Chemodiversity between selected Cardueae and *Anthriscus* plant species: a phytochemical study of their intact tissues and *in vitro* cultures

Ph.D. Dissertation

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

3D	three-dimensional
A549	Human lung cancer cell line
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
AGS	Human gastric cancer cell line
Akt-1	serine-threonine protein kinase
approx.	approximately
ATP	adenosine triphosphate
B16 melanoma 4A5	Mouse melanoma cell line
ca.	circa
CD	circular dichroism
Cdc2	cell division control protein
Cdc25C	Human cell division cycle 25C
CEM/ADR5000	Human leukemia cell line
COSY	Correlation Spectroscopy
COX	cyclooxygenase
CTR	control
DNA	deoxyribonucleic acid
DOP6H	deoxy-podophyllotoxin 6-hydroxylase
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionisation
EtOH	ethanol
FBS	fetal bovine serum
GL331	an epipodophyllotoxin analogue
HCT116	Human colorectal carcinoma cell line
HeLa	Human cervical cancer cell line
HepG2	Human liver cancer cell line
HIV1	Human immunodeficiency virus 1
HL-60	Human leukemia cell line

HMBC	Heteronuclear Multiple Bond Correlation
HPAE	high-purity arctigenin extract
HPLC	high-performance liquid chromatography
HPLC-MS	high- performance liquid chromatography coupled with mass spectrometry
HSQC	Heteronuclear single quantum coherence
IC50	half maximal inhibitory concentration
IDPFG	inverse detection gradient
IEFPCM	integral equation formalism
iNOS	inducible nitric oxide synthase
ITS	Internal transcribed spacer
IUPAC	International Union of Pure and Applied Chemistry
JA	jasmonic acid
LC-MS	liquid chromatography coupled with mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
LPS	lipopolysaccharide
m/z	mass-to-charge-ratio
MCF-7	Michigan Cancer Foundation-7 derived from Human breast cancer cell line
MeJA	methyl jasmonate
MeOH	methanol
MRC-5	Medical Research Council cell strain 5 derived from the lung tissue of an aborted fetus
MS	Murashige and Skoog medium
NCI-H460	Human lung cancer cell line
NF- κB	nuclear factor-κB
NMR	nuclear magnetic resonance
NO	nitric oxide
ORAC	oxygen radical absorbance capacity
p53	tumor protein p53
PCA	principal component analysis

PGD2	prostaglandin D2
PT	podophyllotoxin
RAW 264.7	Murin macrophage cell line
ROS	reactive oxygen species
SA	salicylic acid
sect.	section
SGC-7901	Human gastric cancer cell line
SNU-1	Human gastric carcinoma cell line
sp.	species
SRB	sulforhodamine B
SW480	Human adenocarcinoma cell line
syn.	synonym
TFA	trifluoroacetic acid
TOCSY	Total Correlation Spectroscopy
TOF	time-of-flight mass analyser
UV	ultra violet
WHO	World Health Organisation
Wnt/β	Wnt/β-Catenin signalling pathway
YE	yeast extract
β-peltatin 6OMT	β-peltatin 6-*O*-methyltransferase

1 INTRODUCTION

Nowadays, cancer is globally the second leading disease causing death. Worldwide, one in six deaths occur due to cancer, according to the WHO data (World Health Organisation, 2018), therefore seeking an efficient treatment of cancer is a particularly pressing issue.

In the developed countries an increasing tendency of popularity of phytomedicine and the plant-based therapies can be observed in the last decades, therefore this area is one of the main focus of today's research. Moreover, a broad spectrum of plant-derived secondary metabolites occur in the nature that are used for centuries for human health and a tremendous number of molecules are not discovered yet, which might have a positive effect in cancer treatment, such as the semisynthetic etoposide and teniposide, derived from the lignan podophyllotoxin.

Lignans are one of the most potetntial groups of natural compounds which can play a relevant role in cancer theraphy. They represent a unique group of plant phenolics originating from the phenylpropanoid pathway. Although, their skeleton is formed by coupling of phenylpropane moieties, this group shows high structural diversity due to the presence of aromatic rings, chirality centers and hydroxylations. In addition to their significance in cancer therapy, numerous studies confirm their anti-inflammatory, phytoestrogene, antiviral and several other beneficial effects that are widely researched.

Lignans are commonly distributed in the group of seed plants, over a few hundreds of species accumulates different type of lignans in almost each part of the plant. Although, lignans are ubiquitous in plants, but in general do not provide commercially useful quantities and the cultivation of the most important species are not solved yet.

Cell- and tissue culture technique advances have further expanded the repertoire cost-effective fast production and scale-up of molecules with potential therapeutic importance.

Previously, at my research Institutes, several genera in the Asteraceae family were investigated and high amount of dibenzylbutyrolactone and furofuran-type lignans have been found in the seeds of these species. Continuing this research, we analysed fruits of species from *Cirsium* genus – such as *Cirsium rivulare*, *C. canum*, *C. oleraceum*, *C. brachycephalum*, *C. palustre*, *C. arvense*, *C. vulgare*, *C. eriophorum* and *C. boujartii* –,

and two further other species from this family, namely *Jurinea mollis* as well as *Carduus nutans* endemic in Hungary. Moreover, aryltetralin-type lignan, podophyllotoxin-derivatives containing genus *Anthriscus*, representing three species (*A. cerefolium, A. sylvestris,* and *A. caucalis*) belonging to Apiaceae family was investigated. In addition to the phytochemical study of intact plant organs, we also aimed establishing their *in vitro* cultures and producing the valuable secondary metabolites in *in vitro* cultures.

Taking into consideration that the structure of produced secondary metabolites is often restricted to taxonomically related groups, chemical taxonomy or chemotaxonomy can be a tool in our hand to find better sources of aimed metabolites in a taxonomically related plants.

As a literature overview, the following chapters (1.1 – 1.7) summarizes the most important knowledge about the plant-derived lignans as well as the studied plant species in the focus on our recent study. These sections are based on the biosynthesis of lignans, their availability in the plant kingdom, their biological activity and the opportunity of their production in *in vitro* plant cell and tissue cultures.

1.1 Nomenclature and structural features of lignans

An abundant class of plant-derived phenylpropanoids are the lignans (Umezawa 2003). They are biogenetically related to the phenylalanine metabolism (Moss 2000). The characteristic structural unit of lignans is a phenylpropanoid dimer, linked by the C8 carbons of the side chains of two phenylpropanes (Figure 1) (Umezawa 2003). Their name was introduced by Haworth in 1936. The term of lignans was proposed to extend to those natural compounds with low molecular weight that originate from the oxidative coupling of *p*-hydrxyphenylpropene units (McCredie et al. 1969). Among lignans, compounds with additional rings was named as cyclolignans and discussed in more detail by Weinges et al. in 1978 (Moss 2000). Subsequently, Gottlieb (1972) supplemented the nomenclature with compounds linked in a manner other than C8-C8’ and denominated as neolignans. However further recommendations were made in the classification, the nomenclature mentioned above are widely used and accepted by IUPAC in 2000. Moreover, the higher analogues – composed by three or more units – have analog terminology to terpenes: e.g., sesquineolignans and dineolignans (Moss 2000).

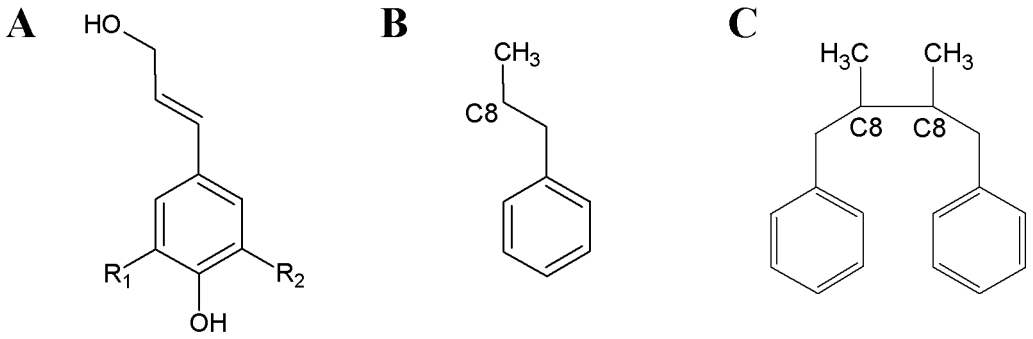

Figure 1. (A) Structures of lignan precursors: p-coumaryl alcohol (R_1=H, R_2=H), coniferyl alcohol (R_1=H, R_2=OCH_3) and sinapyl alcohol (R_1=OCH_3, R_2= OCH_3); (B) Phenylpropane unit; (C) Basic structure of lignans linked between the phenylpropanoid units at the C8-C8'.

Based on the carbon skeleton of lignans they are classified into subgroups, namely furofuran, furan, dibenzylbutane, dibenzylbutyrolactone, aryltetralin, arylnaphthalene, dibenzocyclooctadiene (Figure 2) (Ghisalberti 1997, Chang et al. 2005, Suzuki and Umezawa 2007, Satake et al. 2015). The oxidation levels of both the propil chain and the aromatic ring of each subgroups can vary in a significant level, thus they can be classified further (Suzuki and Umezawa 2007). The basic skeleton of lignans is often containing several side groups, i.e. methylenedioxy, methoxy groups or even different type of sugar moieties. The lignan-glycosides can be either monoglycosides (containing rhamnose, apiose or glucose) (Abe and Yamauchi 1989, Ito et al. 1994) or diglycosides (Ghisalberti 1997).

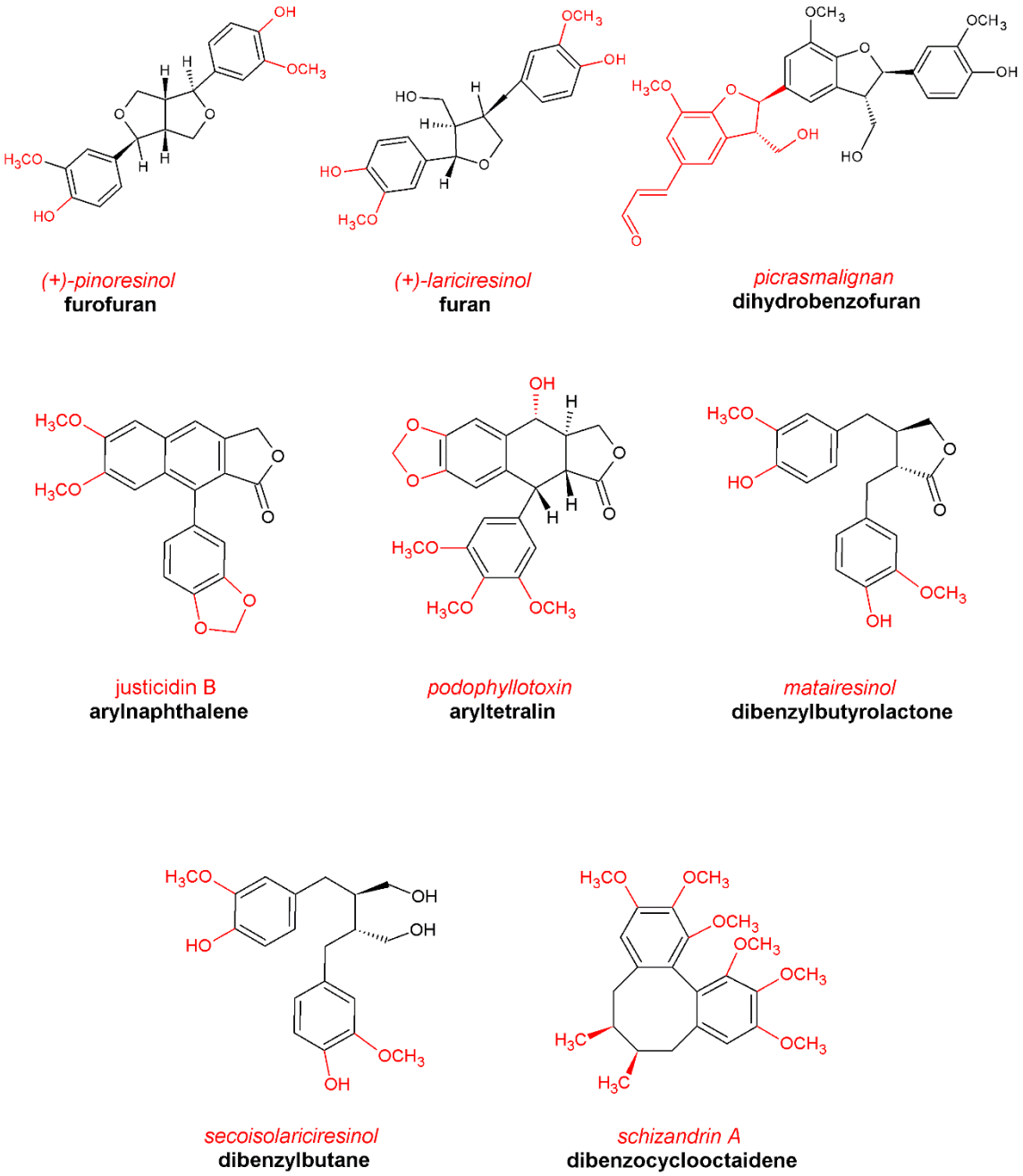

Figure 2. Variation of the lignan skeleton (bold) with example sturctures (red italic).

The optical rotation, enantiomer compositions and also the percentage values of enantiomers of the naturally occurring lignans are also varying substantially with plant species (Umezawa et al. 1997) or even within organs of the same species (Suzuki et al. 2002a).

1.2 Biosynthetic pathways of lignans

The biosynthesis of several lignans has already been well established, but many other biosynthetic pathways still remains unknown (Umezawa 2003). Among the subgroups, biosynthesis of lignans with 9(9')-oxygen is the most well-known example (Chuna et al. 2012, Suzuki and Umezawa 2007).

The skeleton of lignans originate from C6-C3 units that are derived from aromatic amino acids: *L*-phenylalanine and *L*-tyrosine derived from shikimic acid pathway. These amino acids are converted into cinnamic acid derivatives, thereafter, due to reduction *via* coenzyme A, are formed three alkohols, namely *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 1). These alcohols are the main precursors of the lignans (Chuna et al. 2012). The biosynthesis of these precursors – are also called monolignols (Davin and Lewis 2000, Sólyomváry et al. 2017a) – are fully established (Lewis et al. 1999).

For the C8-C8' linked lignans, the biosynthetic patway have been already defined at the metabolic, enzymatic and also molecular levels (Chu et al. 1993, Dinkova-Kostova et al. 1996, Kasahara et al. 2006). Their biosynthesis starts from the radical-radical coupling of two of *E*-coniferyl alcohol yielding (+)-pinoresinol. During the coupling of the two coniferyl alcohol, oxidases (laccase or peroxidase) generate free-radical intermediates, which are presumed to be captured by the so called diringent protein. Although this protein is responsible for the essential stereoselective coupling in the initial steps (Davin and Lewis 2000, Kasahara et al. 2006, Ono et al. 2006), in further metabolic steps the optical rotating isomers can occur in several plant species, or even within on species in different organs (Sólyomváry et al. 2017a). Pinoresinol was proved as a precursor of both secoisolariciresinol and matairesinol. Pinoresinol undergoes sequential reduction by pinoresinol/lariciresinol reductases to generate lariciresinol and secoisolariciresinol (Chu et al. 1993, Katayama et al. 1993, Dinkova-Kostova et al. 1996, Calvo-Flores et al. 2015, Sólyomváry et al. 2017a). These four lignans – pinoresinol, lariciresinol, secoisolariciresinol and matairesinol – were identified as the precursors of the biosynthesis of many other lignans. Further metabolic changes, structural variations are reached from different biosynthetic pathways (Suzuki and Umezawa 2007, Kim et al. 2009, Chuna et al. 2012, Calvo-Flores et al. 2015).

Furofuran lignans – such as sesamin, the main lignan in sesame seeds – was demonstrated to synthetize from pinoresinol (Ono et al. 2006).

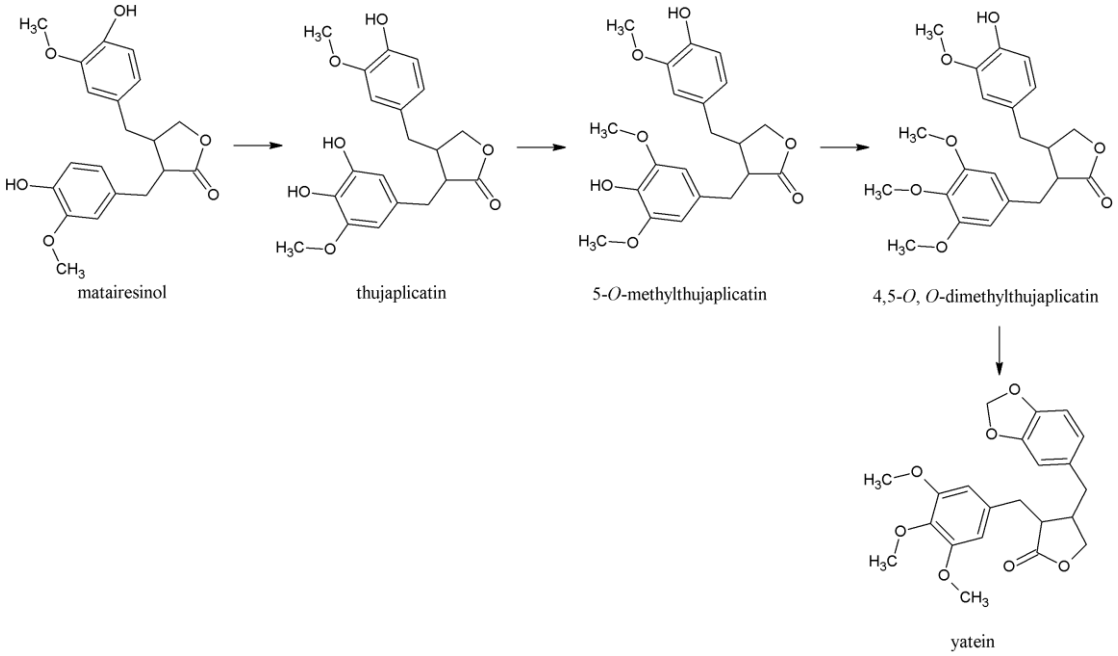

Figure 3. Biosynthetic pathway for yatein via thujaplicatin in *Anthriscus sylvestris* (Umezawa 2003).

A dibenzylbutirolactone lignan yatein was proved to transform from matairesinol *via* thujaplicatin as shown on Figure 3 (Sakakibara et al. 2003). Although the conversion of yatein into podophyllotoxin (PT) *via* deoxy-PT was proposed by Dewick (1989), the enzyme involved the transformation of yatein into deoxy-PT is still unknown. In contrast with the late steps of the biosynthesis of PT, enzymatic data on the earlier steps of its synthesis are not completely understood. Different pathways were proposed in the last decades (Calvo-Flores et al. 2015). Matairesinol was presumed to form yatein in four steps, which implies yatein as an intermedier toward PT and 6-methoxy-PT. Although, these intermediers are detected in plants, the catalysing enzymes are still unidentified (Sakakibara et al. 2003, Broomhead et al. 1991). Deoxy-PT 6-hydroxylase – a P450 dependent monooxygenase – was determined in the first time among the late step enzymes of the biosynthesis of PT and its derivatives. According to theses studies, the structure of 6-methoxy-PT does not proceed *via* PT (Molog et al. 2001, Seidel et al. 2002), but β-peltatin 6-*O*-methyltransferase (Kranz and Petersen 2003) Figure 4. Similar problems with the identification of the actual enzymes involved in the biosynthesis also happen with other alternative pathways (Calvo-Flores et al 2015).

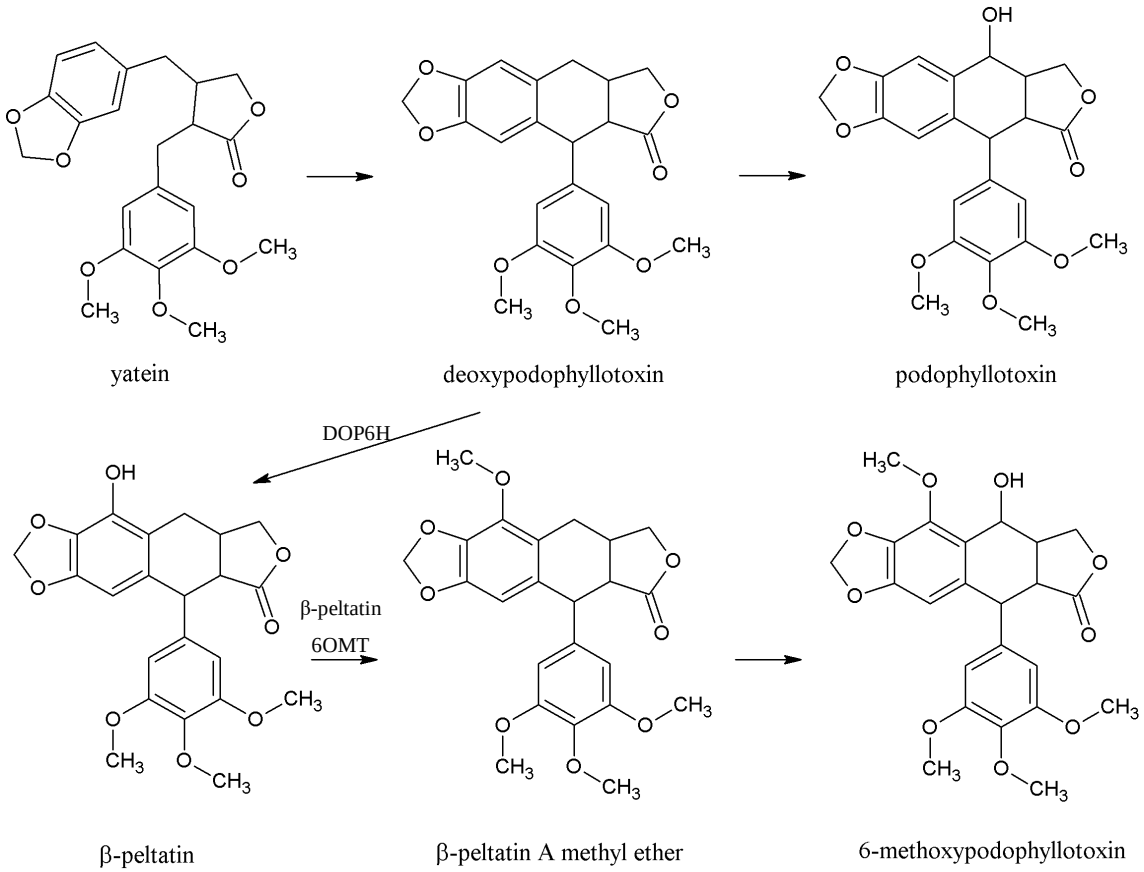

Figure 4. Biosynthesis of podophyllotoxin and 6-methoxypodophyllotoxin, involved the identified enzymes: deoxy-podophyllotoxin 6-hydroxylase (DOP6H) catalyses the deoxy-PT to form β-peltatin, while the transformation of β-peltatin into β-peltatin A methyl ether occurs by β-peltatin 6-*O*-methyltransferase (β-peltatin 6OMT) (Suzuki et al. 2002b)

Umezawa (2003) suggested the arylnapthalene lignans are formed from aryltetralin lignans by dehydrogenation or by hydroxylation due to the structural similarity.

Metabolic steps, enzymes and genes involved in biosynthetic pathway of the structurally more diverse C8-C5' neolignans, are only involved in research in the last decade. In case of neolignans, the initial coupling step resulted dehydrodiconiferyl alcohol, which can be either racemic or optically active, depending upon the plant species (Kasahara et al. 2006). Diringent proteins also participate in the initial coupling, thus they support the chirality of the majority of neolignans.

1.3 Distribution of lignans in the plant kingdom

Lignans are widely distributed in the plant kingdom. A comprehensive study on the distribution of lignans proved the presence of these molecules in 108 families within the Magnoliophyta division, 8 within Gymnospermophyta and 2 within Pteridophyta already in 1990. Since then, in the division of Bryophyta, only p-hydroxycinnamate

dimers have been isolated (Umezawa 2003). According to Gordaliza et al. (2004), lignans can be found also in a large number of plant families belonging to the vascular plants. These molecules have been isolated from different plant parts, including roots, rhizomes, woody parts, stems, leaves, fruits and seeds as well as in exudates and resins (Gordaliza et al. 2004, Saleem et al. 2005). Lignans are also common in food plants. Namely, secoisolariciresinol (Ward 2003), matairesinol (Ward 1997), lariciresinol (Ward 1995), medioresinol (Suzuki and Umezawa 2007), pinoresinol (Saleem et al. 2005), syringaresinol (Macías et al. 2004), sesamin (Moon et al. 2008) and sesamolin (Yun et al. 2011) have been published to be present in various food plants. Compared with the total family number of subclasses, there are some of them, i.e. Magnoliidae, Rosidae and Asteridae, which include many lignan producing families, i.e. Asteraceae, Apiaceae, Rubiaceae, etc. (Umezawa 2003). Although lignans occur typically in vascular plants, these sources normally do not provide commercially useful quantities, but some exceptions occur (Saleem et al. 2005, Calvo-Flores et al. 2015).

1.4 Lignans in plants

Lignans can be occur in plant both as aglycone and glycoside form (Saleem et al. 2005, Sólyomváry et al. 2017a). Glycosides are hydrolyzable by endogenous glycosidase enzymes, if they are also present. These enzymes are separated from their substrates in intact plant tissues. Namely, under normal conditions, enzymes and their substrates are accumulated in the cytoplasm and the vacuole, respectively. This compartmentalization may be related to the protective function of lignans: As is often the case in plants, the biologically active lignans are stored as inactive glycosides. Cell disruption by pests (or by pulverization inexperimental conditions) removes this compartmentalization and allows the hydrolysis of glycosides into their effective aglycones (and saccharide molecules) in aqueous medium. In this process, the glycosidase enzyme catalyzes the hydrolysis of the glycosidic bonds (Sólyomváry et al. 2015a).

Hovewer, function of lignans in plants is not fully understood (Calvo-Flores et al. 2015). Among the lignans and their biological activities, some of their aglycones have potent antimicrobial, antifungal and insecticid activities, thus they probably play an important role in plant defense against several biotic stressors (Fukuda et al. 1985, Figgitt et al. 1989, Moraes et al. 2000, Harmatha and Dinan 2003). The presence of lignans in seeds and fruits is common due to the protection of developing embryos

against pests (Dayan et al. 2003). It is known that many lignans are produced as a response to fungal attack. Their antifungal activity is believed to be an inhibitory effect over the fungal enzymes (Takemoto et al. 1975), and fungal growth (Shain and Hillis 1971). Moreover isolated lignans possess insecticid (Messiano et al. 2008, Taniguchi et al. 1989, Yamauchi et al. 1992a, Yamauchi et al. 1992b, Yamauchi and Taniguchi 1991, Yamauchi and Taniguchi 1992a, Yamauchi and Taniguchi 1992b), antifeedant (Sintim et al. 2009, Garcia and Azambuja 2004, Nitao et al. 1992), larvicidal (Srivastava et al. 2001, Nogueira et al. 2009, Nishiwaki et al. 2011) activity against certain pests or reduce insect development (Nawrot and Harmatha 1994, Schroeder et al. 2006) in a dose dependent manner (Messiano et al. 2008).

Some lignans (e.g., matairesinol and bursehernin) provide protection against nematodes (Gonzalez et al. 1994, Gonzalez et al. 1995) as well as some neolignans (e.g., dehydrodiconiferylic alcohol) express a similar activity as cytokinins, a type of plant growth regulators, but in higher concentration (Orr and Lynn 1992).

1.5 Therapeutic significance of lignans

Lignans are recognised as a class of natural product with wide range of remarkable biological activities (Calvo-Flores et al. 2015). Their main reported biological ativities are the following: antiviral, anticancer, cancer prevention, anti-inflammatory, antimicrobial, antioxidant, immunosuppressive, hepatoprotective and osteoporosis prevention (Chuna et al. 2012).

Some species containing aryltetrahydronaphtalene type podophyllotoxin-derivatives (*Anthriscus* and *Podophyllum* sp.) as well as dibenzylbutirolacone type (*Carthamus*, *Ferrula*, *Piper*, *Trachelospermum*, *Wikstroemia* sp.) lignans are used for centuries in the folk medicine (Chuna et al. 2012, Ayres and Loike, 1990, Li et al. 2014, Elfahmi et al. 2007, Parmar et al. 1997, Yoo et al. 2006, Zhu et al. 2013, Kuehnl et al. 2013, Chen et al. 2009).

Considering the numerous literatures on the biological activities of lignans, the following subchapters are going to focus on the main biological effects of those lignans that are closely related to our research topic (Table 1). Namely, the dibenzylbutyrolactone-type lignan arctigenin and trachelogenin, the furofuran-type lignan pinoresinol and epipinoresinol, the dihydrobenzofuran-type sesquineolignan

picrasmalignan, the aryltetraline-type lignan podophyllotoxin and its derivatives as well as the dihydrobenzofuran-type neolignan balanophonin.

Table 1. Biological effects of the lignans that are closely related to the focus of the thesis.

Effects	Compounds	Used cell lines, viruses, mechanism of actions or assays	References
Anti-tumorual	arctigenin	HL-60	Hirano et al. 1994
		human lung adenocarcinoma cell line (A549)	Gu et al. 2012
		human gastric cancer (SNU-1, AGS)	Jeong et al. 2011
		colon adenocarcinoma cell line (SW480)	Mervai et al. 2015
	trachelogenin	colon adenocarcinoma cell line (SW480)	Mervai et al. 2015
	pinoresinol	colon, leukemia, breast and hepatocarcinoma cells	López-Biedma et al. 2016, Fini et al. 2008, Sepporta et al. 2013, Zhang et al. 2018a
	epipinoresinol	colon adenocarcinoma cell line (SW480)	Sólyomváry et al. 2017b
	balanophonin	B16 melanoma 4A5	Nakashima et al. 2010
		human tumor lines: HeLa, HepG2, SW480, HL-60, MCF-7, SK-MEL-5, SK-OV-3, SGC-7901, SMMC-7721	Lee et al. 2007 and 2008, Li et al. 201, Huang et al. 2013, Shang et al. 2013, Devkota et al. 2014, Woo et al. 2016, Guo et al. 2015, Wang et al. 2010
Antioxidant	arctigenin	ROS level of Raw264.7cells were determined	Xianjuan et al. 2011
	pinoresinol	MCF-7	Chin et al. 2008, López-Biedma et al. 2016
	epipinoresinol	*in vitro* against DPPH	Wang et al. 2018
	balanophonin	on-line HPLC-ABTS and ORAC assays	Mohamadi et al. 2015
Anti-inflammatory	arctigenin	inhibition of the expression of nitric oxide synthase (iNOS) and its enzymatic activity; suppress the mitogen-induced tryptophan breakdown	Zhao et al. 2009; Kuehnl et al. 2013
	trachelogenin	suppress the mitogen-induced tryptophan breakdown	Kuehnl et al. 2013
	pinoresinol	endothelial cells	Spilioti et al. 2014
	balanophonin	RAW 264.7 cells	Ma et al. 2013, Xie et al. 2013
Antiviral	arctigenin	Human immunodeficiency virus 1 (HIV1)	Eich et al. 1996
	trachelogenin	Hepatitis C	Quan et al. 2016
	epipinoresinol	Hepatitis B	Zhao et al. 2014

1.6 Description of plant groups involved in our study

1.6.1 Cardueae tribe

1.6.1.1 Taxonomical classification, spread and general characteristics

The Asteraceae (syn. Compositae) are among the largest families of flowering plants, comprising more than 23,000 species and about 1600 genera. Species of the family is represented worldwide (Jeffrey 2007). The tribe *Cardueae* is a large tribe in this family, comprises about 2500 species organized in 83 genera. Within the tribe *Cardueae*, several large genera are found, such as *Carduus* (approx. 90 species), *Cirsium* (200-300 species), *Onopordum* (approx. 60 species) and *Jurinea* (approx. 200 species) (Häffner and Hellwig 1999).

Hungarian flora comprises only a few species among the large genera mentioned above. Six species of *Carduus*, twelve species of *Cirsium*, two of *Jurinea*, while only one of the *Onopordum* species can be found in Hungary (Simon 2000).

The species are drought tolerant biennial or rarely perennial plants. Their leaves are spiny and the capitulum consists of disk and ray flowers. The fruit wall and the dicotyledonous seed incorporated into a cypsela fruits with hairy pappus.

1.6.1.2 Botanical description

1.6.1.2.1 Cirsium Mill. genus

Cirsium species are widely distributed in the Northern Hemisphere. Spiny, biennial or perennial herbs, rarely annuals (Susanna and Garcia-Jacas 2007). Species which were investigated are *C. arvense* (L.) Scop. (creeping thistle, mezei aszat), *C. boujartii* (Pill. et Mitterp.) Schultz. Bip. (‘no English name’, pécsvidéki aszat), *C. brachycephalum* Juratzka (‘no English name’, kisfészkű aszat), *C. canum* (L.) All. (Queen Anne’s thistle, szürke aszat), *C. eriophorum* (L.) Scop. (woolly thistle, gyapjas aszat), *C. oleraceum* (L.) Scop. (cabbage thistle, halovány aszat), *C. palustre* (L.) Scop. (marsh thistle, mocsári aszat), *C. rivulare* (Jacq.) All. (plume thistle, csermelyaszat) and *C. vulgare* (Savi) Ten. (spear thistle, közönséges aszat) (Figure 5).

Cirsium species are traditionally classified into three sections, namely, Cephalonoplos, Chamaeleon (syn. Cirsium) and Eriolepis. The nine investigated species are grouped to these three sections as follows: Eriolepis: *C. boujartii*, *C. eriophorum*, *C. vulgare*,

Chamaeleon: *C. brachycephylum*, *C. canum*, *C. oleraceum*, *C. palustre*, *C. rivulare* and Cephalonoplos: *C. arvense* (Tutin et al. 1968).

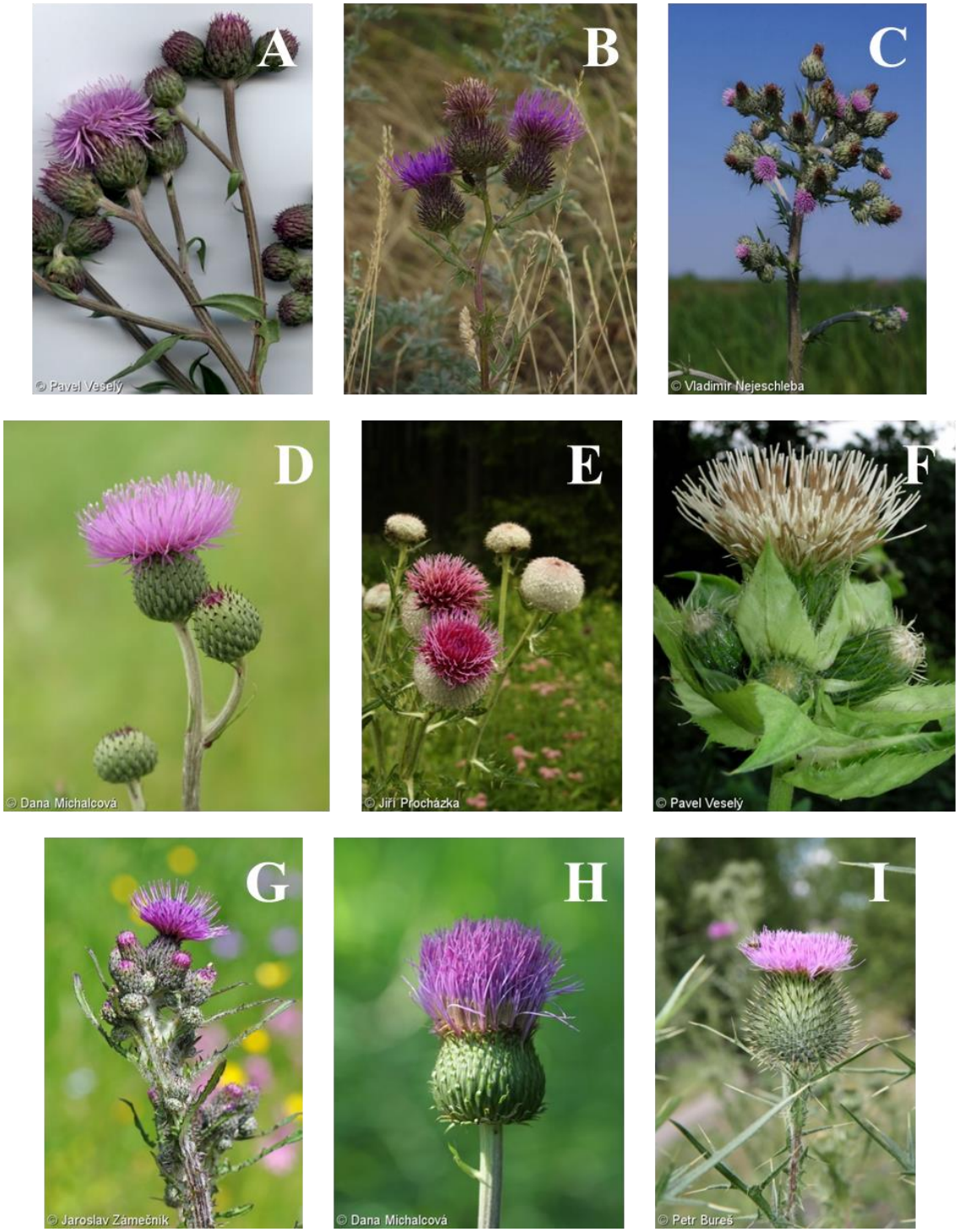

Figure 5. The effusive, radially symmetrical disc flower heads of the nine, investigated *Cirsium* spp. native to Hungary. (A) *C. arvense* – by Pavel Veselý, (B) *C. boujartii* – by Attila Lengyel (from his personal blog), (C) *C. brachycephalum* – by Vladimír Nejeschleba, (D) *C. canum* – by Dana Michalocová, (E) *C. eriophorum* – by Jiří Procházka, (F) *C. oleraceum* – by Pavel Veselý, (G) *C. palustre* – by Jaroslav Zámečník, (H) *C. rivulare* – by Dana Michalocová, (I) *C. vulgare* – by Petr Bureš.

1.6.1.2.2 Carduus L. genus

Carduus species are distributed in Eurasia, mainly in the Mediterranean region, and also in northern Africa. They are annual, biennial or perennial herbs with spiny-winged stems (Susanna and Garcia-Jacas, 2007). Among the six species comprise by the Hungarian flora, one species: *Carduus nutans* L. (musk thistle, bókoló bogáncs) (Simon, 2000) was examined during our studies (Figure 6).

Figure 6. (A) Depressed-globose inflorescence of Carduus nutans, (B) herbarium sheet of *C. nutans* – photos by Dana Michalocová (C) an achene fruit of the plant – by Marie Konečná.

1.6.1.2.3 Jurinea Cass. *genus*

Jurinea species are widely distributed in western and central Asia, Europe and northern Africa. They are unarmed perennial herbs or shrublets (Susanna and Garcia-Jacas 2007). Two species are native in Hungary, among those, *Jurinea mollis* (L.) Reichenb. ('no English name', kisfészkű hangyabogáncs) (Simon 2000) was examined (Figure 7).

Figure 7. (A) Purple inflorescence of *Jurinea mollis* – by Dana Michalocová, (B) the natural habitat of the plant – by Jan Pokorný.

1.6.1.3 Species in the tradicional medicine

Several commonly occurring *Cirsium* species have a crucial role as herbal remedy in the folk medicine (Hawrył et al. 2016), in folk veterinary (*C. arvense* and *C. eriophorum*) (Viegi et al. 2003), and have been used as the part of the Mediterranean diet (*C. arvense*, *C. oleraceum* and *C. vulgare*) (Guarrera and Savo 2016). However, other species are less-used and there is no knowledge on their application. Exclusively, species with folk-medicinal application are listed in the table (Table 2).

Table 2. Species used in folk medicine and their biological effects on human body.

Species	Application	References
Cirsium arvense	healing wounds, sniffing, during pregnancy against vomitting, faintings, giddiness, abdominal pains	Guarrera 2005
	diuretic, haemostatic and anti-inflammatory	Orhan et al. 2013
	dermal inflation or appetizing	Rajaei and Mohamadi 2012
	intestinal worm	Bahmani et al. 2014
Cirsium eriophorum	antiseptic	Bonet et al. 1999
Cirsium oleraceum	diuretic, haemostatic, anti-inflammatory astringent remedy	Nalewajko-Sieliwoniuk et al. 2012
Cirsium rivulare	anxiolytic remedy	Nalewajko-Sieliwoniuk et al. 2012
Cirsium vulgare	anxiolytic remedy	Nazaruk and Jakoniuk 2005, Nazaruk 2008
Carduus nutans	diuretic, cardiotonic, antihemorroidal remedy	Guarrera2003, Zheleva-Dimitrova et al. 2011
	hepatoprotective agent	Aktay et al. 2000
	treating eczema	Mustafa et al. 2012

1.6.1.4 Phytochemical characterisation

Already identified metabolites of *Cirsium*, *Carduus* and *Jurinea* plants have wide structural diversity, representing aldehydes, alkanes, flavonoids, hydrocarbons, lignans, phenolic acids, polyacetilenes, sterols, terpenes (Table 3).

Table 3. Phytochemical characterisation of the species.

Species	Compounds	References
Cirsium arvense	flavonoids, sterols, polyacetilenes, aldehydes	Jordon-Thalen and Louda, 2003
Cirsium brachycephalum	lignans (trachelogenin and tracheloside)	Mervai et al. 2015
Cirsium canuum	sterols, triterpenes, aldehyde	Jordon-Thalen and Louda, 2003
	phenolic acids, flavonoids	Kozyra et al. 2015
	lignans (trachelogenin and tracheloside)	Boldizsár et al. 2010
Cirsium eriophorum	neolignans (balanophonin, prebalanophonin, sesquineolignans (picrasmalignan, prepicrasmalignan)	Sólyomváry et al. 2015
Cirsium oleraceum	flavonoids, sterols, triterpenes, aliphatic aldehydes	Jordon-Thalen and Louda, 2003
Cirsium palustre	flavonoids, triterpenes, β-sitosterol	Nazaruk 2009, Nazaruk and Jablonski 2011, Nazaruk and Galicka 2014
	lignans (arcitenin, arctiin)	Boldizsár et al. 2010
Cirsium rivulare	flavonoids, phenolikc acids	Nazaruk and Jakoniuk 2005
Cirsium vulgare	flavonoids, sterols, triterpenes, phenolic acids	Jordon-Thalen and Louda, 2003, Kozyra and Głowniak 2013
Carduus nutans	flavonoids, sterols, polyacetylenes, hydrocarbons	Bain and Desrochers 1988, Jordon-Thalen and Louda 2003
Jurinea mollis	alkanes	Radulovic and Milotojevic 2012

1.6.2 Anthriscus genus

1.6.2.1 Taxonomical classification and spread

The genus *Anthriscus* belonging to the Apiaceae (syn. Umbelliferae) family (Spalik and Jarvis 1989). *Anthriscus* genus comprises up to 14 species, which occur throughout northern Eurasia from Spain to Japan and in the montainous region of Africa (Spalik 1996). *A. cerefolium* was being cultivated in England at the end of the 16th century and in America in 1800's (Liopa-Tsakalidi and Barouchas 2011). Hungarian flora comprises four species of the genus, among them three was investigated: *A. caucalis* M. Bieb. (burr chervil, borzas turbolya), *A. cerefolium* (L.) Hoffm. (chervil, zamatos turbolya), *A. sylvestris* (L.) Hoffm. (wild chervil, erdei turbolya) The genus has some economic importance. *Anthriscus cerefolium* - chervil is grown as a pot-herb, while *A. caucalis* and *A. sylvestris* are common weeds. At the same time *A. sylvestris* had have special

attention due to their potential medicinal values. The genus was divided into three sections – sect. *Anthriscus*, sect. *Caroides* and sect. *Cacosciadium* – based on habitat and their life history. The last section includes several critical taxa (*A. caucalis* and *A. cerefolium*), called the '*A. sylvestris* group', which may constitute a single polymorphic species (Spalik 1996). Hovewer, regarding to the genetical data, *A. cerefolium* and *A. caucalis* do not appear to be closely related. Even though the ITS sequences are already known, the classification of the species into sections are still not resolved (Downie et al. 2000).

1.6.2.2 Botanical description

The small white flowers of *Anthriscus* sp. form umbels, their leaves are bipinnate or tripinnate. The schizocarp achene fruits separate maturity into two mericarps, each containing a single seed (Figures 8,9 and 10).

***Anthriscus caucalis* M. Bieb.**

Burr chervil's (*A. caucalis*) white flowers form umbels. The tiny, 3-4 mm fruits are covered in hooked spines (Simon 2000).

Figure 8. (A) White flowers of *Anthriscus caucalis* that form several umbels – photo by Barry Breckling on CalPhotos (B) Flowering *A. caucalis* plant in natural habitat, triangular leaves with many leaflets – photo by Keir Morse on CalPhotos, (C) unripen double achene fruits of *A. caucalis* with the hooked spines– photo by Robert. L. Carr. on EWU.

Anthriscus cerefolium (L.) Hoffm.

Chervil (also called French parsley) has black, slender, elongated, 6-8 mm long and glabrous fruits when rippen (Simon 2000) with ridged beak (Vaughan and Geissler 1997). The whole plant smells of anise and tastes a little of petter and of anise (Farooqi and Srinivasappa 2004).

Figure 9. (A) White flowers of *Anthriscus cerefolium* that form umbels – by Pavel Veselý –, (B) bright green, bipinnate leaf of the plant – by Dana Michalocová – and (C) its elongated, dark greyish-black fruits – by Vladimír Motyckal on Botanickafotogalerie.

Anthriscus sylvestris (L.) Hoffm.

Wild chervil (*A. sylvestris*) has fruits with two mericarps, that are 5-10 mm long and 0.9-1.6 mm wide greenish brown to dark brown or black. The two mericarps are glabrous with a short five slight ribs (Darbyshire et al. 1999, Spalik et al. 2001, Magnússon 2011).

Figure 10. (A) White umbel inflorescence of *Anthriscus sylvestris*, (B) wild chervil in its natural habitat and (C) the brown fruits of *A. sylvestris* still on the plant – photos by Dana Michalocová on Botanickafotogalerie.

1.6.2.3 Anthriscus species in the traditional medicine

Two of the three studied species – *A. cerefolium* and *A. sylvestris* – had a crucial role in the folk medicine (Darbyshire et al. 1999, Farooqi and Srinivasappa 2004, Liopa-Tsakalidi and Barouchas 2011).

***Anthriscus cerefolium* (L.) Hoffm.**

Nowadays, principal use of chervil is as a flavouring agent for culinary purposes, but it has been used for several medicinal purposes. A drink made from chervil has been used for several health problems such as an expectorant, a stimulant, a dissolver of congealed blood, a healer of eczema, a digestive and a cure for high blood pressure, gout, kidney stones, pleurisy, dropsy and menstrual problems. The young leaves are used tonics due to their rich vitamins and minerals content (Farooqi and Srinivasappa 2004) (Adams et al. 2011). In Turkish ethnopharmacology, eating the chervil leaves was assumed to treat stomachache (Polat et al. 2013), moreover brewing a tea was used as a shoothing eye wash, while the whole plant was believed to relieves hiccoughs (Farooqi and Srinivasappa 2004). Its ability of lowering the blood pressure as the most persistently recognised up to now, but no clinical studies confirmed this effect (Farooqi and Srinivasappa 2004).

***Anthriscus sylvestris* (L.) Hoffm.**

Wild chervil is reported to be occasionally cultivated and consumed (Darbyshire et al. 1999). The use of wild chervil – both for food and medicinal – was rather result due to the confusion with other Apiaceae species than ethnobotanical knowledge (Alleen and Hatfield 2004). The aerial parts of wild chervil were used as a cure for kidney stones, in Ireland and Tunisia also to treat headaches and in Serbia as a diuretic and tonic. A. sylvestris roots have traditionally been used as an antipyretic, an analgesic, a diuretic and a cough remedy (Yong et al. 2009, Olaru et al. 2015). As an abortifacient and remedy in childbirth, while in Europe the powdered plant was used for healing wounds (Darbishire et al. 1999).

1.6.2.4 Phytochemical characterisation

Among the three studied species, the chemical composition of *Anthriscus sylvestris* was revealed in several phytochemical studies performed on fresh and dried leaves, flowers and roots (Olaru et al. 2015). However, the secondary metabolite profile of *A.*

cerefolium have been less studied (Fejes et al. 2000), while there is no scientific data on the chemical profile of *A. caucalis*.

***Anthriscus cerefolium* (L.) Hoffm.**

Characteristic components of the herb of chervil are flavonoids – such as apiin, luteolin-glycosides (Fejes et al. 2003), naringin, rutin, quercetin and apigenin (Abdulmanea et al. 2012) –, and its essential oil (methylchavicol), while its root contains lignans – such as deoxypodophyllotoxin, anthriscinol methyl ether and angeloyloxymethyl-2-butenoic acid (Fejes et al. 2003). Moreover, the presence of eugenol, stimasterol, β-sitosterol was also represented in the plant (Milovanovic et al. 2009), while Flamini et al. (1993) isolated cumarins (scopoletine, esculetine, acetylesculetine) from the herb of chervil for the first time.

***Anthriscus sylvestris* (L.) Hoffm.**

The major classes of secondary metabolits are terpenoids and lignans (Borg-Karlson et al. 1993, Chen et al. 2014). Amongst the main components of the plant, lignans were the most studied due to their potential cytotoxic activity (Milovanović et al. 2009, Chen et al. 2014).

Monoterpenes represent the major constituents in essential oils of roots and leaves (Boss et al. 2002). Several secondary metabolite groups were identified in the roots, such as coumarins (Jeong et al. 2007), as well as phenylpropene derivatives, polyacetilene derivatives and several lignans (Ikeda et al. 1998, Kramer et al. 2011). Hendrawati et al. (2011a) have found nine lignans and five related stuctures in the roots of *Anthriscus sylvestris*. Metabolic turnover can occur due to the developmental stages of the plant, the habitat the population and the sample preparation, which might cause the low concentration of lignan level. (Kamil and Dewick 1986, Koulman et al. 2003a, Hendrawati et al. 2011b). Other research groups have identified podophyllotoxin in the roots of *A. sylvestris* in trace amount (under 0.01 μg/mg of dry product), small quantities of arctigenin, matairesinol- thujaplicatin-, yatein- and podophyllotoxin-derivatives (Koulman et al. 2003a, Jeong et al. 2007).

Aerial parts of the plant aromatic alcohols, a phenylpropan derivative and terpene derivatives (Olaru et al. 2015), flavonoids (Abdulmanea et al., 2012) and one phytosterol (Koulman et al. 2003a). Although, the lignan content of the aerial parts of *A. sylvestris* is significantly lower than in the roots, deoxypodophyllotoxin, yatein,

secoisolariciresinol, lariciresinol, matairesinol, hinokinin were identified in the aerial parts of wild chervil (Suzuki and Sakakibara 2002).

Lignans were isolated also from the flowers of *A. sylvestris*, such as deoxypodophyllotoxin, yatein, (−)-morelensin, and (−)-hinokinin. Morelensin and hinokinin were reported only in the fruits. Moreover, other compounds were also identified, ie. morinin L, and falcarindiol (Koulman et al. 2003b).

1.7 Plant biotechnology: a key tool of secondary metabolite production

In the coming decades, the development of the next generation of the advanced plant-based pharmaceuticals requires several new technologies, such as biotechnological approaches. Modern biotechnology has ensured the possibility to use plant cells for the production of specific pharmaceuticals (Ionkova 2011). Production of bioactive compounds from intact plants depends on various factors, including the species used, the age and maturity of its parts, its genetic composition and both environmental and physiological conditions (Malik et al. 2014). Today, we are able to establish *in vitro* cell or tissue cultures of plants, using the appropriate medium and growth regulators. Starting from callus tissue, cell suspension cultures can be established that can even be grown in bioreactors. In addition, biotechnology is still the most environmentally friendly tool of the production of the desired plant metabolites (Ionkova 2011). Thus, biotechnology offers quick and efficient methods for producing the high-value medical compounds in cultivated cells and tissues. However, the commercial success is limited by the lack of understanding of the complex biosynthetic pathways leading to the desired endproduct (Ionkova 2011).

1.7.1 Callus cultures

Besides agriculture and horticulture, callus cultures offer a wide range of usages in pharmacology and pharmacy. Callus, a non-differenciated tissue represents a defense mechanism of all major terrestrial plants over a wounded tissue. These cells can be cultivated *in vitro* in the presence of several growth hormones (i.e. auxin, cytokinin) for biotechnological purposes. Explants taken from plant tissues generate *in vitro* on solid medium an amorphous cell mass. Its color can range from colorless to brown due to the lack or presence of chloroplasts. Culture and medium conditions (i.e. photoperiode or

phytohormon concentration) considerably depends on the species. While some callus cultures need dark growth conditions, others grow under specific day-night conditions (Efferth 2019). The induction, growth and phytochemical profile of callus depend *inter alia* on the explants and phytohormones included in the culture medium (Malik et al. 2014).

Focusing on lignans and our studied species, several research groups have found that the culture conditions play a crucial role in the lignan-production, thus optimization of these factors are a necessity (Ionkova 2007, Konuklugil et al 2007, Fuss 2003, Mohagheghzadeh et al. 2002). Hendrawati et al. (2012) found that calli of *A. sylvestris* did not have the ability to produce deoxypodophyllotoxin. Therefore, they concluded that differenciated cells are required to produce this lignan. Callus cultures of several *Cirsium* species were established but not for the purpose of secondary metabolite production (Hu et al. 2009, Lalonde and Shorthouse 1984).

1.7.2 Cell suspension cultures

Friable callus cultures are feasible to generate single-cell cultures that are cultivated in slowly shaken liquid medium, called cell suspension culture (Efferth 2019).

Although callus cultures of *A. sylvestris* were described to be unable to produce lignans due to the lack of the differentiation of the cells (Hendrawati et al. 2012), a trace amount of deoxypodophyllotoxin was detected using cell suspension cultures. Feeding the cells with deoxypodophyllotoxin, yatein and anhydropodorhizol, they have found that yatein was toxic on the cell cultures, anhydropodorhizol was not transformed into any detectable compound but in contrast, deoxypodophyllotoxin was converted into podophyllotoxin yielding significantly higher concentration than measured in the whole plant (Koulman et al. 2003a). Different lighting conditions can also be affect the secondary metabolite production of cell suspensions. *Artemisia* sp. suspension culture showed enhanced production of phenolic compounds in light, while cultures were cultivated in dark showed higher production of flavonoids (Mir et al. 2017).

1.7.3 Elicitation and precursor feeding for lignan production

Using elicitors in order to enhance the biosynthesis of several secondary metabolites in diverse plant species is a frequently applied technique in plant cell cultures. As an elicitation technique, adding precursors to the medium activate the expression of genes

encoding the relevant biosynthetic enzymes. Thus, several enzymes in the biosynthetic chain can be stimulated by precursor-elicitation to enhance product formation (Malik et al. 2014, Efferth 2019).

Since podophyllotoxin is the preferred precursor for the semisynthesis of anti-cancer drugs, the accumulation of predominantly podophyllotoxin is especially interesting (Ionkova 2011). Elicitation can be carried out by adding growth regulators (a.k.a. phytohormones). In the case of *Linum* species, several phytohormone elicitors were studied in order to increase the lignan production in the cell suspensions of *Linum* sp. Both salicylic acid (SA) and methyl jasmonate (MeJa) have enhanced the podophyllotoxin and its related lignan production in the cells. It was found that elicitation by SA has increased the podophyllotoxin production three-fold, without affecting cell growth (Yousefzadi 2010), while MeJa treatment has been also observed to enhance lignan accumulation (van Furden et al. 2005). As a third possibility, abiotic (Ag^{+}, Pb^{2+}, Cd^{2+}) elicitors were also tested. Silver have been found to increasing the podophyllotoxin production, while Cd^{2+} have been inhibited as well as the cell growth (Shams-Ardakani et al. 2005). Finally, Various fungal extracts were also found to be effective to supress the lignan production, they were more effective than pure elicitors (chitin, chitosan and MeJa) (Bahabadi et al. 2011).

2 OBJECTIVES

Our studies were focused on some of the less studied species of Cardueae tribe, which potentially accumulates lignan in their fruits, with the following aims:

1. Our previous studies on *Jurinea mollis* (L.) Rchb. revealed a large amount of arctiin in its fruits. We aimed at designing an enzymatic method utilizing endogenous enzyme treatment for the preparation of arctigenin from the fruits.
2. As a continuation of recent work of our research group, fruit metabolites of *Carduus nutans* were investigated in order to determine its lignan components.
3. Phytochemical characterisation of *Cirsium* spp. (*C. boujartii*, *C. rivulare*), native to Hungary, have been not investigated yet. We aimed at evaluating the chemotaxonomic significance of lignans, neolignans and sesquineolignans in *Cirsium* sections and species and determining the optimal sources from which these compounds can easily be isolated in relatively high amounts (without impurities).

Furthermore, based on the existing knowledge on the lignan content of *Anthriscus sylvestris*, we aimed:

4. to characterise the phytochemical profile of the aerial parts of the available, Hungarian *Anthriscus* spp.
5. We aimed also to generate *in vitro* cultures of *Cirsium* and *Anthriscus* spp. in order to produce valuable metabolites

6. and to confirm the significance of lignans isolated from theitr optimum sources as antiproliferative components.

3 MATERIALS AND METHODS

3.1 Plant material

Ripe fruits of *C. arvense* (L.) Scop., *C. boujartii* (Pill. et Mitterp.) Schultz. Bip., *C. brachycephalum* Juratzka, *C. canum* (L.) All., *C. eriophorum* (L.) Scop., *C. oleraceum* (L.) Scop., *C. palustre* (L.) Scop., *C. rivulare* (Jacq.) All., *C. vulgare* (Savi) Ten. *Carduus nutans* L. and *A. cerefolium* (L.) Hoffm. were collected from different Hungarian locations: in Somogy Country and Buda Hills. Fruits of *Jurinea mollis* (L.) Reichenb. were collected during the ripening phase in 3 stages (unripe (green fruit), nearly ripe and fully ripe) in the Buda Hills. Whole plants of the *Anthriscus caucalis* M. Bieb., *A. cerefolium* (L.) Hoffm. and *A. sylvestris* (L.) Hoffm. were harvested during their flowering stage in Budapest (*A. caucalis* and *A. cerefolium*) and in the Buda Hills (*A. sylvestris*). Additionally, their ripe fruits were also harvested.

The fruits of the *Anthriscus cerefolium* were stored at 4°C for 3 months prior to applications. The aerial parts of *Anthriscus* species were dried and stored at room temperature.

All the voucher specimens are deposited in the Department of Plant Anatomy, Eötvös Loránd University, Budapest, Hungary.

3.2 Extraction and sample preparation

3.2.1 Fruit wall and ebryo separation from the fruit

Fruits of *J. mollis* were separated by manual dissection, resulting in the isolation of an external woody fruit wall (together with the seed coat) and an internal embryo section.

3.2.2 Preparation of enzyme-hydrolyzed fruit samples

Lyophilized and pulverized *Cirsium* fruit samples (0.500 g), as well as whole fruit and embryo tissues of *Jurinea mollis* (75.0 mg) – obtained from about 1 grams of fruits – were suspended in 3 mL (*Cirsium* samples) and 0.5 mL (*Jurinea* samples) of distilled water. Suspensions of *J. mollis* were left for 5, 15, 30, 60, 300, 600 and 900 min at room temperature, while suspensions of *Cirsium* spp. were stirred at 40 °C for 30 min to perform endogenous enzymatic hydrolysis of glycosidic compounds. Afterward, the

samples were lyophilized and extracted according to the protocol shown in the following section.

3.2.3 Extraction procedure

Lyophilized and pulverized plant materials – obtained from several plants, callus and cell suspension cultures and from from about 1 grams of fruits – (0.500 g of *Cirsium* fruits, 75.0 mg of *J. mollis* fruits, 20.0 mg from herbs and *in vitro* cultures of *Anthriscus* and *Cirsium* plants) as well as enzyme-hydrolyzed and lyophilized fruit samples, were extracted consecutively three times with 5 mL of 80% (V/V) methanol at 60 ºC in close capped vials for 30 min, to prepare 15 mL stock solutions, which were used for further analysis.

Lyophilized and pulverized *Carduus* nutans fruits (10 g) were extracted three times with 100 ml of 80% (V/V) MeOH under reflux for 30 minutes. Samples were centrifugated between each step and the insoluble material was reextracted. Supernatants were combined and dried by a vacuum evaporator (at 30-40 °C).

3.2.4 Performing acid treatment

Aliquots of the stock solutions of unhydrolyzed intact *Cirsium* fruit samples were evaporated to dryness in a vacuum evaporator at 30–40°C. Acid treatments were performed with 500 μL of 2 M TFA at 50°C for 15 min. Next, acidified samples were dried by a vacuum evaporator (at 30–40°C). The dried samples were dissolved in 80% (V/V) methanol before analyses and isolations, which were performed by analytical and preparative HPLC.

3.2.5 Preparation of high-purity arctigenin extracts (HPAEs) from *J. mollis* fruits

200 mg of lyophilized and pulverized whole fruit and embryo tissues of *J. mollis* were extracted with 2 mL diethyl ether at room temperature applying 10 min of vigorous shaking. Thereafter, centrifuged tissues were subjected to a second and third extraction procedure, as detailed above. After the third extraction, centrifuged tissues were suspended in 0.5 mL of distilled water and were left for 30 min (whole fruit) or 600 min (embryo tissue) at room temperature to perform enzymatic hydrolysis. The enzymatically hydrolyzed samples were extracted with 5 mL of diethyl ether at room temperature for 1 min applying vigorous shaking prior to centrifugation. The diethyl

ether fractions were dried at 30-40°C and called as high-purity arctigenin extracts (HPAEs).

3.2.6 Isolation of pure arctigenin from *J. mollis*

For isolation purposes, the HPAE obtained from the embryo was dissolved in 2 mL methanol, and 500 μL aliquots were injected repeatedly to isolate arctigenin by preparative HPLC.

3.2.7 Analytical HPLC hyphenated with UV and time of flight mass spectrometric (TOF-MS) detections

The HPLC-UV-TOF-MS analyses of the extracts obtained from *Cirsium* species, *Carduus nutans* and *Jurinea mollis*, were performed on an Agilent 1260 Infinity HPLC system consisting of a G1315C diode array detector, a G1312B gradient pump and a G1367E autosampler) (Agilent Technologies, Waldbronn, Germany). Column: GraceSmart RP18 (5 μm) 150mm×4.6mm (Grace Davison Discovery Sciences Lokeren, Belgium);.flow rate: 1.0 mL/min; eluents A: acetonitrile:water containing 0.07 M acetic acid (15:85, V/V), B: acetonitrile:water containing 0.07 M acetic acid (85:15, V/V). A linear gradient was used as follows: 0.0 min, 15% B; 12.0 min, 44% B. Injected volumes: 10 μL. The chromatograms were acquired at 280 and 348 nm for *Cirsium* spp. at 280 for *C. nutans*. For exact molecular mass determination, the Agilent 6230 time-of-flight mass spectrometer equipped with a Jet Stream electrospray ion source in negative mode was used for *Cirsium* spp. and in positive ion mode for *Carduus nutans*, and *Jurinea mollis*. Jet Stream parameters: drying gas: (N_2), flow rate: 10.0 l/min, temperature 325 °C. Nebulizer gas (N_2) pressure, capillary voltage, sheath gas flow rate and temperature were set as follows: 10 psi, 4000 V, 7.5 L/min, 325°C, respectively. HPLC–TOF/MS parameters: fragmentor voltage: 100 V; skimmer potential: 100 V; OCT 1 RF Vpp: 750 V. Full-scan mass range *m/z* 100 – *m/z* 2500; scan rate: 250 ms/spectrum. Data processing was performed by the Agilent MassHunter B.03.01 software.

3.2.8 Analytical HPLC hyphenated with UV and high-resolution Orbitrap mass spectrometric detections of *Carduus nutans* fruit, *Cirsium boujartii* callus culture and *Anthriscus* samples

For chromatographic separation and high resolution mass spectral analysis of *Anthriscus* spp., a Dionex Ultimate 3000 UHPLC system (3000RS diode array detector (DAD), HPG-3400RS pump, SRD-3400 solvent rack degasser, TCC-3000RS column thermostat, WPS-3000TRS autosampler) was used, hyphenated with a Orbitrap Q Exactive Focus Mass Spectrometer equipped with electrospray ionization (ESI) (Thermo Fischer Scientific, Waltham, MA, USA)..The HPLC separations were performed on a Kinetex C18 column (75 × 3.0 mm; 3.5 μm) (Phenomenex, USA). The mobile phase consisted of formic acid (0.1% V/V) (A) and acetonitrile (B) with the following linear gradient: 0.0 min, 20% B; 12.0 min, 60% B; flow rate: 0.3 mL/min; column temperature: 25 °C; injected volume: 1.0 μL. The ESI source was operated in negative ionization mode and using the built-in software operation parameters were optimized automatically. The working parameters were as follows: spray voltage, 2500 V (-); capillary temperature 320 °C; sheath-, auxiliary- and spare-gases (N2): 47.52, 11.25 and 2.25 arbitrary units, respectively. Full MS scan resolution: 70,000, scan range: *m/z* 100 – *m/z* 1000. MS/MS scan resolution: 35,000 and MS/MS scan range: *m/z* 80 – *m/z* 1000. Collision energy: 10, 20, 30 and 45 eV. DAD spectrum range: 250 – 600 nm.

3.2.9 Preparative HPLC

To isolate compounds, a Nucleosil 100, C18 (10 μm), 15 × 1 cm (Teknokroma,Spain) semipreparative column was connected to our HPLC instrument (Agilent 1260 Infinity HPLC system). The separation method (gradient program, eluents, and detection) was identical with the method used for analytical works (chapter 3.2.7) with the exception the flow rate and injected volume. These parameters were as follows: 3 mL/min and 500 μL, respectively.

3.2.10 Nuclear magnetic resonance (NMR) spectroscopy

NMR data of the isolated compounds derived from *Cirsium* spp. species were recorded in methanol-d4 at 25°C on a Varian DDR spectrometer (599.9 MHz for 1H and 150.9 MHz for 13C) equipped with a dual 5 mm inverse detection gradient (IDPFG) probe-

head. Standard pulse sequences and parameters were used to obtain 1D ^{1}H, 1D ^{13}C and various 2D COSY, TOCSY, [^{1}H ^{13}C] HSQC and [^{1}H ^{13}C] HMBC spectra. ^{1}H and ^{13}C chemical shifts were referenced relative to the solvent resonances (δ_H=3.31 ppm, δ_C=49.15 ppm).

3.2.11 Circular dichroism (CD) spectroscopy

To record the CD spectra of compounds isolated from *Cirsium* spp., a Jasco J720 Spectropolarimeter (Jasco INC, Tokyo, Japan) was used. Solvent: methanol. All spectra were accumulated three times as follows: Bandwidth: 1 nm; scanning steps and speed: 0.2 nm and 50 nm/min, respectively.

3.2.12 Compound quantification

Quantification of compounds in the fruit samples of ***Cirsium* spp.**, *Carduus nutans* and ***Jurinea mollis*** was based on their HPLC-UV separation, by using an external standard method. For quantifying the desmethyl balanophonin and desmethyl picrasmalignan, linear regression analysis was performed in the range of 0.030–0.300 μg of their injected amounts, resulting in appropriate r2 values. The amounts of lignan glycosides (arctiin, tracheloside) and their corresponding aglycones (arctigenin, trachelogenin) were calculated, by using the calibration curve of standard arctigenin.

Additionally, quantification of balanophonin, prebalanophonin, picrasmalignan, prepicrasmalignan and pinoresinol was based on their HPLC-UV separation by using external standard method. Our quantitative method was validated for linearity, precision, limits of quantification (LOQ) and detection (LOD). Low LOD and LOQ values, high recovery data and appropriate r2 values were obtained. Furthermore, intra- and inter-day relative standard deviations (low, mid and high concentrations of the isolated compounds is six parallel runs on the same day and on three successive days, respectively) were less than 2.8% (prepicrasmalignan) and 4.0% (prepicrasmalignan), respectively. Based on these results we could conclude that our HPLC method is appropriate for the quantitation of thes molecules.

Quantification of compounds in the intact and *in vitro* samples of ***Anthriscus* spp.** was also based on on their HPLC-UV separation, by using an external standard method. For quantifying the chlorogenic acid, linear regression analysis was performed in the range of 0.030–0.300 μg of their injected amounts, resulting in appropriate r2 values. The

amounts of the caffeoylquinc acids were calculated, by using the calibration curve of standard cynarine.

3.3 Principal component analysis (PCA)

To perform PCA analysis, the Minitab 18 Statistical Software was used considering nine *Cirsium* species and ten compounds.

3.4 Antiproliferation studies

Cell proliferation was investigated on SW480 colon adenocarcinoma cell line (ATCC, CCL 228). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C in RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. For the proliferation assay, cells were seeded into 96-well plates at a density of 3000 cells/well, containing the isolated compounds: balanophonin, desmethyl balanophonin, picrasmalignan, desmethyl picrasmalignan (each at 25, 50 and 100 μM), prebalanophonin, prepicrasmalignan (each at 50 and 100 μM) or podophyllotoxin (10 and 20 nM) and incubated for 48 h. Control cells were incubated in culture media. To follow cell proliferation, the sulforhodamine B (SRB) colorimetric assay was used. Podophyllotoxin, used as a positive control, showed a cell growth inhibition of 30 and 55% at 10 and 20 nM, respectively.

3.5 Cell- and tissue cultures

3.5.1 Establishing *in vitro* callus cultures

Ripe fruits of *Cirsium boujratii* and *Anthriscus cerefolium* were surface sterilized with 70% (V/V) NaOCl solution containing for twice 10 min, then were rinsed three times into demineralized sterile water (10 min per rinse). The fruits were then blotted on sterile filter paper sheets before cutting them half to ensure a wounded surface. The half fruits were placed on half and whole strength Murashige and Skoog (MS) (Murashige and Skoog 1962) medium supplemented with different growth regulators. Both the applied MS media were modified by the reduction of the iron level. The composition of the used basic media is summarized in the Table 4 and the supplementation in Table 5. All the media formulations contained 3% sucrose and 7% (w/v) agar, pH was adjusted to 5.7 before autoclaving. The autoclaving time was set to 20 minutes at 121°C.

Table 4. Composition of ½ MS and MS basal media (Values are given in mg/l).

		½ MS (mg/l)	MS (mg/l)
Macro elements			
	Potassium nitrate	950	1900
	Ammonium nitrate	825	1650
	Calcium chloride	220	440
	Magnesium sulfate	185	370
	Monopotassium phosphate	85	170
Micro elements			
	Boric acid	3.10	6.20
	Manganese (II) sulfate dihydrate	11.15	22.30
	Zinc sulfate heptahydrate	4.3	8.60
	Sodium molybdate dihydrate	0.125	0.25
	Copper (II) sulfate pentahydrate	0.0125	0.025
	Cobalt(II) chloride hexahydrate	0.0125	0.025
Ferric sodium EDTA		25	25
Potassium iodide		0.83	0.83
Vitamins			
	Inositol	100	100
	Thiamine (B1)	0.1	0.1
	Niacin (B3)	0.5	0.5
	Pyridoxine (B6)	0.5	0.5

The cultures were grown at 12/12h photoperiod with approx. 3000 lux light provided by cool and warm white fluorescent lamps, or in dark at 22°C. Subcultivation occurred monthly.

Table 5. Different media variants: abbreviations and compositions according to the basic medium and growth regulators.

Medium abbreviation	Medium composition
½ MS 7	½ MS basic medium + 1 mg/l ZEA + 2 mg/l NAA
½ MS 8	½ MS basic medium + 1 mg/l ZEA + 2 mg/ml 2,4-D
MS 4	MS basic medium + 2 mg/l 2,4-D + 2 mg/l BAP
4	MS basic medium + 2 mg/l BAP +1 mg/l NAA
MS $^{IVS\text{-}BAP}$	MS basic medium + 0.1 mg/l IBA + 1 mg/l BAP

3.5.2 Cell suspension cultures

Cell suspension culture is based on the calli of *Anthriscus cerefolium* cultivated on MS4 medium. The medium was the same MS4 as mentioned in the previous subchapter, but lacking agar. 2-2 g calli were transferred into each Erlenmeyer lombic, containing 40 mL of medium and was stirred at 100 rpm. Cultures were grown at 22°C on light without dark period (with the same light condition mentioned in the previous subchapter). Subcultivation occurred monthly.

3.5.3 Experiments on elicitation

Cell suspension cultures of *Anthriscus cerefolium* were grown for 7 days, prior elicitation. Elicitors were added to the cultures that were in the linear phase of the cellular-growht curve.

Stock solutions of jasmonic acid (JA) and salycilic acid (SA) were dissolved in EtOH, while yeast extract (YE) in destilled water (DW) and sterile filtered through a 0.22 μm sterile filter (FilterBio; China). The final concentration of the elicitors occurred as follows: JA and SA 10 μM and YE in 50 μg/ml. Equivalent amount of DW were added to the control cultures. The incubation time was 1, 3, 5 and 7 days. The experiment was made in triplicate.

4 RESULTS

4.1 Phytochemical characterisation of the Cardueae fruits

4.1.1 *Anylsis of Jurinea mollis fruit*

To determine the phytochemical profile of *J. mollis* fruits, intact (non-hydolysed) fruit sample as well as enzyme-hydrolysed samples were analysed by HPLC-UV. Figure 11 demonstrate the results of these analyses.

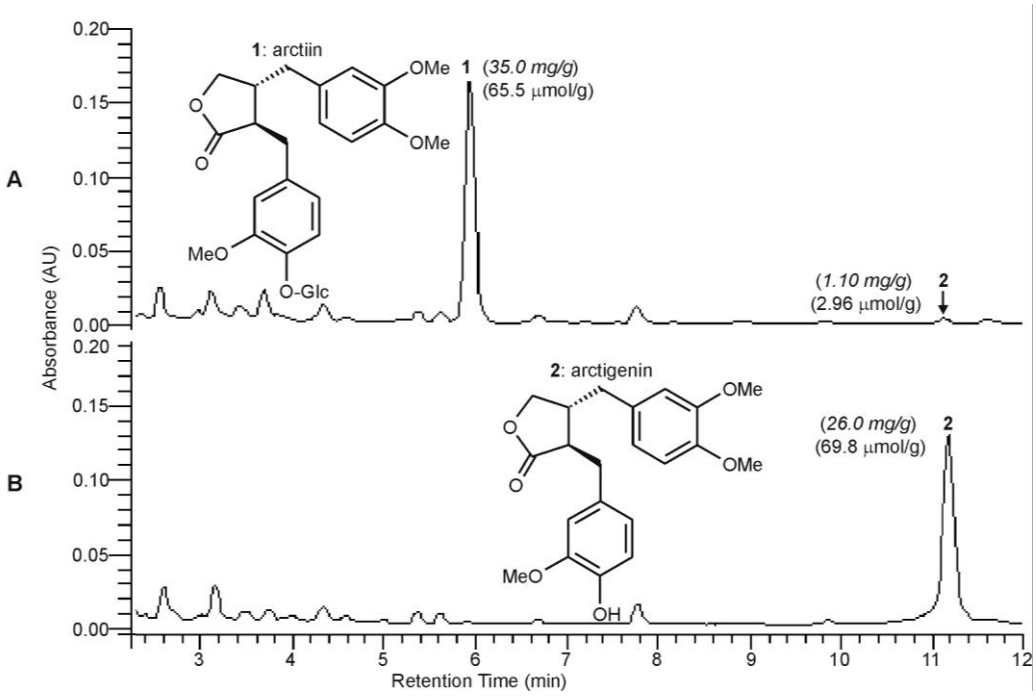

Figure 11. HPLC-UV elution profiles (λ = 280 nm) of the *J. mollis* fruit extracts obtained from the non-hydrolyzed intact (A) and enzymatically hydrolyzed (B) samples, together with the structures of the lignans. Data in parentheses represent the amount of compounds expressed in mg/g and μmol/g values. The amount of arctiin (compound 1) was calculated based on arctigenin (compound 2).

A specific conversion of compound 1 of the intact fruit sample (Figure 11A) into compound 2 during enzymatic hydrolysis (Figure 11B) was followed by LC-UV-MS. Performing LC-MS analysis in positive ion mode, the protonated molecular ions of compound 1 (*m/z* 535) and compound 2 (*m/z* 373) showed the presence of an additional 162 Da moiety (corresponding to a glucosyl unit) in compound 1. These results suggest the presence of the glucoside arctiin and its aglycone arctigenin in *J. mollis* fruit. Arctigenin was isolated using preparative HPLC and additional identification of the isolated compound was carried out by CD and NMR spectroscopy.

For isolation purposes, *J. mollis* fruit composition at three rippening stages were compared (Figure 12).

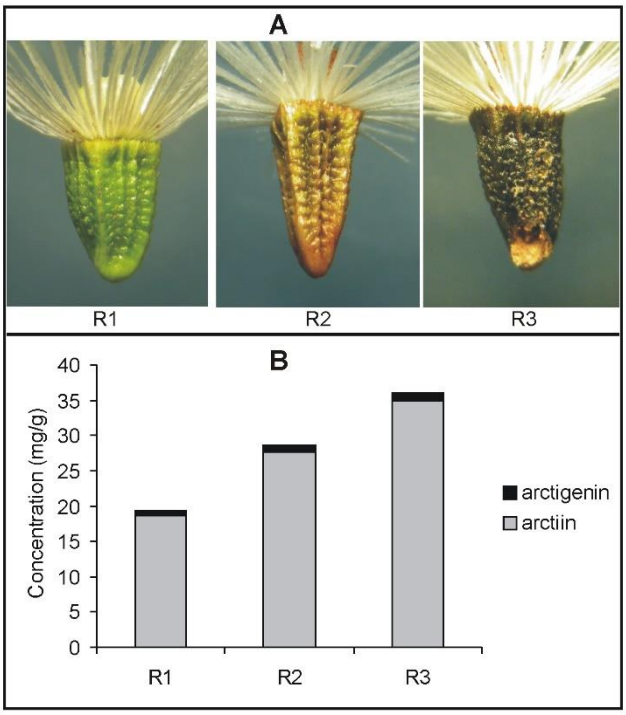

Figure 12. (A) Fruits of *Jurinea mollis* during their ripening stages: unripe, green fruit (R1), nearly ripe fruit (R2) and fully ripe fruit (R3) and (B) the corresponding composition of the lignan content (R1, R2 and R3) in these three ripening stages of fruits. Reproducibility characterization was performed by analysis of additional fruit samples which were collected in R1, R2 and R3 stages (in total, three samples of each, measured in triplicates) and differences were characterized by the RSD percentages, varying from 3.2 RSD% (arctigenin in R3 sample) to 11.7 RSD% (arctigenin in R1 sample).

The highest amount of arctiin was determined in the ripe fruit (R3), thus ripe fruits were used in the following experiments. According to the results above, the ripe fruits were investigated in further studies. The arctiin and arctigenin content of the whole ripe fruits and the embryo as well as the fruit wall were compared by HPLC-UV. Results of this analysis confirmed that both compounds were exclusively found in the embryo part of the fruit, containing 71.5 mg/g arctiin and 2.23 mg/g arctigenin.

4.1.1.1 Optimization of enzymatic hydrolysis

In order to optimise the conditions of the enzymatic hydrolysis, whole ripe fruit and its separated embryo part were investigated. The conversion of arctiin into arctigenin was monitored by HPLC-UV as a function of the hydrolysis time (Figure 13) (In order to characterize the effects of endogenous enzymatic hydrolysis on the conversion of compounds on a comparable basis, the amounts are given in μmol/g values).

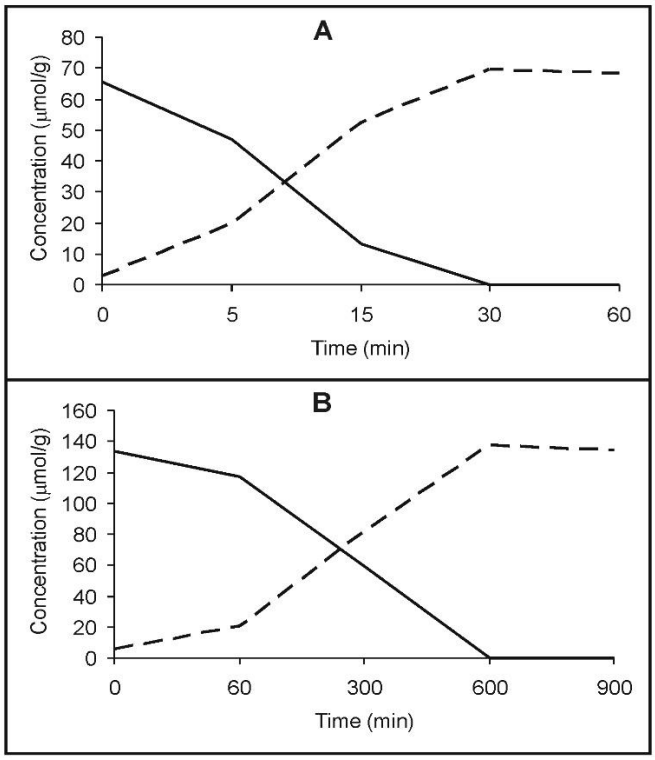

Figure 13. Comparison of arctiin (continuous line) and arctigenin (dashed line) composition in non-hydrolyzed (0) and in enzymatically hydrolyzed whole fruit (A). Hydrolyzation times were as follows: 5 min, 15 min, 30 min and 60 min. While in non-hydrolyzed (0) and in enzymatically hydrolyzed embryo (B) samples the following hydrolyzation times were applied: 60 min, 300 min 600 min and 900 min. Samples were analysed by HPLC-UV, at 280 nm. Results mean values obtained from three parallel experiments; differences were characterized by the RSD values from 1.1 (arctigenin in non-hydrolyzed whole fruit) to 4.3 (arctiin in enzymatically, for 5 min hydrolyzed whole fruit).

The quantitative conversion of arctiin into arctigenin was confirmed in both samples but a significant difference was observed regarding to the time requirement. Total glycoside-aglicon conversion required in whole fruit and in embryo tissues at least 30 minutes and 600 minutes, respectively.

4.1.1.2 Preparing High Purity Arctigenin Extract (HPAE)

To prepare HPAE from the separated fruit parts of *J. mollis*, simple, effective and environmentally friendly isolation technique was introduced, which based on the specific enzymatic hydrolisys of arctiin into arctigenin.

Using this method, from 200 mg whole fruit and from 200 mg embryo tissues, 7.9 mg HPAE containing 57.5% arctigenin and 12.5 mg HPAE containing 74.2% arctigenin were prepared (results represents the average of three paralel measurements).

Theoretically extractable arctigenin content of 200 mg embryo tissues – that means a calculated value, derived from the sum of arctiin and arctigenin amount of intact

embryo tissue – was 10.4 mg. Comapring this value with the arctigenin content of HPAE prepared from 200 mg embryo (9.3 mg, representing 74.2% of 12.5 mg HPAE isolated from 200 mg tissue) demonstrates that the three-step isolation method results in a very high efficiency (89.4%) of arctigenin extraction.

4.1.2 Analysis of *Carduus nutans* fruit

Phytochemical composition of intact (untreated) *Carduus nutans* fruits were investigated (Figure 14).

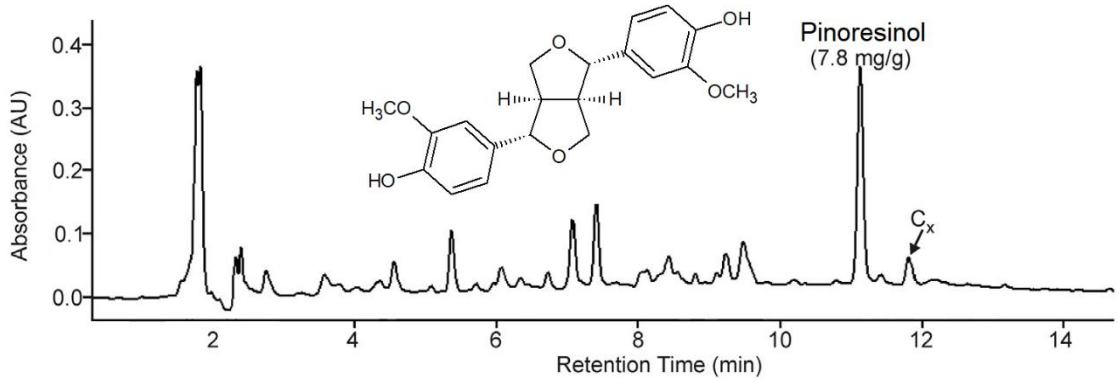

Figure 14. HPLC-UV elution profile (λ=280 nm) obtained from the intact fruit extract of Carduus nutans, together with the structure of pinoresinol. Data in parentheses represents the amount of compound expressed in meg/g (dried fruit).

The HPLC-UV-MS separation (using TOF-MS and Orbitrap-MS detections equally) confirmed the presence of a main compound representing a molecular formula of $C_{20}H_{22}O_6$ (Figure 15). Based on this data, the high-level accumulation of the furofuran lignan pinoresinol was was confirmed for the first time, allowing its effective, high-yield isolation. Further NMR and optical rotation data confirmed its identity unambiguously.

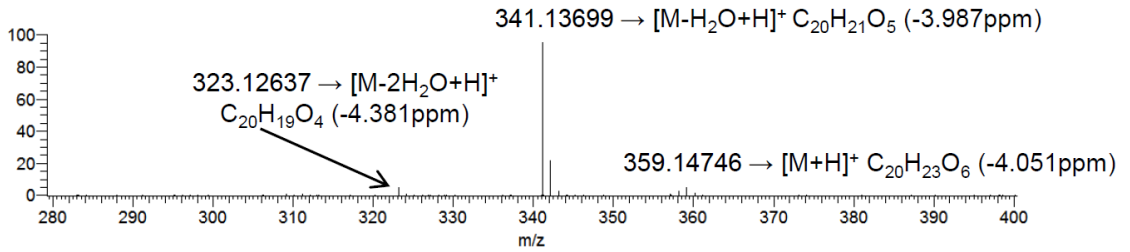

Figure 15. The high-resolution mass spectrum of pinoresinol, obtained by Orbitrap MS detection (using positive ionization mode) from the HPLC separation of intact *Carduus nutans* fruit extract (Note: in addition to the protonated molecular ion $[M+H]^+$, its fragment ions formed by H_2O losses were also determined.

4.1.3 Phytochemical characterisation of *Cirsium boujartii* fruit

The fruit composition of *C. boujartii* was analyzed for the first time. Intact fruit samples and their corresponding acid treated samples were both investigated and four compounds were detected. The acid treatment resulted a characteristic conversion of compounds 1 and 2 into 3 and 4. Comparing the HPLC-UV chromatographic profile and the specific behavior under acidic treatment of *C. boujartii* fruit compounds with those of *C. eriophorum* published previously (Sólyomváry et al. 2015b), the presence of the same four compounds (prebalanophonin prepicrasmalignan, balanophonin and picrasmalignan (Figure 16) was presumed.

Accordingly, the 4′,7-dihydroxy structures (prebalanophonin and prepicrasmalignan), being present in high amounts in the intact fruit (Figure 19), were quantitatively convertible into their 4′,7-epoxy counterparts (balanophonin and picrasmalignan), respectively. Spectral data, obtained by HPLC-UV-TOF-MS, NMR and CD methods, of these compounds were comparable with previously published results, confirming their identity unambiguously as (7R,8S)-balanophonin, (7R,8S,7′R,8′S)-picrasmalignan, (7S,8S)-prebalanophonin and (7S,8S)-prepicrasmalignan.

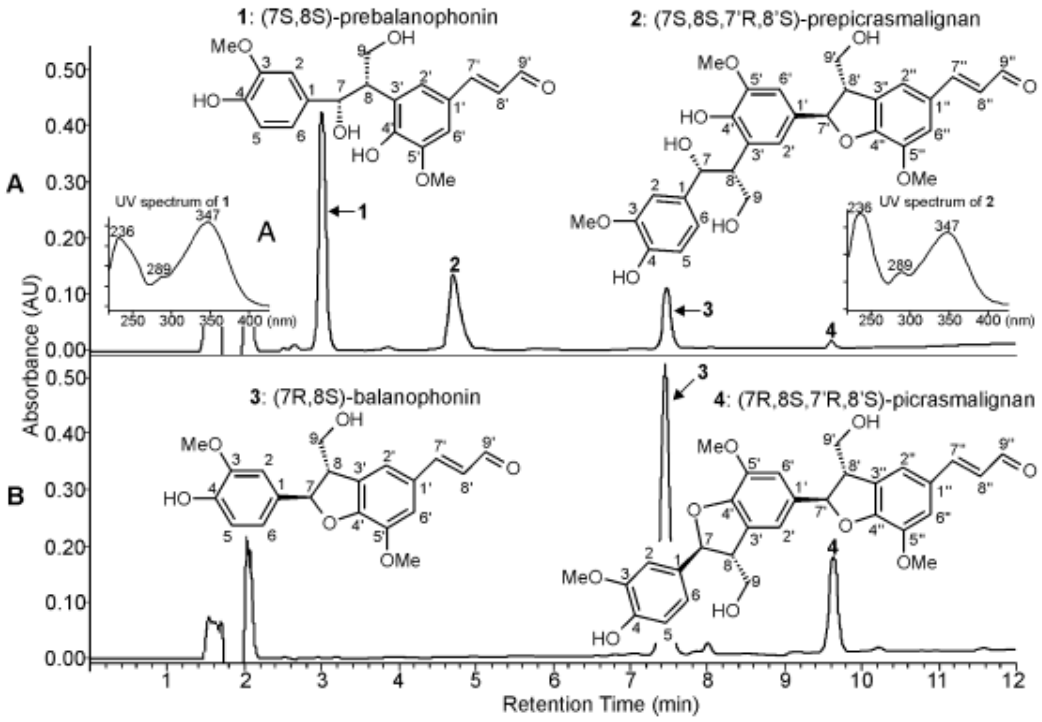

Figure 16. HPLC-UV elution profiles (λ = 347 nm) of the fruit- extracts obtained from the untreated intact (A) or acid treated (at 50◦C) samples (B). together with the structuresof compounds and the UV spectra of prebalanophonin and prepicrasmalignan.

Isolation of the compounds was accomplished by preparative HPLC.

4.1.4 *Cirsium rivulare* fruit composition

In order to gain the phytochemical data of the fruits of *C. rivulare*, intact (untreated) fruit samples and their corresponding hydrolysates, obtained by an endogenous enzyme, were both investigated. The enzyme treatment resulted a characteristic conversion of compounds 1 and 3 into 5 and 6, but compounds 2 and 4 remained untouched as shown on Figure 17.

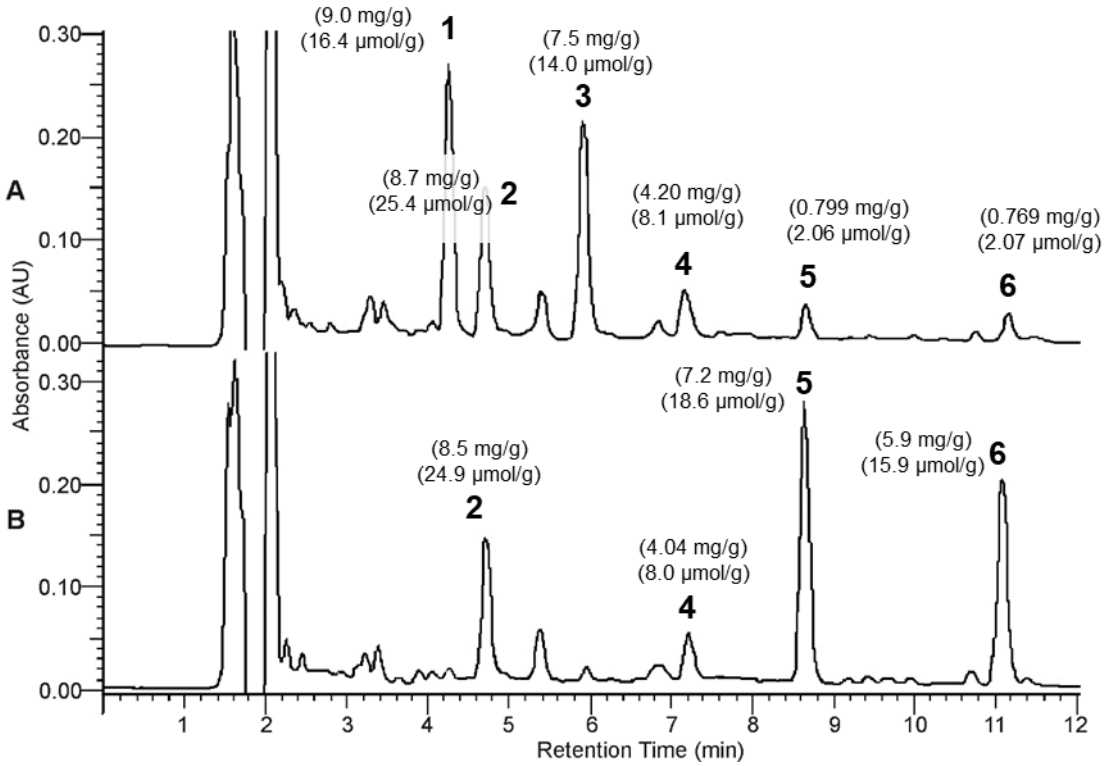

Figure 17. HPLC-UV elution profiles (λ=280 nm) of the fruit extracts obtained from the unhydrolyzed intact (A) and enzyme-hydrolyzed (B) fruit samples of *C. rivulare*. The structures of the compounds, namely tracheloside (1), desmethyl balanophonin (2), arctiin (3), desmethyl picrasmalignan (4), trachelogenin (5) and arctigenin (6), are shown in Figure 18. Data in parentheses represent the content of the identified compounds, expressed in both mg/g and µmol/g values

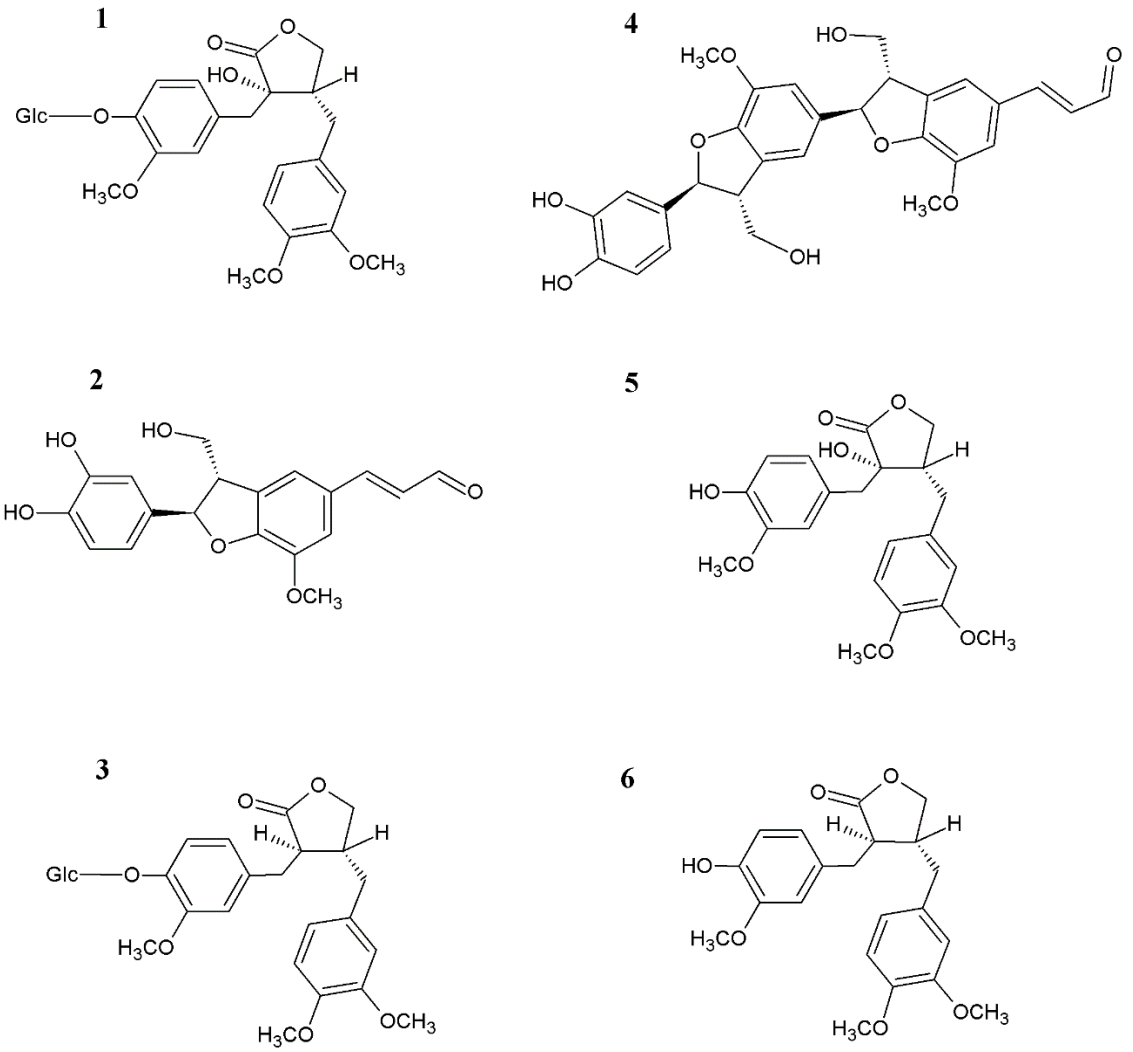

Figure 18. Chemical structures of the identified lignans in *C. rivulare* fruits, namely tracheloside (1), desmethyl balanophonin (2), arctiin (3), desmethyl picrasmalignan (4), trachelogenin (5) and arctigenin (6).

Based on these results and the HPLC-TOF-MS data (Table 6), the unknown compounds were presumed as the lignan glycoside tracheloside (1) and arctiin (2) as well as their corresponding aglycones, trachelogenin (5) and arctigenin (6). According to these results, the compounds were identified unambiguously as (8R,8'R)-arctigenin and (8R,8'R)-trachelogenin.

Table 6. High resolution mass-spectral (ESI, negative ion mode) data of the isolated lignans, neolignans and sesquineolignans.

Compound		Formula	Detected formula	Detected ion	Calculated *m/z*	Found *m/z*	diff (ppm)
No*	**Name**						
2	desmethyl balanophonin	$C_{19}H_{18}O_6$	$C_{19}H_{17}O_6$	$[M-H]^-$	341.1031	341.1020	3.10
4	desmethyl picrasmalignan	$C_{29}H_{28}O_9$	$C_{29}H_{27}O_9$	$[M-H]^-$	519.1661	519.1655	1.12
1	tracheloside	$C_{27}H_{34}O_{12}$	$C_{27}H_{33}O_{12}$	$[M-H]^-$	549.1978	549.1971	1.23
5	trachelogenin	$C_{21}H_{24}O_7$	$C_{21}H_{23}O_7$	$[M-H]^-$	387.1449	387.1441	1.98
3	arctiin	$C_{27}H_{34}O_{11}$	$C_{27}H_{33}O_{11}$	$[M-H]^-$	533.2028	533.2023	0.74
6	arctigenin	$C_{21}H_{24}O_6$	$C_{21}H_{23}O_6$	$[M-H]^-$	371.1500	371.1497	0.78

Compounds 2 and 4 were isolated and the HPLC-TOF-MS results of these molecules indicated a molecular formula of $C_{19}H_{18}O_6$ (compound 2) and $C_{29}H_{28}O_9$ (compound 4). Comparing these data with those of balanophonin and picrasmalignan (identified previously in *C. boujartii* fruit, (section 6.1.3), compound 2 and compound 4 were presumed to be the desmethyl derivatives of balanophonin and picrasmalignan, respectively. To confirm the structures of these desmethyl derivatives, their NMR and CD spectra were compared to (7R,8S)-balanophonin and (7R,8S,7'R,8'S)-picrasmalignan. The NMR data verify that compounds 2 and 4 are the 3-*O*-desmethyl derivatives of balanophonin and picrasmalignan. The broad negative Cotton effect near 330 nm in the CD spectra of 3-*O*-desmethyl balanophonin and 3-*O*-desmethyl picrasmalignan confirmed their absolute configurations. Therefore compounds 2 and 4 of the fruit of *C. rivulare* were identified as (7R,8S)-3-*O*-desmethyl balanophonin [(7R,8S)-3,4,9-trihydroxy-5′-methoxy-4′,7-epoxy-8,3′-neolign-7′-en-9′-al] and (7R,8S,7′R,8′S)-3-*O*-desmethyl picrasmalignan [(7R,8S,7′R,8′S)-3,4,9,9′-tetrahydroxy-5′,5″-dimethoxy-4′,7:4″,7′-diepoxy-8,3′:3″8′-sesquineolign- 7″-en-9″-al], both being new natural compounds.

4.1.5 Chemodiversity of of *Cirsium* fruits

As the result of the analyses performed on *C. boujartii* and *C. rivulare* fruits, three neolignans (desmethyl balanophonin, prebalanophonin, balanophonin), three sesquineolignans (desmethyl picrasmalignan, prepicrasmalignan, picrasmalignan) and

four lignans (tracheloside, arctiin, trachelogenin, arctigenin) were identified. The elution pattern of these compounds summarized in Table 7.

Table 7. Elution of the identified lignans, neolignans and sesquineolignans in *C. boujartii* and *C. rivulare*.

Name	Retention time (min)
prebalanophonin	3.33
tracheloside	4.28
desmethyl balanophonin	4.70
prepicrasmalignan	5.09
arctiin	5.98
desmethyl picrasmalignan	7.23
balanophonin	7.82
trachelogenin	8.63
picrasmalignan	9.95
arctigenin	11.10

The question arose whether some of these compounds (besides to the already identified ones) may also be accumulated in the fruits of the seven further *Cirsium* species investigated recently by our research group (Boldizsár et al. 2010, Boldizsár et al. 2012, Mervai et al. 2015, Sólyomváry et al. 2015b). Thus, all those samples were re-analysed by HPLC-UV-MS confirming the presence of the following neolignans and sesquineolignans as new compounds of these species (Table 8). In addition, comparing the complete lignan profiles of *Cirsium* fruits (Figure 19), the chemotaxonomic significance of these metabolites among *Cirsium* species could also be confirmed.

Table 8. Newly identified neolignan and sesquineolignan compounds and their amounts (expressed in mg/g) in the recently investigated *Cirsium* species.

Species	Identified compound	Quantity (mg/g)
C. canum	desmethyl balanophonin	4.8
C. oleraceum	desmethyl balanophonin	2.3
C. arvense	balanophonin	2.9
C. vulgare	prebalanophonin	6.6
	prepicrasmalignan	5.0
	picrasmalignan	0.80

Considering that all the three traditional taxonomic sections (Cephalonoplos, Chamaeleon and Eriolepis) of *Cirsium* genus are represented in our study, by evaluating the distribution and quantities of the lignans neolignans and sesquineolignans, the chemotaxonomic correlations of these compounds among the sections could be revealed. Figure 19 summarizes the lignan composition of the nine investigated *Cirsium* species.

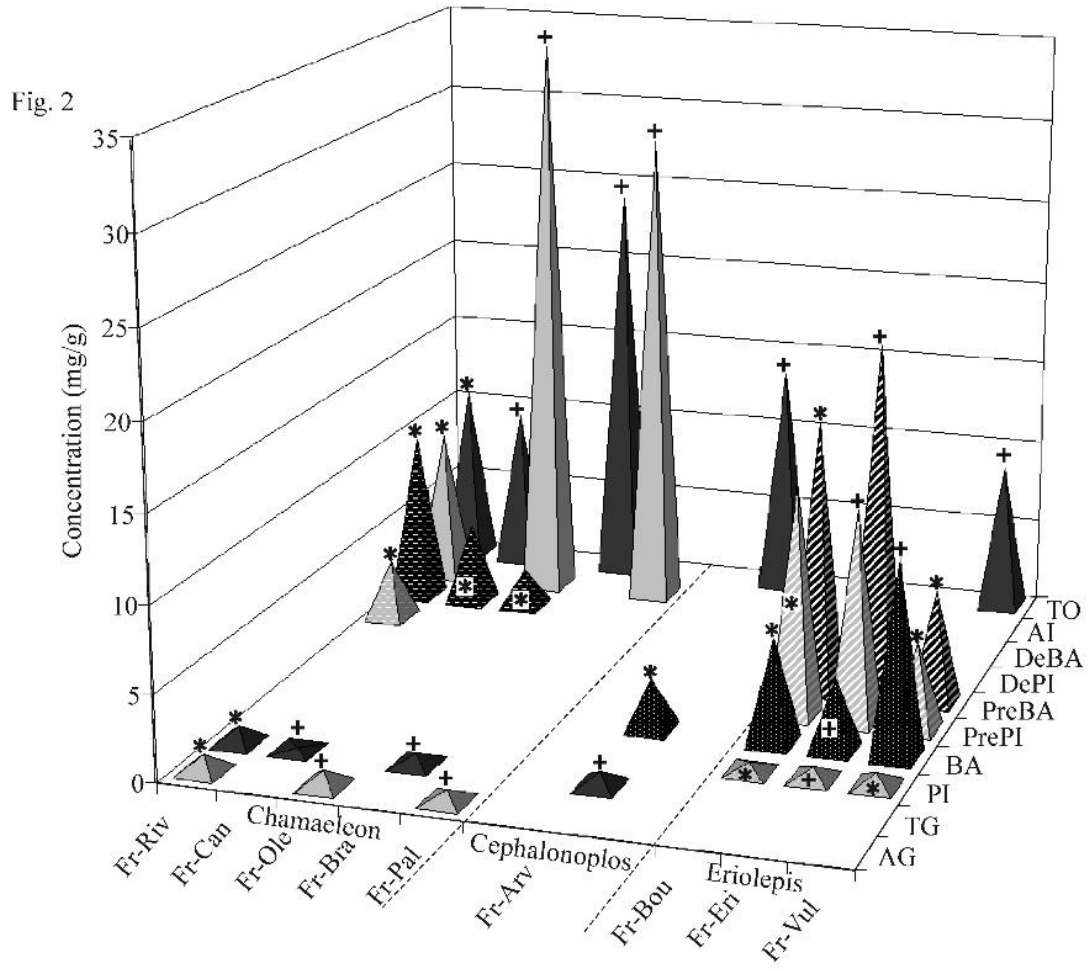

Figure 19. Fruit composition of *Cirsium* species grouped into three sections (namely, Chamaeleon, Cephalonoplos and Eriolepis), according to the traditional taxonomic classification. Fruits of *C. arvense*, *C. boujartii*, *C. brachycephalum*, *C. canum*, *C. eriophorum*, *C. oleraceum*, *C. rivulare* and *C. vulgare* are marked with Fr-Arv, Fr-Bou, Fr-Bra, Fr-Can, Fr-Eri, Fr-Ole, Fr-Riv and Fr-Vul, respectively. Compounds of the given species were determined in this study (columns marked with asterisks) or in our recent works (columns labeled with+sign), for the first time. Amounts are the average values obtained from at least three fruit samples (except *C. canum* and *C. palustre*, represented by two and one sample, respectively), which were harvested from different habitats. Differences could be characterized by the relative standard deviation (RSD) values, ranging from 3.8% (tracheloside in Fr-Can) to 27.7% (desmethyl picrasmalignan in Fr-Riv).

Based on these data, principal component analysis (PCA) of the metabolites among the species revealed a close chemotaxonomic relationship between *C. boujartii*, *C. eriophorum* and *C. vulgare*, belonging to Eriolepis section (Figure 20).

Fig. 4

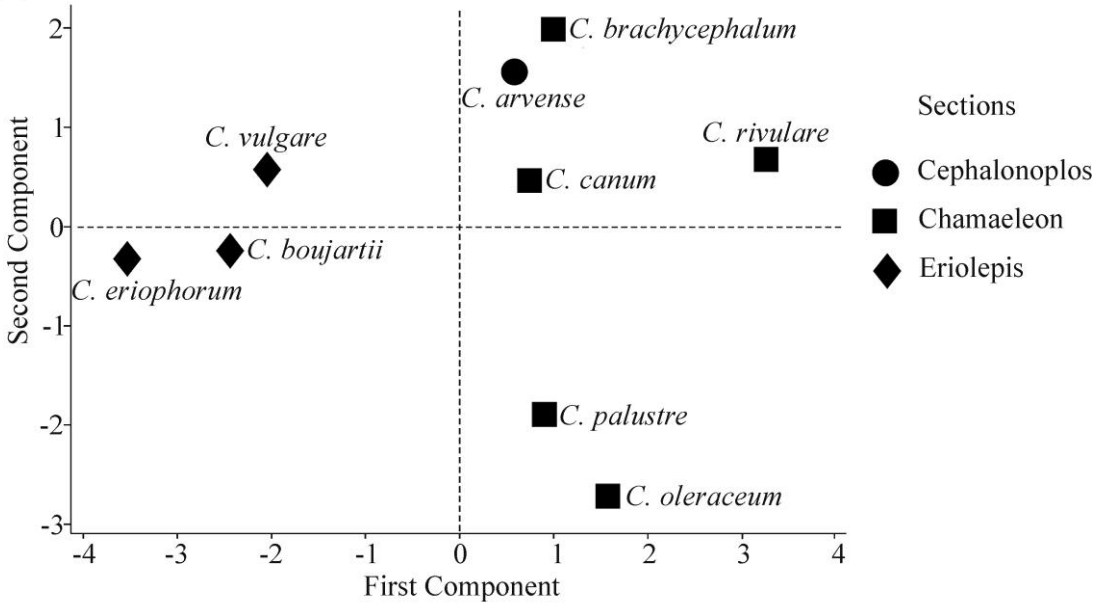

Figure 20. Principal component analysis (PCA) of nine *Cirsium* species belonging to three sections (Cephalonoplos, Chamaeleon and Eriolepis), based on the lignan, neolignan and sesquineolignan composition of their fruits.

4.2 Antiproliferative activity of the isolated neolignans and sesquineolignans against SW480 adenocarcinoma cell line

Literature data revealed the importance of balanophonin, as it has a significant antiproliferative activity on SW480 adenocarcinoma cell lines wit an IC50 of 19 μM (Shang et al. 2013) which is comparable to the antiproliferative activity of the known lignan derived chemotherapeutic agent etoposide, that has an IC50 of 17.1 μM against the same cells (Zi et al. 2013). Therefore, we investigated the antiproliferative activity of all the isolated neo- and sequineolignan compounds, isolated from *Cirsium* species, namely prebalanophonin, balanophonin, desmethyl balanophonin, prepicrasmalignan, picrasmalignan and desmethyl picrasmalignan against the SW480 cell line. Results are summarized in the following chart (Figure 21), confirming a significant, dose- and structure-dependent antiproliferative activity of these compounds

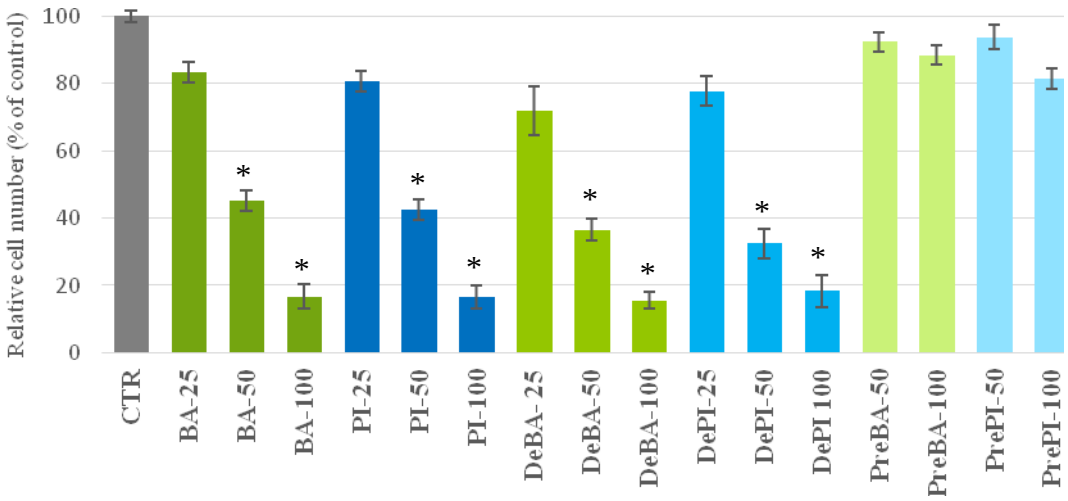

Figure 21. Inhibitory effect of neolignans and sesquineolignans on the SW480 cells, determined after 48-h treatments with 25, 50 and 100 µM balanophonin (BA), picrasmalignan (PI), desmethyl balanophonin (DeBA) and desmethyl picrasmalignan (DePI), as well as 50 and 100 µM prebalanophonin (PreBA) and prepicrasmalignan (PrePI), compared to the untreated control (CTR) cells. The data are represented as the mean ± standard deviation (SD) of eight experiments; *P < 0.001 compared to the control condition.

Significant antiproliferative activity of balanophonin, picrasmalignan and their desmethyl counterparts was observed (50% in 50 µM concentration), while prebalanophonin and prepicrasmalignan were ineffective.

4.3 *In vitro* cultures of the *Cirsium boujartii*

In vitro callus culture was established from *Cirsium boujartii* fruits. Calli were grown on "4" and MS $^{IVS\text{-}BAP}$ medium (Figure 22).

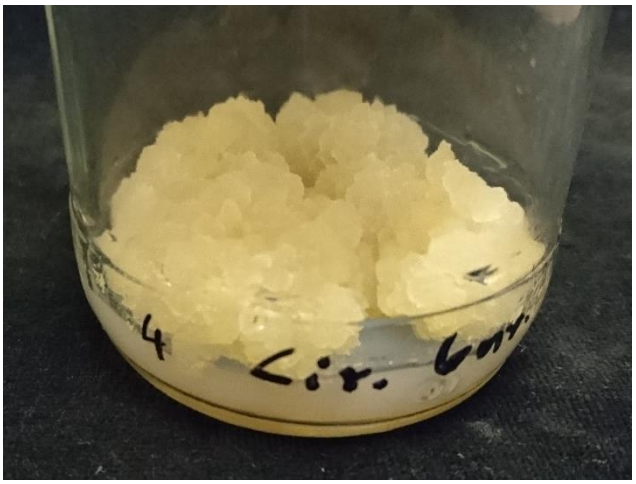

Figure 22. Callus culture of *Cirsium boujartii* grown on medium "4" for four weeks.

These cultures were producing a single compound that has different UV spectrum than lignans. This compound was isolated by preparative HPLC-UV and identified by high-

resolution mass spectrometric detections (TOF-MS and Orbitrap-MS) and NMR techniques as 1,5-dicaffeoylquinic acid.

4.4 Phytochemical characterisation of *Anthriscus* species

The phytochemical profile of the aerial parts of the three *Anthriscus* species: *A. caucalis*, *A. cerefolium* and *A. sylvestris*, were investigated by HPLC-UV-MS. Results confirmed the presence of caffeic acid derivatives; however, lignan type metabolites were not determined (Table 9).

Table 9. Composition of the dried herb samples, determined by HPLC-UV. The species are marked as follows *A. cerefolium* (A. cere), *A. sylvestris* (A. sylv), *A. caucalis* (A. cau).

Samples	Amounts of compounds in the dried *Anthriscus* samples (mg/g)			
	Chlorogenic acid (3-caffeoylquinic acid)	1,5-dicaffeoylquinic acid	Dicaffeoylquinic acid malonyl (1)	Dicaffeoylquinic acid malonyl (2)
A. cere	1.19	1.35	33.2	6.1
A. sylv	22.6	7.6	6.7	1.05
A. cau	0.31	-	1.25	-

Regarding the significance of *Anthriscus* herbs in the accumulation of caffeic acid derivatives, *Anthriscus cerefolium* accumulated the highest amount of dicaffeoylquinic acid malonyl derivatives, in *A. sylvestris* the chlorogenic acid and the 1,5-dicaffeoylquinic acid was characteristic.

4.5 *In vitro* cultures of *Anthriscus cerefolium*

In vitro culture of *A. cerefolium* was established on MS4 medium. In order to enhance the secondary metabolite production of the cultures, jasmonic acid, salicylic acid and yeast extract was selected as elicitors.

Table 10. Composition of the cell suspension samples, determined by HPLC-UV. Cell suspension cultures are marked according to the elicitors: jasmonic acid (JA), salicylic acid (SA), yeast extract (YE), control (CTR), while the numbers represent the duration of the elicitation process, expressed in days.

sa mples		Amounts of compounds in the dried *Anthriscus* samples (mg/g)		
		Chlorogenic acid (3-caffeoylquinic acid)	1,5-dicaffeoylquinic acid	Tricaffeoylquinic acid
Cell susp. control	CTR1	7.9	7.2	0.89
	CTR3	9.4	7.8	1.05
	CTR5	11.3	9.4	1.71
	CTR7	10.0	9.7	0.80
Elicitated cell suspensions	JA1	12.7	10.3	0.94
	JA3	21.4	18.3	2.77
	JA5	24.0	34.6	9.1
	JA7	30.7	39.3	12.3
	SA1	9.3	7.6	0.66
	SA3	11.1	7.6	1.04
	SA5	9.9	8.0	1.18
	SA7	14.6	12.2	1.58
	YE1	4.8	3.71	0.39
	YE3	5.0	3.49	0.42
	YE5	10.1	10.3	1.05
	YE7	10.9	9.8	1.11

As the main compounds, a caffeoylquinic acid (chlorogenic acid) and a dicaffeoylquinic acid (1-5-dicaffeoylquinic acid) were determined by HPLC-MS, while dicaffeoylquinic acid malonyl derivatives were not detectable in the untreated (CTRx) and elicited cultures (Figure 23, Table 10). The cell cultures produced uniquely a tricaffeoylqunic acid derivative and using the different compounds as elicitors resulted an increasing amount of the produced metabolites. Jasmonic acid was the most effective to enhance the concentration of 1,5-dicaffeoylqunic- and tricaffeoylqunic acids.

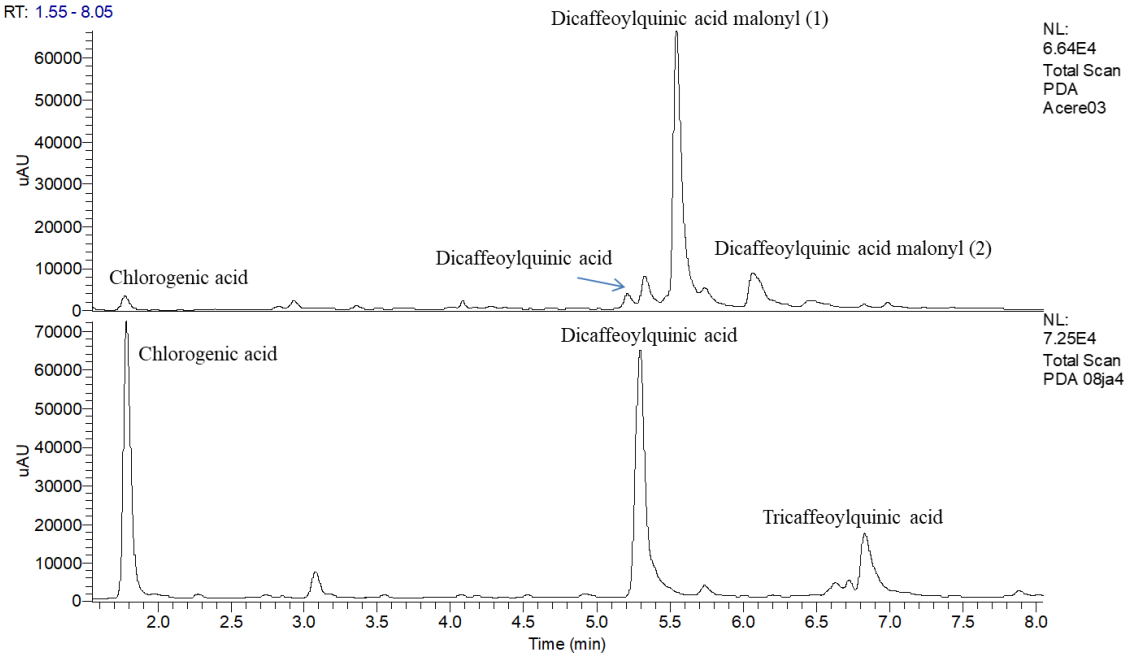

Figure 23. Differences on HPLC-UV chromatogram (λ = 280 nm) between the secondary metabolite profile of intact *A. cerefolium* herba (A) and its cell suspension culture elicited with 10µM jasmonic acid (JA) for 7 days (B).

5 DISCUSSION

5.1 Phytochemical characterisation of the Cardueae fruits

5.1.1 *Jurinea mollis* fruit: the novel source of arctigenin

Identifying the secondary metabolites of *J. mollis* fruits for the first time, intact and enzyme hydrolysed samples were used and during the analysis a specific conversion was followed by LC-MS, which confirmed the identity of the compounds as a pair containing a glycoside arctiin (1) and aglycone arctigenin (2). This conversion could be explained by the specific glycosidase activity expressed in the fruit. Namely, the dibenzylbutyrolactone lignan glycosides can be hydrolyzed by endogenous glycosidase enzymes. These enzymes are separated from their substrates in the intact plant tissues. However, cell disruption (e.g., pulverization) breaks down this compartmentalization and allows the decomposition of glycosides into the corresponding aglycone and sugar(s) in aqueous condition (Mervai et al. 2015, Sólyomváry et al. 2015a, Sólyomváry et al. 2014a, Sólyomváry et al. 2014b, Szokol-Borsodi et al. 2012, Boldizsár et al. 2010).

5.1.1.1 Selection of tissues for isolation of arctigenin

In order to isolate arctigenin from *J. mollis* fruits three rippening stages of the fruits were investigated by HPLC-UV. It was confirmed that the amounts of arctiin and arctigenin increased during fruit ripening, reaching their maximum levels of 35 mg/g and 1.10 mg/g, respectively, in the ripe fruit (Figure 12: R3).

Identifying the location of arctiin and arctigenin in the fruit parts, i.e., in the fruit wall and embryo, the ripe ones were used. These fruit tissues were separated prior to extraction and HPLC-UV measurement. Both compounds were exclusively found in the embryo tissue (arctiin 71.5 mg/g and arctigenin 2.23 mg/g). Taking into consideration these amounts of arctiin and arctigenin measured in the embryo, as well as the proportion of the fruit wall (52.3% (w/w)) and embryo (47.7% (w/w)) in the whole fruit, amounts of arctiin and arctigenin could also be calculated for the whole fruit. The theorytical values of arctiin and arctigenin were consistent with their amounts which were obtained by quantitative HPLC-UV determiation as shown in Table 11.

Table 11. The theoretical (calculated) and the obtained (by HPLC-UV) values of arctiin and arctigenin.

	Calculated values (mg/g)	Quantitavely determined values (mg/g)
arctiin	34.1	35.0
arctigenin	1.06	1.10

These results highlighting the practical utility and excellent reproductibility of quantitative determination of arctiin and arctigenin in *J. mollis* fruits.

The fruit part-specific accumulation of the compounds was also reported previously in several Cardueae species (Sólyomváry et al. 2014a, Sólyomváry et al. 2014b, Szokol-Borsodi et al. 2012, Boldizsár et al. 2010). In addition to the previously reported results of our research group, the current results on *Jurinea* fruits verified that the achene fruits of Cardueae plants accumulate lignan-glycosides in their seeds. This separated accumulation provides the raw material with relatively high amount of arctiin for isolation of arctigenin after enzymatic hydrolysis (Sólyomváry et al. 2014b, Szokol-Borsodi et al. 2012).

5.1.1.2 Optimization of enzymatic hydrolysis

During the optimization of the enzymatic hydrolysis, the results confirmed the quantitative conversion of the lignan glycoside arctiin into its aglycon, arctigenin, but significant difference was observed in the time required for the conversion. While complete hydrolysis in the whole fruit needed only 30 min, this process in embryo required 600 min (Figure 13). On a quantitative basis, the expected amounts of arctigenin in enzyme-hydrolyzed samples could be calculated by summation of the amounts of arctiin and arctigenin measured in the untreated tissues Table 12.

Table 12. Quantitative data on the expected amounts of arctigenin in the treated (enzyme-hydrolised) samples, considering the measured amounts of arctiin and arctigenin in untreated samples.

	arctiin	arctigenin	sum of arctiin and arctigenin:	References
whole fruit	65.5 μmol/g	2.96 μmol/g	68.5 μmol/g	Figure 11A
embryo	133.9 μmol/g	6.0 μmol/g	139.9 μmol/g	Figure 13

These calculated values (68.5 μmol/g and 139.9 μmol/g) were consistent with the amounts of arctigenin measured in the completely enzyme-hydrolyzed whole fruit and

embryo. Namely, the measured arctigenin content was in the whole fruit 69.4 μmol/g, calculating the average of samples hydrolyzed for 30 and 60 min, while in the embryo 136.3 μmol/g, takin the average of samples hydrolyzed for 600 and 900 min.

These results demonstrated that the enzymatic conversions are quantitative processes producing high amounts of arctigenin. In fact, this arctigenin content (136.3 μmol/g, corresponding to 50.7 mg/g) in enzyme-hydrolyzed embryo is one of the highest amounts reported to date in the plant kingdom. To confirm the high amount of arctigenin in *J. mollis* fruit embryo, two additional samples from different habitats were collected and analyzed, subsequently to their enzymatic hydrolysis. High amounts of arctigenin were also found in these samples (48.9 mg/g and 44.2 mg/g), highlighting the importance of *J. mollis* fruit embryo for the isolation of arctigenin.

5.1.1.3 Preparing High Purity Arctigenin Extract (HPAE)

To obtain a HPAE, the enzymatic hydrolysis of arctiin into arctigenin was performed, because this reaction resulted in a change in solubility: the glycosidic form of arctigenin is a hydrophilic compound that is insoluble in non-polar solvents (i.e., diethyl ether), whereas the free aglycon arctigenin is a hydrophobic molecule that is soluble in diethyl ether. Based on this, the HPAE preparation procedure consisted of three consecutive steps: 1) purification of fruit tissue using diethyl ether; 2) the endogenous enzymatic hydrolysis of arctiin in purified fruit tissue, and 3) the extraction of the formed arctigenin from the purified and hydrolyzed fruit tissue using diethyl ether.

If the enzymatic hydrolysis of purified fruit tissue is a selective process, it should result in the formation only of arctigenin as a hydrophobic compound, thus, the non-polar solvent extract prepared in the third step should contain a high level of arctigenin.

Applying the three-step isolation procedure in order to prepare HPAE, fruits were purified with diethyl ether to remove all of their hydrophobic constituents, thus the hydrophyllic arctiin was remained in the tissues. Arctiin was converted into arctigenin in aquaeous medium by endogenous enzymatic hydrolysis prior the extraction with diethyl ether in order to obtain HPAE. To complete enzymatic conversion of arctiin into arctigenin in the whole fruit and embryo of *J. mollis*, 30 min and 600 min hydrolyses were required, respectively (Figure 13). Consequently, this procedure, based on enzymatic hydrolysis allowed the three-step isolation of arctigenin from *J. mollis* fruit samples.

Considering the time requirements of the conversion of arctiin into arctigenin, highlight the differences of the initial plant material (Figure 13) as the complete arctiin hydrolysis in the separated embryo required 5-times more time than that in the whole fruit. However, regarding to the quantitative data, embryo tissue accumulates higher arctigenin content than whole fruit tissue. Thus, despite the more time consumption of arctiin hydrolysis in embryo, our results highlight the importance of embryo separation: thereby offering an efficient (89.4%) way for the preparation of HPAE extracts containing significantly higher levels of arctigenin (74.2%) than the whole fruit (57.5%).

5.1.2 Carduus nutans fruit: the new source of pinoresinol

One of the highest pinoresinol content among plants could be determined in the fruit of *C. nutans* (7.8 mg/g), confirming the significance of this fruit in pinoresinol isolation. In fact, the resin of *Picea abies* and the leaf of *Forsythia intermedia* contain more pinoresinol than the fruit of *C. nutans* (Holmbom et al. 2008, Rahman et al. 1990). In addition, as the HPLC peak profile of the fruit extract shows, pinoresinol is the predominant compound, allowing to perform its simple isolation by one-step preparative HPLC purification.

5.1.3 *Cirsium boujartii* fruit as the abundant source of rarely occurring neolignans and sesquineolignans

Based on our results, two neolignans (prebalanophonin (1) and balanophonin (3)) and two sesquineolignans (prepicrasmalignan (2) and picrasmalignan (4)) were determined for the first time in the fruit of *C. boujartii*) (Figure 16).

As a result of acid-treatment of prebalanophonin and prepicrasmalignan, their acid-catalyzed cyclization could be observed, forming balanophonin and picrasmalignan Accordingly, the amounts of balanophonin and picrasmalignan (minor compounds of intact fruit, as shown in Figure 19) could be increased, enabling their efficient isolation. Thus, the optimum source for the isolation of prebalanophonin and prepicrasmalignan is the intact, untreated fruit, whereas, balanophonin and picrasmalignan can easily be isolated from the acid-treated fruit extract. The amounts of balanophonin and picrasmalignan in the acid-treated *C. boujartii* fruit (22.0 mg/g and 19.0 mg/g, respectively) are comparable to those determined recently in the acid-treated *C.*

eriophorum fruit, confirming these two *Cirsium* species to be the most abundant sources of balanophonin and picrasmalignan, as the highest amounts of these compounds were only 0.027 mg/g (Sy and Brown 1999) and 2.9×10^{-5} mg/g (Jiao et al. 2011), respectively.

5.1.4 New lignans in the fruit of *Cirsium rivulare*

As the result of our analyses, a new neolignan (desmethyl balanophonin) a new sesquineolignan (desmethyl picrasmalignan) and four known lignans (tracheloside, arctiin and their aglycones), were determined in the fruit of *C. rivulare.* Comparing the amounts of compounds in non-hydrolyzed or enzyme-hydrolyzed *C. rivulare* fruit samples, we confirmed the quantitative conversion of glycosides (tracheloside and arctiin) into their corresponding aglycones (trachelogenin and arctigenin), as well as the stability of desmethyl balanophonin and desmethyl picrasmalignan, during enzymatic treatment (Figure 17). The NMR and CD spectral data of the isolated trachelogenin and arctigenin, were comparable to those reported in the literature (Mervai et al. 2015, Sólyomváry et al. 2015a). Accordingly, enzyme treatment is of primary importance in the identification and isolation of *C. rivulare*'s fruit compounds. In the intact fruit extract, desmethyl balanophonin (Figure 17A, peak 2) and tracheloside (Figure 17A, peak 1) eluted with similar retention times, resulting in cross-contamination during isolation by preparative HPLC. Consequently, to isolate desmethyl balanophonin, desmethyl picrasmalignan, trachelogenin and arctigenin, the enzyme-hydrolyzed fruits of *C. rivulare* was applied as the raw material of choice.

5.1.5 Chemodiversity of *Cirsium* fruits

As the result of our phytochemical study performed on the fruits of nine *Cirsium* species, five compounds (prebalanophonin, balanophonin, desmethyl balanophonin, prepicrasmalignan and picrasmalignan) were found in four species (*C. canum*, *C. oleraceum*, *C. arvense* and *C. vulgare*) for the first time. When taking into consideration the fruit composition of *Cirsium* species and the convertibility of the identified lignan-type compounds, optimum sources containing the highest amounts of the required compounds, without close-eluting impurities, could be selected for isolation purposes. Thus using the enzyme-treated *C. rivulare* fruit, it could provide the two new compounds (desmethyl balanophonin and desmethyl picrasmalignan) (Figure 17B).

Furthermore, due to the high prebalanophonin and prepicrasmalignan contents of *C. eriophorum* and *C. boujartii*, fruits of both plants could be equally applied to isolate these compounds (Figure 16A), as well as balanophonin and picrasmalignan from acid-treated fruit sample (Figure 16B). Due to the availability reasons, *C.* boujartii fruits were selected for isolation purposes. Therefore, balanophonin, picrasmalignan, prebalanophonin and prepicrasmalignan, were obtained from the fruit extract of *C. boujartii* for further assays.

Abundant evidence documents similarities in secondary plant metabolites among closely related plants. No recent study, however, has compiled the evidence available on chemical profiles of true thistles (subtribe Carduinae contining *Carduus*, *Cirsium* and *Jurinea* genera) (Jordon-Thaden and Louda 2003).

The accumulation of the 4′,7-dihydroxy structures (prebalanophonin, prepicrasmalignan) and that of their 4′,7-epoxy counterparts (balanophonin, picrasmalignan) was found to be characteristic in the section Eriolepis (*C. boujartii*, *C. eriophorum*, *C. vulgare*) (Figure 19). All representatives of Chamaeleon and Cephalonoplos sections can be characterized by the presence of the dibenzylbutyrolactone lignan glycoside arctiin or tracheloside (*C. arvense*, *C. brachycephalum*, *C. canum*, *C. oleraceum*, *C. palustre*) or by the presence of both glycosides (*C. rivulare*). Furthermore, among the studied species, only some Chamaeleon species contain desmethyl balanophonin (*C. canum*, *C. oleraceum*, *C. rivulare*) and desmethyl picrasmalignan (*C. rivulare*). Thus, these new natural compounds seem to be specific to the Chamaeleon section (Figure 19).

Accordingly, the conventional classification of these species into the same section was also supported for the first time by phytochemical results. However, the section Chamaeleon may be characterized as a heterogeneous group by PCA. based on the presence of tracheloside or arctiin, the species in this section could be delimited into two, well-separated groups: one comprising the arctiin-containing *C. palustre* and *C. oleraceum* and the other, including *C. brachycephalum*, *C. canum* and *C. rivulare*, accumulating tracheloside.

5.2 Antiproliferative activity of the isolated lignan-type metabolites against SW480 adenocarcinoma cell line

Our results confirmed the inhibitory effect of balanophonin against SW480 adenocarcinoma cell line. Furthermore, for the first time, the antiproliferative activity of picrasmalignan and the new compounds, desmethyl balanophonin and desmethyl picrasmalignan, were confirmed, with an efficacy comparable to balanophonin (Figure 21). Although these active compounds bearing 4′,7-epoxy moieties and their corresponding 4′,7-dihydroxy counterparts (prebalanophonin and prepicrasmalignan) are closely related structures, the latter ones were inactive in the concentration range applied. An earlier study analyzing the cytotoxicity of synthetic phenyl-dihydrobenzofuran neolignans confirmed that the presence of an unsaturated propyl side chain attached to the dihydrobenzofuran moiety is of primary importance in their activity (Pieters et al. 1999). Based on this result, the analysis of the structure-activity relationship of our compounds suggests that the phenyl-dihydrobenzofuran, together with an unsaturated propyl moiety (highlighted in bold in the structure of picrasmalignan, Figure 24), may be responsible for the antiproliferative effect (Figure 21).

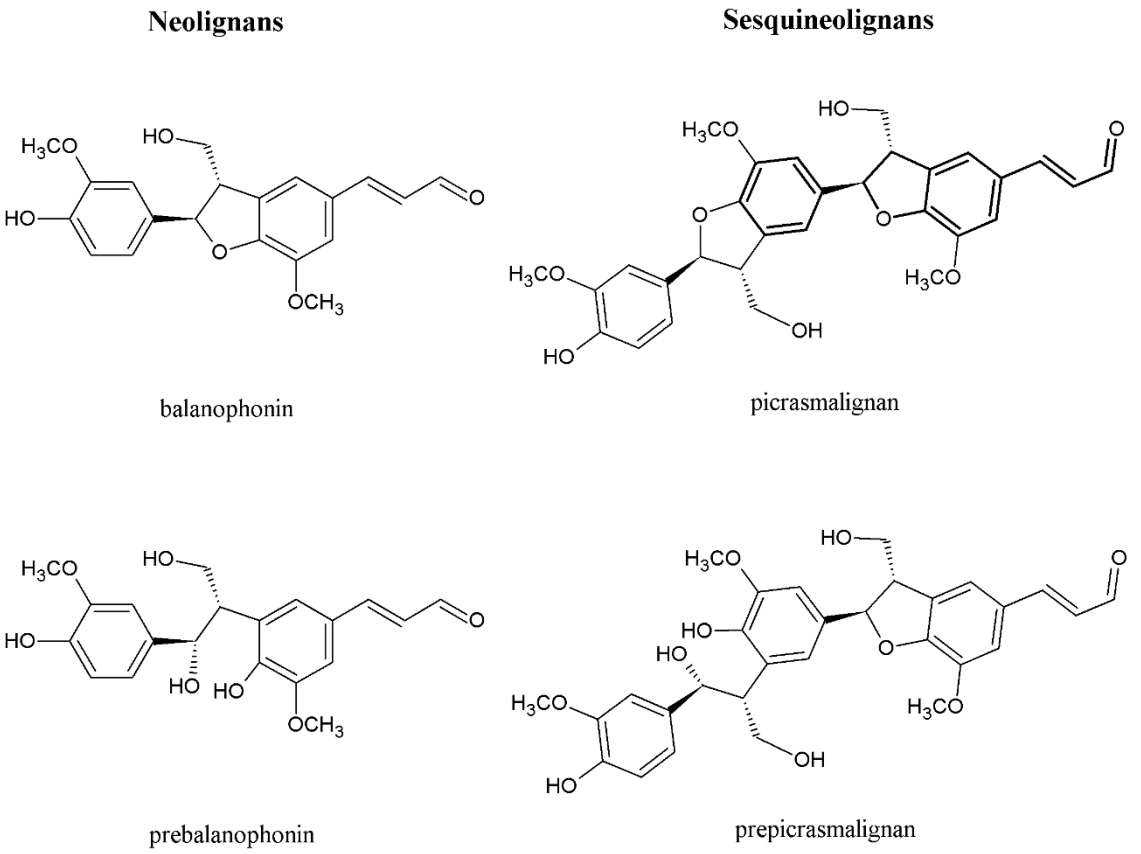

Figure 24. Chemical structures neolignans and sesquineolignans identified in the fruits of *Cirsium* species. The phenyl-dihydrobenzofuran moiety responsible for their antiproliferative effects are highlighted in bold in the structure of picrasmalignan.

If so, the inactivity of prepicrasmalignan, though containing the effective structure, may be related to the strong polarity of the 4′,7-dihydroxy moiety, also present in this molecule (Figure 24).

5.3 *In vitro* cultures of the *Cirsium boujartii*: the source of a caffeic acid derivative

Based on the presence of the effective neolignan and sesquineolignan compounds balanophonin and picrasmalignan, fruits of *Cirsium* boujartii were selected in order to prepare callus cultures, aiming to prepare these valuable metabolites in higher yields. The cultures were grown on two different media and on different light conditions. Despite of the various conditions none of the cultures produced the desired neo- and sesquineolignans. However, a compound, having characteristic UV absorbtion spectrum with absorptions maximum at 330 nm characteristics to caffeic acid derivatives, was

detected. Based on the HPLC-MS/MS data of this compound the presence of a dicaffeoylquinic acid isomer was confirmed (Figure 25). Namely,

a) the deprotonated molecular ion (Figure 25A, $[M-H]^-$), and
b) its fragment ions *m/z* 353.0876 and *m/z* 191.0552 (Figure 25B) corresponding to a caffeoylquinic acid and a quinic acid moiety, confirmed the presence of a dicaffeoylquinic acid isomer.

The configuration of this compound was determined by NMR as 1,5-dicaffeoylquinic acid.

Caffeic acid derivatives are commonly occurring in Asteraceae family. Amongst caffeic acid derivatives, 1,5-dicaffeoylqunic acid was reported in *Lactuca aculeata* as the major compound (Stojakowska and Malarz 2017) identical to our isolated compound (Stojakowska and Malarz 2017). This plant and its *in vitro* cultures were found to be also lignan producing. According to these results we suppose a connection between the biosynthese of lignan type metabolites and 1,5-dicaffeoylquinic acid.

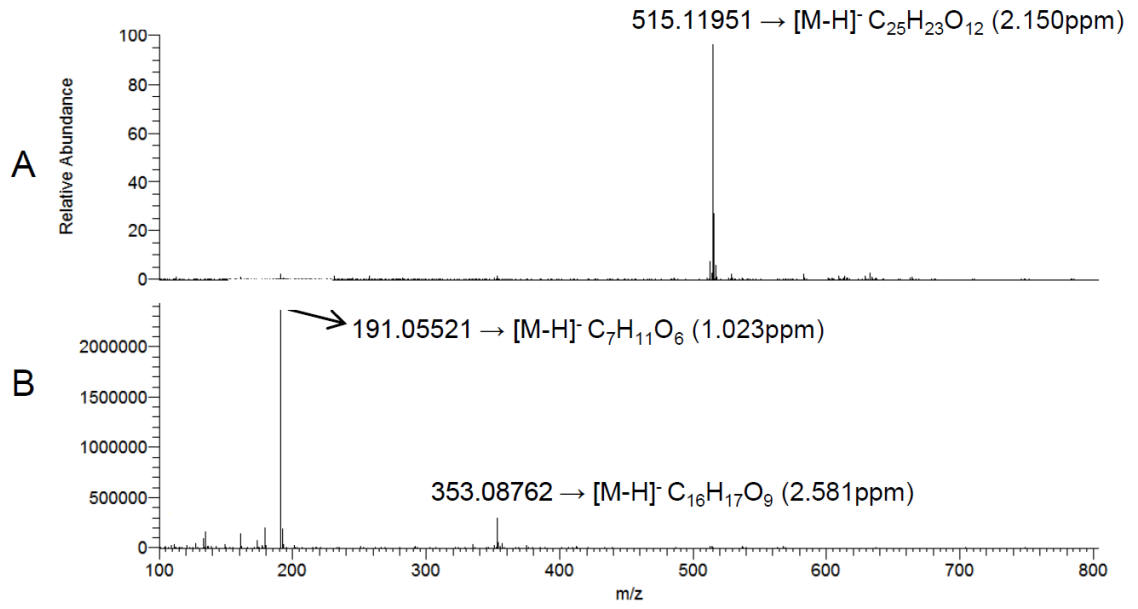

Figure 25. The high-resolution mass spectrum of isolated dicaffeoylquinic acid isomer (spectrum A) and the collision-induced dissociation (CID, stepped, 10, 30 and 45 eV) spectrum (B) generated from the molecular ion of this compound, using negative ionization mode.

5.4 Phytochemical characterisation of *Anthriscus* species

Our results confirmed the presence of caffeoylquinic acid-type metabolites chlorogenic acid (3-caffeoylquinic acid), and dicaffeoylquinic acid malonyl as the main compounds in all *Anthriscus* samples we investigated. In addition, dicaffeoylqunic acid and an

another dicaffeoylquinic acid malonyl were also detectable in *A. cerefolium* and *A. sylvestris*. The dicaffeoylquinic acid of *A. cerefolium* and *A. sylvestris* was identified using external standards cynarin (1,3-dicaffeoylquinic acid, authentic standard) and 1,5-dicaffeoylquinic acid (isolated from *C. boujartii* callus). Since (i) the peak of dicaffeoylquinic acid present in *Anthriscus* species and the peak of cynarin were not overlapping, and (ii) the Rt values of the *Anthriscus'* dicaffeoylquinic acid and that of 1,5-dicaffeoylquinic acid identified in *C. boujartii* callus, were identical, we could identify the compound of *Anthriscus* species as 1,5-dicaffeoylquinic acid.

The malonyl derivatives were present in *A. cerefolium* herb at the highest concentration: 33.2 mg/g and 6.1 mg/g amounts were determined. The occurrence of dicaffeoylquinic acid malonyls are extremely limited in the plant kingdom (Gouveia and Castillho 2009); however, they could be regarded to be valuable natural compounds with predictable antioxidant effects. Therefore, in order to produce these valuable natural compounds, *in vitro* cultures of *A. cerefolium* was introduced.

5.5 Phytochemical study on *in vitro* cultures of *Anthriscus* species

5.5.1 Compounds in the callus cultures

Fruits of *A. cerefolium* were chosen in order to establish callus cultures due to their ability to produce the phenolics at the highest concentration among the species we investigated. The cultures were grown on three different media variants: MS4, ½MS7 and ½MS8 different light conditions both light and dark. The temperature was consistent: 22°C. Despite the several conditions, the cultures have not produced the desired malonyl derivatives, but caffeic- and chlorogenic acid and 1,5-dicaffeoylquinic acid as the callus cultures of *C. boujartii* did (chapter 5.3).

According to the results, we decided to use the culture producing the highest concentration of the detected compounds for further experiments.

5.5.2 Phytochemical composition of cell suspension culture: an elicitation study

The elicited cell suspension cultures also have not produced the malonyl derivatives. Interestingly, all the cultures accumulated a tricaffoylquinic acid that is not found in itact plant. Tricaffoylquinic acid levels were on the 7th day of elicitation 12.3 mg/g (JA), 1,58 mg/g (SA) and 1.11 mg/g (YE), respectively (Table 10). Correspondingly to our results, Tamura et al. (2006) found also a tricaffeoylquinic acid (3,4,5-tricaffeoylquinic

acid) in cultured cells of lettuce, but not in the intact leaves of the plant, and their results show a potent HIV inhibitory effect of this compound. In intact leaves of *Ipomoea babatas* L. (sweetpotato) was reported a significant volume of 3,4,5-tricaffeoylquinic acid (1,4 mg/g, respectively) and its antimutagenic activity was confirmed (Kurata et al. 2011), thus highlighting on the significance of tricaffeoylquinic acid production. A significant increase of 1-5-dicaffeoylquinic acid production was also confirmed during elicitation, reaching 39.3 mg/g level by JA treatment (7 day-elicitation). JA was the most effective to enhance the concentration of 1,5-dicaffeoylqunic (4 times) and the tricaffeoylqunic acid (more then 15 times) level. Elicitation of callus biomass of *Vitis vinifera* using JA resulted an enhanced accumulation of resveratrol was reported (Raluca et al. 2011) and also triggers the production of a wide range of plant secondary metabolites in various *in vitro* cell cultures (Naik and Al-Khayri 2016).

Accordigly, JA-elicited *A. cerefolium* culture could be regarded to be an abundant source of this valuable compound expressing biological significance.

6 CONCLUSIONS

1. Precious butyrolactone lignans, i.e. arctiin and arctigenin were determined for the first time in the furits of *Jurinea mollis*. Arctiin was quantitatively transformed into arctigenin in the fruits by endogenous enzymatic hydrolysis. The fruit part-specific accumulation of these lignans resulted in an extraordinarily high arctigenin level in the enzyme-hydrolyzed embryo part (50.7 mg/g) allowing the efficient preparation of an extract containing 74.2% arctigenin by the simple three-step isolation procedure.

2. *Carduus nutan*s fruit was found to be a new and rich source of the valuable furofuran lignan pinoresinol (7.8 mg/g in dried fruit), allowing its high-level isolation.

3. Two new natural compounds, one new neolignan- and one new sesquineolignan, denominated desmethyl balanophonin and desmethyl picrasmalignan, together with new sources of their structurally related, already known compounds (balanophonin, picrasmalignan, prebalanophonin, prepicrasmalignan, arctiin, arctigenin, tracheloside and trachelogenin) were confirmed during the phytochemical study of *Cirsium* fruits representing nine species. After having recognized the distribution of these metabolites showing chemotaxonomic significance among *Cirsium* species, as well as their transformation properties during acidic and enzymatic treatments, optimum sources for high-yield isolation of pure compounds has been selected. Thus, desmethyl balanophonin and desmethyl picrasmalignan from the enzyme-hydrolyzed fruit of *C. rivulare*, prebalanophonin and prepicrasmalignan from the intact, untreated fruits of *C. boujartii* or *C. eriophorum*, and balanophonin and picrasmalignan from their acid-treated counterparts could be obtained.

4. Among these isolated compounds, the ones bearing a 4′,7-epoxy moiety (balanophonin, picrasmalignan, desmethyl balanophonin and desmethyl picrasmalignan) showed significant, dose-dependent antiproliferative activity against the SW480 cell line; however, their 4′,7 dihydroxy counterparts were

ineffective. These results highlight the significance of *Cirsium* fruits in the production of biologically active lignan-type metabolites and confirm the antiproliferative potential of our new compounds (DeBa, DePi) for the first time.

5. Calli of *Cirsium boujartii* was were found to producing 1,5-dicaffeoylquinic acid as a major compound without lignans. The lack of the lignan content of the calli presumed the fact that the biosynthetic pathway may blocked at some point in undifferenciated cells.

6. Caffeoylquinic acid derivatives were detected in the aerial part of *Anthriscus cerefolium, A. caucalis* and *A. sylvestris,* in *A. caucalis* for the first time. *In vitro* cultures of *A. cerefolium,* after an optimized elicitation (using jasmonic acid for 7 days) accumulated 1,5-dicaffeoylquinic acid as a main component in high level.

7 SUMMARY

Plant secondary metabolites may have biological effect on human health, hence these metabolites are widely researched. Among them, lignans belonging to phenolics, have special relevance in the cancer theraphy. However, their available amount derived from plants is limited and their total synthesis is uneconomical/unresolved. Therefore, finding abundant sources and/or using cell- and tissue cultures in order to expand the properties of the cost-effective way of lignan production is a crucial point of today's research.

In this study:

(i) the phytochemical characterisation of the fruits and herbs of selected plants belonging to *Cardueae* tribe (Asteraceae family) and *Anthriscus* genus was accomplished;

(ii) *in vitro* cultures were established in order to produce lignans and to scale up their production and

(iii) the antiproliferative significance of compounds isolated from their optimal sources was confirmed.

Enzyme hydrolysed embrio of *Jurinea mollis* fruits contained arctigenin at a high concentration allowing the simple three-step isolation procedure to be as efficient as the yield of arctigenin was 74.2% from an extract.

In total, eleven lignan compounds were identified in the fruits of *Carduus nutans* and in nine *Cirsium* species (pinoresinol, desmethyl balanophonin, desmethyl picrasmalignan, balanophonin, picrasmalignan, prebalanophonin, prepicrasmalignan, arctiin, arctigenin, tracheloside, trachelogenin). Amongst these compunds, the desmethyl derivatives of balanophonin and picrasmalignan were published the first time as new natural compounds.

Antiproliferative activity of the neolignans and sesquineolignans of *Cirsium* spp. against SW480 was investigated and desmethyl balanophonin, desmethyl picrasmalignan, balanophonin and picrasmalignan have shown a significant antiproliferative activity, while prebalanophonin and prepicrasmalignan were ineffective.

Herb of *Anthriscus* spp. produced unique malonyl derivatives of dicaffeoylquinic acids.

In vitro callus cultures were prepared from *Cirsium boujartii* and *Anthriscus cerefolium*, confirming their 1,5-dicaffeoylquinic acid producing ability.

8 ÖSSZEFOGLALÁS

A másodlagos növényi anyagcseretermékek jelentős hatásaik miatt intenzíven kutatott vegyületek. Ilyenek fenoloidok közé tartozó a lignánok is, melyeket elsősorban sejtosztódás-gátló hatásaik szempontjából vizsgálják. A növényekből kinyerhető mennyiségük ugyanakkor korlátozott, a szintézisük pedig nem kifizetődő, vagy nem megoldott. Így fontos feladat, olyan bőséges forrásokat találni a természetben és/vagy a sejt- és szövetkultúrák nyújtotta lehetőségek által, melyek ezen vegyületek nagymennyiségű előállítását biztosítják.

Munkánk során:

1) a lignán tartalmukról ismert *Cardueae* nemzetségcsoport (Asteraceae család) és *Anthriscus* nemzetség kiválasztott fajainak fitokémiai jellemzését végeztük el;

2) létrehoztunk *in vitro* kultúráikat lignánok termeltetése céljából és

3) vizsgáltuk az izolált vegyületek sejtosztódás-gátló hatását.

Jurinea mollis termés mag részéből arktigenin nagymennyiségű előállítására egy enzimatikus hidrolízisre épülő, egyszerű, háromlépéses izolációs folyamatot dolgoztunk ki (mely 74,2%-os hatékonyságúnak bizonyult).

Összesen tizenegy lignán összetevőt azonosítottunk a *Carduus nutans* (pinorezinol) és kilenc *Cirsium* faj (dezmetil-balanofonin, dezmetil-pikrazmalignán, balanofonin, pikrazmalignán, prebalanofonin, prepikrazmalignán, arktiin, arktigenin, trahelozid, trahelogenin) termésében. Közülük a dezmetil-balanofonin és a dezmetil-pikrazmalignán új természetes vegyületek. A *Cirsium* fajokban leírt neo- és szeszkvineolignánok sejtosztódásgátló hatását vizsgáltuk SW480 adenokarcinóma sejtvonalon, mely során a balanofonin és a pikrazmalignán és ezek dezmetil-származékai szignifikáns sejtosztódásgátló hatást mutattak.

Az *Anthriscus* fajok föld feletti részeinek vizsgálata során dikaffeoilkínasav malonil származékát azonosítottuk.

A Cirsium boujartii és *A. cerefolium in vitro* kultúráiban 1,5-dikaffeoilkínasav teremelődését mutattuk ki.

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