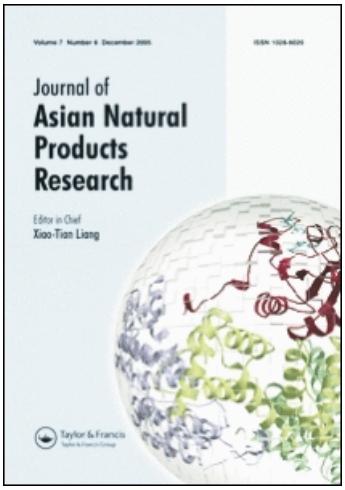

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ANALYSIS OF PHYTOECDYSTEROIDS IN CULTURED PLANTS OF *AJUGA NIPPONENSIS* MAKINO

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Cultured plants of *Ajuga nipponensis* contained cyasterone (**1**), ajugasterone C (**2**), cyasterone-22-acetate (**3**) and 22-dehydrocyasterone (**4**) based on HPLC and NMR data, whereas 20-hydroxyecdysone was not detectable. The presence of compounds **2**–**4** is reported for the first time in this species. Compound **1** is the main phytoecdysteroid component found in both pre-blossom and blossom plants, but the latter contained higher amount than the former. Compared with other parts of the plant, the highest percentage of **1** and **3** occurred in leaves, amounting to 60.1% and 88.0% respectively, whereas the flowers contained mainly **2**, which represented 72.8% of the total amount in whole plant. The contents of phytoecdysteroids in stems were very low.

Keywords: Ecdysteroids; *Ajuga nipponensis*; Cyasterone; Ajugasterone C; Cyasterone-22-acetate; 22-Dehydrocyasterone

INTRODUCTION

The herb plant *Ajuga nipponensis* has been used as a folk medicine in many Asian countries to cure inflammation, ease pain, and remove blood accumulation. After the first report of the occurrence of phytoecdysteroids in this plant by Imai *et al.* in 1969 [1], the phytoecdysteroid constituents of wild

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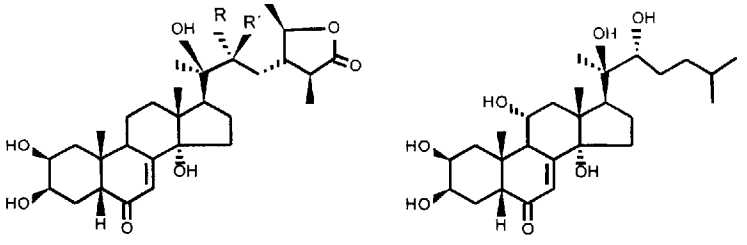
plants grown in China were analyzed and its utilization in sericulture was suggested [2,3]. In late 1970s, large scale extraction of 20-hydroxyecdysone from wild plants was established in factories. However, it was found in practice that the content of 20-hydroxyecdysone varied with materials collected from different growing locations and different seasons, and, in some cases, it only contained cyasterone instead of 20-hydroxyecdysone [4].

Cultivation is one of the solutions in providing sustainable source material with reasonable constant contents of phytoecdysteroids. Field trials of plantation were carried out and the profile of phytoecdysteroids in cultured plants was analyzed.

RESULTS AND DISCUSSION

The repeated analysis of samples collected from different seasons revealed that three major ecdysteroids (**1**–**3**) and a minor one (**4**) occurred in the cultured plants (Fig. 1). Cyasterone (**1**) has been previously reported as one of the main phytoecdysteroid components in this species [1] and it is common in almost all *Ajuga* plants [5], but ajugasterone C (**2**), cyasterone-22-acetate (**3**) and 22-dehydrocyasterone (**4**) are unprecedented in this species. Compounds **2**–**4** were firstly isolated and identified in *A. japonica* [6], *A. turkestanica* [7] and *A. iva* [8], respectively.

The structural assignment of the phytoecdysteroids present in the plant was based on HPLC retention times and relative retention times under two different solvent systems (Table I), compared with those of previously isolated standards and taking 20-hydroxyecdysone as reference. All of them had been confirmed by NMR spectroscopy.



1 R=OH, R'=H
3 R=OCOCH$_3$, R'=H
4 RR'=O

2

FIGURE 1 Structure of phytoecdysteroids isolated in cultured plants.

TABLE I Retention time of phytoecdysteroids on HPLC

Phytoecdysteroid	*System* 1*		*System* 2†	
	Rt (min)	*RRt* (%)	*Rt* (min)	*RRt* (%)
1	4.278	102	4.763	110
2	8.601	206	16.357	379
3	8.160	195	11.415	264
4	15.211	363	18.062	418
20-hydroxyecdysone	4.185	100	4.321	100

*MeOH/H_2O 43 : 57, 1 ml/min, 30.0–35.0°C;
†20% *i*-PrOH: H_2O 60 : 40, 1 ml/min, 55.0 ± 1.0°C.

TABLE II Estimated contents of major phytoecdysteroids in whole plants

Phytoecdysteroid	*Pre-blossom*		*Blossom*	
	Amount (ppm)	*Portion* (%)	*Amount* (ppm)	*Portion* (%)
1	389	64.6	659	51.1
2	66	11.0	415	32.2
3	147	24.4	216	16.7
Total	602		1290	

TABLE III Estimated contents of major phytoecdysteroids in blossom plants

Phytoecdysteroid	*Content* (ppm)*			
	Root	*Stem*	*Leaf*	*Flower*
1	814 (71)	114 (45)	1355 (396)	661 (147)
2	262 (23)	31 (12)	267 (78)	1357 (302)
3	103 (9)	31 (12)	650 (190)	22 (5)
Total	1179 (103)	176 (69)	2272 (664)	2040 (454)

*Data in parentheses are based on dry weight of whole plant.

The phytoecdysteroid contents were estimated according to the measured peak area of 20-hydroxyecdysone standard by HPLC. The data obtained indicate that **1** was the main phytoecdysteroid in both pre-blossom and blossom plants, contributing more than half to the total amount of major phytoecdysteroids (Table II). As shown, the blossom plants contained much higher amount of phytoecdysteroids than the pre-blossom plants, though their chemical patterns were the same. It was also found that compound **2** mainly occurred in blossom plants.

The cultured plants harvested in blossom yielded 88 g roots, 398 g stems, 292 g leaves and 222 g flowers per kilogram dry weight. The contents of major phytoecdysteroids in those parts were measured and their distribution rates were calculated (Table III). It was found that leaves and flowers contained a relatively high amount of phytoecdysteroids (2272 and 2040 ppm, respectively). Generally, roots occupied the second position in

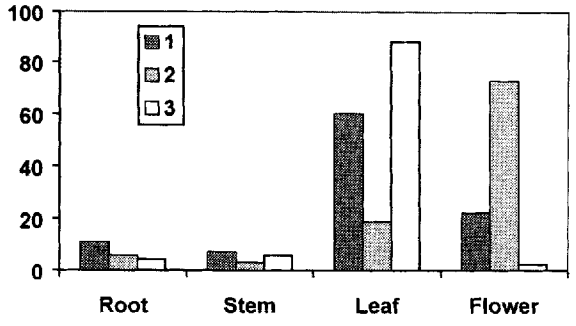


FIGURE 2 Distribution of major phytoecdysteroids in different plant parts.

the amount of phytoecdysteroids. The contents of phytoecdysteroids in stems were very low.

From the distribution pattern, it was noteworthy that 60.1% of **1** and 88.0% of **3** were found in leaves and compound **2** was mainly distributed in flowers, which contained 72.8% of the total amount (Fig. 2). The percentage of distribution in root was lowered by its low weight portion.

Unexpectedly, 20-hydroxyecdysone, the major phytoecdysteroid in wild plants [1,2], was not detectable in all samples tested in our three years work. This phenomenon was also noticed previously [4]. The variability of ecdysteroid production by several samples of one species of *Ajuga* plants (of different geographic origin), grown under the same greenhouse conditions to avoid environmental effects, has been already discussed [9]. Lafont assumed that the genes required for ecdysteroid biosynthesis are present in all plant species, whether they accumulate ecdysteroids or not, and that the difference is rather at the regulatory level [10]. Further investigation is needed to acquire knowledge of the effects of environmental factors on the biosynthesis and accumulation of phytoecdysteroids in plants.

EXPERIMENTAL SECTION

General Experimental Procedures

The HPLC system used in this analysis consists of two pumps for solvent delivery (Applied Biosystems model 400), a mixing injector (Applied Biosystems model 491), a diode array detector (Applied Biosystems model 1000S), a column thermostat (Spark Holland SPH 99) and an integrator (HP3396 series II). A reversed phase column Merck LiChrospher 100RP-18 (LiChroCART, 5 μm, 12.5 × 0.4 cm, N/m = 20000) with a pre-column

TABLE IV ^{1}H-NMR spectral data of compounds **3** and **4** (300 MHz, C_5D_5N, δ, ppm, J = Hz)

H	**1**	**3**	**4**
2	4.16	4.15 dm (13.8, $w_{1/2}$25)	4.16 dm (14.4, $w_{1/2}$24)
3	4.22	4.21 bs ($w_{1/2}$ 9)	4.23 ov*
5	3.01	3.01 dd (12.2, 3.6)	3.01 dd (13.0, 3.6)
7	6.31	6.26 d (2.1)	6.24 d (2.1)
9	3.59	3.58 bm	3.58 ddd (*ca.* 10, 7.2)
12	2.65	2.63 ov*	2.66 ddd (12.5, 12.5, 5.0)
17	2.86	2.83 t (8.4)	
22	3.94	5.47 bd (10.5)	
28	4.01	3.96 dq (8.7, 6.0)	4.23 dq (8.1, 6.3)
Me-18	1.24	1.18 s	1.12 s
Me-19	1.07	1.06 s	1.04 s
Me-21	1.57	1.57 s	1.67 s
Me-27	1.36	1.44 d (7.2)	1.26 d (6.6)
Me-29	1.31	1.32 d (6.0)	1.32 d (6.0)
AcO	–	2.04 s	

*Overlapped signal.

(Bondapak C-18, 10 μm, 24 × 4 mm) is installed. Phytoecdysteroid peaks were detected at 245 nm and co-monitored at 215 and 300 nm. Authentic standard of 20-hydroxyecdysone was used. Its concentration in methanol solution was verified by UV absorption. The contents of phytoecdysteroids in the test samples were estimated by comparing the peak area with the standard curve of 20-hydroxyecdysone. The identity and structure of each phytoecdysteroid isolated were confirmed by NMR spectroscopy (Varian UNITY 300, Varian Associates, Inc., USA). NMR spectral data of **1** and **2** were in good accordance with those previously reported [11–13]. Improved ^{1}H- and ^{13}C-NMR spectral data of **3** and **4** are displayed (Tables IV and V) and data of **1** are included for correlation purposes.

Plant Material

Plants of *A. nipponensis* cultured in the insecticidal plant garden of South China Agricultural University, Guangzhou, China, were collected before flowering as pre-blossom plant sample and during flowering as blossom plant sample. Fresh plants were washed with tap water, dried in open air, and finally dried to constant weight in an air-driven oven below 75°C. Plant powder of 40 mesh was prepared for the next step use.

Extraction and Isolation

Two methods have been used to prepare the methanolic extracts. In the first period of our research, samples of plant powder were exhaustively extracted

TABLE V ^{13}C-NMR spectra data of compounds **3** and **4** (75 MHz, C_5D_5N, δ, ppm)

C	**1**	**3**	**4**
1	38.0t	37.9t	37.97t
2	68.1d	68.1d	68.08d
3	68.0d	68.0d	68.04d
4	32.5t	32.0t	32.42t
5	51.4d	51.3d	51.39d
6	203.4s	203.3s	203.34s
7	121.8d	121.9d	121.90d
8	165.8s	165.5s	165.37s
9	34.5d	34.3d	34.42d
10	38.7s	38.7s	38.71s
11	21.1t	21.5t	21.02t
12	32.0t	31.8t	31.93t
13	48.2s	48.2s	48.16s
14	84.1s	84.1s	84.00s
15	31.9t	32.4t	31.81t
16	21.3t	21.0t	21.58t
17	50.0d	50.3d	51.67d
18	17.9q	17.8q	17.32q
19	24.5q	24.4q	24.41q
20	76.8s	76.1s	81.33s
21	21.0q	21.2q	25.55q
22	73.9d	76.8d	215.00s
23	34.4t	29.9t	39.08t
24	48.6d	48.0d	46.06d
25	42.4d	42.7d	42.12d
26	179.2s	178.7s	178.53s
27	15.9q	15.6q	14.39q
28	79.8d	79.2d	79.79d
29	19.3q	18.9q	9.89q
CO (AcO)		170.8s	
Me (AcO)		21.7q	

with five times v/w of methanol (analytical grade) by Soxhlet at 70 ± 2°C. Later, extraction was made with *ca.* 50 times v/w of methanol under 30 min of ultrasonic bath. The further isolation and purification were carried out by following the previously developed procedure [14]. Compounds **3** and **4** were isolated from the early eluting fractions from vacuum liquid chromatography on silica gel of the ecdysteroid fraction using a chloroform–methanol gradient, followed by preparative HPLC (solvent system 1).

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