

Chlorogenic acid in *in vitro* cultures of *Eleutherococcus senticosus*

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S u m m a r y

The establishment of callus and cell suspension cultures of *Eleutherococcus senticosus* was reported. The four callus lines were obtained from stolon and bud explants from a field-grown plant. They were cultured on Murashige and Skoog (MS) or Schenk and Hildebrandt (SH) agar media, supplemented with 30 g/l sucrose, 2,4-dichlorophenoxyacetic acid (2,4-D) (1 mg/l), α -naphthaleneacetic acid (NAA; 1 mg/l) and 6-benzylaminopurine (BAP; 0.5 mg/l). The cell suspension culture was also maintained in SH liquid medium with the same growth regulators and sucrose (50 g/l). HPLC analysis showed that the cultures produced chlorogenic acid. The highest accumulation of the compound was detected in the cell culture (29.7 mg/g d.w.); it was 15 times the average level reported for starting callus, several times as much as roots, and far more than leaves of intact plants.

Key words: *Eleutherococcus senticosus*, chlorogenic acid, callus tissue, cell suspension culture

Eleutherococcus senticosus Harms (syn. *Acanthopanax senticosus*), a member of *Araliaceae* family, is a slender, woody shrub native to northeastern Asia, primarily occurring in eastern Siberia [1]. It has gained popularity because of tonic, adaptogenic and immunostimulant properties of the cortical tissue of its roots. It has been postulated that the activity of eleuthero extract is equivalent to, or may even surpass that of Asian ginseng root [2]. The eleuthero dried root material has originated from wild stands but a sufficient supply could not be obtained for medicinal use [2]. The species is endangered by overharvesting. It is generally propagated vegetatively by cuttings of the stem and roots. However, the efficiency of the method is low [3]. Propagation of the shrub by seeds has also been unsuccessful because of long natural germination time of zygotic embryos (three years).

Alternatively, stratification of the seeds can shorten the process to 18 months [4]. Thus, field cultivation takes several years to obtain plants with satisfactory content of the pharmacologically interesting compounds.

The main substances responsible for therapeutic effects of *E. senticosus* root are phenolic derivatives, namely eleutheroside B (4-*O*- β -D-glucoside syringin) (0.1-0.5%), lignans including sesamin – eleutheroside B₄ (0.023%) and eleutheroside E (liriodendrin) (0.05-0.34%), coumarins (such as isofraxidin and its 7-*O*-glucoside), sterols and phenolic acids, among them chlorogenic acid [2]. The structure of the compound was established as 3-*O*-caffeoylquinic acid (Fig. 1) by Fisher and Dangschat in 1932 [5]. It is widely distributed in plant kingdom and the main site of chlorogenic acid accumulation are roots of plants [6]. It was also isolated from the roots and rhizomes of *E. senticosus* [7]. Chlorogenic acid has many medicinal properties: it is recognized to be an antioxidant for human LDL; it is also known as a scavenger for reactive forms of oxygen and nitrogen, and an inhibitor against formation of conjugated diene from linoleic acid oxidation [8, 9]. According to Kono et al. [10], chlorogenic acid may be effective not only in protecting against oxidative damage but also in inhibiting potentially mutagenic and carcinogenic reactions *in vivo*. The compound shows an inhibition on stress-induced gastric ulcer and has cholagogic and antiphlogistic properties [11]. Besides that, chlorogenic acid is a glucose-6-phosphatase inhibitor, so it may be useful for the reduction of hepatic glucose output in non-insulin-dependent diabetes [12].

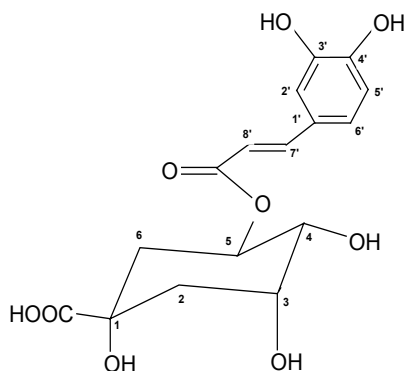


Figure 1. Structure of chlorogenic acid.

So far a few reports concerning chlorogenic acid accumulation in *in vitro* cultures have been published. Meravy [13] obtained callus and cell suspension cultures of *Centaurium erythraea*, exhibiting their ability to synthesize chlorogenic acid. The studies showed that the callus produced higher level of the metabolite (78 $\mu\text{g/g}$ fresh weight) in comparison with the cell culture (53 $\mu\text{g/g}$ fresh weight). Chlorogenic acid was also found in *Eucommia ulmoides* cell culture [14]. In this case, its content was as high as 2.15% of dry weight, which is similar to that detected in the leaves of parent plant (2.34-2.56% of dry weight).

In the present paper, we report the establishment of callus and cell suspension cultures of *E. senticosus* and production of chlorogenic acid by the cultures. The contents of the compound under *in vitro* conditions were compared with its content in leaves and roots of the intact plants.

MATERIAL AND METHODS

Callus induction and maintenance

Stolons and buds of a two-year-old, field-grown *E. senticosus* plant were used as the explant source. The explants were sterilized for 15-20 minutes in 1.5% sodium hypochlorite, then rinsed three times in sterile distilled water and inoculated on Murashige and Skoog (MS) [15] or Schenk and Hildebrandt (SH) [16] agar (0.7%) media supplemented with 3% sucrose, at pH 5.8. 2,4-dichlorophenoxyacetic acid (2,4-D) (1mg/l), α -naphthaleneacetic acid (NAA) (1mg/l) and 6-benzyladenine (BAP) (0.5 mg/l) were used as growth regulators. Cultures were incubated at 26°C with a 16-h photoperiod using cold-white fluorescent lamps at a light intensity of *ca* 40 μ M \cdot m⁻²·s⁻¹. After six weeks, the frequency of callus induction (i.e. percentage of explants forming callus in respect to total explants used) on the surface of the explants was measured. Subcultures were done every five weeks by transferring about 70 mg of callus on MS or SH fresh media (25 ml) containing 2,4-D (1 mg/l), NAA (1mg/l) and BAP (0,5 mg/l). In this way, four callus lines were developed. They were designated as:

- A. stolon-derived callus incubated on SH medium
- B. stolon-derived callus incubated on MS medium
- C. bud-derived callus incubated on SH medium
- D. bud-derived callus incubated on MS medium

The calluses were subcultured for at least four passages to obtain continuous lines before any studies were undertaken.

Cell suspension culture

To induce suspension culture of *E. senticosus* 4 g (fresh weight) of A line callus were transferred into 50 ml of SH liquid medium supplemented with 2,4-D (1 mg/l), NAA (1 mg/l), BAP (0.5 mg/l) and 50 g/l sucrose. The culture was maintained on a rotary shaker (100 rpm) under the same conditions as callus lines and was subcultured every two weeks by transferring 10 ml of culture into 80 ml SH fresh medium.

Growth measurements

For the determination of growth of callus and cell cultures, fresh and dry weights were measured. The initial weight of inoculum was about 70 mg fresh weight (5 mg dry weight) on 25 ml agar solidified medium for callus cultures

and about 950 mg fresh weight (63 mg dry weight) in 80 ml liquid medium for suspension culture. The callus and cells from suspension culture were harvested after five and two weeks, respectively, and final fresh weight and dry weight were determined. The experiments were repeated three times for three successive passages. The results were presented as the growth index (GI): final dry wt – initial dry wt/ initial dry wt.

EXTRACTION AND DETERMINATION OF CHLOROGENIC ACID

Sample preparation

Chlorogenic acid was extracted from samples of four lines of callus (A-D) and cells grown in a suspension culture as well as from organs (leaves and roots) of 2-year-old intact plant. Dried and powdered material (250-500 mg) was extracted with methanol (MeOH) for 24 hours, and then methanol was removed by rotary evaporation under reduced pressure at 40°C. The residue was reconstituted with 10 ml of water and purified by Solid-Phase Extraction [17], described as follows. The C18 Sep-Pak cartridge (Waters Associates) was preconditioned for neutral phenolics by sequentially passing 2 ml dropwise of methanol and distilled water. For acidic phenolics, Sep-Pak was preconditioned by passing 2 ml of 0.01 M HCl instead of distilled water. The water extract (2 ml) was adjusted to pH 7.0 and then passed through neutral Sep-Pak to absorb the neutral phenolic compounds. The effluent portion was adjusted to pH 2.5 with HCl and passed through the acidic SEP-PAK to absorb acidic phenolics. The cartridge was then eluted with methanol and the first 2 ml was collected for the HPLC analysis. The samples were filtered through a 0.45- μ m Minisart SRP 4 filter (Sartorius, Germany) prior to injection of 10 μ l to HPLC.

HPLC determination of chlorogenic acid

Chlorogenic acid was determined using HPLC Knauer system equipped with a UV-Vis detector and a Eurospher-100 C-18 column (25 cm x 4.6 mm; 5 μ m). The binary mobile phase according to Dyrby et al. [18] consisted of water/formic acid (90:10, v/v) (solvent A) and water/acetonitrile/formic acid (40:50:10, v/v/v) (solvent B). The flow rate was 1 ml/min and a total run time was 50 min. The system was run with a gradient program: 0 min: 88% A + 12% B, 26 min: 70% A + 30% B, 40-43 min: 0% A + 100% B, 48-50 min: 88% A + 12% B. The detection of hydroxycinnamic acids was at 320 nm. The chlorogenic acid peak was identified by comparing the retention time value with that of standard and by spiking the sample with standard. Chlorogenic acid was quantified with a calibration curve obtained by plotting the peak area compared with the corresponding concentrations of standard solutions. All measurements were done in triplicate.

RESULTS AND DISCUSSION

Callus tissues were effectively induced when stolons and buds of *E. senticosus* plants were placed on MS or SH agar medium supplemented with sucrose (3%) and growth regulators: 2,4-D (1 mg/l), NAA (1 mg/l) and BAP (0.5 mg/l). The best response in respect to the frequency of callus induction within six weeks of culture was obtained from stolon explants incubated on SH medium. In this case, nearly half of the explants formed callus. The frequency of callus induction decreased to 25% when the explants were cultured on MS medium. On the other hand, this medium was the most effective for callus initiation from the bud explants. On the medium, 34% of the explants produced callus, compared to 24% on SH medium. Such variations in the responses for different explant types can be attributed to the physiological stage of the explants [19]. The calluses induced from stolons and buds on MS or SH media were transferred into fresh media containing the same growth regulators (see above) and subcultured every five weeks for at least four passages. Thus, four continuous callus lines (designated as A-D: see Material and Methods) were obtained and used for subsequent study. The calluses were yellow-green in colour and varied in morphology, growth intensity and production of chlorogenic acid. Those cultured on the SH medium were fragile while the calluses maintained on the MS medium were more compact. It was found that B and D callus lines which grew on MS medium gave higher values of growth index (30 and 33 in respect to dry weight) than those cultured on the SH medium (lines C and A) (GI 23 and 28, respectively) (Table 1).

Table 1.

Growth and chlorogenic acid content in *in vitro* cultures and field-grown plants of *Eleutherococcus senticosus*.

| Plant material | GI | Chlorogenic acid content (mg/g dry weight) |
|------------------------|-------------|--|
| Callus Line | | |
| A | 28(29) ± 11 | 1,7 ± 1.10 |
| B | 30(30) ± 3 | 1.5 ± 0.07 |
| C | 23(24) ± 6 | 2,0 ± 0.08 |
| D | 33(37) ± 9 | 0.6 ± 0.08 |
| Suspension culture | 11(8) ± 2 | 29.7 ± 1.49 |
| Roots of intact plant | - | 5.5 ± 0.09 |
| Leaves of intact plant | - | trace |

Figures in parentheses show GI on the basis of fresh weight. The callus cultures were grown for five weeks on MS agar medium (B and D lines) or SH agar medium (A and C lines). The media were supplemented with 30 g/l sucrose and 2,4-D (1mg/l), NAA (1 mg/l) and BAP (0.5 mg/l). The cell culture was grown for two weeks in SH liquid medium containing 50 g/l sucrose and growth regulators as described above. The results are means ± SE of three independent measurements.

The HPLC analysis confirmed the presence of chlorogenic acid in all four callus lines tested according to the same retention time of 21.84 min as authentic sample. The amounts of the compound in callus lines ranged from 0.6 mg/g dry weight to 2 mg/g dry weight. The highest concentration of chlorogenic acid was detected in C line which was cultured on SH medium and showed the lowest increase of biomass. The lowest content was found in D line which grew on MS medium and reached the highest growth rate (Table 1). This is a well known phenomenon which is attributed to the fact that in *in vitro* cultures of many plants growth and production of secondary metabolites are inversely related [20]. The result may also imply that both growth and chlorogenic acid formation in callus cultures of *E. senticosus* were dependent on nutrient composition of the medium. Two kinds of culture media (SH and MS) used in this study differ mainly in nitrogen concentrations. SH medium contains 27.3 mM of nitrogen with a high ratio NO_3^- to NH_4^+ (9,5:1). In MS medium, the concentration of nitrogen was 60 mM with a ratio NO_3^- to NH_4^+ 2:1. It is known from other cultures that the amount and source of nitrogen can affect a range of culture parameters, among them growth rate and yield of secondary products [21, 22].

Using the most fragile callus tissue (A line), it was possible to initiate cell suspension culture of *E. senticosus*. The cell culture grew well in SH liquid medium that contained the same combination of growth regulators as in solid medium for callus induction and maintenance, and sucrose increased to 50 g/l. The cultures in liquid media have several advantages and are an important step towards a bioreactor culture. In the present study, the concentration of chlorogenic acid in cell culture of *E. senticosus* was 15 times higher than the average level reported for starting callus (Table 1). It is worth noting that such a high accumulation of the compound was reached in 2.5-fold shorter period (14 days) than it was necessary for the callus tissue (five weeks). The cell suspension culture produced over five times higher amounts of chlorogenic acid (29.7 mg/g dry weight) than roots (5.5 mg/g dry weight) and far more than leaves of intact plants. In the latter organs only traces of the compound were detected. The present study opens up a way to further studies for the improvement of biomass production and chlorogenic acid accumulation in cell suspension culture of *E. senticosus*.

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KWAS CHLOROGENOWY W KULTURACH *IN VITRO* *ELEUTHEROCOCCUS SENTICOSUS*

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Streszczenie

Otrzymano kultury kalusowe i zawiesinowe *Eleutherococcus senticosus*. Cztery linie kalusowe uzyskano z rozłogów i pąków rośliny rosnącej w gruncie. Kultury hodowano na agarowym podłożu Murashige-Skooga (MS) lub Schenka i Hildebrandta (SH) wzbogaconym w kwas 2,4-dichlorofenoksyoctowy (2,4-D; 1 mg/l), kwas α -naftylo-1-octowy (NAA; 1 mg/l) i 6-benzyloaminopurynę (BAP; 0,5 mg/l). Kultura zawiesinowa była prowadzona w płynnym podłożu SH z tymi samymi regulatorami wzrostu i ze zwiększoną do 50 g/l zawartością sacharozy. Za pomocą analizy HPLC wykazano, że wszystkie kultury produkowały kwas chlorogenowy. Największą zawartość tego kwasu wykryto w kulturze zawiesinowej (29,7 mg/g suchej masy). Była ona 15 razy wyższa niż w tkance kalusowej, kilkakrotnie wyższa niż w korzeniach i znacznie wyższa niż w liściach roślin gruntowych.

Słowa kluczowe: żeńszeń syberyjski, kwas chlorogenowy, tkanka kalusowa, kultura zawiesinowa.