

Production of Rosmarinic Acid in *Echium amoenum* Fisch. and C.A. Mey. Cell Cultures

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Abstract

Echium amoenum Fisch. and C.A. Mey. (Boraginaceae) is a very popular medicinal plant which is used as a tonic, tranquillizer, diaphoretic, cough remedy, sore throat and pneumonia in Iran's traditional medicine. Callus culture of medicinal plants is one of the ways for production of secondary metabolites. In this study, callus culture of *E. amoenum* and its major secondary metabolite were investigated. The callus culture of *E. amoenum* was initiated and established from seeds in MS media with three different ratios of plant growth regulators: kinetin, 2,4-D and NAA. Methanolic extracts of freeze-dried calluses were compared by TLC and HPLC. The major secondary metabolite was separated by preparative HPLC and the structure of this pure compound was elucidated by UV, IR, one and two dimensional ¹H and ¹³C-NMR and Mass spectroscopy. Rosmarinic acid was identified by various spectroscopic methods from callus culture of *E. amoenum*. Rosmarinic acid is widespread within the plant cell tissue culture of the Lamiaceae and Boraginaceae families, although in insignificant quantities. Rosmarinic acid has an antimicrobial, antiviral, and anti-inflammatory effect, which makes it a valuable product for the pharmaceutical and cosmetic industries.

Keywords: *Echium amoenum*; Callus culture; Rosmarinic acid.

Introduction

Echium genus (Boraginaceae) has 4 species in Iran (1) and only dried violet-blue petals of *Echium amoenum* Fisch. & C.A. Mey. have medicinal uses in Iran (2, 3). *E. amoenum* is a biennial or perennial herb indigenous to the narrow zone of northern part of Iran and Caucasus, where it grows at an altitude ranging from 60 to 2200 m (1). This medicinal plant has long been used as a tonic, tranquillizer,

diaphoretic, a remedy for cough, sore throat and pneumonia in traditional medicine of Iran (2, 4).

The production of secondary metabolites through a cell culture technology of renowned medicinal plants has been a challenging subject for many researchers. It has been established that cell cultures, obtained from plants of Boraginaceae family such as *Lithospermum erythrorhizon* and *Anchusa officinalis*, produce considerable amounts of rosmarinic acid (5, 6) and *L. erythrorhizon* also produces shikonin and a new brown benzoquinone (7, 8). Other secondary metabolites (e.g. quinones and naphthoquinones) were isolated from cell cultures of *Echium*

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lycopsis (9, 10) and even their antimicrobial activities investigated (11).

In this study callus culture of *E. amoenum* and its major secondary metabolite were investigated.

Experimental

Plant Material:

Seeds of *E. amoenum* for production of callus were collected from a farm in Rabor at 200 km South of Kerman in June 2000. Voucher specimens of plant (No. 1001) were authenticated and then deposited in Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.

Initiation of calli and cell cultures:

The callus culture was produced from seedling. The seeds of *E. amoenum* were germinated as follows. First, the surfaces were sterilized by shaking in 30% (w/v) aqueous hydrogen peroxide solution containing 1% (v/v) Tween 20 for 2.5 min and after washing by sterilized water, placed within 96° ethanol for 20 second and then washed again and incubated in 10×90 mm sterilized glass petri dishes lined with two sheets of filter paper which contained 20 ml of distilled water. The petri dishes were kept in the dark at a temperature of 25±2°C until the seeds germinated. After obtaining the strong seedlings (up to 2 cm long), for producing callus, the sterile hypocotyls apical buds and adjacent leaf pairs were cut with an sterile scalpel and then aseptically transferred to screw-cap wide-mouth bottles containing 50 ml of Murashige and Skoog (MS) with Gamborg vitamins solid media, containing 1 mg/l of 1-naphthalen acetic acid (NAA), 0.5 mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5 mg/l of kinetin (kin). Cultures were maintained at 25±2°C under a 16/8 h light / dark photo period, with a light radiation of ca. 10000 lux provided by cool white fluorescent tubes (GE, 20 W). After three subcultures, a homologous callus mass was divided to three parts and then transferred to MS with Gamborg vitamins solid media with three different combinations of growth regulators, as follows: NAA 2 mg/l+kin 0.7 mg/l (S1); NAA 1 mg/l+2,4-D 0.5 mg/l+kin

0.7 mg/l with 15% coconut milk (S2), and NAA 1 mg/l+2,4-D 0.5 mg/l+kin 1.2 mg/l (S3).

Preparation of medium:

Murashige and Skoog (MS) with Gamborg vitamins medium (Sigma, 4.4 g), sucrose (BDH, 30 g), agar-agar (Merck, 12 g), de-ionized water and growth regulators (as needed, Sigma) were employed for preparation of one liter of solid medium and then pH adjusted to 5.7. Finally 50 ml of the medium poured into containers, then autoclaved at 121°C / 1.8 bar for 15 min (12).

Preparation of callus tissues and extraction:

Every 21 days the young and healthy callus culture were subcultured. In 44 sequential subcultures, sufficient amounts of three different calluses (according to difference of growth regulators): S1, S2 and S3, were dried by a freeze drier (Snijders-Ly-5-FM). Their dried weight percentage were then calculated. They were then extracted with methanol in a Soxhlet apparatus under reduced pressure at a temperature of 30°C, separately. The resulting methanolic extracts were filtrated and concentrated in vacuum and percentage of total dried extracts determined.

Phytochemical analysis:

TLC analysis- The S1, S2 and S3 callus extracts were analyzed by TLC (40 µl of 100 mg extract/ 10 ml methanol) on Merck TLC GF₂₅₄ plates (10×10 cm) with 1, 2-dichloroethane- methanol-acetic acid-water (54:28:11:7) as the eluent. TLC plates were sprayed by Natural Products (NP) reagent (ROTH) (0.6% in methanol) and the spots visualized under UV₃₆₆ light.

High performance liquid chromatography (HPLC) analysis- A Waters HPLC system, equipped with preparative LC-4000, and UV-Vis dual λ 2487 spectrophotometric detector, was used for analytical and preparative HPLC analysis of isolated fractions by column chromatography. An analytical µBondapak C₁₈ (10 µm) stainless steel column (4.6×250 mm) was used for this purpose. Flow rate was 1 ml/min and the injected volume was 20 µl of 100 mg extract/10 ml methanol. The column used for preparative HPLC was a PrepLC™ µBondapak C₁₈ (10 µm) (25×200 mm). The injected

volume was 1000 μ l and flow rate was 5 ml/min. Mobile phase was methanol (A) and 5% formic acid (B), and a linear gradient from 100% B to 45% A in 175 min was applied. The detector was preset at 280 nm and 350 nm. The major compound was collected by Waters II fraction collector and after removal of the solvent, the residue was subjected to spectroscopic methods.

Ultraviolet (UV) spectroscopy- The UV absorption spectra (220-400 nm) of purified compound (in methanol) was recorded using a Secomam S-1000 UV/Vis spectrophotometer.

Infrared (IR) spectroscopy- The IR spectrum of the purified compound (in KBr) was recorded using a Perkin-Elmer 650 IR spectrophotometer.

Electron impact-mass spectroscopy (EI-MS)- Mass spectra of the purified compound (in DMSO) were recorded using an electron impact (EI) mode at 45 and 70 eV in Finnigan-mat TQS 70EI and Shimadzu Qp 1100EX EI quadruple mass, respectively. The source, probe and scanning temperatures used in this study, were 200, 100-300 and 25-30°C, respectively.

Nuclear magnetic resonance (NMR) spectroscopy- The NMR spectra were recorded using a Bruker DRX 500 Avance spectrometer. $^1\text{H-NMR}$ (at 500 MHz) and $^1\text{H-}^1\text{H}$ (COSY) and $^1\text{H-}^{13}\text{C}$ (HETERO-COSY) correlation, DEPT 135° and $^{13}\text{C-NMR}$ (125 MHz) spectroscopic data were collected at room temperature in d_6 -DMSO. Chemical shifts (δ , ppm) were reported relative to tetramethylsilane (TMS) as the internal standard.

Results and Discussion

Callus cultures

The percentage of high growth rate, dried weights and total dried extracts of *E. amoenum* calluses were obtained on the S1 (table 1). It seems that on increase in all three cases depends on a high amount of NAA. Coconut milk as a source of cytokinins, compared to the use of higher amounts of kinetin, (S2 vs. S3) results in an increase in all three cases. The calluses of S1, S2 and S3 extracts were fragile and yellowish white; solid and greenish yellow; proportionally fragile and creamy, respectively.

Table 1. Effect of growth regulators on the growth rate, dried weight and total dried extract of *E. amoenum* calluses.

G.R.C. ^A	Callus growth ^B	Dried weight (%)	Total dried extract (%)
S1	3 ⁺	4.6	48
S2	2 ⁺	3.6	44
S3	2 ⁺	3.4	36

^A G.R.C. = Growth Regulators Combination

^B 1⁺ = slight growth; 2⁺ = moderate growth; 3⁺ = profuse growth

S1= NAA 2 mg/l + kin 0.7 mg/l

S2= NAA 1 mg/l + 2,4-D 0.5 mg/l + kin 0.7 mg/l with 15% coconut milk

S3= NAA 1 mg/l + 2,4-D 0.5 mg/l + kin 1.2 mg/l

Secondary metabolites

In TLC analysis of S1, S2 and S3 calluses extracts, at an R_f of 0.7, a major spot with quenching in UV_{254} and bright yellow fluorescence in UV_{366} (after spraying the NP reagent), was appeared. S1 had the largest spot. By HPLC, this major compound ($R_t = 110$ min) was separated and its' structure elucidated by UV, IR, NMR and Mass spectra. All the data confirmed that this compound is rosmarinic acid (figure 1). Absorption maxima of UV spectra were 330 nm and a shoulder at 290 nm, as reported for this compound in the literature (13). The IR spectrum showed that the compound has OH groups (3180 and four peaks at 3350-3550 cm^{-1} , acidic C=O (1740 cm^{-1}), esteric C=O (1720 cm^{-1}) and aromatic rings (1615,1540,1465 cm^{-1}). The mass fragment profile at 45 eV showed a very small molecular radical ion ($M^{\cdot+}$) at m/z of 360.2 (intensity = 0.1%) which was not present as it decomposed at 70 eV. $m/z=123$ was the base peak at 45 eV and had 75% intensity at 70 eV, belonging to $\text{C}_7\text{H}_7\text{O}_2^+$ (13). NMR data of the compound confirming the structure of rosmarinic acid (13, 14) were as follow:

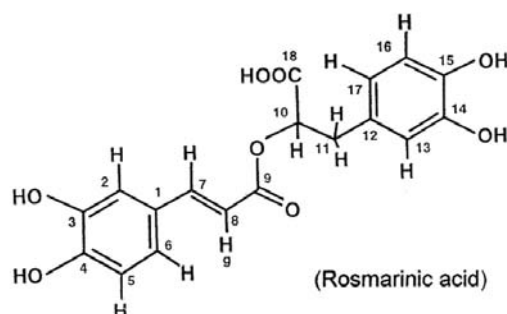


Figure 1. Chemical structure of rosmarinic acid (13).

¹H-NMR (500 MHz in d₆-DMSO): δ12.90 (1H, s, OH-18), 9.68 (1H, s, OH-4), 9.20 (1H, s, OH-15), 8.81 (1H, s, OH-3), 8.75 (1H, s, OH-14), 7.47 (1H, d, *J*=16_{(7,8)Hz}, H-7), 7.06 (1H, s, H-2), 7.01 (1H, d, *J*=8_{(6,5)Hz}, H-6), 6.77 (1H, d, *J*=8_{(5,6)Hz}, H-5), 6.69 (1H, s, H-13), 6.64 (1H, d, *J*=7.6_{(14,15)Hz}, H-16), 6.53 (1H, d, *J*=7.6_{(15,14)Hz}, H-17), 6.24 (1H, d, *J*=16_{(8,7)Hz}, H-8), 5.03 (1H, dd, *J*=8.6_{(17,16)Hz}, *J*=4_{(17,16)Hz}, H-10), 2.99 (1H, dd, *J*=14_{(16,16)Hz}, *J*=4_{(16,17)Hz}, H-11), 2.91 (1H, dd, *J*=14_{(16,16)Hz}, *J*=8.6_{(16,17)Hz}, H-11).

¹³C-NMR and DEPT 135° (125 MHz in d₆-DMSO): δ171.84 (s, C-18), 166.81 (s, C-9), 149.49 (s, C-15), 146.73 (d, C-7), 146.47 (s, C-14), 145.79 (s, C4), 144.85 (s, C-3), 128.28 (s, C12), 126.20 (s, C-1), 122.45 (d, C-6), 120.90 (d, C-17), 117.55 (d, C-13), 116.64 (d, C-5), 116.25 (d, C-16), 115.74 (d, C-2), 114.18 (d, C-8), 73.84 (d, C-10), 37.01(t, C-11).

Rosmarinic acid is accumulated in undifferentiated plant cell cultures such as *Zataria multiflora* (15), *Coleus blumei* (16), *Salvia fruticosa* (17), *Thymus vulgaris* (18) and *Lavandula officinalis* (19) from the Lamiaceae family and *Lithospermum erythrorhizon* and *Anchusa officinalis* from the Boraginaceae family (5, 6). This study is the first report on the accumulation of rosmarinic acid in one of the *Echium* species. In previous reports, quinone derivatives have been obtained from cell cultures of these plants (9, 10).

For the first time rosmarinic acid has been reported from other species of *Echium*, *Echium vulgare* (20) and according to the chemtaxonomic rules, isolation rosmarinic acid from callus culture of *E. amoenum* could be expected. An increase in the level of rosmarinic acid accumulation was shown to be dependent on the sucrose content (5, 15, 16) and nitrate ions (19). In this investigation, increasing the NAA, as a growth regulators, or the addition of coconut milk, as a nutrient containing cytokinins, could increase the accumulation of rosmarinic acid in callus culture cells of *E. amoenum*.

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