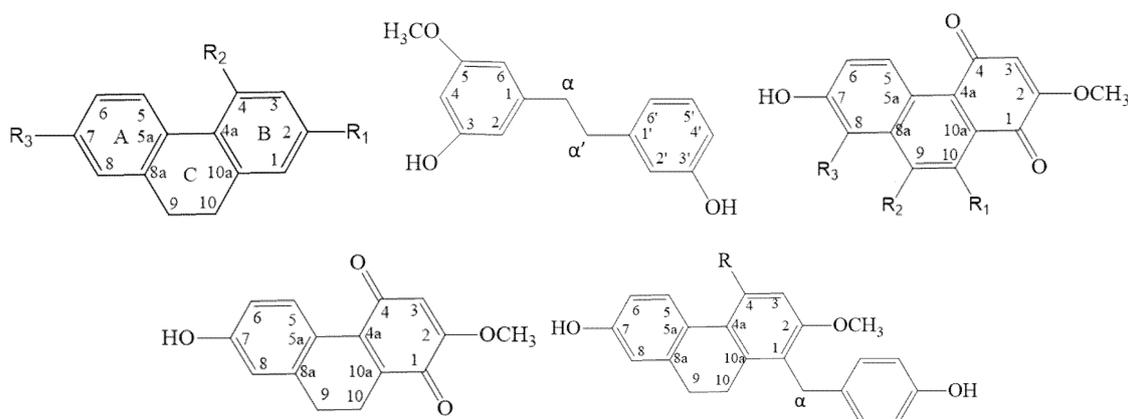
## 1. Introduction

*Arundina graminifolia* (D. Don) Hochr., a species of *Arundina*, was widely distributed in Yunnan province in China, known as “Bai-yang-jie” [1]. It was a detoxifier with the clinical application of heat-clearing and detoxifying, removing blood stasis and relieving pain [2], and was performed as one of principal components of the compound formulation called Baogan capsule which was used to treat liver damage and hepatic fibrosis in Dai hospital.

Previous studies indicated that hepatic fibrosis was the compensatory reaction in the process of liver tissue repair with excessive deposition of extracellular matrix (ECM) after suffering from various chronic liver damages [3, 4], ending up with hepatic cirrhosis, even hepatocellular carcinoma, and earlier intervention of hepatic fibrosis is more meaningful to the treatment and prevention of liver diseases, which increased the interest in *Arundina graminifolia* (D. Don) Hochr. The preliminary phytochemical investigation indicated that stilbenoids and phenols were the main structural types of the plant [5]. In order to clarify the relationship between the chemical structures and their pharmacological activities, the further studies were carried out on the plant.



In the present paper, we described the separation, structural identification, and anti-hepatic fibrosis bioactivity of ten stilbenoids obtained from the ethyl acetate extract of this plant (**Figure 1**). Their structures were decided as 7-hydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene (1), 4,7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene (2), 2,7-dihydroxy-4-methoxy-9,10-dihydrophenanthrene (3), 3,3'-dihydroxy-5-methoxy-bibenzyl (4), 7-hydroxy-2,8-dimethoxy-phenanthrene-1,4-dione (5), 7-hydroxy-2,10-dimethoxy-phenanthrene-1,4-dione (6), 7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene-1,4-dione (7), 7-hydroxy-2-methoxy-phenanthrene-1,4-dione (8), 7-hydroxy-1-(*p*-hydroxybenzyl)-2,4-dimethoxy-9,10-dihydroxy-phenanthrene (9), 4,7-dihydroxy-1-(*p*-hydroxybenzyl)-2-methoxy-9,10-dihydroxy-phenanthrene (10). Compounds **5** and **6** were identified from this plant for the first time. In addition, the isolated compounds were firstly examined for their anti-hepatic fibrosis activity against HSC-T6 *in vitro*.



**Figure 1.** Structures of compounds 1~10

- 1**  $R_1 = R_2 = \text{OCH}_3$ ,  $R_3 = \text{OH}$ ,  
**2**  $R_1 = \text{OCH}_3$ ,  $R_2 = R_3 = \text{OH}$ ,  
**3**  $R_1 = R_3 = \text{OH}$ ,  $R_2 = \text{OCH}_3$ ,  
**4**.  
**5**  $R_1 = R_2 = \text{H}$ ,  $R_3 = \text{OCH}_3$ ,  
**6**  $R_1 = \text{OCH}_3$ ,  $R_2 = R_3 = \text{H}$ ,  
**7**.  
**8**  $R_1 = R_2 = R_3 = \text{H}$ ,  
**9**  $R = \text{OCH}_3$ ,  
**10**  $R = \text{OH}$ .

## 2. Experimental Section

### 2.1. Plant material

The whole plant of *Arundina graminifolia* was bought from Dai hospital of Xishuanbanna in Yunnan Province, China. A specimen was collected in Beijing BIT&GY Pharmaceutical R&D Co. Ltd (batch number: 20111128).

### 2.2. General Experimental Procedures.

Column chromatography was performed using silica gel (200~300 mesh, 300~400 mesh, Qingdao Marine Chemical, Inc., Qingdao, People's Republic of China), TLC was used to monitor fractions, and spots were visualized by UV (TH-1810) in 254nm and 365nm and heating silica gel plates sprayed with 5%  $\text{H}_2\text{SO}_4$  in ethanol. NMR spectra were detected on a DRX-500 nuclear magnetic resonance (NMR) spectrometer with TMS as internal standard. Mass spectrum (MS) data was performed on a

Agilent 1100-6210 TOF LC/MS. Semi-preparative high performance liquid chromatography (HPLC) was performed on a Shimadzu LC-6AD preparative liquid chromatograph with YMC C<sub>18</sub> reverse phase column (250 × 20mm, 5μm) and a Shimadzu LC-20A analytical liquid chromatograph with Agilent C18 reverse phase column (250 × 4.6mm, 5μm). Mobile phase were purified water and methanol with chromatographic grade, which were bought from Merck. Organic reagents were analytical grade (Beijing Chemical Works, Beijing). RPMI-1640 medium, penicillin-streptomycin, and trypsinase were bought from Solarbio, Beijing, China and fetal bovine serum were purchased from Sijiqing, Hangzhou, China.

### 2.3. *Extraction and isolation*

The powdered plant (8.0 kg) was decocted with 80% ethanol (3 times, 2h/time) at room temperature and extracting solution was merged. After filtration and solvent evaporation, the crude extraction was further extracted with petroleum ether, chloroform, ethyl acetate and n-butanol to obtain the corresponding fractions. The ethyl acetate extract was chromatographed on a silica gel column, eluting with a PE-EtOAc gradient system (6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 0:1, CH<sub>3</sub>OH) to give eight fractions **I–VIII**, respectively. Fraction **II** was subjected to silica gel column and semi-preparative HPLC eluted by 80% CH<sub>3</sub>OH- 20% H<sub>2</sub>O to yield compound **1** (24 mg). Fraction **IV** and **V** was subjected to silica gel column and semi-preparative HPLC eluted by 70% CH<sub>3</sub>OH-30% H<sub>2</sub>O and 60% CH<sub>3</sub>OH-40% H<sub>2</sub>O, respectively, to yield compounds **2** (29 mg), **3** (8 mg), **4** (11 mg), **5** (5 mg), **6** (9 mg), **7** (2 mg), **8** (3 mg), **9** (11 mg) and **10** (6 mg).

### 2.4. *Cell culture*

HSC-T6, which was a cell line isolated from a normal rat's liver, was cultivated in RPMI-1640 medium supplemented 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C incubators in an atmosphere of 5% CO<sub>2</sub>. The cells were trypsinized and passaged to new plates every three days.

### 2.5. *Cell proliferation inhibition assays*

Cell proliferation inhibition assays were evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method. Ten stilbenoids were dissolved in DMSO (≤ 0.1% volume) and diluted at various concentrations (10, 50, 100μg/mL). In 96-well plates, HSC-T6 were seeded at the density of 1 × 10<sup>4</sup> cells/well for 24h. Sample was then added into the corresponding groups and each group got 6 parallels. After incubating for another 24 hours, OD values were measured by MTT method and detected at 540nm by an ELISA reader. 0.1% DMSO was considered as blank control and legalon (silymarin capsules) as positive control. The inhibition rate of the control cells were used as the control values at 0% and IC<sub>50</sub> was calculated in origin Pro 8.0.

## 3. Results & discussion

In order to further obtain the effective constituents and clarify the pharmacological material basis, the separation of components was carried out on the ethyl acetate extract portion by silica gel column and semi-preparative HPLC and the structures of the isolated natural products were determined mainly by NMR and MS methods.

### 3.1. *Structure Elucidation*

In the study, 10 stilbenoids were obtained, and their structures were identified by <sup>1</sup>H-NMR and MS data, combined with the literatures. All these compounds showed positive FeCl<sub>3</sub> and phosphomolybdic acid reactions, suggesting the presence of phenolic hydroxyl groups. The <sup>1</sup>H-NMR data were listed in **Table 1**.

Compound **1–3** were obtained as colorless crystal, pink powder and white oblong crystal, respectively. The negative HRESIMS gave the quasi-molecular ion peaks at *m/z* 255.1053 [M-H]<sup>-</sup>, 241.0912 [M-H]<sup>-</sup> and 241.0897 [M-H]<sup>-</sup>, corresponding the molecular formulas C<sub>16</sub>H<sub>16</sub>O<sub>3</sub>, C<sub>15</sub>H<sub>14</sub>O<sub>3</sub> and C<sub>15</sub>H<sub>14</sub>O<sub>3</sub>, respectively. Their <sup>1</sup>H-NMR data showed the same characteristic signals, including three

protons signals of benzene ring ABX coupling system at  $\delta_{\text{H}}$  8.11 (1H, d, H-5,  $J = 8.5\text{Hz}$ ), 6.74 (1H, dd, H-6,  $J = 8.5, 2.5\text{Hz}$ ) and 6.71 (1H, H-8,  $J = 2.5\text{Hz}$ ) in A ring, two protons signals at  $\delta_{\text{H}}$  6.45 (1H, d, H-1,  $J = 2.5\text{Hz}$ ) and 6.41 (1H, d, H-3,  $J = 2.5\text{Hz}$ ) on the B ring, and two methylene protons at  $\delta_{\text{H}}$  2.72 (2H, m) and 2.73 (2H, m) in C ring. The major differences were the number and combining position of OCH<sub>3</sub> and OH groups, combined with the MS analysis. Compound **1** showed two OCH<sub>3</sub> groups at  $\delta_{\text{H}}$  3.87 (3H, s, H-2) and 3.84 (3H, s, H-4), and one OH group; compound **2** showed one OCH<sub>3</sub> group at  $\delta_{\text{H}}$  3.85 (3H, s, H-2), and two OH groups; compound **3** showed one OCH<sub>3</sub> group at  $\delta_{\text{H}}$  3.80 (3H, s, H-4), and two OH groups (Figure 1). Thus, compared with orchinol, lusianthridin, and coelonin reported in the literatures [6, 7], the structures of compounds **1**~**3** was established as 7-hydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene, 4,7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene, 2,7-dihydroxy-4-methoxy-9,10-dihydrophenanthrene.

Compound **4** was obtained as white needle-like crystal. The negative HRESIMS showed quasi-molecular ion peaks at  $m/z$  243.1046 [M-H]<sup>-</sup>, corresponding the molecular formula C<sub>15</sub>H<sub>16</sub>O<sub>3</sub>. The <sup>1</sup>H-NMR spectrum showed four aromatic protons at  $\delta_{\text{H}}$  7.09 (1H, t, H-5'), 6.68 (2H, t,  $J = 2.5\text{Hz}$ , H-2, H-6'), and  $\delta_{\text{H}}$  6.65 (1H, dd,  $J = 9.5\text{Hz}, 2.5\text{Hz}$ , H-4') and three protons' signals at  $\delta_{\text{H}}$  6.34 (1H, m, H-2'), 6.32 (1H, m, H-6) and 6.25 (1H, t,  $J = 2.8\text{Hz}$ , H-4). One OCH<sub>3</sub> signal at  $\delta_{\text{H}}$  3.72 (3H, s, H-5) and two methylene protons combining  $\alpha$  and  $\alpha'$  at  $\delta_{\text{H}}$  2.80 (4H, m) were observed in <sup>1</sup>H-NMR spectrum. Thus, compared with batatasin III reported in the literature [8, 9], the structure of compound **4** was established as 2,7-dihydroxy-4-methoxy-9,10-dihydrophenanthrene.

Compound **5** and **6** were obtained as orange-red powder. The negative HRESIMS showed quasi-molecular ion peaks at  $m/z$  283.0644 [M-H]<sup>-</sup> and 283.0645 [M-H]<sup>-</sup>, corresponding the same molecular formulas C<sub>16</sub>H<sub>12</sub>O<sub>5</sub>, respectively. In the assignment of <sup>1</sup>H-NMR data, compound **5** showed one proton at  $\delta_{\text{H}}$  6.19 (1H, s, H-3), two couple protons signals of benzene ring AB coupling system at  $\delta_{\text{H}}$  9.20 (1H, d, H-5,  $J = 9.5\text{Hz}$ ), 7.33 (1H, d, H-6,  $J = 9.5\text{Hz}$ ) and 8.34 (1H, d, H-9,  $J = 9.0\text{Hz}$ ), 8.08 (1H, d, H-10,  $J = 9.0\text{Hz}$ ), and compound **6** showed two protons at  $\delta_{\text{H}}$  6.08 (1H, s, H-3) and 7.44 (1H, s, H-9) as well as three proton signals of benzene ring ABX coupling system at  $\delta_{\text{H}}$  9.40 (1H, d, H-5,  $J = 9.5\text{Hz}$ ), 7.24 (1H, dd, H-6,  $J = 9.5\text{Hz}, 2.7\text{Hz}$ ), 7.53 (1H, d, H-8,  $J = 2.7\text{Hz}$ ). According to the analysis of <sup>1</sup>H-NMR and MS spectra, both compound **5** had two OCH<sub>3</sub> and one OH groups, which replaced in different positions. Thus, compared with the compound reported in the literature [10], the structure of compound **5** was established as 7-hydroxy-2,8-dimethoxy-phenanthrene-1,4-dione, and compound **6** compared with cypritibetquinone B reported in the literature [11], the structure of compound **6** was established as 7-hydroxy-2,10-dimethoxy-phenanthrene-1,4-dione.

Compound **8** was obtained as orange-red powder. The negative HRESIMS showed quasi-molecular ion peaks at  $m/z$  253.0547 [M-H]<sup>-</sup>, corresponding the molecular formulas C<sub>15</sub>H<sub>10</sub>O<sub>4</sub>. The <sup>1</sup>H-NMR data showed the similar proton signals with those of compounds **5** and **6**, except the loss of one OCH<sub>3</sub> signal. Thus, compared with cypritibetquinone A reported in the literature [6, 7, 11], the structure of compound **8** was established as 7-dihydroxy-2-methoxy-phenanthrene-1,4-dione.

**Table 1.**  $^1\text{H}$  NMR Data of Representative Compounds ( $\delta$  in ppm, J in Hz)

No.	Comp.1	Comp.4	Comp.5	Comp.7	Comp.9
1	6.45(1H, d, 2.5)				
2	3.84(3H, s)	6.68(2H, t, 2.5)	3.91(3H, s)	3.84(3H, s)	3.84(3H, s)
3	6.41(1H, d, 2.5)		6.19(1H, s)	5.98(1H, s)	6.62(1H, s)
4	3.87(3H, s)	6.25(1H, t, 2.8)			3.88(3H, s)
5	8.11(1H, d, 8.5)	3.72(3H, s)	9.20(1H, d, 9.5)	7.90(1H, d, 9.4)	8.00(1H, d, 8.5)
6	6.74(1H, dd, 8.5, 2.5)	6.32(1H, m)	7.33(1H, d, 9.5)	6.69(1H, dd, 9.4, 2.5)	6.60(1H, m)
7					
8	6.71(1H, 2.5)		3.90(3H, s)	6.68(1H, d, 2.5)	6.61(1H, m)
9	2.72(2H, m)		8.34(1H, d, 9.0)	2.72(1H, m)	2.58(2H, m)
10	2.73(2H, m)		8.08(1H, d, 9.0)	2.62(1H, m)	2.50(2H, m)
2'		6.34(1H, m)			6.88 (2H, d, 8.5)
3'					6.64(2H, d, 8.5)
4'		6.65(1H, dd, 9.5, 2.5)			
5'		7.09(1H, t, 9.5)			6.64(2H, d, 8.5)
6'		6.68(2H, t, 2.5)			6.88 (2H, d, 8.5)
$\alpha$ ( $\alpha'$ )		2.80(4H, m)			3.93(2H, s)

Compound 1~3 were tested in  $\text{CDCl}_3$ ; Compound 4~10 were tested in  $\text{CD}_3\text{OD}$ .

Compound **7** was obtained as dark red powder. The negative HRESIMS showed quasi-molecular ion peaks at  $m/z$  255.0704 $[\text{M}-\text{H}]^-$ , corresponding the molecular formula  $\text{C}_{15}\text{H}_{12}\text{O}_4$ . The  $^1\text{H}$ -NMR data showed the similar signals with those of compound **8**. The most difference was that the presence of two methylene protons at  $\delta_{\text{H}}$  2.72 (2H, m) and 2.62 (2H, m) in compound **7**, which indicated the double bond at  $\Delta_{9,10}$  in compound **8** was hydrogenated. Thus, compared with densiflorol B reported in the literature [6, 10], the structure of compound **7** was established as 7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene-1,4-dione.

Compound **9** and **10** were obtained as needle crystal. The negative HRESIMS showed quasi-molecular ion peaks at  $m/z$  361.1478  $[\text{M}-\text{H}]^-$  and 347.1324  $[\text{M}-\text{H}]^-$ , corresponding the molecular formulas  $\text{C}_{23}\text{H}_{22}\text{O}_4$  and  $\text{C}_{22}\text{H}_{20}\text{O}_4$ . On the basis of the  $^1\text{H}$ -NMR data analysis, compound **9** possessed the same structural skeleton with compound **3**, expect the difference of the substituent groups located at C-1 and C-2. One *p*-hydroxybenzyl and one  $\text{OCH}_3$  group was observed in the  $^1\text{H}$ -NMR spectrum. Thus, compared with arundinaol reported in the literature [12], the structure of compound **9** were assigned as 7-hydroxy-1-(*p*-hydroxybenzyl)-2,4-dimethoxy-9,10-dihydroxy-phenanthrene. Meanwhile, the  $\text{OCH}_3$  signal of compound **9** at C-4 disappeared in the  $^1\text{H}$ -NMR spectrum of compound **10**. According to molecular weight and molecular formula, one OH group was confirmed. Thus, compared with isoshancidin reported in the literatures [8], the structure of compound **10** were assigned as 2,7-dihydroxy-1-(*p*-hydroxybenzyl)-4-methoxy-9,10-dihydroxy-phenanthrene, respectively.

### 3.2. Cell proliferation assays

Ten isolated compounds were evaluated for their anti-hepatic fibrosis activity against HSC-T6 *in vitro* by MTT method. Legalon (silymarin capsules) was taken as positive control. The results listed in **Table 2** indicated that most of the analyzed compounds had slight growth-inhibiting intensity, and compounds **4** and **5** showed moderate growth inhibitory effects with  $\text{IC}_{50}$  61.9  $\mu\text{g}/\text{mL}$  and 52.7  $\mu\text{g}/\text{mL}$ , respectively.

**Table 2.** 10 stilbenoids' IC<sub>50</sub> values of inhibiting proliferation of HSC-T6 cells

Compound.	Postive drug	1	2	3	4	5	6	7	8	9	10
IC <sub>50</sub> (μg/mL)	254.1	69.7	67.8	69.9	61.9	52.7	141.6	271.6	121.4	68.8	73.8

Most of the isolated compounds were performed to inhibiting efficacy against HSC-T6, which expressed specifically in the growth inhibition rate. Most compounds were higher than positive drug within the range of 10~100μg/mL, especially compounds **4** and **5**. All of these compounds were stilbenoids with OH and OCH<sub>3</sub> groups. Compound **4** was the only phenanthrene with C bibenzyl ring opening in all of compounds and had a better activity than most of the analyzed compounds, which might play an important role in the growth inhibition. Compounds **7** and **8** showed weaker activities than other compounds, which indicated that the number of OH and OCH<sub>3</sub> groups might increase the effects against HSC-T6. The work corresponding to the synthesis and chemical structural transformation of natural products had been carried out, and the structure and activity relationship will be clearer.

#### 4. Conclusion

In the present study, ten stilbenoids were separated and identified from this plant. All these compounds were tested for anti-hepatic fibrosis activity against HSC-T6 *in vitro*, and compounds **4** and **5** performed significant efficacy. Although pharmacological studies indicated that *Arundina graminifolia* (D. Don) Hochr. had antitumor, antioxidant and antiviral activities, anti-hepatic fibrosis activity was firstly tested for this plant, which will be helpful for the further research to elucidate the pharmacological mechanism.

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