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Anthraquinone production by callus cultures of *Rubia cordifolia*

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Abstract

Munjistin and purpurin were identified as the major components of anthraquinone pigments produced by callus cultures of *R. cordifolia*. Anthraquinone content in calluses was 0.62–1.22% (by dry wt.) depending on the source of explants. Selection of coloured aggregates yielded a cell line with twofold increase in anthraquinone production.

Keywords: *Rubia cordifolia*; Callus culture; Anthraquinones

1. Introduction

Root extracts of *R. cordifolia* L. (Rubiaceae) possess hepatoprotective activity, antineoplastic properties and are considered to be useful for the disintegration and elimination of urinary stones [1–3]. The low content of biologically active substances in this plant has served as an incentive for the development of cell cultures

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of *R. cordifolia* capable of long-term cultivation and synthesis of secondary metabolites. In the present work we demonstrate results of developing *R. cordifolia* callus cultures which can be exploited as an alternative source of anthraquinones.

2. Experimental

2.1. Plant material

Plants of *R. cordifolia* L. collected in June 1996 from the southern Primorsky Region of the Russian Far East and identified in the Botany Department of the Institute of Biology and Soil Science, were used as explant sources.

2.2. Culture conditions and selection

After rinsing in water, explants of *R. cordifolia* were sterilized with a 0.1% solution of commercial Diocide for 3 min and washed three times with sterile distilled water. They were then placed on solid W-O medium [4] containing 0.5 mg/l 6-benzyladenin and 2.0 mg/l α-naphthaleneacetic acid (W-B/A medium) and cultured in the dark in 100-ml flasks containing 20 ml of medium. Calluses emerging on the explants were excised and cultivated on the same medium at 25°C for 1-month periods.

To select high-producing callus cultures, the deeper coloured small cell aggregates (2–3 mm) were isolated from the stock yellow calluses and further subcultured on W-B/A medium for 1-month periods. Callus tissues of these selected strains were subcultured for a further 6 months before they were characterized quantitatively for pigment content.

2.3. Extraction and isolation of anthraquinones

Dried, ground calluses impregnated with 5 N HCl were extracted with MeOH. After evaporation of the solvent the residue was partitioned between chloroform and water. The chloroform soluble fraction was extracted with 1 N NaOH which was then acidified with HCl and extracted with $CHCl_3$. The final chloroform extract contained almost exclusively quinone. This extract was chromatographed on Sephadex LH-20 (in $CHCl_3$–MeOH 10:1) to yield two main anthraquinones which, according to UV, MS and NMR data, were identified as purpurin [5] (**1**), m.p. 262°C and munjistin [5] (**2**), m.p. 231–233°C.

2.4. Quantitative determination

One hundred milligrams of dried powdered calluses impregnated with 0.3 ml 5 N HCl were extracted with 3 ml ethanol for 20 h at room temperature. The purpurin content of the ethanol extract was determined by absorption at λ 515 nm in a

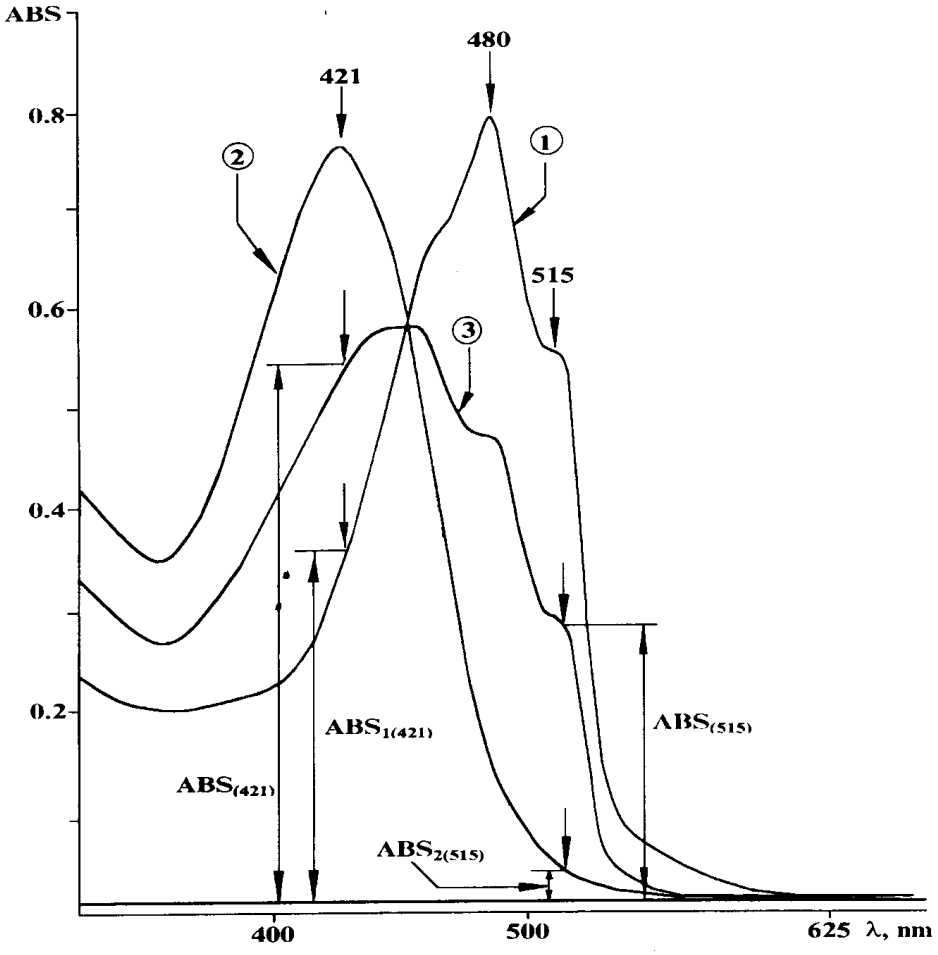

Fig. 1. Visible spectrum of the ethanol solution: (1) purpurin (0.212 mg/ml, l = 1 mm); (2) munjistin (0.384 mg/ml, l = 1 mm); and (3) mixture purpurin + munjistin (1:1.63, l = 1 mm).

1-mm quartz cell. The content of munjistin was quantified by absorption at λ 421 nm, subtracting the absorption due to purpurin at this wavelength.

3. Results

3.1. Callus line establishment and anthraquinone determination

Calluses of *R. cordifolia* aerial parts were obtained as described in Section 2 and were further selected for fast growth and yellow pigmentation to produce high-yielding cell lines. Attempts to develop callus tissue from the roots were unsuccessful. The ability of bioregulators to support growth and pigment formation of the anthraquinone-producing callus cultures was also investigated. 6-Benzyladenin and

α-naphthaleneacetic acid were found to be the most effective for *R. cordifolia* cells (data not shown). At the end of the culture period calluses were collected and analyzed. Purpurin (**1**) and munjistin (**2**) were identified as the main anthraquinones produced by all lines, representing almost 90% of total anthraquinones.

Absorption maxima of **1** and **2** were quite different in the visible spectrum, being λ 480 and λ 421 nm, respectively (Fig. 1). Moreover, the molar extinction coefficients (ε) of **1** and **2** at the specified maxima differed by a factor of almost 2 [$\varepsilon_{1(480)} = 9660 \pm 190$ mol$^{-1} \times$ cm^{-2} and $\varepsilon_{2(421)} = 5680 \pm 110$ mol$^{-1} \times$ cm^{-2}, respectively]. Clearly, quantitative analysis of **1** and **2** cannot be carried out at the same wavelength. We therefore conclude that the determination of anthraquinone concentrations in the callus extracts at the same wavelength (as has been done in a number of previous studies) [6–9] would result in a high degree of error. Our calculations suggest that the error could be 30% and higher.

We have therefore proposed a new procedure for the determination of **1** and **2** in our cultures using different wavelengths. The content of **1** in the ethanol extracts is conveniently determined by absorption of a shoulder at λ 515 nm (Fig. 1), where the contribution from absorption by **2** [$ABS_{2(515)}$, see Fig. 1] is small (less than 3%). The molar extinction coefficient of the standard **1** at 515 ± 2 nm, averaged from three independent measurements, was: $\varepsilon_{1(515)} = 6685 \pm 130$. Anthraquinone **2** is determined in ethanol extracts by absorption at 421 nm, taking into account the absorption of **1** [$ABS_{1(421)}$, see Fig. 1] at the same wavelength [$\varepsilon_{1(421)} = 4200 \pm 100$]. The molar extinction coefficient of the standard **2** at λ 421 nm is equal to $\varepsilon_{2(421)} = 5680 \pm 110$.

Mixtures of the standards **1** and **2** in various molar ratios (from 2:1 to 1:2) were prepared. Determination of **1** and **2** in these mixtures by the new method yields a relative error not exceededing ± 2%.

The quantity of **1** and **2** (mg/ml) in the ethanol extracts was determined as:

$$C_1 = \varepsilon_{1(515)} \times ABS_{(515)} \times 10/l \times Mm_1$$

$$C_2 = \varepsilon_{2(421)} \times [ABS_{(421)} - C_1 \times \varepsilon_{1(421)}] \times 10/l \times Mm_2$$

where Mm_1 and Mm_2 are molecular mass of **1** and **2**, respectively; and l is the cell path lengh (mm).

Examination of the anthraquinone content in the roots of wild-growing *R. cordifolia* plants revealed the presence of the same anthraquinones **1** and **2** as the major components of pigments isolated (0.023 and 0.17% dry wt., respectively).

3.2. Anthraquinone production by callus cultures derived from different parts of R. cordifolia

It was of interest to see what effect explant source would have on the anthraquinone production. The leaf petiole- and stem-derived calluses accumulated higher anthraquinone levels compared with stem apex (apical meristem) and

Table 1
Biomass accumulation and anthraquinone production by callus cultures derived from different organs of *R. cordifolia*[a]

Source of explant	Fresh biomass (g/l)	Dry biomass (g/l)	**1** + **2** content (% dry wt.)	**1** + **2** production (mg/l)
Stem	360 ± 26	12.3 ± 0.7	1.08 ± 0.11	133
Stem apex	339 ± 22	12.9 ± 0.8	0.62 ± 0.07	80
Leaf	307 ± 31	12.0 ± 1.1	0.81 ± 0.06	97
Leaf petiole	247 ± 22	10.0 ± 0.7	1.22 ± 0.13	122

[a]Mean values ± S.E. based on at least four separate replicate samples obtained at 3-month intervals; **1**, purpurin, **2**, munjistin.

leaf-derived calluses (Table 1). Although the biosynthesis of anthraquinone pigments in plant cell cultures has been studied extensively, little is known about the dependence of anthraquinone formation on the source of explants. Recently, Rady and Nazif [10] have demonstrated using *Cassia acutifolia* cultures, that anthraquinone production depends on explant origin, which is in agreement with our results.

3.3. Cell aggregate selection

To evaluate the possibility for establishment of a high-yielding cell line by selection of deeper colored aggregates, using an approach originally applied to *Lithospermum* cells [11], we chose two callus cultures possessing the high growth rate and derived from different organs of the plant. From the data presented in Table 2, it could be concluded that the anthraquinone content in the selected (orange) calluses was 1.89% and 1.65% dry wt. for 1–1 (stem apex derived) and 2–7 (leaf derived) lines, respectively, i.e. 2.5 and 2.1 times higher than that of yellow calluses. Increase in anthraquinone production is due mainly to an increase of the munjistin yields (Table 2). In contrast to leaf-derived calluses, the stem-apex selected calluses did not have lower biomass accumulation compared with yellow calluses (Table 2). Increased anthraquinone production by selected calluses was stable during long-term cultivation.

Table 2
Anthraquinone production by initial (yellow) and selected (orange) calluses of *R. cordifolia*[a]

Cell line	Fresh biomass (g/l)	Dry biomass (g/l)	**1** + **2** content (% dry wt.)	Munjistin (% of **1** + **2**)	**1** + **2** production (mg/l)
1–1 yellow	322 ± 26	10.5 ± 1.9	0.76 ± 0.14	62 ± 3.0	80
1–1 orange	340 ± 22	13.0 ± 1.4	1.89 ± 0.17	77 ± 0.7	246
2–7 yellow	476 ± 35	13.5 ± 1.1	0.81 ± 0.06	69 ± 1.4	109
2–7 orange	233 ± 38	11.0 ± 1.4	1.65 ± 0.07	75 ± 1.0	182

[a]Mean values ± S.E. based on five separate replicate samples obtained at 3-month intervals; **1**, purpurin, **2**, munjistin.

4. Discussion

It is interesting to compare the current data with available information from other studies on anthraquinone-producing plant cell cultures. Although pigment content of *R. cordifolia* calluses was nine times higher than that of the root tissues (1.89 vs. 0.2% dry wt.), the total anthraquinone yield was less then has been reported for *Rubiaceae* cell cultures, i.e. for *R. fruticosa* (13.3% dry wt.), *Morinda citrifolia* (10% dry wt.), *R. tinctorum* (3.8% dry wt.) [6,7]. It is noteworthy that *R. cordifolia* calluses produce only two major anthraquinones, with munjistin and purpurin representing 90% of the total anthraquinone yield. This is in contrast to other *Rubiaceae* cultures, which produce a broad spectrum of quinones [8,12]. This observation is important because munjistin and purpurin do not possess mutagenic activity [13]. By a simple manipulation (the visual selection of cell aggregates) an increase in yield of quinones could be achieved without a decrease in accumulation of cell biomass.

References

[1] Gilani AH, Janbaz KH. Phytother Res 1995;9:372.
[2] Itokawa H, Takeya K, Mori N, Hamanaka T, Sonobe T, Mihara K. Chem Pharm Bull 1984;32:284.
[3] Keller J, Grölich B. Z Urol 1944;38:1.
[4] Bulgakov VP, Zhuravlev YuN, Fedoreyev SA et al. Fitoterapia 1996;67:238.
[5] Thomson RH. Naturally occurring quinones. London, New York: Academic Press 1971;407:383.
[6] Zenk MH, El-Shagi H, Schulte U. Planta Med Suppl 1975;79.
[7] Schulte U, El-Shagi H, Zenk MH. Plant Cell Rep 1984;3:51.
[8] Verpoorte R, Wijnsma R, Mulder-Krieger Th, Harkes PAA, Baerheim Svendsen A. Primary and secondary metabolism of plant cell cultures. Berlin, Heidelberg: Springer-Verlag, 1985:196–208.
[9] Kuzovkina IN, Mantrova OV, Alterman IE, Jakimov SA. Fiziol Rast (Plant Physiol Rus) 1996;43:291.
[10] Rady MR, Nazif NM. Fitoterapia 1997;68:349.
[11] Mizukami H, Konoshima M, Tabata M. Phytochemistry 1978;17:95.
[12] Wijnsma R, Verpoorte R, Mulder-Krieger Th, Baerheim Svendsen A. Phytochemistry 1984;23: 2307.
[13] Kawasaki Y, Goda Y, Yoshihira K. Chem Pharm Bull 1992;40:1504.