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QUANTITATIVE DETERMINATION OF TOTAL POLYPHENOLS AND FLAVONOIDS FROM INDIGENOUS SPECIES OF *EPILOBIUM* OF WILD ORIGIN AND '*IN VITRO*' REGENERATED PLANTLETS

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Abstract: The aim of this study was to determine the total polyphenol and flavonoid content in six *Epilobium* species from the wild flora of Transylvania (*E. hirsutum*, *E. parviflorum*, *E. montanum*, *E. palustre*, *E. alsinifolium*, *E. nutans*), *Chamerion angustifolium*, and *in vitro* regenerated plantlets of *E. hirsutum* (Onagraceae). All samples contain high concentrations of these active principles (9.19–17.15% polyphenols and 1.9–4.3% flavonoids in the species from wild flora, and 11.11–19.35% total polyphenols in plantlets). The flavonoid content is about 25% of total polyphenols in the indigenous species, whereas in *in vitro* regenerated plantlets it is less then 0.01%. The absence of UV-B radiation in the neon tube light could explain lack of biosynthesis of flavonoids in the plantlets.

Keywords: *Epilobium* spp., polyphenols, flavonoids, *in vitro* regenerated plantlets

Introduction

Fourteen species of *Epilobium* (*Onagraceae*) and two *Chamerion* (syn. *Epilobium*) species are widespread in the Romanian native flora; they occur in humid places and marshes, and felled forests. The aerial parts (*Epilobii herba*) harvested in the flowering period are used in folk medicine.

The vegetal products contain galotannins, flavonoids (quercetol, myricetol and kaempferol derivatives), triterpenic acids, polyholosides, pectins, esters and glycosides of β-sitosterol [3, 4, 5]. An infusion of *Epilobium herba* is used to treat benign prostate hyperplasia (BPH) and associated micturition problems, and for its anti-inflammatory [3, 4, 6, 7, 8] and antibacterial properties [9].

The medicinal product *Epilobii herba* may be obtained from small-flowered willowherb species such as *E. parviflorum* Schreb., *E. hirsutum* L., *E. montanum* L., *E. roseum* L., *E. collinum* L. [3, 4], whereas *Chamerion angustifolium* (L.) Holub. (syn. *Chamaenerion angustifolium* (L.) Scop., *Epilobium angustifolium* L.) is considered an adulterant, even when the water extract of this last species had a stronger inhibitory effect on prostaglandin release in the rat-paw oedema test [3].

Obtaining phenolic compounds by means of induced cell and tissue cultures of *Epilobium* has not been investigated until the present study. However, Japanese researchers have succeeded in obtaining callus tissue cultures or shoot cultures from several *Oenothera* species (belonging within the same family plant, Onagraceae, as *Epilobium*). These cultures proved capable of biosynthethis of hydrolysable tannins, of which the macrocyclic dimers – ellagitannins and oenothein B – as well as the oenothein A trimer are produced in large amounts, sometimes with even higher concentrations than the whole plant. The same authors cultured these shoots in liquid medium flasks kept on rotative mixers. Following these experiments, the authors have ascertained that by cultivating the tissues in a liquid medium their capacity to biosynthesize phenolic compounds remains unchanged [10].

Because the *Epilobium* species contain flavonoids, polyphenolic derivatives of galic and catechic acids, polyphenol carboxylic acids (C_6-C_3 type) and even some acid phenols, and because they have important antioxidant properties [11, 12], the aim of this study was to perform a quantitative analysis of these compounds both in native *Epilobium* species from Transylvania and in regenerated *in vitro* plantlets of *E. hirsutum.*

Materials and Methods

The *Epilobium* species were collected during the flowering stage from the native flora of Transylvania (Table 1); they were dried at room temperature (20°C), and powdered to the IVth sieve mesh [13].

The total polyphenol content was estimated by the European Pharmacopoeia 2008 technique [14], and the results were expressed in galic acid, using Folin-Ciocâlteu Reagent (Merck). 0.5g of vegetal product was extracted with 50 mL 50° ethanol for 30 minutes on a water bath at 70°C. After cooling, the solution was filtered and made up to 50 mL in a volumetric flask with the same solvent. 2,5 mL of this solution was diluted in a 25 mL volumetric flask with the same solvent. To 2,0 mL of this solution was added 1 mL Folin-Ciocâlteu Reagent, 10 mL distilled water and made up to 25 mL in a volumetric flask with 290 g/L sodium carbonate solution.

The absorbances of the solutions were determined at 760 nm, using a UV-VIS JASCO V-530 spectrophotometer and distilled water as compensation liquid, after 30 minutes storage in darkness.

The standard curve was obtained using 0.2% galic acid solution in distilled water. 1mL of this solution was diluted to 100 mL in a 100 mL volumetric flask (0.002% solution). In five 25 mL volumetric flask we added 1, 2, 3, 4 and 5 mL of 0.002% galic acid solution, then 1 mL Folin-Ciocâlteu Reagent, 10 mL distilled water and made up the volume with 290 g/L sodium carbonate solution. The absorbances of the solutions were determined under the same conditions as described above, after 30 minutes storage in darkness.

The quantitative determination of flavonoids from *Epilobium* species was carried out by the spectrophotometric method, as described in the Romanian Pharmacopoeia 10th Edition [13], using 2.5% $AlCl_3$ solution as a colour reagent, and the results were expressed in rutoside (%).

Epilobium hirsutum cultivation in a stirred liquid medium

In order to initiate shoot cultures in a stirred liquid medium, nodes as well as shoot apices derived from *E. hirsutum* plantlets cultivated on semi-solid medium (employing agar) were used as explants. These explants were inoculated in 200 mL Erlenmeyer flasks with 25 mL Murashige-Skoog (MS) liquid medium ([15]) supplemented with 3% sucrose and plant growth regulators (2iP + ANA) (2-isopentyl adenine and naphthalene acetic acid, respectively). These basal media were further supplemented with a series of antioxidative agents (ascorbic acid – AAS; citric acid – CTA and cysteine – CY), adsorbants (polyvinyl pyrrolidone – PVP), and also with growth inhibitors (paclobutrazol – PBZ) in the following variants:

19 = MS + 0,5 mg/L PBZ; 15 days culture harvest (sample)
20 = MS + 1,0 mg/L PBZ; 15 days culture harvest
23 = MS + 1,0 mg/L PBZ; 20 days culture harvest
27 = MS + antioxidative agents - (AAS + CTA + CY) 100 mg/L
29 = MS + antioxidative agents - (AAS + CTA + CY) 100 mg/L + 100 mg/L PVP; 15 days culture harvest
30 = MS + antioxidative agents - (AAS + CTA + CY) 100 mg/L + 250 mg/L PVP; 15 days culture harvest
31 = MS + antioxidative agents - (AAS + CTA + CY) 100 mg/L + 350 mg/L PVP; 15 days culture harvest

33 = MS + antioxidative agents - (AAS + CTA + CY) 100 mg/L + 500 mg/L PVP; 15 days culture harvest
34 = MS + antioxidative agents - (AAS + CTA + CY) 100 mg/L; 20 days culture harvest
35 = MS + antioxidative agents - (AAS + CTA + CY) 100 mg/L; 25 days culture harvest

The flasks were kept on a rotative stirrer (80 rpm) at 24±2^{o}C under a 16 hour photoperiod. Grown-on shoots were harvested and they were washed and desiccated at 60^{0}C for 24 hours at periods of 15, 20 and 25 days in order to determine their growth (in g/L).

The *in vitro* regenerated plantlets of *E. hirsutum* were powdered, and the extracted solutions of these samples were obtained in the same way as described for the others, as well as the quantitative determinations of total polyphenols.

Because the flavonoid content in *in vitro* cultivated plantlets was very small, these active principles could not be assessed by the spectrophotometric method.

Results and Discussion

We present the concentrations of total polyphenols and flavonoids of *Epilobium* species of wild origin (Table 1), and total polyphenol content in *Epilobium hirsutum* regenerated plantlets (Table 2).

Table 1: Total polyphenol and flavonoid content in *Epilobium* spp. from of wild origin

Species, harvesting place	Total polyphenols (% galic acid)	Flavonoids (% rutoside)	% flavonoids from total polyphenols
1. *Epilobium hirsutum* – Cluj county	**17.15**	**4.3**	25.07
2. *E. parviflorum* – Valea Drăganului, CJ	15.33	3.1	20.22
3. *E. montanum* – Valea Arieşul Mare, AB	12.38	4.3	34.73
4. *Chamerion angustifolium* – Valea Arieşul Mic, AB	15.02	3.4	22.63
5. *E. palustre* – Vf. Şesuri, MM	7.38	1.9	25.74
6. *E. alsinifolium* – Valea Bila-Izvoare, MM	15.33	3.8	24.78
7. *E. nutans* – Mt. Mare, CJ	9.19	2.4	26.11

Table 2: Concentration of total polyphenols in *Epilobium hirsutum* plantlets

Sample	Total polyphenols (% galic acid)
19.	11.11
20.	12.00
23.	15.00
27.	18.75
29.	**19.35**
30.	17.64
31.	17.18
33.	16.66
34.	11.32
35.	12.74

The results obtained show a high polyphenol content in native *Epilobium* species; the concentrations were between 7.38% (in *E. palustre*), 9.19% (in *E. nutans*) and 17.15% (in *E. hirsutum*).

We also determined a high content of total polyphenols in *Chamerion angustifolium* (15.02%). Previous studies showed 19.44% polyphenols in aerial parts of *C. angustifolium* [16].

The flavonoid content was about 25% of total polyphenols (Table 1), the differences being represented by tannoid polyphenols, polyphenol carboxylic acids, proanthocyanins, and simple phenolic acids.

We also observed a direct proportionality between the total polyphenol and flavonoid content, within the context of the species with lowest polyphenol content (*E. palustre*, *E. nutans*), which had also the lowest flavonoid content, while the highest concentrations of both total polyphenols and flavonoids were determined in *E. hirsutum* (Table 1).

In our experiments, *E. hirsutum* was cultivated in a liquid MS medium (supplemented with 2iP and ANA) with the aim to study the influence of immersed culture conditions over both biomass growth of the shoots obtained from micro-propagation of nodes and apices, used as explants and biosynthesis of polyphenolic compounds. In most cases, in *in vitro* cell culture for which a nutritive liquid medium is employed, a vitrification (hyperhydricity) phenomenon occurred, clearly visible by the onset of stem (shoot) or root malformations [17]. With the purpose of preventing or at least ameliorating the effects of hyperhydricity, most of the time some plant growth retardants are employed such as ancymidol, abscisic acid, paclobutrazol, daminozyde, chloromecvate, etc. [18,19].

Another process which could readily be ascertained in the case of *in vitro* culture of some plant species synthesizing high amounts of polyphenols under natural conditions (*in situ*) is the excretion of these compounds into their culture media. This phenomenon was also noted in *E. hirsutum* immersed culture, which, only a few days after inoculation, displays an intense browning of the liquid medium, entirely due to the high amount of tannins exuding into the medium from the explant tissues. For this particular reason, a significant inhibition occured of both shoot multiplication rate and biomass growth. This sort of process may be counteracted by adding some antioxidative compounds, as well as several adsorbant agents, to the culture medium. For the antioxidative agents, we used ascorbic acid, citric acid and cysteine mix, in some of the variants supplemented with PVP (as adsorbant agent) at various concentrations.

Even if in some plant species cultivated in a liquid medium, their multiplication was stimulated by paclobutrazol [20]; however, our results indicate that this compound, irrespective of its concentration, exerted a negative influence not only on shoot propagation and growth in *E. hirsutum* but also on polyphenolic biosynthesis (Table 2). In turn, shoot cultivation on a MS medium supplemented with antioxidative agents and PVP resulted in a beneficial effect over the biosynthesis of polyphenolic compounds; hence, after 15 days culture (sample 29), *E. hirsutum* shoots accumulated in their tissues an increased amount of whole polyphenols, even higher (19.35%) than *E. hirsutum* individuals collected from the native flora (17.15%) (Table 1).

Conclusions

Because such a low content of flavonoids has been obtained from *E. hirsutum* plantlets regenerated *in vitro*, we were unable to determine their concentrations by the spectrophotometrical method (content below 0.01%). A possible explanation of these extremely reduced flavonoid concentrations displayed by *E. hirsutum* shoots cultivated on stirred liquid media is due to the fact that the culture flasks were exposed to neon tube light (cool-white fluorescent light) which does not emit UV-B radiations. In this context it has been ascertained that the flavonoid concentration level increases, in most cases, following exposure higher UV-B intensity [21].

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DETERMINAREA CANTITATIVĂ A POLIFENOLILOR TOTALI ŞI A FLAVONOIDELOR DIN SPECII INDIGENE DE *EPILOBIUM* DIN FLORA SPONTANĂ ŞI DIN PLANTULE REGENERATE *IN VITRO*

(Rezumat)

Scopul acestui studiu a fost determinarea conţinutului de polifenoli totali şi de flavonoide din şase specii de *Epilobium* din flora spontană a Transilvaniei (*E. hirsutum*, *E. parviflorum*, *E. montanum*, *E. palustre*, *E. alsinifolium*, *E. nutans*), din *Chamerion angustifolium* (*Onagraceae*), şi din plantule regenerate *in vitro* de *E. hirsutum*. Toate probele conţin concentraţii mari ale acestor principii active (9.19-17.15% polifenoli şi 1.9-4.3% flavonoide în speciile din flora spontană şi 11.11-19.35% polifenoli totali în plantule). Flavonoidele reprezintă 25% din polifenolii totali în speciile indigene, în timp ce în plantulele regenerate *in vitro* au o concentraţie sub 0.01%. O explicaţie a lipsei flavonoidelor din biosinteza plantulelor ar putea fi absenţa radiaţiilor UV-B din lumina de neon.

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