THESES OF PHD THESIS

Identification, Biosynthesis Monitoring of Dibenzylbutyrolactone Lignans in Fruits of *Arctium*, *Centaurea*, *Cirsium* and Quantification in Fruits and *in vitro* Cell Cultures of *Centaurea* Species

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I. Introduction and Aims

Active ingredients of natural origin have gained worldwide attention from the pharmaceutical industry. These compounds belong mainly to the secondary metabolite pool of plants. The significance of plant phenolic compounds including lignans, has increased, due to their various medicinal utilization and the improvement of analytical methods. Lignans derive from synthesis reaction of two cinnamyl alcohols, or their biogenetic equivalents. Lignans, such as arctigenin, matairesinol, trachelogenin and their corresponding glycosides, are important research subjects.

The major role of lignans in plants is considered to be in defense as antimicrobial, antiviral, antifungal, and antioxidant metabolites. However, in animals they exhibit anticancer, anti-HIV, anti-inflammatory, hepato- and neuroprotective, and antioxidant properties. The therapeutic utilization is broad (Ayres and Loike 1990).

Arctigenin, matairesinol, trachelogenin, and their corresponding glycosides, occur in high quantities in many genera like *Arctium*, *Centaurea, Cirsium, Ipomoea* and *Forsythia*. Based on the literature data and their local availabilities, *Arctium lappa*, 11 species of *Centaurea* and *Cirsium arvense* were the subjects of our investigations.

Lignans accumulate in glycoside or aglycone forms in different organs of plants, but high quantities of their glycoside forms are stored. Since the free aglycones have significantly higher biological properties than the glycoside forms, plants hydrolyze the glycosides with β-glucosidase enzyme into the corresponding aglycone and saccharide molecules in effective defense processes against pathogens (Yoo et al. 2010).

In recent years, the increasing commercial interest for products with natural ingredients has resulted in the utilization of plant cell cultures for the production of secondary metabolites, in addition to the extraction from intact plant material, because useful pharmaceuticals can be produced under controlled, optimized conditions, and the isolation is rapid and efficient.

The aim of this thesis was to identify and monitor the biosynthesis of the above mentioned lignans and their corresponding glycosides in *Arctium*, *Centaurea,* and *Cirsium* species during ripening and germination, in the achenes and *in vitro* cultures of *Centaurea* species, to produce these agents in pure forms and high quantities for a subsequent application in the pharmaceutical industry.

The aims of our research were the following:

1) Literature data clearly revealed that dibenzylbutyrolactone lignans are valuable pharmaceutical compounds, and their chemical synthesis has not been discovered or it is very expensive. In the first part of our work we investigated intact plant materials for the extraction and accumulation of lignans. Based on recent literature data, fruits of *Arctium lappa, Cirsium arvense, Centaurea scabiosa,* and another 10 species of *Centaurea*, were selected for further research. HPLC-UV and HPLC-ESPI-MS methods were used for the identification, quantification, and separation of lignans.
2) To evaluate the changes in lignan compositions and contents, we wanted to identify the distribution of lignans within the parts of the achenes, as well as at different stages during ripening and germination. We wanted to investigate also the occurrence of β-glucosidase enzyme responsible for the hydrolysis of glycosides into aglyones and the physiological conditions of the hydrolysis.
3) In the second part of the thesis, establishment of callus and suspension cultures from the above mentioned species of *Centaurea* were planned in order to extract lignans economically. Due to the continuous growth and differentiation of the cultures, a long-term lignan production can be expected.
4) We studied the correlation between the differentiation of callus cultures and the lignan composition and content of the cells committed to different directions. To observe cell differentiation, light microscopy, transmission- and scanning electron microscopy techniques were used; in parallel to analytical methods.

II. Materials and Methods

Plant materials

The following species were investigated: *Arctium lappa*, *Cirsium arvense*, *Centaure adjarica*, *C. americana*, *C. calcitrapa*, *C. cyanus*, *C. dealbata*, *C. jacea*, *C. montana*, *C. pannonica*, *C. scabiosa*, *C. solstitialis*, *C. triumfetti*. The lignan composition was determined in fruits of the mentioned species. Qualitative changes in the lignan contents were determined at several stages, during ripening and germination.

In vitro cell cultures were established from eight species of *Centaurea.* Primer calli were placed on solid Gamborg B5 (0.5 mg l^{-1} 2,4-diclorofenoxiacetic acid) (Gamborg et al. 1968) medium, secondary calli on a modified Murashige-Skoog (2 mg l^{-1} 1-naphthaleneacetic acid (NAA), 0,2 mg l^{-1} kinetin) (Murashige and Skoog 1962) medium. The callus cultures were maintained at room temperate (20-25 ^{o}C) with low intensity, diffuse natural light (10-20 µmol $m^{-2}s^{-1}$) filtered through the laboratory window. Photooxidation did not take place under these circumstances, but different forms of cell differentiation were observed. This medium initiated calli differentiation, which supported the investigation of the relationship between cell differentiation and lignan production.

Morphological, anatomical and ultrastructural analysis of differentiated cultures

In our work, the morphology of cells was investigated by stereo-, the anatomy by light- and scanning-, and the ultrastructure by electron microscopic methods.

Analytical studies

Lyophilized samples were extracted with 80% (v/v) methyl alcohol at 60 °C, applying reflux condenser.

Performing trifluoroacetic acid hydrolysis, aliquots of stock solutions were evaporated to dryness on a vacuum evaporator. Hydrolysis was performed with 0.2 ml 2 mol l^{-1} trifluoroacetic acid, samples were dried on a vacuum evaporator again, and the dried samples were distilled in 80% (v/v) methyl alcohol.

Performing hydrolysis with endogenous enzymes, lyophilized, pulverized and homogenized plant samples were suspended in 1 ml distilled water. Thereafter, the samples were lyophilized and extracted according to the protocol detailed above.

The identification of lignans was performed with high performance liquid chromatography (HPLC) with mass spectrometry (MS), and the quantification of lignan content with UV detection.

III. Results

Identification of lignans with liquid chromatography

The identification of lignans in *Arctium lappa*, *Centaurea scabiosa*, and *Cirsium arvense* ripe fruits was performed with high performance liquid chromatography (HPLC) with mass spectrometry (MS), and quantification of lignan contents with UV detection. *Arctium lappa* contained 78 mg g^{-1} arctiin, *Centaurea scabiosa* 41.7 mg g^{-1} matairesinoside, and *Cirsium arvense* 14 mg g^{-1} tracheloside.

The lignan compositions were determined both in the intact and in the acidic and enzyme hydrolysed plant samples, confirming the identity of the glycoside-aglycone pairs under different conditions. This provided relevant information on the completeness, and quantitativeness of the hydrolysis and showed that dibenzylbutyrolactone-type lignan aglycones remained stable even after acidic hydrolysis.

Simultaneous identification and quantification of lignan glycoside-aglycone pairs, the arctiin-arctigenin, matairesinol-matairesinosid and trachelosid-trachelogenin, was performed from different samples of the species, verifying the method to be suitable for lignan identification and quantification.

Changes in the lignan composition during fruit ripening and germination, and their distribution between the fruit wall and embryo parts of ripe achenes

The yields of lignans were investigated at different developmental stages of the fruits of *Arctium lappa*, *Centaurea scabiosa*, and *Cirsium arvense* and their distribution between different parts of the fruit as well. During the ripening process the amounts of lignans were evaluated from full bloom stage to ripe fruits and during germination of all three species.

Arctiin, matairesinoside, and tracheloside contents continuously increased during ripening in *Arctium lappa* and *Cirsium arvense*, while this quantitative increase has stopped earlier in case of *Centaurea scabiosa*. The unripe fruits of *Arctium* and *Cirsium* were found to be suitable for the isolation of dibenzylbutyrolactone lignan glycosides, because the yield of lignan glycosides is almost as high as in the ripe fruits, and earlier in case of *Centaurea*. The yield of lignan aglycones was well below of the glycosides. The glycoside and aglycone lignan quantities remained unchanged during the germination of all three species.

Our previous results showed that high amounts of lignan glycosides accumulate in the ripe achenes, thus we investigated the proportion of lignans distributed in different parts of the fruit, separately. The composition of dibenzylbutyrolactone lignans, investigated

independently for each species, revealed that the majority of lignans accumulate in the embryo part in the intact sample. If a β-glucosidase enzymatic hydrolysis took place, the glycosides completely hydrolyzed into their corresponding aglycones in *Arctium* and *Cirsium*, on the contrary to the ripe fruits of *C. scabiosa*. The results showed that the function of β-glucosidase enzyme in *C. scabiosa* is associated with the germination process.

The germinated fruits are the optimum sources for the glycoside and aglycone extractions, because the yield of lignan glycosides is high in the embryo part of the fruits. The separation of the fruit wall from the embryo part occurs naturally during germination, thus facilitating the extraction of metabolites.

Lignan composition of *Centaurea* ripe fruits

The heterogeneity of lignan compositions was investigated in 11 *Centaurea* species, at the same state of maturity of the fruits.

All investigated fruits contained lignans, but qualitative and quantitative differences were identified in the distribution and contents of the lignan pairs. The total amount of lignans also differed in the samples.

Arctiin was identified in all, except for 2 species (*C. scabiosa* and *C. solstitialis*), the most abundant in the intact samples, and in highest quantity in the fruits of *C. calcitrapa* (59.6 mg g^{-1}). Our previous results showed that the fruits of *Arctium lappa* contained higher amount (73.9 mg g^{-1}). This species is the most suitable for arctiin isolation. Arctigenin can be extraced from the enzyme hydrolysed samples of *A. lappa*. Matairesinoside can easily be extracted from the fruits of *C. scabiosa* and the most cost-effective method for matairesinol extraction is the hydrolysis. Tracheloside was not significant in the samples, unlike in *Cirsium arvense*, meaning this species is the most suitable for tracheloside extraction, and trachelogenin if hydrolysis is performed.

This information about the content and distribution of naturally occurring lignans is important before the establishment of *in vitro* callus cultures. Heterogeneity was observed in the quantities of lignans present in each species, but lignan extraction is efficient from certain species, such as *Centaurea scabiosa* and *Centaurea calcitrapa*.

Establishment of tissue cultures, the composition and quantity of lignans and morphology of *Centaurea* species

Cell cultures can produce useful compounds under controlled conditions independent of climatic changes or soil conditions; the control of cell growth is automated. *In vitro* callus cultures were successfully established from 8 *Centaurea* species.

Callus cultures were kept on MSA30 medium for maintenance and differentiation purposes. It was observed that the different genotypes responded to the same medium in various ways. Some species formed shoot-like (*C. jacea*), shoot- and root-like (*C. adjarica*), and some only root-like (*C. americana* and *C. scabiosa*) organs, while e.g. *C. solstitialis* did not show any organogenesis, similar to the other species.

Structural and analytical investigations were performed on different developing organlike structures. *C. adjarica* callus showing shoot-like differentiation contained the highest amount of arctiin, 2.96 mg g^{-1}. *C. adjarica* callus with adventitious roots contained the highest amount of arctigenin, 1.96 mg g^{-1}. *In vitro* cultures, compared to intact samples, accumulate lower amount of lignans (0.01-4%), in accordance with literature data.

Total lignan contents were significantly influenced by the state of organogenesis. The *in vitro* cell cultures contained the same lignans as accumulated in the fruits, but the yield was well below of those, and also the lignan compositions differed.

IV. Conclusions

1) The results of this work confirmed that the fruits of *Arctium lappa, Centaurea scabiosa* and *Cirsium arvense* are suitable for the production of lignans in the pharmaceutical industry. HPLC-UV and HPLC-ESPI-MS methods were used for the identification and quantification of three lignan glycoside-aglycone pairs, arctiin-arctigenin, matairesinol-matairesinoside and tracheloside-trachelogenin. Lignan glycosides accumulate mainly in the fruits, from which the biologically advantageous aglycones can be produced via hydrolysis quantitatively with trifluoroacetic acid or β-glucosidase.

2) Our results proved that the lignan contents varied during ripening and germination in *Arctium lappa, Centaurea scabiosa* és *Cirsium arvense* species, and accumulated mainly in the embryo part of the fruits; the unripe fruits should be harvested during field collection. The ripe fruits of *Arctium lappa* and *Cirsium arvense* had functional β-glucosidase, enzyme responsible for the hydrolysis of lignan glycosides; however the enzyme was activated only during the germination in *Centaurea scabiosa*.

3) Investigating the arctiin-arctigenin, matairesinosid-matairesinol, and trachelosid-trachelogenin lignan glycoside/aglycone contents and ratios of the ripe fruits of 11 *Centaurea* species, heterogeneity was identified. The investigations presented in this thesis, provide a good basis further large scale research. Thus the establishment of *in vitro* cultures from all available species seemed to be promising for the further investigations in tissue cultures.

4) *In vitro* cell cultures were successfully established from eight *Centaurea* species. The yield of total lignan was well below of those of the intact plant samples, and also the lignan compositions differed. The use of *in vitro* cell cultures for the production of biological compounds is promising on a long-term, because the field samples showed great heterogeneity, the lignan contents varied in the fruits of the same species.

5) The differentiation pathways of the species were heterogeneous, and different organogenic pathways were observed in case of the species, grown on the same medium. The lignan composition and content of the organogenic callus was diverse, compared to the undifferentiated cultures. The tissue cultures were morphologically and anatomically heterogeneous even if grown on the same media. Lignan production was influenced by the cell differentiation stage. *C. adjarica* callus showing shoot-like differentiation contained the highest amount of arctiin, and with adventitious roots the highest amount of arctigenin. This shows the importance of lignan distribution.

6) *Centaurea* cell cultures can be suitable for the long-term production of lignan, if the production can be enhanced. The results presented in this thesis demonstrated the theoretical possibilities of *in vitro* lignan production. Although high amounts of lignans can nowadays be extracted exclusively from intact plant samples, the use of *in vitro* cell cultures in the pharmaceutical industry is promising on the long-term. Continuous lignan production can be achieved, because a large-scale fermentation technology can operate economically in a controlled environment.

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List of publications containing the results of this thesis

Papers in refereed journals:

Sedlák, É., Boldizsár, I., Borsodi, L., Füzfai, Z., Molnár-Perl, I., Preininger, É., & Gyurján, I. (2008). Identification and quantification of lignans, carboxylic acids and sugars in the leaves of Forsythia species and cultivars. *Chromatographia*, *68*(1), 35-41.

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Poster presented at: 7th Balaton Symposium on High-performance Separation Methods In Memorian Szabolcs Nyiredi, Siófok, September 5-7.

Sedlák, É., Boldizsár, I., Borsodi, L., Preininger, É., & Gyurján, I. (2008) Lignans in leaves of Forsythia species, cultivars and its in vitro cultures. Brochure page 43.
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Poster presented at: Magyar Mikrobiológiai Társaság 2008. évi Nagygyűlése és a XI. Fermentációs Kollokvium, Keszthely, October 15-17.

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