INVESTIGATION OF LIGNANS IN THE ACHENE FRUITS OF SPECIES BELONGING TO THE ASTERACEAE FAMILY

PhD thesis

Anna Sólyomváry

Doctoral School of Pharmaceutical Sciences

Semmelweis University

Supervisor: Dr. Boldizsár Imre, Ph.D., senior lecturer

Official reviewers:
Dr. Csupor Dezső, Ph.D., senior lecturer
Dr. Völgyi Gergely Ph.D., associate professor

Head of the Final Examination Committee:
Dr. Török Tamás, professor emeritus

Members of the Final Examination Committee:
Dr. Papp Nóra, Ph.D., senior lecturer
Dr. Ludányi Krisztina, Ph.D, associate professor

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1. Introduction

During my PhD work, lignan (Li), neolignan (Neli) and sesquineolignan (SeNeLi) plant secondary metabolites – which are made up of phenilpropane units – were studied chemically (by chromatographic and spectroscopic methods) and pharmacologically (in vitro inhibition of tumor cell proliferations). The species which were investigated are belonging to the thistle – *Cynareae* (*Carduae*) tribe of the the Asteraceae family and their composition was no or little-known. We selected the thistle tribe since recent results revealed the accumulation of these type of metabolic products in akin species, especially in the fruit. In our investigations we chose species that are native in Hungary (*Cirsium brachycephalum*, *C. eriophorum* - woolly thistle, *C. vulgare* - spear thistle, Serratula tinctoria - saw-wort) or cultivated (*Cnicus benedictus* - blessed thistle, *Leuzea carthamoides*), thus the plant raw materials were relatively readily available.

Beside the structure characterization of two new natural compounds (the NeLi prebalanophonin and SeNeLi prepicrasmalignan) we also identified new sources of 13 known component (Li-s of the dibenzylbutyrolactone (DBBL) group: arctiin, arctigenin, carthamoside, carthamogenin, matairesinol, tracheloside and trachelogenin; the SeNeLi lappaol and isolappaol A and C, picrasmalignan; and the NeLi balanophonin). To characterize the transformation reactions of the ingredients (hydrolysis and cyclization), we developped and optimized methods like acid treatment and enzymatic hydrolysis. Using these methods and the separation of the fruit parts (pericarp and embryo), optimal conditions and raw material were found to each compound for the isolation from the new plant source. Thus, big amounts of the components could be isolated in pure form, without the presence of interfering contaminants for the structure analyses and pharmacological experiments.

Most of the ingredients identified by us and some related compounds (eg. podophyllotoxin) are significant antitumor agents. Based on this fact, during our pharmacological investigations we demonstrated the inhibitory effect of carthamogenin on cell division for the first time, as well as new mechanisms of action of trachelogenin and arctigenin.

2. Objectives

Our aim was (listed in paragraphs 1-6.)

(1) The examination of Li-s and related compounds (Neli-s and SeNeLi-s) in some species belonging to the *Cynareae* group. In two of the species (*Cnicus Benedictus* and *Leuzea carthamoides*), lignans have already been identified, but their quantities have been left undefined. In the case of the other four species (*Cirsium brachycephalum, C. Eriophorum, C. vulgare* and *Serratula tinctoria*) not any information were available about the fruit composition. Accordingly, we aimed the identification and quantification of the components in any case. After the analysis (qualitative and quantitative) of the compounds and the characterization of

(2) specific transformation reactions (enzymatic hydrolysis and acid catalyzed cyclization) and

(3) specific accumulation in the fruit parts, we aimed to develop

(4) optimal methods to isolate them in pure form.

(5) After the structure characterization of the obtained pure compounds,

(6) we intended to explore their anti-tumor effects and the contributing mechanism of action.

3. Methods

Plant material

Fruit samples of *Leuzea carthamoides* and *Cnicus benedictus* were obtained from the Research Institute for Medicinal Plants, Budakalász, Hungary. *Cirsium brachycephalum, C. eriophorum, C. vulgare* and *Serratula tinctoria* fruits were collected in their natural habitat.

Chromatographic methods

HPLC-UV MS/MS and TOF MS

For HPLC-UV-TOF-MS analyses Agilent 1260 Infinity HPLCsystem (diode array detector) was used. Column: GraceSmart RP18 (5 μm), 150 mm × 4.6 mm. Eluents: eluent A, ACN: 0.07 M aqueous acetic acid (15:85, v/v), eluent B, ACN:0.07 M aqueous acetic acid (85:15, v/v). Solvent gradient: 0 min, 15% B; 5 min, 30% B; 12 min, 44% B. Flow rate: 1.0 mL/min. Detection: 200-600 nm (evaluation: 280 nm or 347 nm).

Tandem mass spectrometric analyses (MS/MS) were made on triple quadrupole mass spectrometer equipped with JetStream Elektrospray (ESI) ion source. For exact determination of molecular weight the Agilent 6230 time-of-flight mass spectrometer equipped with a Jet Stream electrosprayion source.

For preparative purpose the analytical HPLC instrument was connected to a preparative HPLC column: Nucleosil 100, C18 (10 μm), 150 mm × 10 mm. The gradient program and detection were the same as that detailed above, the flow rate was 3.0 mL/min.

Gas Chromatography Mass Spectrometry (GC-MS)

The apparatus was the Saturn II GC–MS system of Varian, supplied with an ion trap detector (ITD) system. A SGE BPX5 (30 m, 0.25mm id, 0.25 μm film thickness) capillary column was used (30 m, 0,25 mm; 0,25 μm).

Dry sample residues were treated with 250 μL hydroxylamine hydrochloride containing pyridine (2,5 g hydroxylamine hydrochloride/100 mL), at 70°C, for 30 min. Thereafter silylation was performed with 450 μL hexamethyldisylazane + 50 μL trifluoracetic acid, and heated at 100°C for 60 min. After dilution with hexamethyldisylazane, samples were injected into the GC-MS system.

NMR spectroscopy

NMR data of the isolated compounds were recorded in $CDCl_3$, methanol-d_4 or DMSO-d_6 on a Varian VNMRS spectrometer (599.9 MHz for ^{1}H and 150.9 MHz for ^{13}C NMR). Standard pulse sequences and parameters were used to obtain 1D (^{1}H-NMR, ^{13}C-NMR, NOE) és a 2D (COSY, TOCSY, [^{1}H-^{13}C] HSQC and [^{1}H-^{13}C] HMBC) spectra.

Absolute configuration

The conformation analysis of possible structures were carried out, configuration were determined by the comparison of the computed and measured CD spektra. The geometries and thermochemistry were computed at M06-2X/6-31G(d)/IEF-PCM(methanol) level of theory, CD spectra were computedat M06-2X/6-311+G(2d,p)/IEF-PCM(methanol) level of theory and were simulated with overlapping Gaussian functions with SpecDis program. All quantum chemical computations were carried out with Gaussian09 package.

Performing hydrolysis with endogenous enzyme

Fruit samples were suspended in distilled water and were held at 40°C applying 5-480 min.; thereafter samples were dried in a vacuum evaporator (at 35–40°C).

Preparation of plant extracts

Lyophilised, pulverised and homogenised fruit samples were extracted three times. First, they were extracted with 80% (v/v) methylalcohol at 60°C for 60 min. Thereafter, the insoluble, centrifuged material was extracted for second and third time, as described earlier. The combined supernatants were adjusted to stock solutions.

Acid treatment

Aliquots of the stock solutions were evaporated to dryness on a vacuum evaporator at 35–40°C. Acid treatments of the dried samples or isolated compounds were performed with 500 µL of 2M trifluoroacetic acid at 50°C or 100°C applying 5-120 min. The treated samples were dried in a vacuum evaporator (at 35–40°C) and dissolved in 80% methylalcohol.

Effects on SW480 colon adenocarcinoma cell-line

SW480 colon adenocarcinoma cell line (American Type Cul-ture Collection: CCL-228) was used for our experiments. Cells were cultured in 5% CO_2 atmosphere at 37°C, in

RPMI medium supplemented with 10% (v/v) FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin and were seeded

1.) into 96-well plates at a density of 3000 cells/well for proliferation assay (suforhodamine-B, SRB method)

2.) on cover slides at a density of 3×10^4 cells/well for immunocytochemistry

3.) in flasks at a density of 10^5 cells/flask for fluorescence-activated cell sorting (FACS) analysis and

4.) in flasks at a density of 4×10^5 cells/flask for the investigation of proteins (β-katenin, foszfo-β-katenin, c-Myc, foszfo-c-Myc, GSK3, foszfo-GSK3α, foszfo-GSK3β, β-tubulin) by Western-blot analysis.

After 48 h incubation following the seeding, culture media were replaced with media containing isolated lignans, standard lignans or podophyllotoxin. Control cells were incubated in culture media without lignans. After the treatment time SRB, imuncytochemistry, FACS and Western-blot were carried out (description of the experiments are detailed in [3] and [5]).

4. Results

1.) In the extract of the whole fruit of *Leuzea carthamoides* six components were identified: traheloside, carthamoside, teo feruloyl-serotonin isomers, trachelogenin and carthamogenin. We found that the glycosides (tracheloside and carthamozide) accumulate in the embryo, while the alkaloids were found only in the wall. The acid and enzymatic hydrolysis resulted the decomposition of the total amount of Li glycosides and the formation of corresponding aglycones.

2.) In the extract of *Cirsium brachycephalum* and *Serratula tinctoria* fruits, HPLC and TOF MS data confirmed the presence of tracheloside – trahelogenin (*C. brachycephalum*) and arctiin – arctigenin (*S. tinctoria*) glycoside – aglycone pairs. Glycoside components which were detected in the intact unhydrolised extracts, transformed into their correspondant aglycons during acid and enzymatic treatment.

3.) *Cirsium vulgare* fruit yielded different types of components accumulating separately in the fruit parts: the pericarp contains the NeLi balanophonin, while the DBBL Li glycoside traheloside occurs in the embryo, where, after enzymatic treatment, completely hydrolyse to trachelogenin.

4.) In the wall-part of *Cirsium eriophorum* fruit, NeLi and SeNeLi components were identified. Those which have diol structure (prebalanophonin, prepicrasmalignan) - which are not yet identified structures - could be formed with acid treatment at 50°C into their corresponding dihydrobenzofurane structure pairs (balanophonin and picrasmalignan, respectively).

5.) Examining the fruits of *Cnicus benedictus* revealed that the Li DBBL arctiin glycoside is present in only the embryo part and during enzymatic hydrolysis it transforms completely to arctigenin. DBBL Li matairesinol aglycone can be detected in the embryo and also in the wall.

In the fruit wall SeNeLi-s were detected, and, examining their acid-catalyzed cyclisation during acid treatment – using different temperature and duration – we concluded that both lappaol and isolappaol C transformed to lappaol A and isolappaol A, respectively. The transformation could be observed after 5 minutes at both temperatures, and the resulting lappaol A and isolappaol A remaind stable at 50°C (even after 120 min.) treatment, however, it decomposed at 100 °C.

6.) The antiproliferative effect of three of the isolated components, the trachelogenin, carthamogenin and arctigenin were examined. While carthamogenin had moderate antiproliferative effect, trachelogenin proved to be the most effective of the three compounds.

Study on the Wnt/β-catenin signaling pathway showed that in the case of proliferation-promoting proteins (β-catenin, c-Myc) the level of the inactivated – phosphorylated – form increased, while the proliferation-inhibiting protein GSK3 was inactivated in lignan treated cell-line. Compared with podophyllotoxin standard, it was found the arctigenin and trachelogenin enhanced multinucleated giant cell apoptosis and blocked the microtubular system.

Table 1. Composition of the whole fruit and separated fruit-parts, embryo (embr.) and pericarp (peric.) of *Leuzea* (*L.*) *carthamoides*, *Cirsium* (*C.*) *brachycephalum* (*C. brach.*), *C. vulgare*, *C. eriophorum*, *Serretula tinctoria* (*S. tinct.*) and *Cnicus benedictus* in mg/g.

Type	Compound	Treat-ment*	*L. carthamoides*			*C. brach.*	*S. tinct.*	*C. vulgare*			*C. eriophorum*			*Cnicus benedictus*		
			embr.	peric.	whole	whole	whole	embr.	peric.	whole	embr.	peric.	whole	embr.	peric.	whole
Dibenzylbutyrolactone lignan	arctiin	no	-	-	-	-	**75,4**	-	-	-	-	-	-	**47,5**	0	23,5
		enz.-h.	-	-	-	-	0	-	-	-	-	-	-	0	0	0
	arctigenin	no	-	-	-	-	6,6	-	-	-	-	-	-	0	0,38	0,18
		enz.-h.	-	-	-	-	**56,0**	-	-	-	-	-	-	**33,5**	-	-
	cartamoside	no	**45,2**	8,5	26,6	-	-	-	-	-	-	-	-	-	-	-
		enz.-h.	0	0	0	-	-	-	-	-	-	-	-	-	-	-
	carthamogenin	no	0,93	1,00	1,04	-	-	-	-	-	-	-	-	-	-	-
		enz.-h.	**31,5**	6,2	18,5	-	-	-	-	-	-	-	-	-	-	-
	tracheloside	no	**33,0**	3,91	18,1	**24.4**	-	**20,3**	0	8,7	-	-	-	-	-	-
		enz.-h.	0	0	0	0	-	0	0	0	-	-	-	-	-	-
	trachelogenin	no	0,89	4,66	3,30	0,60	-	0	0	0	-	-	-	-	-	-
		enz.-h.	**24,1**	7,9	15,7	**17,2**	-	**12,6**	0	5,7	-	-	-	-	-	-
	matairesinol		-	-	-	-	-	-	-	-	-	-	-	1,75	7,7	4,58
neolignan	balanophonin	no	-	-	-	-	-	0	**23,2**	13,0	0	11,5	6,1	-	-	-
		acid-t.	-	-	-	-	-	-	-	-	-	**52,7**	-	-	-	-
	prebalanophonin	no	-	-	-	-	-	-	-	-	0	**45,7**	22,6	-	-	-
		acid-t.	-	-	-	-	-	-	-	-	-	0	-	-	-	-
Seskquineolignan	picrasmalignan	no	-	-	-	-	-	-	-	-	0	1,40	0,68	-	-	-
		acid-t.	-	-	-	-	-	-	-	-	-	**31,0**	-	-	-	-
	prepicrasma-lignan	no	-	-	-	-	-	-	-	-	0	**28,8**	14,0	-	-	-
		acid-t.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	lappaol C + isolappaol C	no	-	-	-	-	-	-	-	-	-	-	-	0	**23,1**	12,2
		sav-k.	-	-	-	-	-	-	-	-	-	-	-	-	0	-
	lappaol A + isolappaol A	no	-	-	-	-	-	-	-	-	-	-	-	0	8,7	4,61
		acid-t.	-	-	-	-	-	-	-	-	-	-	-	-	**31,1**	-

* the amounts were determined in without intact (no), enzyme-hydrolysed (enz.-h.) or acid-treated at 50°C temperature (acid-t.) fruit samples

5. Conclusions

During my PhD work lignan (Li), neolignan (NeLi) and sesquineolignan (SeNeLi) type plant metabolites were studied in a chemical (by chromatographic and spectroscopic methods) and pharmacological (inhibition of tumor cell proliferation in vitro) point of view, in the fruits of selected species belonging to the thistle tribe.

1.) The composition of fruits of *Cirsium eriophorum* (woolly thistle), *C. brachycephalum*, *C. vulgare* (spear thistle) and *Serratula tinctoria* (saw-wort) was determined for the first time, and, beside quantifying the already known compounds of *Cnicus benedictus* (blessed thistle) and *Leuzea carthamoides*, in blessed thistle yet unidentified metabolites were also detected.

2.) Beside the characterization of two new natural products (NeLi prebalanophonin and SeNeLi prepicrasmalignan), new sources of 13 known molecules (the dibenzylbutyrolacton – DBBL – structure arctiin, arctigenin, carthamoside, carthamogenin, matairesinol, tracheloside, trahelogenin; the SeNeLi lappaol A and C, isolappaol A and C, picrasmalignan;and the NeLi balanophonin) have been identified.

3.) Examining the composition of the separated embryo and pericarp sections, specific accumulation of ingredients was shown: while DBBL Li glycosides had been found in the embryo, NeLi and SeNeLi components occur in the fruit wall.

4.) For converting the components (by hydrolysis or cyclization) several methods (acid treatments and enzymatic hydrolysis) were optimized:

4.1.) Neli and SeNeLi components based on their behavior in acidic medium can be arranged in pairs because the open-chain diol-structure forms a dihydrobenzofuran ring system during H_2O elimination by heating with acid. The diol/dihydrobenzofuran pairs are the followings: prebalanofonin/balanofonin, prepicrasmalignan/picrasmalignan, lappaol C/lappaol A and isolappaol C/isolappaol A.

With acid treatment on 50°C temperature of diol-structure NeLi and SeNeLi ingredients of the fruit wall (prebalanophonin, prepicrasmalignan, lappaol C and isolappaol C) can be converted quantitatively into their dihydrobenzofuran structure pair (balanophonin, picrasmalignan, lappaol A and isolappaol A).

4.2.) DBBL Li glycoside components of the embryo can be quantitatively converted into their corresponding aglycone molecules by enzymatic hydrolysis.

5.) The specific accumulation of the components and their quantitative conversion with enzyme or acid treatment has enabled the preparation of fruit samples (enzyme or acid-treated embryo or pericarp) free of contaminants (which hinder or prevent the isolation), that contain the selected components in large quantities for the isolation.

6.) Antiproliferative effect of the isolated arctigenin, carthamogenin and trachelogenin on SW480 colon adenocarcinoma cell-line, and the mechanism of action have been proved: inhibition of cell proliferation-enhancing proteins (β-catenin, c-Myc), activation of the proliferation-inhibiting GSK3 protein. In addition, properties like enhancing of multinucleated giant cell formation and apoptosis, and anti-tubular efficiency were also demonsrated.

6. Bibliography of the candidate's publications

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