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Characteristic of Anthraquinone Production in Plant Roots and Cell Suspension Cultures of *Rubia tinctorum* and *R. akane*

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Constituents of the plant roots of *Rubia tinctorum* and *R. akane* were compared by HPLC-photodiode array analyses with those of the corresponding cell suspension cultures. Although, anthraquinones could be identified in the extracts of the plant roots and those of the cell suspension cultures of both plants, not only the proportion of constituents was different between the plant roots and the corresponding cell suspension cultures but also the constituents themselves were different. Mollugin (**1**) which is a non-anthraquinonyl compound was found only in the extracts of the plant roots. In contrast, amounts of the oxidized anthraquinones *i. e.* munjistin (**16**) and pseudopurpurin (**18**) were greater in the cell suspension cultures.

Introduction

The roots of *Rubia tinctorum* L. and *R. akane* Nakai (=*R. cordifolia* L. var. *mungista* Miq.) are used for sources of natural dyes-mostly anthraquinones[1-5]. In the course of our continuous studies of natural dyes, we estabished their cell suspension cultures which also produce anthraquinone pigments[6,7]. But detailed composition of anthraquinones in the cell suspension cultures seemed to be different from those in field-grown plants, since the color tones of the extracts of the cell suspension cultures were more reddish than those of plans. In recent years, the differences between secondary metabolites produced by mother plants and those of the corresponding cell cultures has drawn the attention of many researchers. For example, kinobeon, which has never been detected in the mother plants, is commonly identified in cell cultures of Compositae plants[8]. Cell cultures of *Cinchona ledgeriana* (Rubiaceae) produce anthraquinones, which have never been identified in the mother plant[2,9]. We report here our comparative studies of pigments in mother plants of *R. tinctorum* and *R. akane* and those of corresponding cell suspension cultures.

Materials and Methods

Authentic Samples

1, 3, 6-Trihydroxy-2-methylanthraquinone (**8**), 1, 3, 6-trihydroxy-2-menthylanthraquinone-3-*O*-

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α-rhamnosyl-(1 → 2)-β-glucoside (**9**) and 1, 3, 6-trihydroxy-2-menthylanthraquinone-3-*O*-(6′-*O*-acetyl)-α-rhamnosyl-(1 → 2)-β-glucoside (**10**) were kindly supplied by Prof. H. Itokawa (Tokyo College of Pharmacy). 1, 3-Dihydroxy-2-acetoxymethylanthraquinone (**13**) and munjistin (**16**) were kindly supplied by Dr. Y. Hirose (Univ. of Kumamoto). Pseudopurpurin (**18**) was kindly supplied by Dr. H. Suzuki (Japan Tobacco Inc.). Mollugin (**1**), 1-hydroxy-2-methylanthraquinone (**5**), rubiadin (**7**), lucidin (**11**), lucidin-3-*O*-primeveroside (**12**), 1, 3-dihydroxy-2-ethoxymethylanthraquinone (**14**), nordamnacanthal (**15**) and munjistin methyl ester (**17**), and 2-ethoxycarbonyl-1-hydroxyanthraquinone (**6**) were isolated from *Rubia tinctorum* and *R. akane*, respectively. Identification of each of these compounds has been described previously[10,11]. Tectoquinone (**2**) was purchased from Tokyo-Kasei Co. Alizarin (**3**) and purpurin (**19**) were purchased from Wako Pure Chemical Co. Ruberythric acid (**4**) was purchased from Funakoshi Chemical Co.

Plant material and cell suspension cultures.

R. tinctorum and *R. akane* plants were cultivated at the Tsukuba Medicinal Plant Research Station, NIHS.

Leaf segments (5×5 mm²) of *R. tinctorum* cut from the aseptic plants, placed on Murashige and Skoog (MS) medium[12], that contained 5 μM NAA and 0.1 μM kinetin and was solidified with 0.2% gellan gum. These were cultured at 27°C in the dark. Callus tissues were transferred to MS medium, that contained 5 μM NAA and 0.1 μM kinetin and was solidified with 0.15% gellan gum to provide a soft solid medium. The callus tissues thus obtained were inoculated into the MS liquid medium containing 5 μM NAA and 0.1 μM kinetin and allowed to stand for one month to establish the cell suspension cultures. The cultures were maintained at 27°C in the dark on a rotary shaker (100 rpm) and were subcultured weekly. The cell suspension cultures (18 g fresh weight) were transferred to Linsmaier and Skoog (LS) liquid medium[13] (10 *l* of medium) containing 0.5 μM NAA and 0.1 μM kinetin in a 10 *l* jar fermenter and cultured at 27°C in the dark. After 20 days of culture cell suspension cultures were harvested (*ca.* 113 g fresh weight).

Leaf segments (5×5 mm²) of *R. akane* cut from the aseptic plants, placed on MS medium that contained 0.5 μM 2, 4-D and was solidified with 0.2% gellan gum. These were cultured at 27°C in the dark. Callus tissues were inoculated to the MS liquid medium with 0.5 μM 2, 4-D and allowed to stand for 2 weeks. The cultures were maintained at 27°C in the dark on a rotary shaker (100 rpm) and were subcultured weekly. The established cell suspension cultures (35 g fresh weight) were transferred to the LS liquid medium (10 *l* medium) containing 0.5 μM NAA in the 10 *l* jar fermenter and cultured at 27°C in the dark. After 10 days of culture cell suspension cultures were harvested (*ca.* 77 g fresh weight).

Sample preparation and hydrolysis of MeOH extracts

The plant roots were washed with tap water and dried in the dark at room temperature. Dried roots were cut into pieces. The cell suspension cultures were freeze-dried. The plant roots and the cell cultures were extracted at room temperature with $CHCl_3$ and MeOH. The extraction periods for the plant roots and the cell cultures were one month and one day, respectively. After filtration, the extracts were evaporated to dryness to afford HPLC samples. Part of MeOH extracts were hydrolyzed with 5% H_2SO_4 at 80°C for 12 hr. The hydrolysates were extracted with EtOAc. The extracts were evaporated to dryness. Samples and hydrolysates were dissolved in DMSO or a mixture of DMSO and $CHCl_3$ for HPLC analyses.

HPLC conditions

HPLC was carried out with the gradient solvent system, using a TOSOH TSK-gel ODS 120 T (4.6×250 mm) column, which was kept at 40°C. The flow rate was 0.8 m*l*/min throughout. The

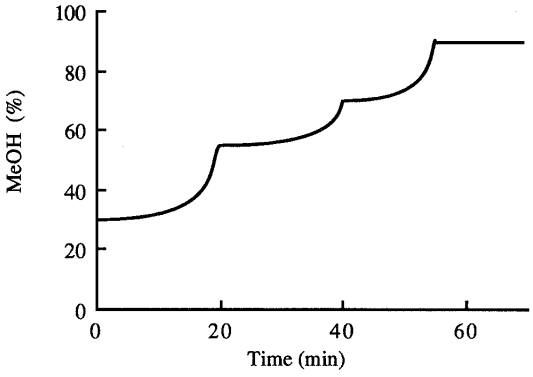


Fig. 1 HPLC gradient system.

solvent used for gradient elution was MeOH and 10% HOAc. The gradient condition is showed in **Fig. 1**. The effluent was monitored by a photodiode array (PDA) detector (SPD-M1A, Shimadzu).

Results and Discussion

$CHCl_3$ and MeOH were used as extractants in order to effectively recover the aglycons and the glycosides. HPLC analyses of the extracts of the plant roots of *Rubia tinctorum* and *R. akane* were compared with those of the corresponding cell suspension cultures. The anthraquinones, which

Table 1. HPLC analyses of *R. tinctorum* extracts.

Substances	Retention time (min.)	% Area*1			
		Plant roots		Cell suspension cultures	
		$CHCl_3$ ext.	MeoH ext.	$CHCl_3$ ext.	MeOHext.
4	16.4	—*2	36	—	15
12	17.3	—	100	—	100
9	26.0	—	—	—	—
16	26.5		27*3	—	27*3
18	26.5	5	4*3	100	11*3
11	31.5	26	trace*4	7	9
3	32.0	72	16	54	64
10	36.4	—	—	—	—
19	44.1	trace	—	—	—
8	45.3	—	—	—	—
13	45.5	—	14	—	—
17	49.3	trace	—	—	—
2	50.3	trace	—	—	—
14	51.2	—	—	—	—
6	51.2	—	—	—	—
7	52.9	7	—	2	—
15	57.0	100	—	15	14
5	60.7	—	—	—	—
1	63.6	26	—	—	—
Numbers of unknown peaks*5		5	2	1	1

*1 Area percent relative to largest peak as 100%.

*2 Not detected.

*3 % area was estimated on the basis of the chromatograms of the hydrolysates.

*4 Below 5% peak area.

*5 Peaks of 5% area or more were counted.

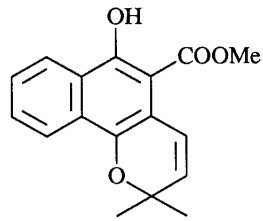
Table 2. HPLC analyses of *R. akane* extracts.

Substances	Retention time (min.)	% Area*1			
		Plant roots		Cell suspension cultures	
		$CHCl_3$ ext.	MeoH ext.	$CHCl_3$ ext.	MeOHext.
4	16.4	—*2	—	—	—
12	47.3	—	—	—	—
9	26.0	—	100*3	—	—
16	26.5	—	26*3	—	100*3
18	26.5	—	—	100	6*3
11	31.5	—	—	—	11
3	32.0	—	—	17	28
10	36.4	—	34	—	12
19	44.1	—	—	—	—
8	45.3	—	—	—	—
13	45.5	—	—	—	—
17	49.3	—	—	—	—
2	50.3	—	—	14	9
14	51.2	—	—	—	—
6	51.2	100	—	—	—
7	52.9	—	—	—	—
15	57.0	—	—	—	—
5	60.7	61	—	—	—
1	63.6	17	—	—	—
Numbers of unknown peaks*5		9	2	0	3

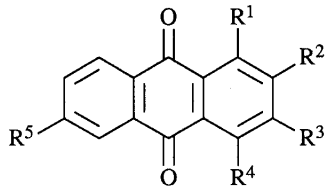
See footnotes for **Table 1.**

seemed to be the major constituents, exhibited absorption maxima around 280 nm. Therefore, detection at 280 nm was used for comparative studies of HPLC patterns. Identification of constituents was accomplished by comparing their PDA spectra (UV-Vis: 230-600 nm) and retention times with those of authentic samples. In addition, we counted the number of the unknown peaks which were more than 5% of the largest peak in each chromatogram. On the basis of PDA spectra, most of the unknown peaks seemed to be anthraquinones. Under our HPLC conditions, **16**, **18** and **9** were not separated well. Fortunately, **16** was stable under hydrolytic conditions that transformed **18** to **19**, and **9** to 8. Since 8, **16** and **19** were well separated from each other by HPLC, **16** was chromatographically distinguishable from **18** and **9** after hydrolysis of extracts. The results of the HPLC analyses are summarized in **Tables 1** and **2**. The structures of authentic samples and their trivial names are shown in **Fig. 2**.

As shown in **Table 1**, in the extracts of *R. tinctorum* roots, **1** and nine anthraquinones (**3, 4, 7, 11, 12, 13, 15, 16**, and **18**) were detected together with some unknown peaks. Of those compounds, **1** and **13** were not detected in the extracts of *R. tinctorum* cell cultures. In case of *R. akane*, **1** and five anthraquinones (**5, 6, 9, 10** and **16**) were detected in the extracts of the plant roots together with some unknown peaks. Of those compounds, only two anthraquinones (**10** and **16**) could be detected in extracts of cell suspension cultures; however, four other anthraquinones (**2, 3, 11** and **18**), were found. Suzuki and Matsumoto[14] reported that **3, 4, 14, 18** and **19** were isolated from the cell cultures of *R. akane*. However we could not detect **14** and **19** in non-hydrolyzed extract of *R. akane* cell cultures (**Tables 1** and **2**). After acid hydrolysis, **19** was detectable. Therefore **19** was presumed to be converted from **18**. These results suggest that not only the proportion of constituents is different between mother plant and corresponding cell suspension cultures but also the constituents them-



Mollugin (1)



	Trivial name	R^1	R^2	R^3	R^4	R^5
2	Tectoquinone	H	Me	H	H	H
3	Alizarin	OH	OH	H	H	H
4	Ruberythric acid	OH	O-glc^6→^{1}xyl	H	H	H
5		OH	Me	H	H	H
6		OH	COOEt	H	H	H
7	Rubiadin	OH	Me	OH	H	H
8		OH	Me	OH	H	OH
9		OH	Me	O-glc^2→^{1}rham	H	OH
10		OH	Me	O-(6'-OAc)glc^2→^{1}rham	H	OH
11	Lucidin	OH	CH_2OH	OH	H	H
12	Lucidin-3-*O*-primeveroside	OH	CH_2OH	O-glc^6→^{1}xyl	H	H
13		OH	CH_2OAc	OH	H	H
14		OH	CH_2OEt	OH	H	H
15	Nordamnacantal	OH	CHO	OH	H	H
16	Munjistin	OH	COOH	OH	H	H
17	Munjistin methylester	OH	COOMe	OH	H	H
18	Pseudopurpurin	OH	COOH	OH	OH	H
19	Purpurin	OH	OH	OH	OH	H

Fig. 2 Structures and trivial names of authentic samples.

selves are different.

Compound **18** was a minor aglycon in *R. tinctorum* roots and not detected in *R. akane* roots, but it was a major aglycon in both cell cultures. Since it absorbs at 530 nm, the reddish color of the extracts of the cell suspension culture may be attributed to this compound.

Comparison of the components of the plant roots of both *R. tinctorum* and *R. akane* with those of the cell cultures, reveals common features of both species.

1) The chromatograms of the extracts of the cell cultures were simpler than those of the plant roots. In other words, fewer peaks were observed in the cell culture extracts than in the extracts of the plant roots. This tendency was noted in the $CHCl_3$ extracts which contained primarily aglycons. This suggests that the biosynthetic pathway is simplified by dedifferentiation.

2) Compound **1** was detected in the extracts of both plant roots only. In contrast, **1** was isolated as another secondary metabolite from plant[15] and cell cultures[16] of *Galium mollugo* L. Itokawa *et al.*[17] speculated that genera of *Rubia* and *Galium* are closely related plants since **1** is found in both.

Therefore, our results are interesting from a chemotaxonomic point of view.

3) Characteristic anthraquinones from each mother plant, i. e. **4** and **15** from *R. tinctorum* [10], and **5, 6, 9** and **10** from *R. akane* [11,18], were reduced or absent in the cell cultures, while the more highly oxidized anthraquinones, *i. e.* **18** for *R. tinctorum*, and **16** and **18** for *R. akane*, were more prominent or appeared as major components. Suzuki and Matsumoto[14] also found that **18** is a major product from the cell cultures of *R. akane*. It seems that **18** is an end product of the anthraquinonyl biosynthetic pathway of anthraquinones because of its oxidative state. Therefore, our results suggest the hypothesis that cell cultures of *Rubia* genus tend to produce biosynthetically terminal anthraquinones.

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《和文要約》

セイヨウアカネ (*Rubia tinctorum* L.) およびアカネ (*R. akane* Nakai) の植物根と液体懸濁培養細胞におけるアントラキノン類生産の特性

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セイヨウアカネ (*Rubia tinctorum* L.) およびアカネ (*R. akane* Nakai) の植物根と液体懸濁培養細胞の成分について，フォトダイオードアレイ検出器付高速液体クロマトグラフィーによる比較を行った．両植物において植物根と培養細胞のいずれの抽出物からも anthraquinone 類が検出されたが，植物根と培養細胞では，成分組成だけでなく anthraquinone 類の種類も異なっていた．非 anthraquinone 類である mollugin は植物根のみから検出された．一方，培養細胞では munjistin や pseudopurpurin といった酸化度の進んだ anthraquinone 類の増加が認められた．