

New Sesquiterpene and Polymethoxy-Flavonoids from *Artemisia annua* L

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ABSTRACT

Our previous study revealed that the polymethoxy-flavonoids, as main components of *Artemisia annua*, could improve the antimalarial activity of Artemisinin. Here, we described the isolation, elucidation, constituent analysis, flavonoids enrichment of the extracts of *A. annua*. A total of 20 compounds were isolated including a new sesquiterpene (compound 12) and five (1, 5, 6, 7, 15) afforded for the first time from *A. annua*. The elucidation of eight flavonoids may be a useful phytochemical data and chemical foundation for further mechanism studies on improving the anti-malarial action of artemisinin. Furthermore, the antitumor activities of the compounds were assayed using four different kinds of human cancer cell lines.

Key words: Antitumor activities, artemisinin, polymethoxy-flavonoid, sesquiterpene

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INTRODUCTION

Artemisia annua L. (Qinghao), a traditional Chinese herb with pharmacological functions on clearances of heat and toxic materials as well as on antimalarial bioactivity, belongs to genus *Artemisia* (Composite family) and distributes mainly in the northern parts of Guangxi and Sichuan provinces in China. However, this plant now grows wild in Europe and America. Phytochemical studies of *A. annua* revealed the presence of terpenoids, flavonoids, aliphatic hydrocarbons, aromatic ketones, aromatic acids, phenylpropanoids,^[1] alkaloids and coumarins.^[2]

Our previous study showed that the polymethoxy-flavonoids of *A. annua* had the effects on improving the antimalarial activity of artemisinin. Using liquid chromatographic tandem mass spectroscopy method for the determination of the antimalarial drug, artemisinin, in rat plasma using arteannuin B as internal standard (I.S.), the results demonstrated that different doses of CHR significantly

increased the areas under the plasma concentration-time curve (AUC) ($P < 0.01$) compared with artemisinin alone and suggested that co-administration of CHR may be an efficient way to increase the anti-malarial action of artemisinin.

Here, we report the phytochemical research result of *A. annua*, including the isolation and elucidation of a new sesquiterpene (compound 12), together with four known sesquiterpenes (compounds 1, 2, 8 and 11), eight flavonoids (compounds 7, 13, 14, 15, 16, 17, 19 and 20), two coumarins (compounds 3 and 6) and two acetophenones (compounds 4 and 5).

MATERIALS AND METHODS

General

NMR spectra were measured with Bruker ARX-300 and ARX-600 spectrometers (Bruker Corporation, Germany), using DMSO- d_6 and $CDCl_3$ as solvent and TMS as an internal standard. HR-EST-MS was performed on Bruker micro TOF-Q mass spectrometer in m/z (rel.%) (Bruker Daltonics Inc., Germany). ESI-MS was performed on a Finnigan LCQ mass spectrometer (Thermo Electron, California, USA). Silica gel (200-300 mesh) and silica gel G (Qingdao Marine Chemical Group Co. Ltd, Qingdao, China) were used for column chromatography and TLC, respectively.

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Extraction and Isolation

The acetone extracts of *A. annua* (200 g) was provided by Department of Pharmaceutics, Ningxia Medical University. Then it was subjected to HPD-100 macroporous adsorption resin and eluted with EtOH/H₂O (3:2, 7:3, 4:1, 19:1) to yield 4 fractions (F1-F4), and these fractions were subjected to silica gel column chromatography (CC), Sephadex LH-20, polyamide CC, ODS CC and PHPLC to yield 20 compounds: 3 α -hydroxy-1-deoxyartemisinin (1, 32.0 mg, colorless crystal in CH₂Cl₂: MeOH 3:1),^[5] α -epoxy-dihydroartemisinic acid (2, 52.0 mg, colorless crystal in CH₂Cl₂: MeOH 1:1),^[4] scopoletin (3, 116.0 mg, light yellow crystal in CH₂Cl₂: MeOH 2:1),^[5] 4, 6-dihydroxy-2-methoxy acetophenone (4, 26.0 mg, colorless crystal in CH₂Cl₂: MeOH 3:1) (Brown, 1992),^[6] 6-hydroxy-2,4-dimethoxy acetophenone (5, 17.2 mg, colorless crystal in CH₂Cl₂: MeOH 1:1) (Brown, 1992),^[6] esculetin (6, 13.6 mg, light yellow crystal in CH₂Cl₂: MeOH 2:1),^[5] quercetagenin-3,6,3',4'-tetramethyl ether (7, 24.4 mg, yellow crystal in CH₂Cl₂: MeOH 2:1),^[3] arteannuin M (8, 98.4 mg, colorless crystal in CH₂Cl₂: MeOH 1:1),^[7] salicylic acid (9, 11.0 mg, colorless crystal in MeOH), β -sitosterol (10, 10.0 mg, white crystal in CH₂Cl₂: MeOH 10:1), artemisinin (11, 109.1 mg, colorless crystal in CH₂Cl₂: MeOH 2:1),^[8] artemisilactone B (12, 42.0 mg, white crystal in MeOH), salvigenin (13, 114.2 mg, yellow crystal in CH₂Cl₂: MeOH 2:1),^[9] 5,4'-dihydroxy-3,3',7-trimethoxy flavone (14, 226.4 mg, yellow crystal in CH₂Cl₂: MeOH 2:1),^[10] artemetin (15, 158.6 mg, yellow crystal in CH₂Cl₂: MeOH 4:1),^[3] chrysosplenetin (16, 389.0 mg, yellow crystal in CH₂Cl₂: MeOH 2:1),^[3] rutin (17, 56.5 mg, yellow crystal in MeOH), daucosterol (18, 32.1mg), 5-hydroxy-3,7,3',4'-tetramethoxy flavone (19, 33.3 mg, yellow crystal in CH₂Cl₂: MeOH 4:1),^[11]

and 5-hydroxy-6,7,8,4'-tetramethoxy flavone (20, 12.0 mg, yellow crystal in CH₂Cl₂: MeOH 3:1).^[12]

Compounds 1-8, 11-16 and 19-20 were identified by comparison of their physical and spectroscopic data (EIMS, IR, ¹H and ¹³C NMR) with those reported in the literature [Figure 1].

In vitro cytotoxicity bioassay

A549, HL60, U87, DU145 cells were purchased from American Type Culture Collection (#HB-8065, ATCT, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (GIBCO, NY, USA) supplemented with 10% fetal calf serum (FCS) (Shengma Yuanheng, Beijing, China), 100 mg/L streptomycin, 100 IU/mL penicillin, and 0.03% L-glutamine, and maintained at 37°C with 5% CO₂ in a humidified atmosphere.

Compounds 1-20 were resolved in dimethyl sulfoxide (DMSO) to make a stock solution. The DMSO concentration was kept below 0.10% throughout the cell culture period and did not exert any detectable effect on cell growth or cell death. The HeLa, MCF-7 and HepG2 cells were incubated at 6 × 10³ cells/well in 96-well plates, respectively. The cells were incubated with the five compounds at 1, 10, 50, 100 μ M for 48 hours. Cell growth was measured by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The percentage of cell growth inhibition was calculated as follows:

$$\text{Cell growth inhibition (\%)} = \frac{[A_{490}(\text{control}) - A_{490}(\text{compound})]}{[A_{490}(\text{control})]} \times 100$$

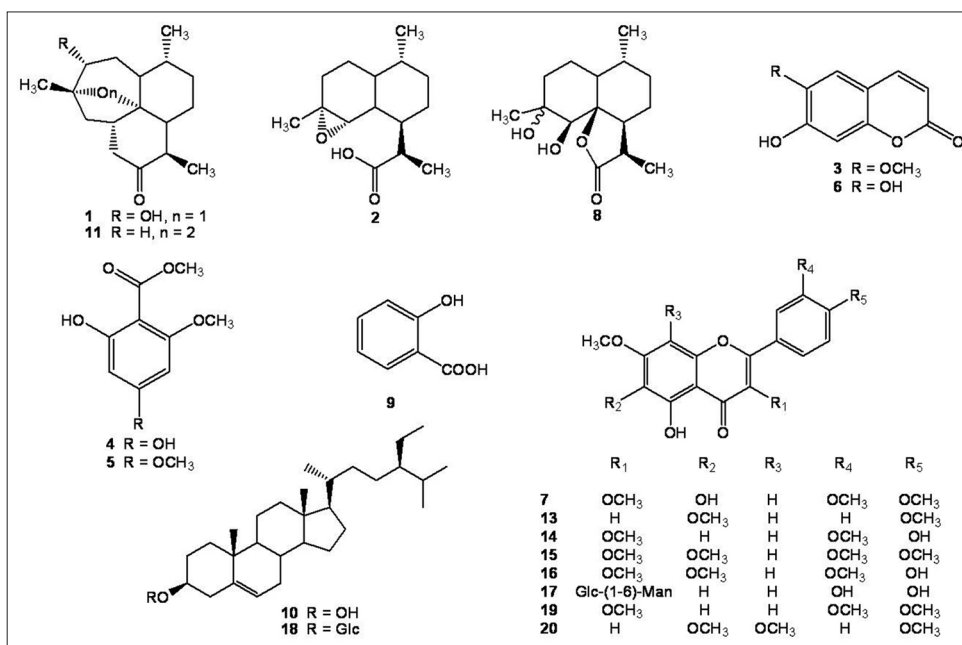


Figure 1: Structures of compounds isolated from *A. annua*

RESULTS AND DISCUSSION

Compound 12 was obtained as a white crystal (MeOH). The molecular formula was established as C₁₅H₂₂O₃ on the basis of ion peaks at *m/z* 273.1466 [M + Na]⁺ (calcd. for C₁₅H₂₂O₃Na, 273.1466) and 251.1640 [M + H]⁺ (calcd. for C₁₅H₂₃O₃, 251.1646) in the HR-ESI-MS, with the help of NMR spectra. Its IR spectra showed absorption bands for hydroxyl groups (3427 cm⁻¹), -CH₂- (2921 cm⁻¹), a terminal double bond (1630 cm⁻¹) and a six-membered ring lactones unit (1720 cm⁻¹). In the ¹H NMR spectrum of 12 [Table 1], two methyl groups at δ 1.53 and δ 0.91 (3H, d, *J* = 5.4 Hz), a O-bearing methylidyne group at δ 4.47 (1H, d, *J* = 12.0 Hz), one pair of olefinic proton signals at δ 4.88/4.73, were observed. The ¹³C NMR spectrum [Table 1] of 12 showed 15 carbon signals that could be assigned to the sesquiterpene moiety. A lactone carbonyl (δ 175.1), a pair of olefinic signals with exo-cyclic double bond at δ 147.8 (101.9), which were assigned C-4 (15), three tertiary carbon signals at δ 49.0/46.2/41.4, a O-bearing tertiary carbon signal at δ 79.3 and a O-bearing quaternary carbon signal at δ 87.8, were observed in the ¹³C NMR spectrum of 12. Thus, these data above allowed the skeleton of 12 to be deduced as a cadinanolide-type sesquiterpene derivative. Furthermore, an examination of the 2D-NMR spectra (HMQC, HMBC) of 12 indicated that it was similar to artemisilactone,^[13] but differed from artemisilactone at C-11 and C-4 [Table 1 and Figure 2]. The HMBC experiment showed ¹H/¹³C correlations between H-15 and C-3, C-5, between H-14 and C-1, C-9, and between H-13 and C-7, C-12. Because the H-C (1), H-C (6) and 14-Me of cadinanolide-type are defined in the α-configuration, H-C (5) is defined in the β-configuration. Additionally, the NOESY experiment showed correlations between H-1 and H-2, 6, 7, 9, 14, H-5 and H-2, 3, 8, 10, H-6 and H-1, 2, 7, 13, H-7 and H-1, 6, 8, 9, 13, H-13 and H-7, H-14 and H-1, 2, 9, 10. So 7-H and 13-Me were defined as α-configuration and 13-OH was defined as β-configuration. As a result, those elucidation allowed the structure of compound 12 [Figure 2] to be deduced.

Using A549, HL60, U87 and DU145 cell lines, the antitumor activities of compounds 1-20 were evaluated in vitro. Compounds 16 showed significant cytotoxic activities against the A549, HL60 and U87 cell lines with IC₅₀ value at 15.76, 13.65 and 22.37 μM, respectively. Compounds 4 and 5 exhibited moderate antitumor activities against HL60 cell line with an IC₅₀ value at 84.07 and 50.60 μM.

CONCLUSION

The finding of the new sesquiterpene (compound 12) will provide reference for the taxonomy of *A. annua*, and the

Table 1: The ¹H-NMR and ¹³C-NMR spectroscopic data and HMBC correlations of compound 12 (300 MHz for ¹H; 75 MHz for ¹³C, CDCl₃)

Position	δH	δC	HMBC
1	1.61	41.4	C-3, 5, 7, 9, 14
2α	1.98 (1H, m)	28.8	C-4, 6, 10
2β	1.47 (1H, m)		
3α	2.02 (1H, m)	29.5	C-1, 5, 15
3β	2.19 (1H, m)		C-1, 3, 7, 15
4	-	147.8	
5	4.47	79.3	
6	2.04	49.0	C-2, 4, 8, 10, 11
7	2.22	46.2	C-1, 5, 9, 12, 13
8α	1.84 (1H, m)	24.0	C-6, 10, 11
8β	1.31 (1H, m)		
9α	1.02 (1H, m)	34.2	C-1, 7, 14
9β	1.74 (1H, m)		
10	1.56	28.6	C-2, 6, 8
11	-	87.8	
12	-	175.1	
13	1.53	26.1	C-7, 12
14	0.91	19.9	C-1, 9
15a	4.73 (1H, s)	101.9	C-3, 5
15b	4.88 (1H, s)		

HMBC: Heteronuclear multiple bond correlation

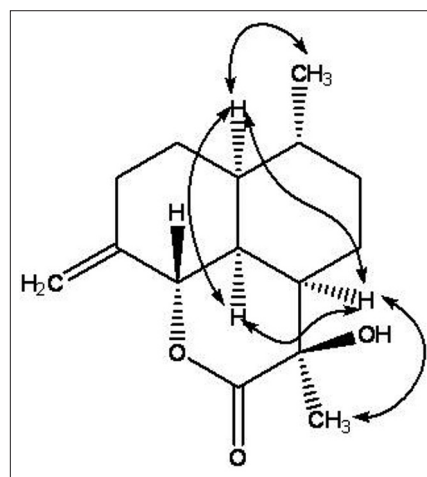


Figure 2: Key NOESY correlations of compound 12

flavonoids may be a useful phytochemical data and chemical foundation for further mechanism studies on improving the anti-malarial action of artemisinin. Furthermore, the characteristic constituent 16, polymethoxy substituted flavonoid, is a potential antitumor agent.

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