Recovery of new diarylheptanoid sources in Betulaceae

Characterisation of the phenolic profile of *Corylus* species by HPLC-ESI-MS methods

Ph.D. Dissertation

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Budapest
2016

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1. LIST OF ABBREVIATIONS

4CL	4-coumarate-CoA ligase
ABTS	2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
AD	Alzheimer's disease
AIC	apoptotic proteinase-activating factor
amu	atomic mass unit
BDE	bond dissociation energy
C3H	cinnamate-3-hydroxylase
C4H	cinnamate-4-hydroxylase
CABE	*Corylus avellana* bark ethyl acetate extract
CABM	*Corylus avellana* bark methanolic extract
CALE	*Corylus avellana* leaves ethyl acetate extract
CALM	*Corylus avellana* leaves methanolic extract
CCBE	*Corylus colurna* bark ethyl acetate extract
CCBM	*Corylus colurna* bark methanolic extract
CCLE	*Corylus colurna* leaves ethyl acetate extract
CCLM	*Corylus colurna* leaves methanolic extract
CE	collision energy
CID	collision-induced dissociation
CMBE	*Corylus maxima* bark ethyl acetate extract
CMBM	*Corylus maxima* bark methanolic extract
CMLE	*Corylus maxima* leaves ethyl acetate extract
CMLM	*Corylus maxima* leaves methanolic extract
CoA	coenzyme A
COX	cyclooxygenase
CURS	curcumin synthase
DAD	diode array detector/detection
DCS	diketide-CoA synthase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ERK	extracellular signal-regulated kinase
ESI	electrospray ionisation
EtOAc	ethyl acetate
GIT	gastrointestinal tract
HAT	hydrogen atom transfer
HCT	hydroxycinnamoyl transferase
HDL	high density lipoprotein
HIF1	Hypoxia-inducible factor 1
HIV	Human Immunodeficiency Virus
HPLC	high-performance liquid chromatography
HPLC-MS	high- performance liquid chromatography coupled with mass spectrometry

IC_{50}	half maximal inhibitory concentration
ICAM	intercellular adhesion molecule
IκB	the inhibitor of κB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IP	ionisation potential
LDL	low density lipoprotein
LOD	limit of detection
LOQ	limit of quantitation
LOX	lipoxygenase
LPS	lipopolysaccharide
MeOH	methanol
MIC	minimum inhibitory concentration
mRNA	messenger ribonucleic acid
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge-ratio
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor-κB
NO	nitric oxide
OMT	*O*-methyltransferase
PAF	platelet-activating factor
PAL	phenylalanine ammonia-lyase
Ph.Hg.	Pharmacopoea Hungarica
PI3K	phosphoinositide 3-kinase
ROS	reactive oxygen species
RNA	ribonucleic acid
RNS	reactive nitrogen species
RP-HPLC	reversed-phase high-performance liquid chromatography
SD	standard deviation
SET	single electron transfer
SEPP	selenoprotein P
Smac	second mitochondria-derived activator of caspase
Sp-1	specificity protein-1
TNF α	tumour necrosis factor α
TNF β	tumour necrosis factor β
TOF	time-of-flight mass analyser
UV	ultra violet
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLDL	very-low-density lipoprotein
$[Y_0\text{-H}]^{-\bullet}$	radical aglycone ion
Y_0^-	aglycone ion

2. INTRODUCTION

Natural products have been used to treat human diseases since ancient times. In recent years compounds of plant origin still play an important role in therapeutic drug discovery by providing large chemical diversity and covering an alternative chemical space compared with synthetic derivatives. Consequently, nowadays a significant part of phytochemical researches focuses on screening for potential lead molecules among herbal extracts. Orientation of these researches is mainly based on ethnopharmacology: identification of the major and minor bioactive constituents of traditionally used medicinal plants is a clearly promising way of discovering novel lead compounds. In addition, screening for structural analogues of natural lead molecules in different plants based on taxonomic relatedness also forms an important part of phytochemical studies. However, the cost- and time-consuming procedures of isolation and identification of natural compounds with beneficial biological activity have limited their use in the pharmaceutical industry in the past two decades.

On the other hand, the popularity of herbal remedies is increasing nowadays which comes together with the upsurge of necessity to assure quality, efficacy and safety of these products. Since plant extracts are complex matrices with numerous different constituents, the development of sophisticated analytical methods is a crucial point in the quality control of phytotherapeutics.

Diode-array detection (DAD) and mass spectrometry (MS) together with high-performance liquid chromatography (HPLC) separation offers great selectivity and sensitivity for the qualitative and quantitative analysis of complex plant samples. In addition, coupling bioassays to these analytical procedures (mainly HPLC-MS) allows the rapid and efficient identification of the bioactive constituents of plant extracts.

The aim of our work was the phytochemical evaluation of the *Corylus* (Betulaceae) species native to Hungary: *Corylus avellana* L, *Corylus colurna* L. and *Corylus maxima* Mill. Despite the long-term use of these plants in traditional medicine, their phytochemical exploration is still incomplete. Previous studies on the phenolic constituents of *C. avellana* kernels and leaves focused on the main flavonoid and caffeic acid derivatives, while there is no report concerning the detailed phytochemical composition of *C. colurna* and *C. maxima*. Numerous studies of Betulaceae species

revealed that besides other phenolic constituents, diarylheptanoid-type compounds also show a frequent occurrence among these plants; however presence of these constituents is not reported in the *Corylus* species mentioned above. Diarylheptanoids have been proved to possess various pharmacological effects: e.g. antioxidant, anti-inflammatory, anticancer, anti-adipogenic and antiviral activities suggest their potential utilisation in clinical practice. Therefore, our experiments focused on screening for structural analogues of these compounds in the selected *Corylus* species.

HPLC-ESI-TOF-MS (high-performance liquid chromatography coupled to electrospray ionisation-time-of-flight mass spectrometry) and HPLC-ESI-MS/MS (high-performance liquid chromatography coupled to electrospray ionisation-tandem mass spectrometry) methods were chosen for the simultaneous structural characterisation of the phenolics present in the *Corylus* extracts.

Moreover, in order to broaden information on their biological activity, studies on *in vitro* radical scavenging activity were carried out.

The first chapters (**2.1**-**2.3.**) of the Literature overview summarise the most important knowledge about diarylheptanoids, the plant-derived compounds in the focus of our study. The following sections present the botanical, phytochemical and pharmacological properties of the *Corylus* (Betulaceae) species examined in order to search for new natural sources of the previously mentioned diarylheptanoid compounds (**2.4**-**2.6**).

2.1. Structural features and biosynthesis of diarylheptanoids

Diarylheptanoids are a group of plant-derived phenolic compounds bearing an 1,7-diphenylheptan skeleton (Fig. 1). Based on the structure of the C7 alkyl chain two main groups exist: linear- and cyclic-diarylheptanoids. Linear-diarylheptanoids can fatherly be classified into five sub-types: those possessing namely a heptane chain, an -oxy bridge or a flavonoid moiety, dimeric linear-diarylheptanoids and unusual structures, respectively (Lv and She 2011, Keserű and Nógrádi 1995). Furthermore, the variability of substituents in different positions on the alkyl chain and on the aromatic rings leads to numerous possible structure variants that can be associated with various biological effects.

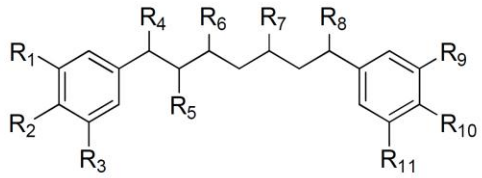


Figure 1. Structure of the 1,7-diarylheptan skeleton of linear diarylheptanoids marking the possible positions of substituents marked with $R_{1\text{-}11}$ (Lv and She 2011).

Until the present day, studies on the biosynthesis of diarylheptanoids are confined to curcuminoids, a group of diarylheptanoid-type molecules possessing the most promising biological effects. Investigations on the possible precursors of curcuminoid biosynthesis suggested very early that the backbone consists of two phenylpropanoids which are connected by an acetate derived carbon unit. However these studies did not manage to make clear distinction between two possible pathways: 1) starter phenylpropanoid-coenzyme A, five extensions with malonyl-CoA, then ring-closure and further modifications; and 2) biosynthesis from two phenylpropanoid-CoA units and one malonyl-CoA (Roughly and Whiting 1973). Much later Schröder proposed that the biosynthesis of these compounds might start with a type III polyketide synthase reaction (Schöder 1997). A more recent study (Katsuyama et al. 2009) on the biosynthesis of curcuminoids of *Curcuma longa* L. might confirm this presumption. The authors propose a pathway, which includes two novel type III polyketide synthases,

namely diketide-CoA synthase (DCS), and curcumin synthase (CURS). According to the report, the starter substrates, cinnamoyl-CoA, *p*-coumaroyl-CoA, or feruloyl-CoA, are synthesized from phenylalanine by the phenylalanine ammonia-lyase (PAL), the 4-coumarate-CoA ligase (4CL), the cinnamate-4-hydroxylase (C4H), the hydroxycinnamoyl transferase (HCT), the cinnamate-3-hydroxylase (C3H), and the *O*-methyltransferase (OMT). The formation of feruloyldiketide-CoA is catalyzed by the diketide-CoA synthase (DCS) via condensation of feruloyl-CoA and malonyl-CoA. Besides, curcumin synthase (CURS) catalyzes the formation of curcuminoids from cinnamoyldiketide-*N*-acetylcystamine (a mimic of the CoA ester) and feruloyl-CoA (Fig. 2).

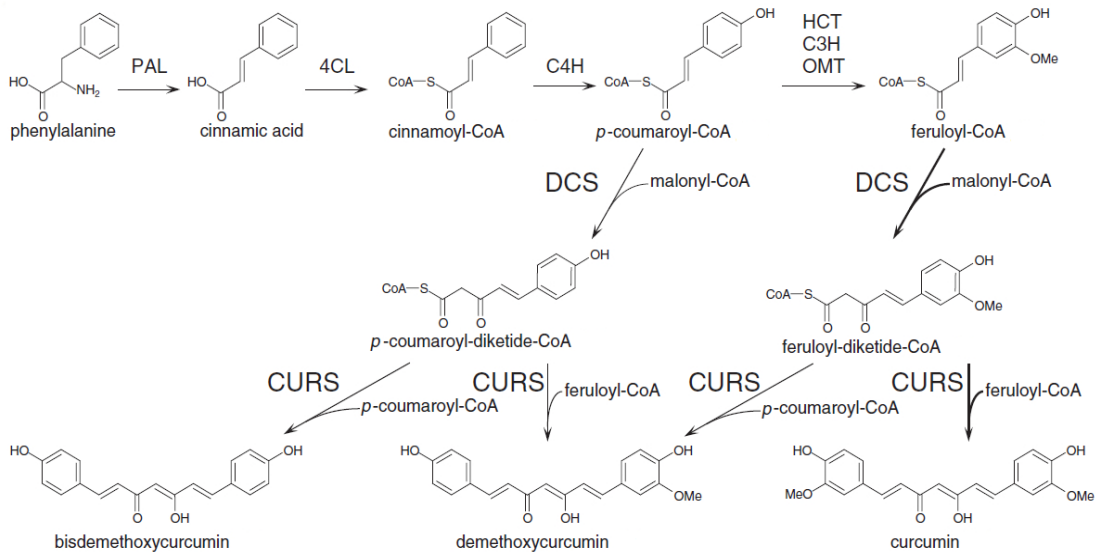


Figure 2. Biosynthesis of curcuminoids in *Curcuma longa* L. (obtained from Katsuyama et al. 2009)

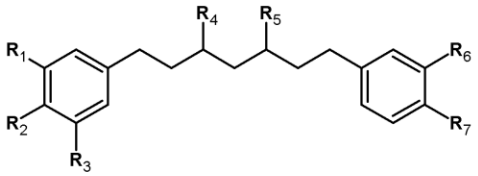
2.2. Distribution of diarylheptanoids in the plant kingdom

The firstly discorvered diarylheptanoid, namely curcumin, was isolated from the traditional herbal remedy and dietary spice, turmeric *(Curcuma longa* L., Zingiberaceae) two hundred years ago by Vogel in 1815 (Vogel and Pelletier 1815). Ever since hundreds (more than 400 different molecules) of new compounds with 1,7-diphenylheptan structures were identified in various plants. Nowadays, studies on natural sources of diarylheptanoids mainly focus on species of Zingiberaceae and Betulaceae families. Numerous plants of the *Curcuma* and *Alpinia* genera of the Zingiberaceae, and of the *Alnus* genus of Betulaceae have been proved to accumulate diarylheptanoids as secondary metabolites (Lv and She 2011). Furthermore, the presence of diarylheptanoids was also reported in species of the *Betula* (Betulaceae) (Matsuda et al. 2008, Lee at al. 2012, Mshvildadze et al. 2007), *Myrica* (Myricaceae) (Kim et al. 2014, Ting et al. 2014, Yoshimura et al. 2012, Akazawa et al. 2010, Wang and Liu 2008, Morihara et al. 1997) *Cymodocea* (Cymodeaceae) (Kontiza et al 2008, Kontiza et al 2005), *Juglans* (Juglandaceae) (Yao et al. 2015, Yang et al. 2011, Li et al. 2008, Gao et al. 2003), *Acer* (Aceraceae) (Akihisa et al. 2012, Yonezawa et al. 2011), and *Pyrostria* (Rubiaceae) (Beniddir et al. 2012) genera. The distribution of diarylheptanoids in various plants is presented in Figure A1., including 102 compounds but limited to the most relevant species.

Nonetheless, researches on new natural sources of diarylheptanoids are still of great interest nowadays, since these molecules are considered as potential therapeutic agents due to their several beneficial physiological effects (see section **2.3.**). These include anti-inflammatory, antioxidant, anti-tumour, leishmanicidal, melanogenesis inhibitor, hepatoprotective and neuroprotective activities (Lv and She 2011).

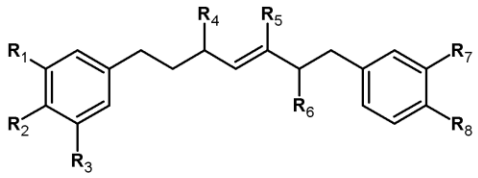
The next chapter (**2.3.)** summarises the most recent knowledge on biological activities of diarylheptanoids isolated from various plant species.

The structures of the most representative diarylheptanoid aglycones for the species discussed in the next chapters are depicted in this section in Figure 3 (**i1-i21**).



i9 R_1= H; R_2= H; R_3= H; R_4= H; R_5= O; R_6= OCH_3; R_7= OH

i12 R_1= H; R_2= H; R_3= H; R_4= OH; R_5= OH; R_6= H; R_7= H



i1 R_1= H; R_2= OH; R_3= H; R_4= O; R_5= H; R_6= H; R_7= H; R_8= H

i4 R_1= OCH_3; R_2= OH; R_3= OH; R_4= O; R_5= H; R_6= H; R_7= H; R_8= OH;

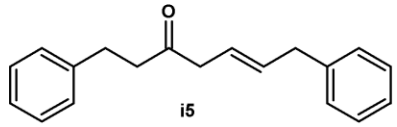
i7 R_1= H; R_2= H; R_3= H; R_4= O; R_5= H; R_6= OH; R_7= H; R_8= H

i11 R_1= OCH_3; R_2= OH; R_3= H; R_4= O; R_5= H; R_6= H; R_7= H; R_8= H

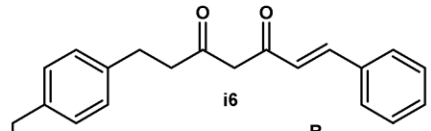
i22 R_1= H; R_2= OH; R_3= H; R_4= O; R_5= OXyl; R_6= H; R_7= H; R_8= OH

1 R_1= OH; R_2= OH; R_3= H; R_4= O; R_5= H; R_6= H; R_7= OH; R_8= OH

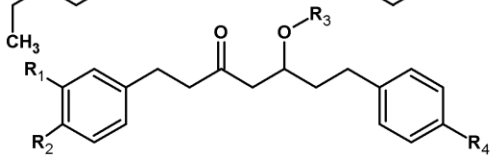
2 R_1= OH; R_2= OH; R_3= H; R_4= O; R_5= OXyl; R_6= H; R_7= OH; R_8= OH



i5

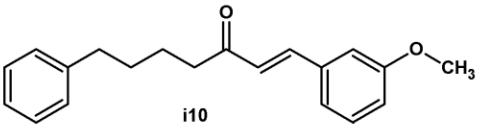


i6

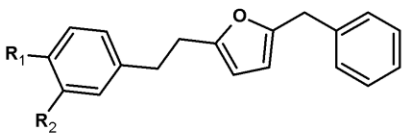


i8 R_1= H; R_2= H; R_3= CH_3; R_4= H;

i21 R_1= OCH_3; R_2= OH; R_3= H; R_4= OH

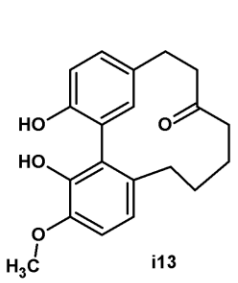


i10

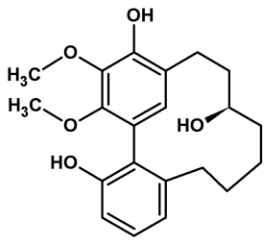


i2 R_1= OH; R_2= OCH_3

i3 R_1= H; R_2= H



i13



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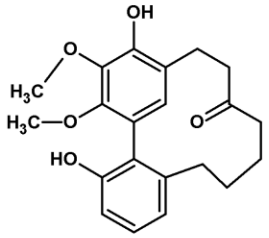
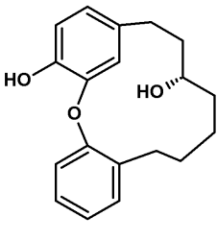
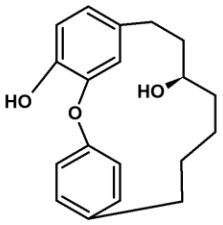


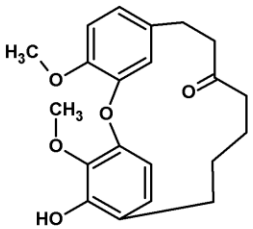
i15



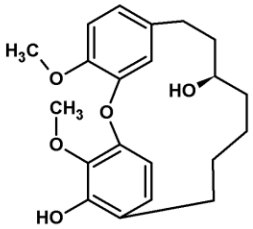
i16



i17



i19



i20

Figure 3. Structures of the most representative diarylheptanoids for the species discussed in section **2.3**.

2.3. Biological activities of diarylheptanoids

2.3.1. *Curcuma* genus (Zingiberaceae)

Considering biological activities, there is no doubt that the most promising and explored molecules of this type are curcumin and its analogues. Curcumin presents almost all the effects that can be associated with diarylheptanoid compounds (summarised in Table A1 in section **13.1.**), therefore, the first part of the discussion focuses on these activities by introducing the pharmacological properties of curcumin.

Curcumin (Fig. 4) is a diarylheptanoid-type polyphenol responsible for the yellow colour of turmeric (*Curcuma longa* L., Zingiberaceae), a curry spice. The yellow-pigmented fraction of turmeric contains also other curcuminoids mainly demethoxycurcumin and bis-demethoxycurcumin that are related to its principal ingredient, curcumin (Wongcharoen and Phrommintikul 2009).

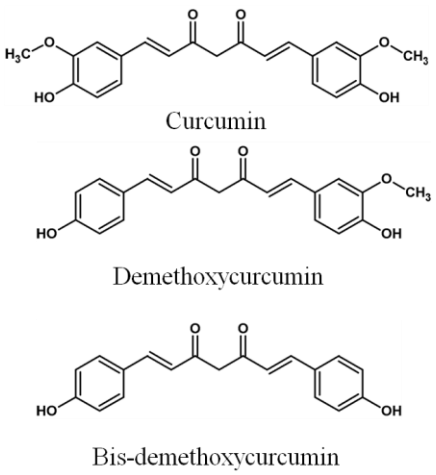


Figure 4. Structures of curcumin, demethoxycurcumin and bis-demethoxycurcumin.

Curcumin has been shown to possess several beneficial biological activities via interaction with various molecular targets (Fig. 5). Taking a look at the site http://clinicaltrials.gov (September, 2015) clearly indicates its promising therapeutic potential: 124 clinical trials are registered using curcumin, some completed, some still ongoing and some recruiting patients. The most relevant clinical trials (Phase II or III) investigate its efficacy in different types of cancer, such as colon carcinoma and pancreas carcinoma in monocomponent therapy or in combination with conventional

chemotherapeutics, e.g. gemcitabine; and in cognitive dysfunctions related to schizophrenia and Alzheimer's disease. These human studies also confirmed the non-toxicity of curcumin up to oral dose of 8 g pro die.

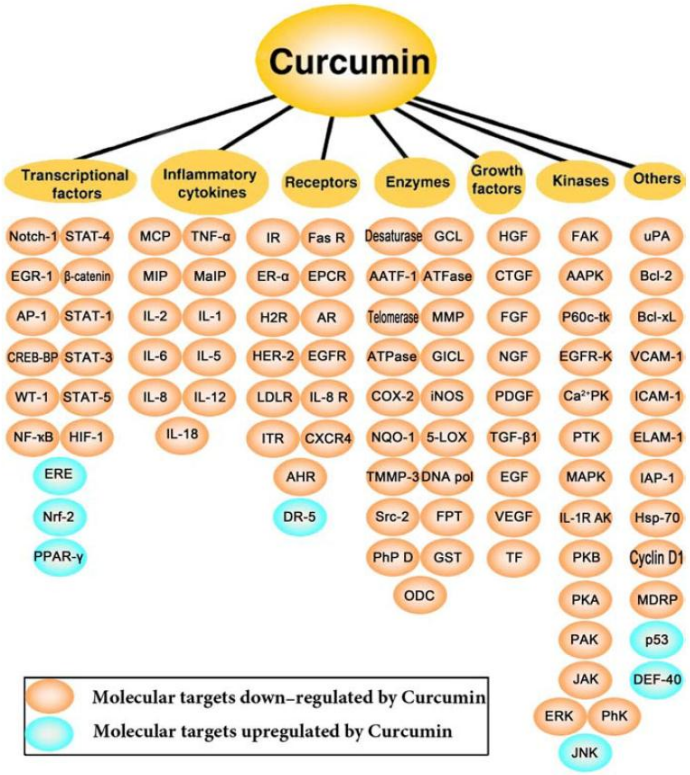


Figure 5. Molecular targets of curcumin

Abbreviations: NF-κB, nuclear factor-kappa B; AP-1, activating protein1; STAT, signal transducers and activators of transcription; Nrf-2, nuclear factor 2-related factor; Egr-1, early growth response gene-1; PPAR-γ, peroxisome proliferator-activated receptor-gamma; CBP, CREB-binding protein; EpRE; CTGF, connective tissue growth factor; EGF, epidermal growth factor; EGFRK, epidermal growth factor receptor-kinase; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor; AR, androgen receptor; Arh-R, aryl hydrocarbon receptor; DR-5, death receptor-5; EGF-R, epidermal growth factor-receptor; EPC-R, endothelial protein C-receptor; ER-α, estrogen receptor-alpha; Fas-R, Fas receptor; H2-R, histamine (2)- receptor; InsP3-R, inositol 1,4,5-triphosphate receptor; IR, integrin receptor; IL-8-R, interleukin 8-receptor; LDL-R, low density lipoprotein-receptor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase-3; iNOS, inducible nitric oxide oxidase; COX-2, cyclooxygenase-2; LOX, lipoxygenase; Gcl, glutamatecysteine ligase; NAT, arylamine N-acetyltransferases; IAP, inhibitory apoptosis protein; HSP-70, heat-shock protein 70; TNF-α, tumour necrosis factor alpha; IL, interleukin; MCP, monocyte chemoattractant protein; MIF, migration inhibition protein; MIP, macrophage inflammatory protein; ERK, extracellular receptor kinase; IARK, IL-1 receptor-associated kinase; cAK, autophosphorylation-activated protein kinase; CDPK, Ca2+-dependent protein kinase; cPK, protamine kinase; JAK, janus kinase; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; TK, protein tyrosine kinase; FAK, focal adhesion kinase; PhK, phosphorylase kinase; pp60c-src, pp60c-src tyrosine kinase; PKA, protein kinase **A**; PKB, protein kinase **B**; PKC, protein kinase **C**; FPTase, farnesyl protein transferase; GST, glutathione S-transferase; HO, hemeoxygenase; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ELAM-1, endothelial leukocyte adhesion molecule-1; SHP-2, Src homology 2 domain-containing tyrosine phosphatase 2, uPA, urokinase-type plasminogen activator. (obtained from Noorafshan and Ahkani-Esfahani 2013 without modification).

2.3.1.1. Structural features and bioavailability of curcumin

Curcumin, 1,7-bis-(4-hydroxy-3methoxyphenyl)-1,6-heptadien-3,5-one, is a small, symmetric and lipophilic molecule, which allows its rapid permeation through biological membranes. The simple molecular structure with various synthetically accessible positions makes it an outstanding target for structure-activity relationship and lead optimisation studies (Noorafshan and Ahkani-Esfahani 2013). Since curcumin shows poor bioavailability and stability due to limited absorption, rapid metabolism and fast systemic elimination (Anand et al. 2007), several analogues have been introduced and investigated in order to improve its bioavailability, selectivity and stability. Besides, pre-formulation approaches have also been performed including the use of adjuvants such as piperine, that attenuates glucuronidation in the liver, the utilisation of cyclodextrines, liposomal curcumin and curcumin-loaded nanoparticles (Dey et al. 2016, Menon et al. 2015, Patial et al. 2015). These studies aim not only the improvement of stability, but also the transportation to the target molecules, e.g. penetration across the blood-brain barrier that is essential for the efficacy in pathological states of the central nervoius system (Mourtas et al. 2014, Cheng et al. 2013, Quitschke et al. 2013).

The next chapters (**2.3.1.2.-2.3.1.6**) discuss the mechanisms of the most important biological activities of curcumin in details: the antioxidant, anti-inflammatory and anti-tumoural effects and the positive impact on neurodegenaretive diseases that contribute to almost all the therapeutic utilisation approaches of the compound. The sections start with a brief summary of the molecular mechanisms of oxidative stress, inflammation apoptosis and neurodegeneration, the processes related to the previously mentioned activities.

2.3.1.2. Antioxidant activity of curcumin

The main factor contributing to the development of the oxidative stress is the production of free radical species. The eukaryotic cells present several protection mechanisms against free radicals. The primary defence system involves enzymes, such as the superoxide-dismutase (SOD), the catalases, peroxydases, the glutathione-S-transferase, DT-diaforase and reductases. The secondary defence system consists of the vitamins

(vitamin-A -C -E -K) and cofactors (e.g. coenzyme Q) possessing scavenger activity, carotenoids, cysteine and methionin. Trace elements, such as Cu, Zn, Mn and Se and small dietary molecules, e.g. flavonoids also play important role in the cell protection mechanism against free radicals. Nevertheless, oxidative stress emerges if the balance between the free radical producing and the defence mechanisms is disrupted. It is considered one of the main risk factors for the development of various illnesses including diabetes, cancer, cardiovascular and neurodegenerative diseases. Several studies have been performed in order to investigate the positive pharmacological effects of curcumin in the above mentioned pathological states. The antioxidant potential of curcumin plays a major role in these activities. It is reported to be a highly potent free radical scavenger, a reducing agent and DNA damage inhibitor (Antunes et al. 2005, Patro et al. 2002, Jovanovic et al. 2001). It has been proved that curcumin inhibits nitric oxide (NO) and reactive oxygen species (ROS) production in macrophages. Besides, oxidative stimulation of G-proteins in human brain tissues is attenuated by this compound, as well as lipid peroxydation in liver microsomes of rats (Hatcher et al. 2008).

2.3.1.3. Anti-inflammatory effect of curcumin

Inflammation plays dual role in the human organism. On the one hand it forms an important part of the defence system that aims the elimination of the extrinsic and intrinsic harmful effects; on the other hand many substances released during the inflammation process have tissue-damaging effects, therefore, chronic inflammation can lead to the impair of normal tissue integrity. The initiation of the process can be attributed to exogenous and also endogenous stimuli. These cause the release of histamine from the basophile granulocytes and the mast cells, and also the liberation of arachidonic acid from the plasma membrane via the phospholipase A_2 enzyme. Arachidonic acid is converted to prostaglandynes and leukotriens by the cycloxygenase (COX) and lipoxygenase (LOX) enzymes, respectively. These play important role in the emergence of pain, inflammation and fever. Besides, adhesion molecules such as intercellular adhesion molecule (ICAM1) and the vascular cell adhesion molecule (VCAM1) also contribute to the process by facilitating the accumulation of leucocytes, platelets and the endothelial cells in the inflammation centrum. The role of cytokines in

inflammation is diverse; the IL-2, IL-6, IL-8, IL-12, IL-18, the tumour necrosis factor α and β, and the interferonγ are considered as pro-inflammatory cytokines; while the IL-4, IL-10, IL-13 have anti-inflammatory effect. The nuclear factor-κB (NF-κB) is considered one of the main factors contributing to the inflammation process. It is a dimeric transcription factor that induces the expression of several genes that are responsible for the production of inflammatory agents, such as cytokines: the IL-1β, IL-2 and TNF-α; adhesion molecules and enzymes: the inducible nitric oxide synthase (iNOS) the COX-2 and 5-LOX. The NF-κB is present in the cells in inactive form; the activation is induced by extracellular factors such as UV-light, or the IL-1. In the non-stimulated cells the NF-κB is placed in the cytoplasm, bounded to its inhibitor, the inhibitor of κB-kinase (IκB). Pro-inflammatoric effects cause the phosphorilation of IκB, whereupon it gets an ubiquitine signal and degrades in the proteosome, thus giving the ability to the NF-κB to enter the nucleus and exert its effect. This inflammatory process plays pivotal role in the pathogenesis of chronic diseases, such as rheumatoid arthritis, arteriosclerosis, asthma or the *Helicobacter pylori* gastritis. Nitric oxide (NO) is responsible for several processes occurring in acute inflammation, by causing vasodilatation, increasing capillary permeability, thus enhancing the acute inflammatory reaction and stimulating the generation of prostaglandynes. In this case it is synthesised by the iNOS enzyme (Gyires 2007).

Members of the *Curcuma* genus have been used to treat inflammatory malfunctions for thousands of years. Since the isolation and identification of curcumin, the main compounds present in the rhizome of these plants, it has been considered to be primarily responsible for this anti-inflammatory effect. Curcumin has been shown to attenuate the activation of the NF-κB, as well as other activation pathways, such as NO generation and COX-2, 5-LOX expression (Ma et al. 2015, Bengmark 2006). It also induces down-regulation of several pro-inflammatory cytokines, such as TNF, IL-1, IL-8, interferonγ and other chemokines (Gao et al. 2004, Surh 2002). Human trials also proved the anti-inflammatory effect of curcumin in many inflammatory diseases (http://clinicaltrials.gov) affecting different body systems (Table A1).

2.3.1.4. Anti-tumoural properties of curcumin

The most promising results regarding the therapeutic effects of curcumin are related to its anticancer activity. Several ongoing clinical trials (http://clinicaltrials.gov) strengthen the relevance of this statement.

Most of the reports agree that the anti-inflammatory, antioxidant, apoptosis inducing and anti-angiogenic activities all contribute to its anti-tumoural effect (Shanmugam et al. 2011, Bengmark et al. 2009, Kunnumakkara et al. 2008, Thomasset et al. 2007, Bemis et al. 2006).

Apoptosis is an intrinsic programme that leads to cell-death. It is governed by different signal-transduction mechanisms that can be initiated two different ways. In the extrinsic process the so-called death ligands bind to the death receptors, such as CD95 or TRAIL that leads to the activation of caspase-8, which transmits the "death signal" to the effector caspases, e.g. caspase-3. During the intrinsic process apogenetic factors, such as cytochrome c, the second mitochondria-derived activator of caspase (Smac) or the apoptotic proteinase-activating factor (AIF) are transferred to the cytosol from the mictochondrial intermembrane space. This also promotes the production of effector caspases. The increase in the permeability of mitochondrial membranes play significant role in this process. The substrates of the effector caspases are enzymes, such as the endonucleases, nuclear lamines, the PARP (the DNS-dependent polymer kinase), gelsolin and fodrin. The activation of the former initiates the farther processes of apoptosis and consequently, leads to cell-death (Saelens et al. 2004).

As it was previously mentioned, curcumin down-regulates the NF-κB transcription factor, which results in the suppression of BcL-2 and Bcl-X_L anti-apoptotic genes, thus the promotion of apoptosis induction (Sandur et al. 2007). Curcumin also inhibits the Akt protein kinase, an other apoptosis inhibitor enzyme (Yamaguchi et al. 2001). The enhanced expression of p53 gene, an apoptosis mediator, was also observed after administration of curcumin in several cancer cells (human basal cells, human hepatoblastoma and human breast cancer cells (Choudhuri et al 2005). This effect was revealed to be tissue-specific based on the fact that in colorectal carcinoma cells the p53 expression decreased, while the heat-shock protein 70 level increased after curcumin treatment (Bush et al. 2001). Recent studies have shown that the suppression of Sp-1

and its downstream targets, calmodulin and SEPP1 may also contribute to the anti-apoptotic effect of curcumin (Vallianou et al 2015).

Besides the anti-apoptotic effect, other cell death mechanisms have also been published related to the anticancer effect of curcumin (Gali-Muhtasib et al. 2015). These include autophagy that has been shown to play role in the anti-tumour effect on, e.g. glioma, colorectal, breast carcinoma, leukemia and ovarian carcinoma. This activity is based on modification of different signalling mechanisms (p53 degradation, inhibition of Act kinase, activation of ERK1/2) and also the generation of ROS. The other non anti-apoptotic effect of curcumin that leads to cell death is programmed necrosis. This was characterised by the induction of ROS and caspase-independent cell death. This activity was proved in prostate, bladder, colorectal, medulloblastoma, pancreatic and cervical cancer cells. Curcumin has been shown to act trough senescence, which implicates morphological, functional, and behavioural modifications and irreversible growth arrest. The importance of this mechanism was proved in breast cancer cells.

COX-2 inhibition is an other effect that is considered to play role in the anti-tumoural activity of curcumin, which was studied in the treatment of colonic tumours (Hatcher et al. 2008).

The enhanced survival, growth and metastasis of tumour cells mainly depend on angiogenesis. Curcumin was shown to attenuate many sub-processes involved in this process. These include the inhibition of the fibroblast growth factor-induced neurovascularisation, ligands of vascular endothelial growth factor, and angiopoitein 1 and 2. Curcumin also has the ability to down-regulate adhesion molecules, such as the leukocyte adhesion molecule-1, intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 (Bhandarkar et al. 2007).

2.3.1.5. The positive effects of curcumin in neurodegenerative diseases

The protective effect of curcumin in neurodegenerative diseases, such as Alzheimer's and Parkinson's disease has been proved *in vivo* (Fu et al. 2015, Ghosh et al. 2015, Zhang et al. 2015).

Alzheimer's disease (AD), a progressive neurodegenerative brain disorder, affects more and more elderly people in the world. There is an ongoing scientific debate upon the pathomechansim of AD, whether the amyloid-β plaques or the phosphorylated tau-

protein is mainly responsible for the neurodegeneration. However, there is consensus among researchers regarding the important role of oxidative damage and the abnormal accumulation of metal ions in several neurodegenerative diseases. Besides the notable antioxidant activity of curcumin that is originated from the free radical scavenging effect, inhibition of lipid peroxydation and increment of SOD action; it also has the ability to chelate metal ions, also to down regulate the secretion of amyloid peptide and prevent amyloid toxicity on neurons (Chen et al. 2011). Curcumin-conjugated nanoliposomes showed even higher affinity for amyloid-β deposits than curcumin, which makes this preformulation approach suitable for diagnostic and therapeutic applications as well (Lazar et al. 2013).

Dementia is one of the main symptoms of AD and also several other neuronal diseases. Curcumin has been reported to improve memory functions in both animal models and human trials. The mechanism of action includes the antioxidant activity, cholinesterase inhibition and also effect on brain insulin receptors (Noorafshan and Ahkani-Esfahani 2013).

2.3.1.6. The dark side of curcumin

In the last decade, several papers have been published reporting the beneficial biological effects of curcumin in various diseases (see above). Numerous clinical trials are ongoing with this natural product (http://clinicaltrials.gov) in order to evaluate its activity. However, to see clear it is important to deal with its negative properties as well. One of the most important doubts about the efficacy of curcumin is that most of the evidence that support its therapeutic potential is based on *in vitro* data. In these studies it was tested in micromolar concentrations, but limited absorption, rapid metabolism in the intestine and liver, as well as fast systemic elimination result in very low plasma concentrations, typically in the nanomolar range (Ireson et al. 2001, Ireson et al. 2002). These data suggest that the therapeutic potential of curcumin administered *per os* is limited. Although, as it was previously mentioned, several approaches have been introduced for increasing the bioavailability of curcumin.

Curcumin is facing clinical trials in order to test its potential to sensitise tumour cells to the effects of conventional chemotherapeutics, e.g. gemctiabine. However it has been previously reported that curcumin can either increase or decrease the efficiency of

chemotherapy depending the concentration utilised (Somasundaram et al. 2002), thus the outcome of these studies is uncertain.

Although curcumin is considered non-toxic, some evidence contradicts this statement. In 1993 the National Toxicology Program published an extensive study on the carcinogenic properties of turmeric containing 80-95% curcumin. Rats and mice were treated with turmeric administered *per os* for two years. The report concluded that there was equivocal evidence of the carcinogenetic activity of curcumin, based on the increased incidences of glitorial gland adenomas, carcinomas of the small intestine and hepatocellular adenomas. This effect might be contributed to the ability of curcumin to increase topoisomerase II-mediated DNA damage, and inactivation of p53 tumour suppression factor (Burgos-Moron et al. 2010). It has also been proved that high concentrations of curcumin can increase ROS levels by irreversibly modifying the antioxidant enzyme thioredoxin reductase (Fang et al. 2005) that can also play role in the carcinogenetic effect. The average daily those of curcumin in these studies was circa 0.2 mg/kg body weight (NTP 1993), while in human studies it can reach 1g/kg body weight.

The next chapters (**2.3.2.-2.3.7.**) briefly introduce the remaining important plant genera reported to contain diarylheptanoid compounds that have been proven to show beneficial biological activities. In the final sections, the Betulaceae species belonging to the *Alnus* and *Betula* generea are presented as the closest relatives of the investigated *Corylus* species.

2.3.2. *Alpinia* genus (Zingiberaceae)

Alpinia is a genus of the Zingiberaceae family, which comprises flowering plants native to Asia, Australia, and the Pacific Islands, where they occur in tropical and subtropical climates. It is named after Prospero Alpini, a 17th-century Italian botanist specialized in exotic plants (Bruneton 2001). Some members of the genus were reported to accumulate diarylheptanoids with various pharmacological effects (see sections **2.3.2.1-2.3.2.6**).

2.3.2.1. Effect on neuronal differentiation

A diarylheptanoid from the plant *Alpinia officinarum* Hance 7-(4-hydroxyphenyl)-1-phenyl-4-hepten-3-one (**i1**), exhibited potent activities on neuronal differentiation and neurite outgrowth. It induced differentiation of neuroblastoma cells into a neuron-like morphology, and accelerated the establishment of axon-dendrite polarization of cultured hippocampal neurons. Moreover, it promoted neurite extension in both Neuro-2a cells and neurons. The authors showed that the effects on neuronal differentiation and neurite growth were specifically dependent on the activation of extracellular signal-regulated kinases (ERKs) and phosphoinositide 3-kinase (PI3K)-Akt signaling pathways. Importantly, intraperitoneal administration of the diarylheptanoid promoted the differentiation of new-born progenitor cells into mature neurons in the adult hippocampal dentate gyrus (Tang et al 2015).

2.3.2.2. Selective cytotoxic effect

Two dimeric diarylheptanoids, namely alpinin C and D that were isolated from the rhizomes of *Alpinia officinarum* were evaluated for their cytotoxicity against human tumour cell lines HepG2, MCF-7, T98G and B16-F10. Alpinin C showed notable and selective cytotoxicity against cell lines of MCF-7 and T98G with IC_{50} values of 8.46 and 22.68 μg/ml, respectively (Liu et al. 2014). Bioassay-guided fractionation of the cytotoxic MeOH extract from the rhizomes of *Alpinia officinarum* Hance led to the isolation of two new diarylheptanoids named alpinoid D (**i2**) and E (**i3**), together with fifteen known linear diarylheptanoids. The cytotoxic activity of the isolated diarylheptanoids was evaluated against the IMR-32 human neuroblastoma cell line. Among the tested compounds, 5-hydroxy-1-(4–hydroxy-3-methoxy)phenyl-7-phenlyheptan, 5-methoxy1-(4–hydroxy-3-methoxy)phenyl-7-phenlyheptan, and 1-(4–hydroxy-3-methoxy)-phenyl-7-phenly-hept-4-3-one exhibited the most potent activities with IC_{50} values of 0.83, 0.23 and 0.11 μM, respectively. The authors could conclude that the linear diarylheptanoids possessing a methoxyl at C3 and a hydroxyl function at C4 on the benzene ring were essential for potent cytotoxic activity (Sun et al. 2008).

2.3.2.3. Antibacterial effect

Three diarylheptanoids that were isolated from the ethanolic extract of the rhizomes of *Alpinia officinarum* by Zhang et al were elucidated as 7-(4,5-dihydroxy-3-methoxyphenyl)-1-phenyl-4-heptene-3-one (**i4**), 1,7-diphenyl-5-heptene-3-one (**i5**) and 4-phenethyl-1,7-diphenyl-1-heptene-3,5-dione (**i6**). All of the compounds showed antibacterial activity against *Helicobacter pylori* with MIC values of 9-30 μg/ml (Zhang et al. 2010). These results suggest the potential use of the investigated diarylheptanoids for the treatment of peptic ulcer and related diseases.

2.3.2.4. Platelet-activating factor receptor binding inhibitory activity

The bioassay-guided purification of ether extracts of *Alpinia officinarum* led to the isolation of two new compounds 6-hydroxy-1,7-diphenyl-4-en-3-heptanone (**i7**) and 6-(2-hydroxy-phenyl)-4-methoxy-2-pyrone as well as two known diarylheptanoid compounds 1,7-diphenyl-4-en-3-heptanone (**i1**) and 1,7-diphenyl-5-methoxy-3-heptanone (**i8**). All three diarylheptanoids exhibited potent platelet-activating factor (PAF) receptor binding inhibitory activities with an IC_{50} of 1.3, 5.0, and 1.6 μM, respectively. The authors concluded that their studies have identified diarylheptanoids as a novel class of potent PAF antagonists (Fan et al. 2007).

2.3.2.5. Anti-angiogenic activity

Gao et al. investigated the anti-angiogenic activity of two diarylheptanoids, namely Yakuchinone A (**i9**) and B (**i10**) isolated from the fruit of *Alpinia oxyphylla* Miq., together with a structure analogue, curcumin. The activity and toxicity of these three compounds were compared using transgenic zebrafish as *in vivo* model and human umbilical vein endothelial cell as *in vitro* model. The results suggested that in both *in vitro* and *in vivo* assays, curcumin exerted the most potent anti-angiogenic effect with the lowest toxicity among these compounds; Yakuchinone A was the second potent; Yakuchinone B has the lowest activity but with the highest toxicity in all three compounds. (Gao et al. 2015).

2.3.2.6. Anti-inflammatory activity

The *in vitro* iNOS inhibitor properties in lipopolysaccharide-activated macrophages of the extract prepared with acetone from the rhizome *Alpinia officinarum* has also been reported (IC_{50} of 35μg/ml) (Matsuda et al. 2006). The successive isolation of the flavonoid galangin and two diarylheptanoid compounds [7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhept-4-en-3-one (**i11**) and 3,5-dihydroxy-1,7-diphenylheptane (**i12**) has also been carried out. Both the compounds inhibited NO production in LPS-activated mouse peritoneal macrophages with IC_{50} values of 62, 55 and 33 μM, respectively. Investigation of the structure-activity relationships regarding the previously mentioned compounds together with other diarylheptanoids led to the following conclusions: an enone moiety at the 3-5 positions suggested to be important for the activity; methylation of the 4',4"-hydroxyl groups tended to reduce the effect; the double bonds and/or enone moiety at the 1-7 positions are considered important for the activity. The authors concluded that the diarylheptanoid compounds can contribute to the iNOS inhibitor, thus anti-inflammatory activity of the extract.

2.3.3. *Morella* and *Myrica* genera (Myricaceae)

Myrica and *Morella* species are taxonomically very closely related trees or shrubs with edible fruit that exhibit relevant applications in traditional medicine. Extracts of the roots, bark and fruit have been used for the treatment of various diseases, such as diarrhoea, stomach pain, bleeding, asthma, coughing, headache, fevers and inflammation. Several different cyclic diarylheptanoids have been identified in the plants, e.g. *Morella adenophora* Hance, *Morella arbores* Hutch, *Morella nana* A. Chev., *Morella cerifera* L., *Myrica gale* L. and *Myrica rubra* Lour (Silva et al. 2015).

2.3.3.1. Antioxidant activity

Antioxidant activity of *Myrica* and *Morella* extracts and 36 isolated compounds (diarylheptanoids, flavonoids and pentacyclic triterpenoids) have been investigated in several *in vitro* assays, e.g. the DPPH, ABTS and nitroblue tetrazolium tests, with the most often used positive control being ascorbic acid. 13 components were found to be more potent antioxidant than ascorbic acid in the DPPH test. The analyses of the results

obtained in this assay allowed the authors to draw conclusions about diarylheptanoid antioxidant action: 1) only two investigated compounds, Myricananin C (**i13**) and Myricanol (**i14**) 5-*O*-β-D-(6'-*O*-galloyl)-glucopyranoside exhibited IC_{50} values below 20 μM and were more active than ascorbic acid; 2) a hydroxyl group at C-11 position instead of carbonyl did not improve the activity; 3) an extra hydroxyl group at carbon C-5 is also irrelevant; 4) the loss of a methyl group causes a strong increase in the antioxidant effect; 5) it seemed that the presence of a sugar moiety, as well as the type and localisation of the sugar also interfere with the antioxidant activity (Silva et al. 2015).

2.3.3.2. Anti-inflammatory activity

According to the results of several studies, the anti-inflammatory activity of *Myrica* and *Morella* extracts seems to be remarkable. The diarylheptanoids isolated from these extracts, myricanone (**i15**) and myricanol (**i14**) were proved to be very active iNOS inhibitors; Myricanin A showed TNFα inhibitory effect, while Juglanin-BB-11-*O*-sulphate decreased IL-6 levels (Silva et al. 2015), typically with IC_{50} values being in the micromolar range.

2.3.3.3. Anticancer activity

A study carried out by Dai et al. explored the inhibitory effect and mechanism of myricanol (**i14**) on lung adenocarcinoma xenografts in nude mice. The results showed that the protein expression of Bcl-2, VEGF, HIF-1α, and survivin were consistently downregulated, whereas that of Bax was upregulated after myricanol treatment. Myricanol also significantly upregulated the mRNA expression of Bax and downregulated that of Bcl-2, VEGF, HIF-1α, and survivin in a dose-dependent manner. These data suggested that myricanol could significantly decelerate tumour growth *in vivo* by inducing apoptosis (Dai et al. 2015).

2.3.3.4. Positive effect in neurodegenerative diseases

Microtubule-associated protein tau accumulates in more than 15 neurodegenerative diseases and is most closely linked with postsymptomatic progression in AD. An extract from *Myrica cerifera* potently reduced both endogenous and overexpressed tau protein levels in cells and murine brain slices. The bayberry flavonoids myricetin and myricitrin were confirmed to contribute to this potency, but a diarylheptanoid, myricanol (**i14**), was the most effective anti-tau component in the extract, with potency approaching the best targeted lead therapies. (+)-a*R*,11*S*-Myricanol, isolated from *M. cerifera* as the naturally occurring aglycone, was significantly more potent than commercially available (±)-myricanol. Accordingly, myricanol may represent a novel scaffold for drug development efforts targeting tau turnover in AD (Jones et al. 2011).

2.3.4. *Acer* genus (Aceraceae)

2.3.4.1. Anti-inflammatory effect

Acer nikoense Maxim. is a small deciduous tree native to Japan and China. In the traditional medicine it has been used to treat hepatic malfunctions and eye diseases. The extracts of the bark were proved to contain several polyphenol compounds, including diarylheptanoids, such as acerogenin and acerosides. *In vitro* studies have proved the degranulation inhibitor effect of the extracts on basophil granulocytes, besides; they attenuated NO synthesis in macrophages (Akihisa et al 2006).

2.3.4.2. Effect on osteoblast differentiation

Osteogenic activity of six diarylheptanoids, acerogenin A (**i16**), (R)-acerogenin B (**i17**), aceroside I (**i18**), aceroside B_1 aceroside III and (-) centrolobol and two phenolic compounds; (+)-rhododendrol and (+)-cathechin, isolated from the stem bark of *Acer nikoense* (Nikko maple) was evaluated by Yonezawa et al., using alkaline phosphatase (ALP) activity as a marker for early osteoblast differentiation. The diphenyl ether-type cyclic diarylheptanoids promoted ALP activity in mouse preosteoblastic MC3T3-E1 cells without affecting cell proliferation, but the linear-type diarylheptanoid (-) centrolobol and the investigated other phenolic compounds did not. Diphenyl ether-type

cyclic diarylheptanoids also increased protein production of osteocalcin, a late stage marker for osteoblast differentiation, and induced osteoblastic mineralization. Structure–activity relationships of these compounds demonstrated that the stimulative efficacy of aglycones was higher than that of its glycosides. The authors speculated that this phenomenon is caused by differences in cell membrane permeability between glycosilated compounds and aglycones. Taken together, diphenyl ether-type cyclic diarylheptanoids promote early- and late-stage osteoblastogenesis, which may open the possibility for the development of novel osteogenic agents (Yonezawa et al. 2011).

2.3.4.3. Anti-diabetic effect

The Na^+-glucose cotransporter (SGLT) is a membrane protein that plays an important role in the re-absorption of glucose in the kidneys. SGLT is known to have three isoforms (SGLT1, SGLT2, and SGLT3). The inhibition of SGLT results in a decrease in blood sugar level. Cyclic diarylheptanoids acerogenin A (**i16**) and acerogenin B (**i17**) isolated from the methanolic extract of *Acer nikoense* bark have shown selective inhibitory effect on the sodium-glucose transporter (with IC_{50} values of 20 and 26 μM, respectively). The authors proved that the extent of this effect might depend on the position of the sugar moieties and the stereochemistry of the molecules (Morita et al. 2010). The appropriate torsion between two aromatic planes as well as their conformation might be important to show inhbition of SGLT.

2.3.5. *Juglans* genus (Juglandaceae)

The plant genus *Juglans* is the type genus of the family Juglandaceae. The 21 species in the genus range across the north temperate from southeast Europe, and more widely in the amercian continent from southeast Canada south to Argentina. The seeds of the trees belonging to the genus are referred to as walnuts (Rushford 1999).

2.3.5.1. Neuroprotective and antioxidant effect

A diarylheptanoid, juglanin C, was isolated from the 80 % methanolic extract of the leaves and twigs of *Juglans sinensis* Dode together with three known diarylheptanoids, juglanin A (**i19**), juglanin B (**i20**), and (5R)-5-hydroxy-7-(4- hydroxy-3-methoxyphenyl)-1(4-hydroxyphenyl)-3-heptanone (**i21**) by Yang et al., using

bioactivity-guided fractionation and chromatographic techniques. Juglanin C and A have shown notable neuroprotective activity against glutamate-induced toxicity in HT22 cells. These two diarylheptanoids significantly reduced the overproduction of cellular peroxyde in glutamate-injured HT22 cells and significantly maintained antioxidative defence systems, including glutathione, glutathione reductase, and glutathione peroxydase, under glutamate-induced oxidative stress in HT22 cells (Yang et al. 2011).

2.3.6. *Alnus* genus (Betulaceae)

Almost all the species belonging in *Alnus* genus have been traditionally used in Ayurveda, Unani, and Chinese folk medicine; traditional Korean medicine utilised them for haemorrhage, burn injuries, fever, diarrhoea and alcoholism; contemporary indigenous healers also use primarily the bark of *Alnus* species for the preparation of various therapeutical teas (Turner and Hedba 1990).

Different parts of the plants like stems, bark, seeds, leaves, roots, fruits, tree cones, buds and blossoms are known to possess therapeutical activity and they are characterised by a remarkable number of compounds such as triterpenoids, saponins, flavonoids, diarylheptanoids, phenols, steroids and tannins (Sati et al. 2011).

These molecules exhibited antioxidant (Dinic et al. 2014), anti-inflammatory (Lai et al. 2012), antiviral (Tung et al. 2010), cytotoxic (León-Gonzales et al. 2014, Novaković et al. 2014, Choi et al. 2008), inhibitory activity against nuclear factor kappa activation, nitric oxide and tumour necrosis factor-production, human umbilical vein endothelial cells, farnesyl protein transferase, cell-mediate low density lipoprotein oxidation, and HIV -1 - induced cytopathic effect in MT-4 cells (Sati et al. 2011).

2.3.6.1. Antioxidant activity

Evaluation of the antioxidant activity of two diarylheptanoids, platyphylloside 5(S)-1,7-di(4-hydroxyphenyl)-3-heptanone-5-*O*-β-D-glucopyranoside and its analogue, 1,7-di(4-hydroxyphenyl)-5-*O*-β-D-[6-(E-p-coumaroylglucopyranosyl)]heptane-3-one, both isolated from the bark of *Alnus glutinosa* L. has been carried out by Dinic et al. (Dinic et al. 2014). The published results indicated that neutralization of reactive oxygen species is an important mechanism of diarylheptanoid action, although these compounds exert a considerable anticancer effect. Therefore, the authors concluded that

these compounds may serve as protectors of normal cells during chemotherapy without significantly diminishing the effect of the applied chemotherapeutics.

Antioxidant and antimicrobial activities of methanolic extracts from the leaves and barks of three *Alnus* species (*Alnus glutinosa* L., *Alnus incana* L. and *Alnus viridis* Chaix) was evaluated by Dahija et al. Antioxidant activity of the extracts was determined using the DPPH radical scavenging method. All of the extracts showed antioxidant activity higher than that of thymol, which was used as a positive control (Dahija et al. 2014).

The ethanolic extract prepared from the leaves of *Alnus formosana* Burk showed notable antioxidant activity in *in vitro* assays using DPPH, hydroxyl and superoxide free radicals (Lee et al. 2006). This study did not include the identification of the antioxidant constituents, but other papers reported the presence of oregonin (**2**) as the main compound in *Alnus formosana* bark extracts (Lee et al. 2005), which previously has been proved to be a potent antioxidant (Keserű and Nógrádi 1995).

Oregonin (**2**) and hirsutenone (**1**) (see structures in section **6.3.1.**) isolated from *Alnus japonica* Thunb. inhibited LDL peroxydation in human cells *in vitro* with much higher activity than the positive control probucol. This effect suggests therapeutical potential in arteriosclerosis and related diseases (Lee et al. 2005).

2.3.6.2. Anticancer activity

The protective effects towards doxorubicin damaging activity of two diarylheptanoids isolated from the bark of *Alnus glutinosa*: platyphylloside (**i22**), 5(S)-1,7-di(4-hydroxyphenyl)-3-heptanone-5-*O*-β-D-glucopyranoside and its newly discovered analog 5(S)-1,7-di(4-hydroxyphenyl)-5-*O*-β-D-[6-(E-p-coumaroylglucopyranosyl)]heptane-3-one were studied by Dinic et al. HaCaT cells were employed, which are non-cancerous human keratinocytes commonly used for skin regenerative studies. Diarylheptanoids significantly antagonised the effects of doxorubicin by lowering the sensitivity of HaCaT cells to this drug. (S)-1,7-di(4-hydroxyphenyl)-5-*O*-β-D-[6-(E-p-coumaroylglucopyranosyl)]heptane-3-one prevented doxorubicin-induced cell death by activating autophagy. Both the diarylheptanoids protected HaCaT cells against doxorubicin-induced DNA damage. They significantly promoted migration and affected F-actin distribution. These results indicate that chemo-protective effects of

diarylheptanoids may occur at multiple subcellular levels. Therefore, these diarylheptanoids could be considered as protective agents for non-cancerous dividing cells during chemotherapy (Dinic et al. 2015).

A study of secondary metabolites from the bark of *Alnus glutinosa* led to the isolation of fourteen diarylheptanoids: oregonin (**2**), platyphylloside, rubranoside A, rubranoside, B hirsutanolol, hirsutenone (**1**), hirsutanonol-5-*O*-β-D-glucopyranoside, platyphyllonol-5-*O*-β-D-xylopyranoside, aceroside VII, alnuside A, alnuside B, 1,7-bis-(3,4-dihydoxyphenyl)-5-hydroxy-heptane-3-*O*-β-D-xylopyranoside, (5S)-1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)- 5-*O*-β-D-glucopyranosyl-heptan-3-one, and (5S)-1,7-bis-(3,4-dihydroxyphenyl)-5-*O*-β-D-[6-(3,4-dimethoxycinnamoyl-glucopyranosyl)]-heptan-3-one. All isolated compounds were analysed for *in vitro* protective effect on chromosome aberrations in peripheral human lymphocytes in 1-2 μg/ml concentrations, using Amifostine as positive control. The majority of them, exerted pronounced effect in decreasing DNA damage. The authors observed that those compounds that possessed pronounced protective activity had a 3-keto group in the heptanoid chain and also catechol moieties. The protective effect strongly correlated with the antioxidant activity of the compounds (Novakovic et al. 2014).

2.3.6.3. Anti-inflammatory activity

Diarylheptanoids from *Alnus hirsuta* Spach. exhibited inhibitory activity on the production of nitric oxide, on reactive oxygen species and on the expression of various pro-inflammatory molecules (Weicheng et al. 2011).

Previous studies of *Alnus formosana* Burk's leaves revealed a rich amount of diarylheptanoid glycosides, including oregonin (**2**) (Lee et al. 2006) that demonstrated to be a significant COX-2 inhibitor (Lee et al. 2000); active against LPS-induced NO production (Lai et al. 2012) and also considered an antioxidant agent.

2.3.6.4. Immunosuppressive activity

The effect of hirsutenone (**1**) isolated form *Alnus japonica* on the processes involved in the induction and maintenance of atopic dermatitis was investigated by Joo et al. Hirsutenone (**1**) inhibited the proliferation of T- and B-cells in a dose dependent

manner. The authors assumed that the main mechanism of action involves the inhibition of the dephosphorilation of NFATc2, a transcription factor responsible for the emergence of atopic dermatitis. The immunosuppressive effect of hirsutenone (**1**) was comparable to that of cyclosporine, a well-known calcineurin inhibitor. These results suggested that the effect of hirsutenone (**1**) on the cytokines produced by the T-cells originates from calcineurin inhibition (Joo et al. 2010).

2.3.6.5. Other effects

An extensive research was carried out on the anti-adipogenic effect *of A. hirsuta f. sibirica*: following the isolation of a new diarylheptanoid, the study indicated its inhibitory effect on the induction of peroxysome proliferator activated receptor γ protein expression and on the adipocyte differentiation in 3T3-L1 cells (Lee et al. 2013).
Anti-influenza activity was reported about platyphyllon isolated from the bark of *Alnus japonica* that showed antiviral effects against KBNP-0028 (H2N2) (Tung et al. 2010).

2.3.7. *Betula* genus (Betulaceae)

Several diarylheptanoid compounds have been identified in *Betula* species, most of them in *Betula platyphylla* Sukaczev and *Betula papyrifera* Marsh., both native to China and Japan; Besides, *Betula pendula* Roth., which is native to Hungary has also been reported to accumulate diarylheptanoid-type secunder metabolites (Matsuda et al. 2008).

2.3.7.1. Antifibrotic activity

A chemical investigation of the n-butanol fraction of the inner bark of *Betula platyphylla* led to the isolation of seven diarylheptanoids, (-)-centrolobol, aceroside VII, aceroside VIII, (3R)-1,7-bis-(4- hydroxyphenyl)- 3-heptanol-3-*O*-[2,6-bis-O-(β-D-apiofuranosyl)-β-D-glucopyranoside, 1,7-bis-(4-hydroxyphenyl)-5-hepten-3-one, platyphyllone, and platyphylloside. The antifibrotic effects of these isolates were evaluated with HSC-T6 cells by assessing cell proliferation. Among them, four compounds inhibited the proliferation of HSCs in a dose dependent manner at concentrations from 10 μM to 100 μM. Based on these results, the authors concluded,

that the antifibrotic activity of *B. platyphylla* and its constituents might suggest therapeutic potential against liver fibrosis (Lee at al. 2012).

2.3.7.2. Selective cytotoxic activity

The methanolic extract of *Betula papyrifera* bark showed notable and selective cytotoxic activity *in vitro* on human lung carcinoma and colorectal adenocarcinoma cells. The extract was reported to contain diarylheptanoids (papyriferoside, platyphylloside), lignans and flavonoids (Mshvildadze et al. 2007).

2.4. *Corylus avellana* L. (Common hazel, Betulaceae)

2.4.1. Ethimology

The scientific name *avellana* was selected by Linnaeus: it derives from Leonhart Fuchs's *De historia stirpium commentarii insignes* (1542), where the species was described as "*Avellana nux sylvestris*" that means "wild nut of Avella", from the Italian town of Avella well known for its nuts flourishing production. Whereas the English name hazel derives from the Anglo-Saxon 'haesel knut', where haesel means cap or hat, and refers to the papery cap of leaves on the nuts (Mitchell 1982).

2.4.2. Taxonomic classification

The hazels (*Corylus*) are a genus of 14-18 species of deciduous trees and large shrubs native to the temperate Northern Hemisphere. It comprises three species native to Hungary: the common hazel (*Corylus avellana* L.), the Turkish hazel (*Corylus colurna* L.) and the large filbert (*Corylus maxima* Mill.). *C. avellana* is a large shrub, widely distributed throughout Europe, reaching as far east as the Ural Mountains in Russia, and from Scandinavia in the north to Spain, Italy and Greece in the south. In Hungary it occurs frequently in Transdanubia. Within its large distribution it grows unevenly and it typically grows as an underbrash component of deciduous forests, together with oaks and conifers. It is also cultivated for its edible nuts (Bruneton 2001, Simon 2000, Launert 1986).

2.4.3. Morphology

C. avellana is a large shrub, rarely grown as a small tree, with a smooth reddish-brown peeling bark. The twigs are densely covered with reddish glandular hairs. The leaves are alternate, simple, petiolate (the petiole is 0.4–1.1 cm long, covered with glandular hairs), almost globular from a heart-shaped base, with the apex acuminate, shallowly lobed or double-toothed / serrulate, pubescent or glabrous. The flowers of each sex are different inflorescences: the male flowers in 1–4 pendulous catkins up to 8 cm long with 4 stamens. There are only a few female flowers, arranged in erect short spikes up to 6 mm long, with red styles. It blooms from January to March. The fruit is a nut, globose or ovoid, up to 2 cm wide, surrounded by a lobed green involucre (Simon 2000, Launert 1986) (Fig. 6).

Figure 6. *Corylus avellana* L. (http://www.wikipedia.org)

2.4.4. Traditional use

The main products of *C. avellana* are kernels, mainly utilized by the confectionary industry, while the leaves have been used for phlebitis, varicose veins and haemorrhoidal symptoms, and also for their slight anti-microbial effect (Bruneton 2001, Fraisse 1999, Frerichs 1925). The buds have been applied in gemmotherapy against hepatic, respiratory, circulatory and inflammatory diseases (Peev et al. 2007). In Hungarian traditional folk medicine the catkin has been used for the treatment of fever, while kernels as a haematogenous remedy (Simon 2000). In Britain the dried or fresh leaves have been used for stimulating bile production. Infusion prepared from the leaves has been taken for 3–4 times a day against diarrhoea (Launert 1986). In traditional medicine hazel bark has been used as a remedy for intermittent fever. Veterinary use is

also known: the pollen has been used against diarrhoea (Dragendorff 1898). Hazelnut oil is used in present days not only as food but also in cosmetics, for its astringent and emollient properties (Contini 2011).

C. avellana was already cultivated by the ancient Greeks and Romans, remains of hazel were explored at the ruins of Pompeii. Its popularity is announced by the fact that it was referred to by numerous ancient sources, such as Hippocrates, Theophrastus, Diuscurides, Cato, Plinius, Vergilius, Arabic and even Chinese authors (Hegi 1957, Dragendorff 1898). In the antiquity it was used as a remedy for sore throat, chronic cough and baldness. Several habits and believes were attached to hazel. It was believed by the Celts to give wisdom and inspiration: this probably explains the similarity between the Gaelic word for nut, '*cno*' and the word for wisdom, '*cnocach*'. Furthermore, "The Hazel Branch" from Grimms' Fairy Tales claims that hazel branches offer the greatest protection from snakes and other things that creep on the earth (Contini 2011).

2.4.5. Pharmacological effects

The ethanolic extract of *C. avellana* kernels showed significant antioxidant activity in total antioxidant and radical scavenging tests, which can be the consequence of the relatively high total phenolic content of the extract (Delgado et al. 2010). The antioxidant capacity of hazel byproducts, such as tree leaves has also been evaluated and was found to possess even higher activity than the kernels (Shahidi et al. 2007). Hazelnut-enriched diet has been proved to contribute to human nutrition and health as a source for natural antioxidant supplementation and for its effects on plasma cholesterol and lipoprotein profile. Frequent consumption of nuts, in fact, is associated with favourable plasma lipid profiles and has been proved to reduce risk of coronary heart disease. The results of this study, led on fifteen hypercholesterolemic men, showed a decrease of the concentration of VLDL cholesterol (29.5%), triacylglycerol (31.5%) and apolipoprotein (9.2%) while it showed an increase on HDL cholesterol concentrations (12.6%). (Mercanligil et al. 2006, Andreoni et al. 2001, Durak et al. 1999). *In vitro* antimicrobial activity *C. avellana* was also proved (Oliveira et al. 2007). Two studies regarding determination of the hazelnut's antimicrobial potential have been carried out, one with hazelnut kernels (Oliveira et al. 2008) and one with the leaves (Oliveira et al.

2007). In both studies their antimicrobial activity was checked against Gram-positive (*Bacillus cereus, B. subtilis, Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa. Escherichia coli. Klesbiella pneumoniae*) and fungi (*Candida albicans, Cryptococcus neoformans*) (Oliveira et al. 2008). When comparing both studies, hazelnut kernels and leaves showed different antimicrobial activities. In the case of leaves, the samples revealed antimicrobial activity against all microorganisms with exception of *P. aeruginosa* and *C.albicans*. In the case of aqueous extracts of hazelnut kernels, high antimicrobial activity was only found against Gram-positive bacteria (*B.cereus, B.subtilis, S. aureus*) (Ramalhosa et al. 2011). The leaf of *C. avellana* is official in the 10th edition of the French Pharmacopoea.

2.4.6. Phytochemical characterisation

In whole hazelnut kernels, as the main products of hazel harvesting, flavan-3-ols, benzoic acids, flavonols and a dihydrochalcone were reported. The main phenolic compounds were identified as: myricetin-3-*O*-rhamnoside, quercetin-3-*O*-rhamnoside, gallic acid, protocatechuic acid, phloretin-2'-*O*-glucoside, (+)-catechin, (-)-epicatechin and procyanidins (Jakopic et al. 2011). The occurrence of caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid in hazelnut kernels was also proved (Shahidi Et al. 2007, Alasalvar Et al. 2006). Hazel kernels contain 50–60% oil with the main fatty acids being oleic acid, linoleic acid, palmitic acid, stearic acid and smaller amounts of palmitoleic acid, linolenic acid and margaric acid (Bruneton 2001). Monounsaturated fatty acids are the most abundant, followed by polyunsaturated fatty acids, while saturated fatty acids were only minor components (Bachetta et al 2013).

The previous investigations on the phenolic constituents of *C. avellana* leaves focused on the main flavonoid and caffeic-acid derivatives (Amaral et al., 2005). The occurrence of myricetin-3-*O*-rhamnoside, quercetine-3-*O*-rhamnoside, kaempferol 3-*O*-rhamnoside, 3-caffeoylquinic acid, 5-caffeoylquinic acid, caffeoyltartaric acid, *p*-coumaroyltartaric acid in hazelnut leaves has been proved and the seasonal variations in the phenolic profile have been studied by Amaral et al. (Amaral et al. 2010, Amaral et al. 2005).

The pollen of common hazel in general is characterized by accumulation of large amounts of secondary plant products, especially carotenoids and flavonoids. The main

flavonoid glycosides from the pollen of *Corylus avellana* have been characterised as quercetin 3-*O*-glycosides (quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rhamnoside) (Starck et al. 1984). Two hydroxycinnamic acid amides from the pollen of *C. avellana* have been identified as (E)-caffeoyl-(E)-feruloyspermidine and di-(E)-feruloyspermidine. Production of these acylated amines may be linked to the physiology of flowering. They may also play a fundamental role as antiviral agents or antibiotics against bacteria. Starck et al. and Hoffman et al. reported detection of the antitumour drug paclitaxel and other related taxanes in stems and branches of hazel (Starck et al. 1984, Hoffman et al. 1998).

2.5. *Corylus colurna* L. (Turkish hazel, Betulaceae)

2.5.1. Morphology

C. colurna is the largest species of Hazel, reaching 25 m tall, the crown is slender and conical at the beginning, and becomes broad with age. The bark is pale grey and shiny, with a thick texture. The deciduous leaves are rounded and can be around 6-15 cm long; leaves are softly hairy on both surfaces with a coarsely double-serrate to shallowly lobed margin. The flowers are produced very early in spring before the leaves, and are monoecious, with single-sex catkins; the male is 5-10 cm long and pale coloured, the female is small and largely concealed in the buds. The fruit is a 1-2 cm long nut, held within a deeply fringed husk which encloses all but the tip of the nut; the nuts are carried in groups of 3-8 in tight clusters, with the involucres fused at the base (Rushforth et al. 1986) (Fig.7).

Figure 7. *Corylus colurna* L. (http://www.wikipedia.org)

2.5.2. Traditional use

The nuts, smaller than common hazel nuts, are edible, but the hard, thick nut shell reduced their commercial value. However this tree is important in commercial hazelnut orchards and is widely cultivated as an ornamental tree in Europe and North America.
Its popularity has increased thanks to its tolerance of difficult growing, particularly in urban planting schemes.

2.5.3. Pharmacological effects

C. colurna leaves extracts (prepared with petroleum ether, dichloromethane, methanol and water) has been reported to posses antibiotic activity against Gram positive bacteria (*Staphylococcus aureus, Enterococcus faecalis, Bacillus subtilis, Listeria monocytogenes*) and Gram negative bacteria (*Escherichia coli, Salmonella typhimurium* and *Klebsiella pneumonia*) by Ceylan et al (Ceylan et al. 2103). The results showed that the dichloromethane extract of *C. colurna* had the highest antimicrobial activity against *S. typhimurium*.
The antioxidant and the free radical scavenging activities of a natural mixture of flavonoids (F_4) have been investigated by Benov et al (Benov and Georgiev 1994). The composition of F_4 was not reported. The mixture was found to possess strong antioxidant activity comparable to that of quercetin and myricetin and higher than that of rutin. It was found to be a potent superoxide radical scavenger, more efficient than quercetin and myricetin or their mixture. It is also a strong inhibitor of ischaemia/reperfusion-induced peroxydation in the brain and liver. In addition to these advantages the mixture had low toxicity and could be readily isolated. The authors could conclude that these advantages make it an alternative to the more toxic synthetic antioxidants for the treatment of pathological conditions in which free radicals are involved.
Hepatoprotective effects of the same natural mixture of flavonoids isolated from *Corylus colurna* were studied on 30 male albino rats with tetrachloromethan - induced hepatitis by Radev et al. (Radev et al. 1997). The authors concluded that the flavonoids possessed hepatoprotective effect similar or identical to that of Carsyl. This effect was most probably due to the decreased cell- membrane permeability and the high antioxidant activity inherent in these compounds.

2.5.4. Phytochemical characterisation

The detailed investigation on the previously mentioned mixture of flavonoids (F_4) has not been reported, besides, no data has been found about the phenolic profile of *C. colurna*.

The fatty acid composition and some physical properties of nuts of *C. colurna* were investigated by Erdogan et al (2005). Average nut weight, diameter, shell thickness, percent kernel, and moisture content were determined. Based on the results of this study, oil content ranged between 64.48% and 71.92%. Oleic and linoleic acids were found to be the predominant fatty acids, together representing 91.7% of the total. The amount of palmitic and stearic acids was low while palmitoleic, margaric, margaroleic, linolenic, arachidic, and gadoleic acids were present in trace amounts.

2.6. *Corylus maxima* Mill. (Filbert, Betulaceae)

2.6.1. Ethymology

The term 'filbert' derives from Norman French: the plant was possibly renamed after Saint Philibert's feast day, since it ripens about August 20 in England.

2.6.2. Morphology

C. maxima is a deciduous shrub 6-10 m tall. The green leaves are rounded, 5-12 cm long by 4-10 cm broad, with a coarsely double-serrated margin. The flowers are catkins that grow in late winter and are wind-pollinated; the male catkins are pale yellow, longer than the female catkins which are bright red and only 1-3 mm long. The fruits are edible nuts, similar to the hazelnut, produced in clusters of 1-5 together; each nut is covered by a tubular and it is 1.5-2.5 cm long. (Rushforth 1999).

Filbert is a close relative of the common hazelnut (*Corylus avellana*) but it differs in having the nut fully enclosed by the hask, longer and narrower (Fig. 8).

Figure 8. *Corylus maxima* Mill.

2.6.3. Traditional use

The filbert is often cultivated for its edible seeds which contains up to 65% of non-drying oil, used in paints and cosmetics, whole seeds are very effective in polish use. Seeds are large and well flavoured, they are usually eaten raw, cooked in cakes and pies or used to obtain the edible oil. Filbert leaves have been used in Turkish traditional medicine both internally and externally; in a decoction for eczemas, and the pieces of leaves for treating the swelling and rash (Amaral et al. 2010).

2.6.4. Phytochemical characterisation

No previous literature data has been found dealing with the phytochemical composition of *C. maxima*.

3. OBJECTIVES

The primary aim of our study was to characterise the phenolic profile of the *Corylus* species native to Hungary: *Corylus avellana* L, *Corylus colurna* L. and *Corylus maxima* Mill. with particular attention to diarylheptanoids, on the one hand in order to screen for structural analogues of diarylheptan derivatives presented in different Betulaceae plants, concerning their various beneficial pharmaceutical effects; on the other hand to revaluate the phytotherapeutic potential of these plants. Literature data on the phenolic composition of the *Corylus* species mentioned above is limited to the kernels and leaves of *C. avellana* focusing on caffeic acid and flavonoid derivatives and not concerning diarylheptanoid compounds. The phenolic composition of *C. colurna* and *C. maxima* has not been studied in details yet. Considering the fact that in the bark of numerous Betulaceae plants a wide range of pharmaceutically important natural compounds, such as diarylheptanoids, were reported, we aimed the phytochemical investigation of both the leaves and bark of the three *Corylus* species.

1. In order to gain preliminary information about the phenolic constitution of the *Corylus* crude drugs, we aimed to determine their total phenolic, flavonoid, and tannin contents according to the methods of Ph. Hg. VIII.

2. Plant phenolics are widely reported to act as antioxidants by scavenging free radicals, thus comparison of *in vitro* scavenging activity of the *Corylus* extracts prepared with ethyl acetate and methanol to those of well-known antioxidant phenolics was chosen as the starting point for further analyses.

3. For qualitative analysis of the phenolic composition of the *Corylus* extracts we aimed to optimise high-performance liquid chromatographic separation of the compounds and acquire structural information about the constituents by different detection methods: diode array detection, electrospray ionisation-time-of-flight mass spectrometry and electrospray ionisation-tandem mass spectrometry. Since literature

data on the structural characterisation of diarylheptanoids by mass spectrometry is limited, we also aimed detailed investigation of their mass spectrometric behaviour.

4. Our objective was to develop and validate high-performance liquid chromatographic-tandem mass spectrometric methods for the quantitative determination of the main phenolic compounds in the *Corylus* extracts.

5. We aimed to reveal the contribution of certain compounds of the *Corylus* extracts to the antioxidant activity by developing a high-performance liquid chromatographic-mass spectrometric method coupled with DPPH free radical scavenging assay.

4. MATERIALS AND METHODS

4.1. Plant material

- Leaves and bark of *Corylus avellana* L. were collected in Nógrád, Nógrád county, Hungary (June 2010).
- Leaves and bark of *Corylus colurna* L. were collected in Göd, Pest county, Hungary (August 2012).
- Leaves and bark of *Corylus maxima* Mill. were collected in Pálfiszeg, Zala county, Hungary (August 2013).

For all the plant material mentioned above, 50-50 g samples were collected from three trees after flowering stage. Plant samples were authenticated in the Department of Pharmacognosy, Semmelweis University, Budapest, where voucher specimen are deposited.

4.2. Solvents and chemicals

Hirsutenone, oregonin, myricetin-3-*O*-rhamnoside, quercetin-3-*O*-rhamnoside, rosmarinic acid, quercetin, kaempferol, ascorbic acid, trolox and caffeic acid standards, DPPH and ABTS free radicals, Folin - Ciocalteu's reagent, potassium peroxodisulfate, aluminium chloride, hexamethylenetetramine and HPLC gradient grade methanol were acquired from Sigma-Aldrich (St. Louis, USA). Hide powder was supplied by Filk GmbH (Freiburg, Germany). HPLC grade acetonitrile were purchased from Merck (Darmstadt, Germany). *n*-Hexane, chloroform, ethyl acetate, acetic acid, acetone, methanol, hydrochloric acid of reagent grade and spectroscopic grade ethanol were purchased from Reanal-Ker (Budapest, Hungary). HPLC grade water was prepared with a Millipore Direct Q5 water purification system (Bedford, MA, USA). All aqueous eluents for HPLC were filtered through MF-Millipore membrane filters (0.45 µm, mixed cellulose esters) (Billerica, MA, USA) and degassed in an ultrasonic bath before use.

4.3. Extraction and sample preparation

Soxhlet extraction was performed using a laboratory-scale apparatus. Dried and milled plant samples (10 g each) were extracted consecutively with 250-250 ml of *n*-hexane at 70 ºC, chloroform at 65 ºC, ethyl acetate at 80 ºC and methanol at 65 ºC, for 6 h each. The extracts were evaporated to dryness under reduced pressure in a rotary evaporator at 50 ºC. The dried extracts were dissolved in HPLC grade methanol to obtain sample solution concentrations of 25-30 mg/ml. The solutions were filtered through Phenex-RC 15 mm, 0.2 µm syringe filters (Gen-Lab Ltd, Budapest, Hungary).

4.4. Quantitative phytochemical analyses

Determination of total phenolic, tannin and flavonoid contents of the *Corylus* samples was performed according to the guidance of Ph. Hg. VIII. (2003). Measurements were carried out in three parallels. Total polyphenol and tannin contents are expressed as pyrogallol, and flavonoid content as hyperoside (g/100 g dry plant sample).

4.5. Antioxidant activity assays

Antioxidant activity of the *Corylus* extracts and hirsutenone, quercetin, kaempferol, caffeic acid, ascorbic acid, myricetin-3-*O*-rhamnoside and trolox standards was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS {2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)}, as free radicals. The method developed by Re et al. (1999) and improved by Arts et al. (2004), as well as the method described by Brand-Williams et al. (1995) were applied with some modifications. Stock solutions were prepared by dissolving 10 mg of DPPH in 25.0 ml HPLC grade methanol and 10 mg ABTS in 2.6 ml HPLC grade water. To produce ABTS radical cation ($ABTS^{\bullet+}$), stock solution was reacted with 1.7 mg potassium peroxodisulfate. The solutions were diluted just before measuring by HPLC methanol and spectroscopic grade ethanol, respectively, so that the absorbance of the diluted free radical solutions was 0.900 (±0.005). In their radical forms, DPPH and ABTS have absorption maxima at characteristic wavelengths of 515 nm and 734 nm, respectively. During the assay, 50 µl of the samples of five different concentrations, in three parallels were added to 2.5 ml of

the free radical solutions. The decrease of the absorbance was measured at 30, 40, 50, 60, 90, 120, 150, 180, 210, 240, 300 and 360 seconds by a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan) at the characteristic wavelengths. The absorbance was recorded against a blank sample (methanol and ethanol for DPPH and ABTS, respectively). The final absorbance (at t=360 sec) was extrapolated by numerically solving the simplest possible reaction kinetics model. The percentage of free radical scavenging activity was calculated as: Inhibition % = $(1\text{-}A/A_0)\cdot 100\%$, where A=absorbance of the free radical solution after reacting with the sample and A_0=absorbance of the solution containing only the free radical. The percentages of inhibition were plotted vs. the concentrations. The concentrations belonging to the half maximal inhibition (IC_{50} value as μg/ml), were determined by linear regression. Data were analyzed at significance level of $p < 0.05$. The results are expressed as mean values and standard deviation (SD). Comparison between the different *Corylus* extracts was made by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc HSD test. Differences with $p < 0.05$ between experimental groups were considered statistically significant.

4.6. Characterisation of phenolics in the *Corylus* extracts by HPLC-DAD-MS

4.6.1. HPLC-DAD-QMS conditions

For chromatographic separation an Agilent 1100 HPLC system (G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector) was used (Agilent Technologies, Waldbronn, Germany). The *Corylus* samples were separated on a Kinetex-XB C18 column (150 × 4.6 mm, 2.6 μm; Agilent Technologies, Waldbronn, Germany) maintained at 40°C. Injection volume was 10 μl. The following gradient elution program was applied at a flow rate of 0.5 ml/min; where eluent A was 0.2 % (v/v) acetic acid, eluent B was acetonitrile:

- 0 min: 0% (v/v) B
- 5 min: 5% (v/v) B

- 15 min: 10% (v/v) B
- 30 min: 20% (v/v) B
- 60 min 50% (v/v) B
- 70 min 100% (v/v) B

Chromatograms were acquired at 254, 280 and 350 nm, as the most selective wavelengths for the detection of flavonoids and diarylheptanoids. UV spectra were recorded between 200 and 400 nm.

Mass spectrometric analyses were performed on an Agilent 6120 single quadrupole system equipped with an electrospray ion source (ESI). The Agilent MassHunter B.01.03 software was used for data acquisition, and for qualitative analyses. Mass spectrometric parameters were as follows:

- Ion source: ESI, negative
- Drying gas (N_2) temperature: 350°C
- Drying gas (N_2) flow rate: 12 l/min
- Nebuliser gas (N_2) pressure: 10 psi
- Fragmentor voltage: 120 V
- Capillary voltage: 4000 V

During the LC-MS analyses ESI was operated in the negative ion mode, which provided better sensitivity due to the phenolate group of the investigated compounds.

4.6.2. HPLC-DAD-TOF-MS conditions

Accurate mass data were acquired on an Agilent 6230 time-of-flight mass spectrometer equipped with a Jet Stream electrospray ion source (ESI) (Agilent Technologies, Palo Alto, CA). Samples were introduced by the Agilent 1260 Infinity HPLC system (G1312B binary gradient pump, G1367E autosampler, G1315C diode array detector)

(Agilent Technologies, Waldbronn, Germany). Mass spectra were processed by Agilent MassHunter B.03.01 software.

The *Corylus* samples were separated on a Zorbax SB C18 column (150 × 3.0 mm, 3.5 µm; Agilent Technologies, Waldbronn, Germany) maintained at 25°C. Injection volume was 10 µl. The following gradient elution program was applied at a flow rate of 0.4 ml/min; where eluent A was 0.2 % (v/v) acetic acid, eluent B was methanol:

- 0 min: 30% (v/v) B
- 20 min: 100% (v/v) B
- 22 min: 100% (v/v) B
- 23 min: 30% (v/v) B

Chromatograms were acquired at 254, 280 and 350 nm, as the most selective wavelengths for the detection of flavonoids and diarylheptanoids. UV spectra were recorded between 200 and 400 nm.

Time-of flight mass spectrometric parameters were as follows:

- Ion source: ESI, negative
- Drying gas (N_2) temperature: 325 °C,
- Drying gas (N_2) flow rate: 10 l/min
- Nebuliser gas (N_2) pressure: 10 psi
- Sheath gas flow 7.5 l/min
- Sheath gas temperature: 325 °C
- Fragmentor voltage: 170 V
- Capillary voltage: 4000 V
- OCT 1 RF Vpp: 750 V.

Full-scan mass spectra were acquired over the *m/z* range of 100-2500 at an acquisition rate of 250 ms/spectrum. Reference masses of *m/z* 112.9855 and 1033.9881 were used to calibrate the mass axis during analysis.

4.6.3. HPLC-DAD-MS/MS conditions

For chromatographic separation an Agilent 1100 HPLC system (G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector) was used (Agilent Technologies, Waldbronn, Germany). The same column maintained at 25 °C, as well as the same eluents and gradient program at a flow rate of 0.3 ml/min were utilised as for HPLC-TOF-MS analyses of the extracts (see section **4.6.2.**).

Tandem mass spectrometric (MS/MS) analyses were performed on an Agilent 6410 triple quadrupole system equipped with an electrospray ion source (ESI) (Agilent Technologies, Palo Alto, CA). The Agilent MassHunter B.01.03 software was used for data acquisition, and for qualitative and quantitative analyses.

Triple quadrupole mass spectrometric parameters were as follows:

- Ion source: ESI, negative
- Drying gas (N_2) temperature: 350 °C
- Drying gas (N_2) flow rate: 9 l/min
- Nebuliser gas (N_2) pressure: 40 psi
- Fragmentor voltage: 120 V
- Capillary voltage: 3500 V

Full-scan mass spectra were acquired over the *m/z* range of 50-1000 (1 scan/sec). Collision energy was changed between 10–50 eV, according to differences in molecule structures (high purity nitrogen was used as collision gas).

For structural characterisation of the compounds retention times, accurate molecular masses and calculated molecular formulas, UV and mass spectral data were compared to literature data and to those of authentic standards, where available.

4.7. Quantitative analyses by HPLC-MS/MS

Quantification of myricetin-3-*O*-rhamnoside, quercetin-3-*O*-rhamnoside, hirsutenone and oregonin in the *Corylus* extracts was performed by the external standard method. Quantities were determined by HPLC-ESI-MS/MS in MRM (multiple reaction monitoring) mode. The same HPLC-MS conditions were applied as described above in section **4.6.3.** Before quantification both fragmentor voltage and collision energy were optimized with the use of the sample extracts by parameter ramping, from 70 to 250 V, with steps of 10 V and from 5 eV up to 50 eV, with steps of 5 eV, respectively.

4.7.1. Determination of diarylheptanoids

Standard solutions for the calibration were prepared at concentrations of 0.01, 0.1, 1, 10, and 50 μg/ml, quality control samples at concentrations of 0.01, 1 and 50 μg/ml of hirsutenone authentic standard in gradient grade methanol. Oregonin standard solutions were prepared at concentrations of 0.1, 1, 10, 50 and 100 μg/ml, quality control samples at concentrations of 0.1, 10 and 100 μg/ml.

The quantifier and qualifier ions were designated by the evaluation of the mass spectra of the compounds. For hirsutenone the fragmentor voltage was set to 120 V, collision energy to 10 eV. Fragment ion *m/z* 205.2 (transition *m/z* 327.1 → *m/z* 205.2) was chosen as quantifier and *m/z* 121.0 (transition *m/z* 327.1 → *m/z* 121.0) as qualifier ions, respectively. In the case of oregonin the fragmentor voltage was set to 120 V, collision energy to 10 eV. Fragment ion at *m/z* 327.1 (transition *m/z* 477.1 → *m/z* 327.1) was chosen as quantifier ion and that at *m/z* 205.2 (transition *m/z* 477.1 → *m/z* 205.2) as qualifier ion. The qualifier/quantifier ion ratio remained within the ± 20% range of the determined value.

4.7.2. Determination of flavonoids

Standard solutions for the calibration were prepared at concentrations of 1, 10, 50, 150 and 300 μg/ml, quality control samples at concentrations of 10, 150 and 300 μg/ml

using myricetin-3-*O*-rhamnoside and quercetin-3-*O*-rhamnoside authentic standards in gradient grade methanol.

The quantifier and qualifier ions were designated by the evaluation of mass spectra of the compounds. In both the case of myricetin-3-*O*-rhamnoside and quercetin-3-*O*-rhamnoside fragmentor voltage was set to 160 V and collision energy to 20 eV. The most intense product ion *m/z* 316.1 (transition *m/z* 463.0 → *m/z* 316.1) was chosen as quantifier ion and *m/z* 316.9 (transition *m/z* 463.0 → *m/z* 316.9) as qualifier ion for myricetin-3-*O*-rhamnoside, and fragment ions *m/z* 300.1 (transition m/z 447.1 → m/z 300.1) as quantifier ion and *m/z* 301.0 (transition m/z 447.1 → m/z 301.0) as qualifier ion for quercetin-3-*O*-rhamnoside. The qualifier/quantifier ion ratio remained within the ± 20% range of the determined value for each analyte.

4.7.3. Method validation – Calibration, precision, accuracy and quality control samples

Standard solutions for the calibration were prepared in triplicate and injected once. Calibration plot was constructed by plotting peak areas against corresponding concentrations. Linearity was determined by analyzing the standards at five concentrations, each in triplicate. Slope, intercept and correlation coefficient were determined by least squares polynomial regression analysis. LOD and LOQ parameters were determined at 3/1 and 10/1 signal to noise ratios, respectively. Retention time repeatability was checked with six successive runs of the *Corylus* extracts. Quality control samples were prepared in three different concentrations for each standard solution. These were used to determine both the intra-day and inter-day precision and accuracy (low, mid and high concentrations of the standards in three parallel runs on the same day and on three successive days, respectively). Blank samples (pure solvents) were analyzed to check the occurrence of any impurity or co-elution with the same *m/z* as that of the analytes.

4.8. HPLC-based DPPH scavenging assay

4.8.1. Sample preparation

100 µl of the *Corylus* extracts of certain concentrations (Table 1.) dissolved in methanol and 100 µl of the DPPH solution (1.2 mg/ml in methanol) were mixed and incubated for 30 minutes at room temperature, protected from light. Then the reaction mixture was directly analysed by HPLC-DAD-QMS (see section **4.6.1.**). The concentration of the extracts used for the analyses was adjusted by the evaluation of the chromatograms after mixing them with the DPPH solution in different concentrations within the range of 1.0-5.0 mg/ml. The concentration, where the decrease in the peak area of myricetin-3-*O*-rhamnoside (**21**), or if that was present in small amounts, that of quercetin-3-*O*-rhamnoside (**23**) reached its maximum, was chosen. The control samples were prepared by the addition of 100 µl methanol to 100 µl of the extracts.

Table 1. Sample preparation for the HPLC-based DPPH measurements

Sample name	Sample concentration (mg/ml)
C. avellana leaves EtOAc	1.9
C. avellana leaves MeOH	2.0
C. avellana bark EtOAc	2.5
C. avellana bark MeOH	1.9
C. colurna leaves EtOAc	1.6
C. colurna leaves MeOH	2.0
C. colurna bark EtOAc	1.7
C. colurna bark MeOH	1.6
C. maxima leaves EtOAc	1.8
C. maxima leaves MeOH	2.1
C. maxima bark EtOAc	2.5
C. maxima bark MeOH	2.8

The chromatograms of the control and sample solutions were compared with regard to the changes in the peak areas of certain compounds.

5. RESULTS

5.1. Quantitative phytochemical analyses

In order to gain preliminary information about the phenolic constitution of the *Corylus* crude drugs, total phenolic, flavonoid, and tannin contents were determined according to the methods of Ph. Hg. VIII. (2003). Results are presented in Table 2 as mean ± standard deviation of three parallel measurements.

Table 2. Quantitative determination of phenolics in the *Corylus* crude drugs

Quantitative determination of phenolics in the *Corylus* crude drugs (expressed in g/100g crude drug)

(Mean±SD% of three parallel measurements)

	C. avellana		*C. colurna*		*C. maxima*	
	Leaves	**Bark**	**Leaves**	**Bark**	**Leaves**	**Bark**
Total phenolic content*	2.61±0.11	2.27±0.02	0.94±0.22	2.42±0.43	2.43±0.22	3.63±0.27
Tannin content*	1.37±0.05	1.18±0.02	0.38±0.01	1.33±0.37	0.10±0.01	0.51±0.06
Flavonoid content**	1.16±0.04	1.54±0.05	0.49±0.13	0.43±0.05	0.96±0.13	0.82±0.09

*expressed as pyrogallol
** expressed as hyperoside

5.2. Antioxidant activity assays

The antioxidant activity of the *Corylus* extracts was determined by *in vitro* tests using DPPH (2,2-Diphenyl-1-picrylhydrazyl) and ABTS {2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)}, as free radicals (conditions see in text, section **4.5.**). Polyphenol constituents of the crude drugs were extracted by Soxhlet method (conditions see in text, section **4.3.**). In order to enrich the extracts in phenolic compounds, extraction with *n*-hexane and chloroform was carried out first as a purification step. Diarylheptanoid-type molecules, which are in the focus of our interest, show relatively high solubility in ethyl acetate, therefore, during the extraction procedure ethyl acetate and methanolic fractions were also prepared.

Scavenging activity of the samples of interest was compared to that of well-known antioxidant standards, a diarylheptanoid aglycone: hirsutenone, two flavonol aglycones: quercetin and kaempferol, a flavonol-glycoside: myricetin-3-*O*-rhamnoside, and caffeic acid, ascorbic acid and trolox (Tables 3 and 4.).

5.2.1. Scavenging activity on DPPH free radical

Results of the determination of scavenging activity of the *Corylus* extracts and the standards on DPPH free radical are presented in Table 3 as mean±SD of three parallel experiments.

Table 3. Scavenging activity of the *Corylus* extracts and the standards on DPPH free radical (mean±SD of three parallel experiments)

Sample	**IC_{50} (µg/ml) DPPH**
Hirsutenone	0.6±0.0
***Corylus colurna* bark ethyl acetate extract**	**2.7±0.2**
***Corylus colurna* bark methanolic extract**	**3.1±0.8**
Quercetin	3.4±0.2
Ascorbic acid	3.9±0.2
Myricetin-3-*O*-rhamnoside	4.7±0.2
Caffeic acid	5.1±0.3
Trolox	5.3±0.2
***Corylus maxima* bark methanolic extract**	**17.9±1.2**
***Corylus avellana* bark methanolic extract**	**14.4±1.0**
***Corylus avellana* leaves methanolic extract**	**15.3±0.9**
***Corylus avellana* bark ethyl acetate extract**	**15.3±1.1**
***Corylus avellana* leaves ethyl acetate extract**	**15.3±1.0**

***Corylus colurna* leaves ethyl acetate extract**	**18.0±0.8**
Kaempferol	19.1±0.9
***Corylus maxima* leaves methanolic extract**	**20.5±4.1**
***Corylus colurna* leaves methanolic extract**	**27.1±1.5**
***Corylus maxima* leaves ethyl acetate extract**	**48.0±3.9**
***Corylus maxima* bark ethyl acetate extract**	**50.7±4.3**

5.2.2. Scavenging activity on ABTS free radical

Results of the determination of scavenging activity of the *Corylus* extracts and the standards on ABTS free radical are presented in Table 4 as mean±SD of three parallel experiments.

Table 4. Scavenging activity of the *Corylus* extracts and the standards on ABTS free radical (mean±SD of three parallel experiments)

Sample	**IC_{50} (µg/ml) ABTS**
Hirsutenone	0.4±0.0
Quercetin	1.2±0.1
***Corylus maxima* bark methanolic extract**	**1.3±0.1**
Caffeic acid	1.4±0.1
***Corylus maxima* leaves methanolic extract**	**1.7±0.1**
Trolox	2.1±0.1
***Corylus colurna* bark ethyl acetate extract**	**3.7±0.2**
Ascorbic acid	3.7±0.2
***Corylus avellana* bark ethyl acetate extract**	**3.7±0.2**
***Corylus avellana* bark methanolic extract**	**3.9±0.3**

***Corylus colurna* bark methanolic extract**	**4.1±0.1**
Myricetin-3-*O*-rhamnoside	4.8±0.3
***Corylus avellana* leaves ethyl acetate extract**	**6.5±0.3**
***Corylus avellana* leaves methanolic extract**	**7.1±1.2**
***Corylus maxima* bark ethyl acetate extract**	**10.5±0.9**
***Corylus colurna* leaves methanolic extract**	**16.4±0.9**
***Corylus maxima* leaves ethyl acetate extract**	**17.2±1.1**
Kaempferol	19.1±1.3
***Corylus colurna* leaves ethyl acetate extract**	**44.1±3.8**

5.3. Characterisation of phenolics in the *Corylus* extracts by HPLC-MS

A RP-HPLC method was developed for the separation of phenolics in the *Corylus* extracts (see section **4.6.2.**), which provided a uniform platform for the investigation of both flavonoid and diarylheptanoid-type compounds (see the chromatograms in Figures 9-20.). The efficiency of the applied chromatographic method (see section **4.6.2.**), proved to be outstanding: even circa 100 (CMBM) chromatographic peaks could have been separated in 60 minutes. For the characterisation of the compounds UV spectral data, obtained by LC-DAD, accurate molecular mass and formula, acquired by LC-ESI-TOF and fragmentation pattern, given by LC-ESI-MS/MS analyses were compared to those of authentic standards and to literature data (Ablajan et al. 2006, Jiang et al. 2006, Ma et al. 1997). In the case of those compounds with matching standards, identification was performed also by spiking the sample solutions with the standards in two different chromatographic systems (see sections **4.6.1.** and **4.6.2.**).

Note that numbering of the compounds refers to the order of discussion of the structural characterisation (see section **6.3.**) which is based on structural groups, not on plant species.

5.3.1. *Corylus avellana* L.

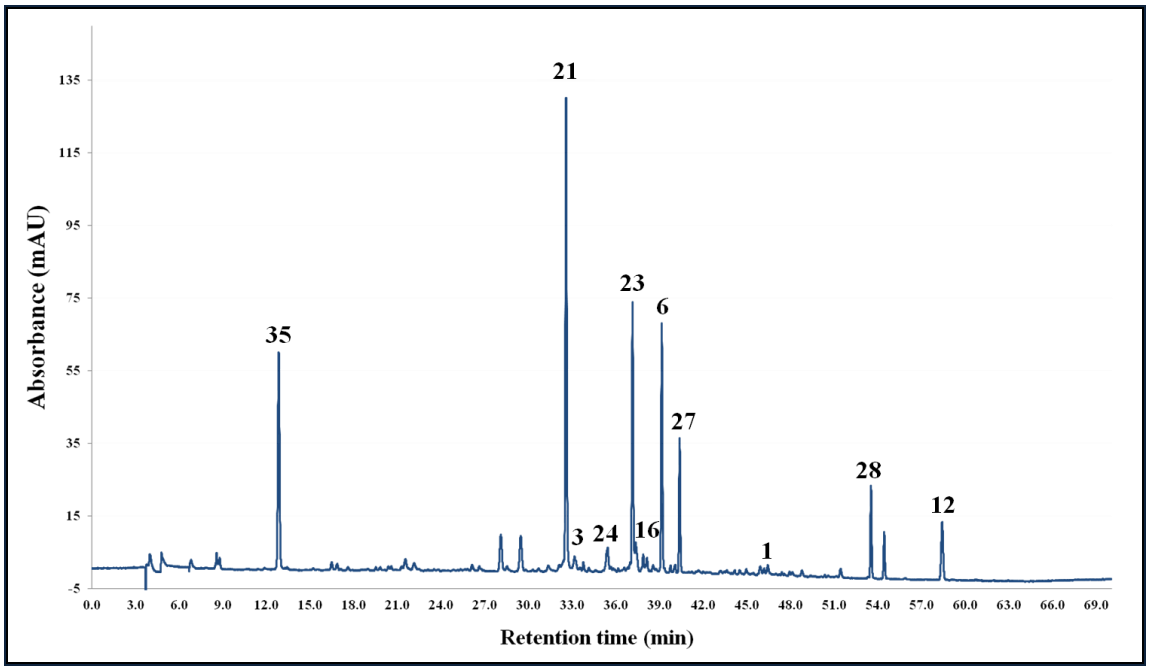


Figure 9. HPLC-UV chromatogram of *C. avellana* leaves ethyl acetate extract (see the chromatographic method in section **4.6.1**.).

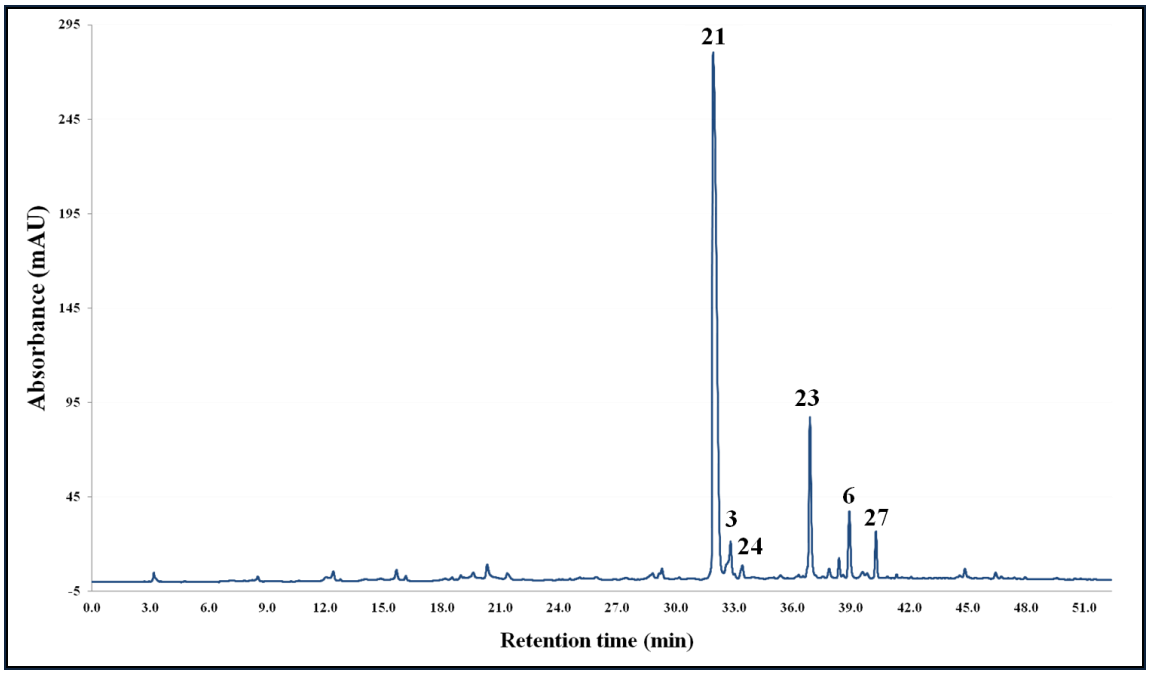


Figure 10. HPLC-UV chromatogram of *C. avellana* leaves methanolic extract (see the chromatographic method in section **4.6.1.**).

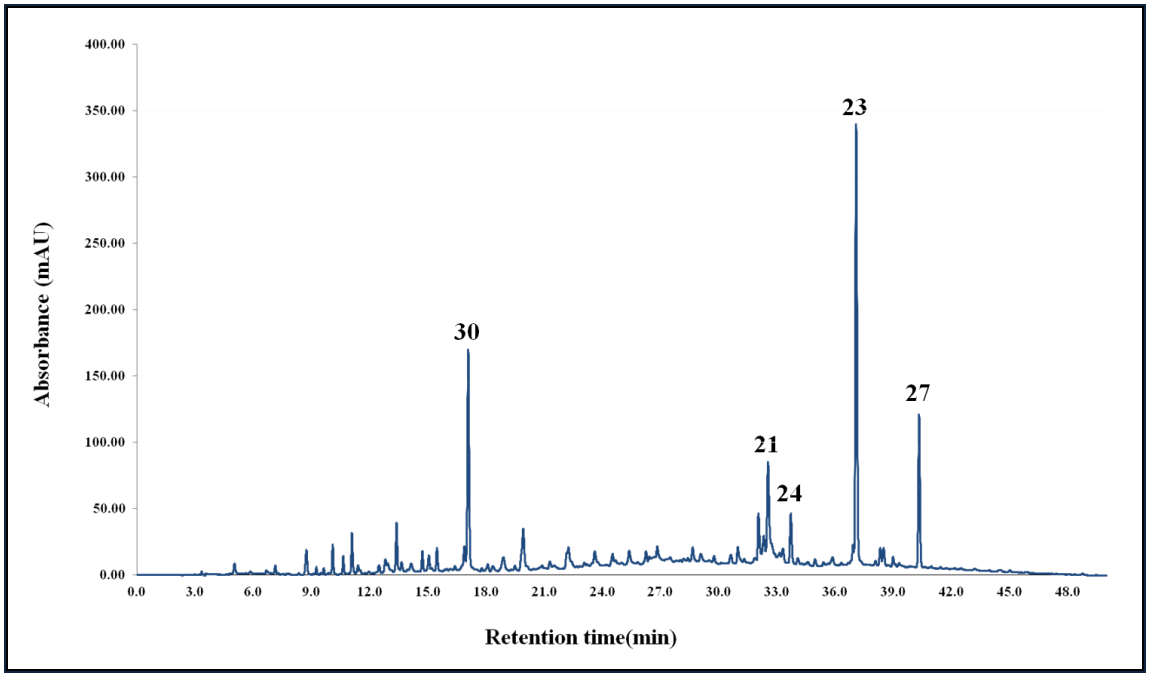


Figure 11. HPLC-UV chromatogram of *C. avellana* bark ethyl acetate extract (see the chromatographic method in section **4.6.1.**).

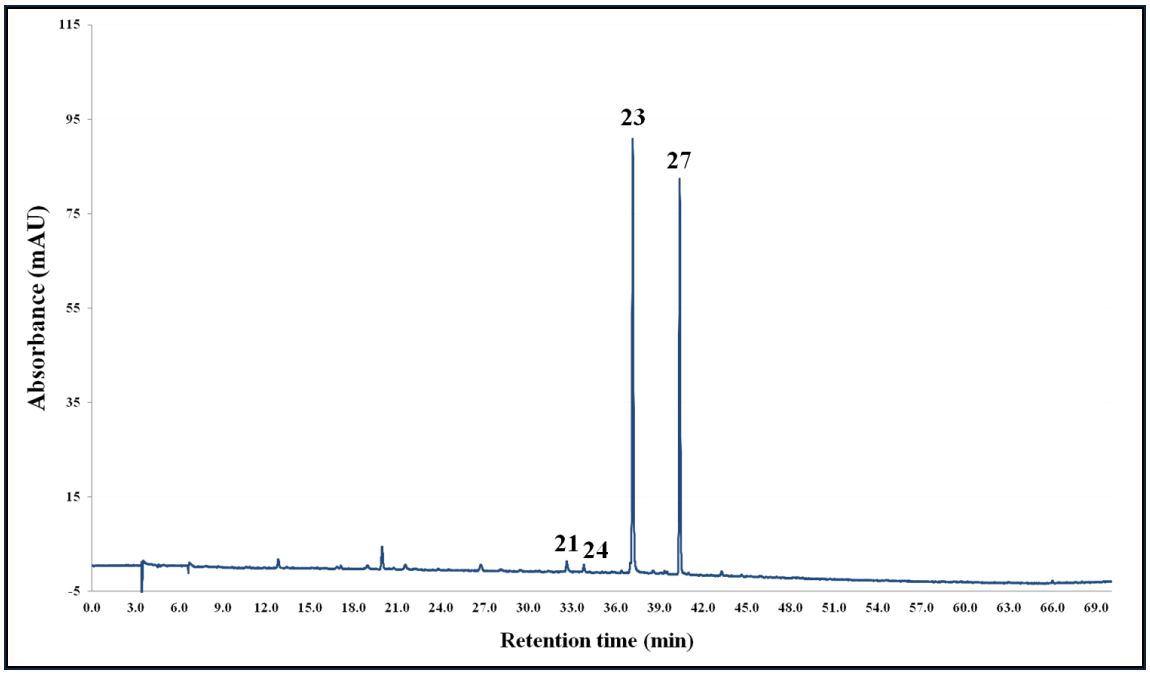


Figure 12. HPLC-UV chromatogram of *C. avellana* bark methanolic extract (see the chromatographic method in section **4.6.1.**).

The studies on the extracts of *C. avellana* leaves revealed that flavonoids were the major constituents of the samples, but in accordance with our presumptions, diarylheptanoid-type compounds were also detected (Table 5, Figures 9-12). In the methanolic extract six flavonoid-glycosides (compounds **21, 24, 27, 28**), five diarylheptanoids (compounds **1, 3, 6, 12, 14**) and two caffeic acid derivatives (compounds **30** and **31**) were detected. Among them myricetin-3-*O*-hexoside (**22**), myricetin-3-*O*-rhamnoside (**21**), quercetin-3-*O*-hexoside (**24**), quercetin-3-*O*-rhamnoside (**23**), kaempferol-3-*O*-rhamnoside (**27**), kaempferol–di(desoxyhexoside) (**28**) Platyphyllonol-hexoside (**8**), 5-hydroxy-1,7-bis-(4-hydroxyphenyl)-hept-6-en-3-one-hexoside (**6**), 1,7-bis-(3,4-dihydroxyphenyl)-4-hepten-3-one (hirsutenone) (**1**), 1,7-bis-(4-hydroxyphenyl)-4,6-heptadien-3-one (**12**), rosmarinic acid (**35**) and a caffeic acid derivative (**30**) were characterised. Based on chromatographic data the main compound of the extract was myricetin-3-*O*-rhamnoside (**21**). In the ethyl-acetate extract of *C. avellana* leaves, besides five flavonoids (compounds **21, 23, 24, 27, 28**), rosmarinic acid (**35**) and six diarylheptanoids (compounds **1, 3, 6, 12, 17, 18**) also revealed in the methanolic extract, four other diarylheptanoids: compound **16**, 5-hydroxy-1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-heptan-3-one (**14**), 5-hydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-one (**15**), 1,7-bis-(4-hydroxyphenyl)-4-hepten-3-one-hexoside (**6**) were detected. The main compound of this extract was also identified as myricetin-3-*O*-rhamnoside.

In the extracts of *C. avellana* bark four flavonoid glycosides (compounds **21, 23, 24, 27**), and a caffeic acid derivative (**30**) were identified. In contradiction to our expectations, neither in the methanolic nor in the ethyl acetate extracts of the bark were diarylheptanoid-type molecules detected. During the analyses of the chromatograms quercetin-3-*O*-rhamnoside (**23**) and kaempferol-3-*O*-rhamnoside (**27**) were found as the most abundant constituents in the ethyl acetate extract. In the methanolic extract a caffeoyl-hexoside derivative (**30**) was predominant.

The presence of myricetin-3-*O*-rhamnoside (**21**) quercetin-3-*O*-hexoside (**24**) quercetin-3-*O*-rhamnoside (**23**) and kaempferol-3-*O*-rhamnoside (**27**) was found to be characteristic for both the leaves and bark extracts (Riethmüller et al. 2013).

Table 5. Phenolic compounds detected in the *C. avellana* extracts (Note that the results of HPLC-DAD-QMS, HPLC-DAD-TOF-MS and HPLC-DAD-MS/MS experiments are combined, LE: leaves ethyl acetate extract, LM: leaves methanolic extract, BE: bark ethyl acetate extract BM: bark methanolic extract)

t_R (min)	$[M-H]^-$ m/z calculated	$[M-H]^-$ m/z experimental	Error (ppm)	Molecular formula	Fragment ions	Compound	No.	LE	LM	BE	BM
12.8	359.1136	359.1126	2.84	$C_{19}H_{20}O_7$	329,269,191,161	**Rosmarinic acid**	(35)	x	x		
16.7	377.0885	377.0875	-1.47	$C_{18}H_{18}O_9$	341,215,179,161	**Caffeic acid derivative**	(30)		x	x	x
31.5	479.0831	479.0821	2.27	$C_{21}H_{20}O_{13}$	317,316,271,255	**Myricetin-3-*O*-hexoside**	(22)		x		
32.6	463.0882	463.0871	2.28	$C_{21}H_{20}O_{12}$	317,316,271,255	**Myricetin-3-*O*-rhamnoside**	(21)	x	x	x	x
33.1	507.1872	507.1855	3.24	$C_{25}H_{32}O_{11}$	327,205	**Hirsutanolol-hexoside**	(3)	x	x		
33.7	463.0882	463.0871	2.28	$C_{21}H_{20}O_{12}$	301,300,271,179,151	**Quercetin-3-*O*-hexoside**	(24)	x	x	x	x
37.1	447.0933	447.0919	3.20	$C_{21}H_{20}O_{11}$	301,300,179,151	**Quercetin-3-*O*-rhamnoside**	(23)	x	x	x	x
37.3	343.1187	343.1173	3.86	$C_{19}H_{20}O_6$	325,269,239,211	**Diarylheptanoid aglycone 1**	(16)	x			
37.9	327.1238	327.1228	3.15	$C_{19}H_{20}O_5$	309,239,211,193	**Diarylheptanoid aglycone 2**	(17)	x			
38.1	311.1289	311.1278	3.32	$C_{19}H_{20}O_4$	211	**Diarylheptanoid aglycone 3**	(18)	x	x		
38.5	329.1394	329.1339	-2.39	$C_{19}H_{22}O_5$	179,165,149,121	**5-hydroxy-1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-heptan-3-one**	(14)	x			
38.5	329.1394	329.1339	-2.39	$C_{19}H_{22}O_5$	179,165,149,121	**5-hydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-one**	(15)	x			
39.1	473.1817	473.1797	4.18	$C_{25}H_{30}O_9$	293,89	**5-hydroxy-1,7-bis-(4-hydroxyphenyl)-hept-6-en-3-one-hexoside**	(6)	x	x		
39.7	475.1974	475.1952	3.96	$C_{25}H_{32}O_9$	295,189,89	**Platyphyllonol-hexoside**	(8)	x			
40.3	431.0984	431.0939	3.52	$C_{21}H_{20}O_{10}$	285,284,255,227	**Kaempferol-3-*O*-rhamnoside**	(27)	x	x	x	x
46.4	327.1238	327.1228	3.04	$C_{19}H_{20}O_5$	254,205	**Hirsutenone**	(1)	x	x		
53.5	577.1351	577.1335	2.96	$C_{36}H_{26}O_{12}$	514,285,284,255,227	**Kaempferol-di(desoxyhexoside)**	(28)	x	x		
58.4	293.1172	293.1183	3.76	$C_{19}H_{18}O_3$	251,225,211,83	**1,7-bis-(4-hydroxyphenyl)-4,6-heptadien-3-one**	(12)	x	x		

5.3.2. *Corylus colurna* L.

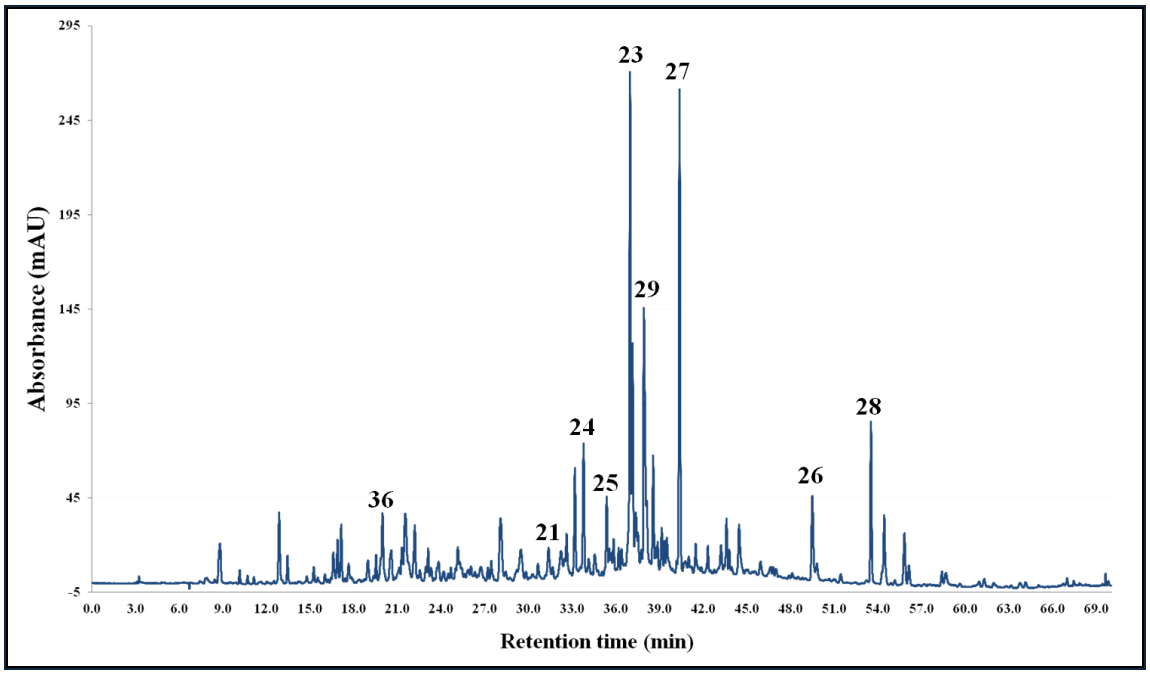


Figure 13. HPLC-UV chromatogram of *C. colurna* leaves ethyl acetate extract (see the chromatographic method in section **4.6.1.**)

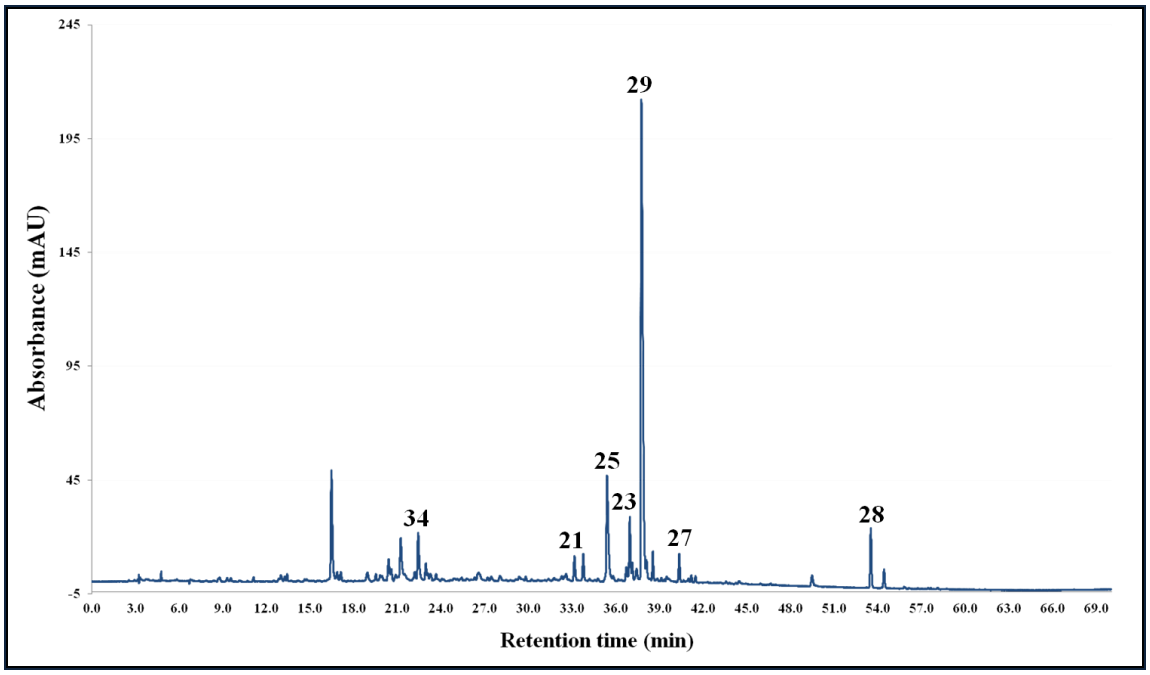


Figure 14. HPLC-UV chromatogram of *C. colurna* leaves methanolic extract (see the chromatographic method in section **4.6.1.**)

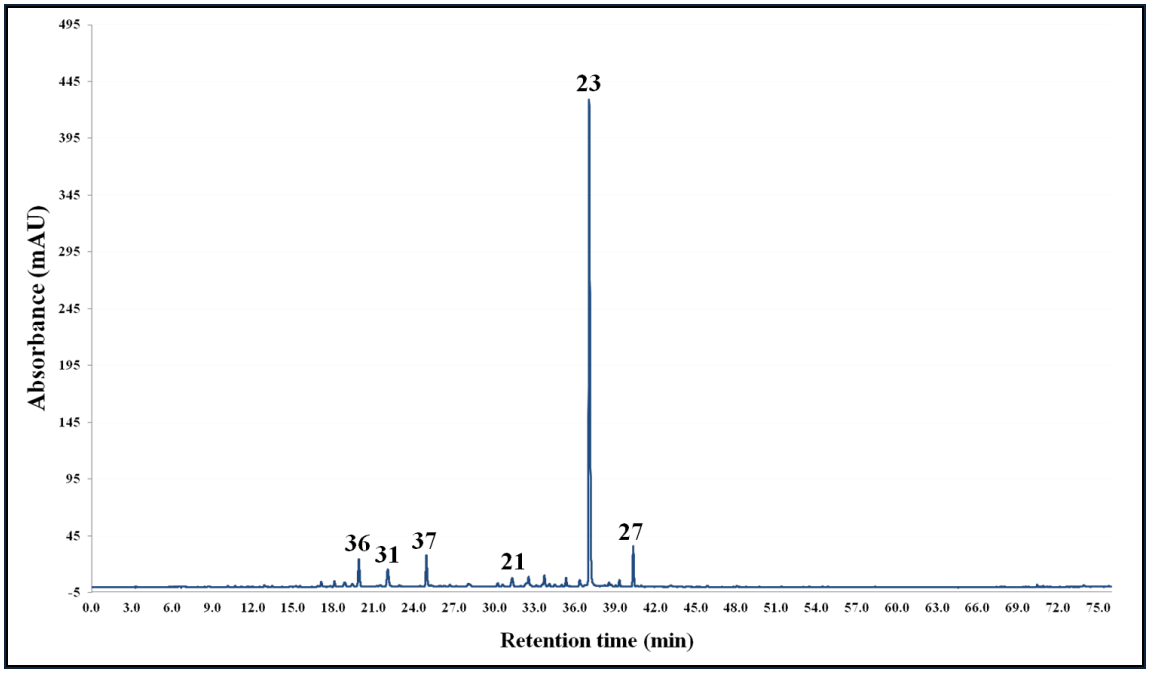


Figure 15. HPLC-UV chromatogram of *C. colurna* bark ethyl acetate extract (see the chromatographic method in section **4.6.1.**).

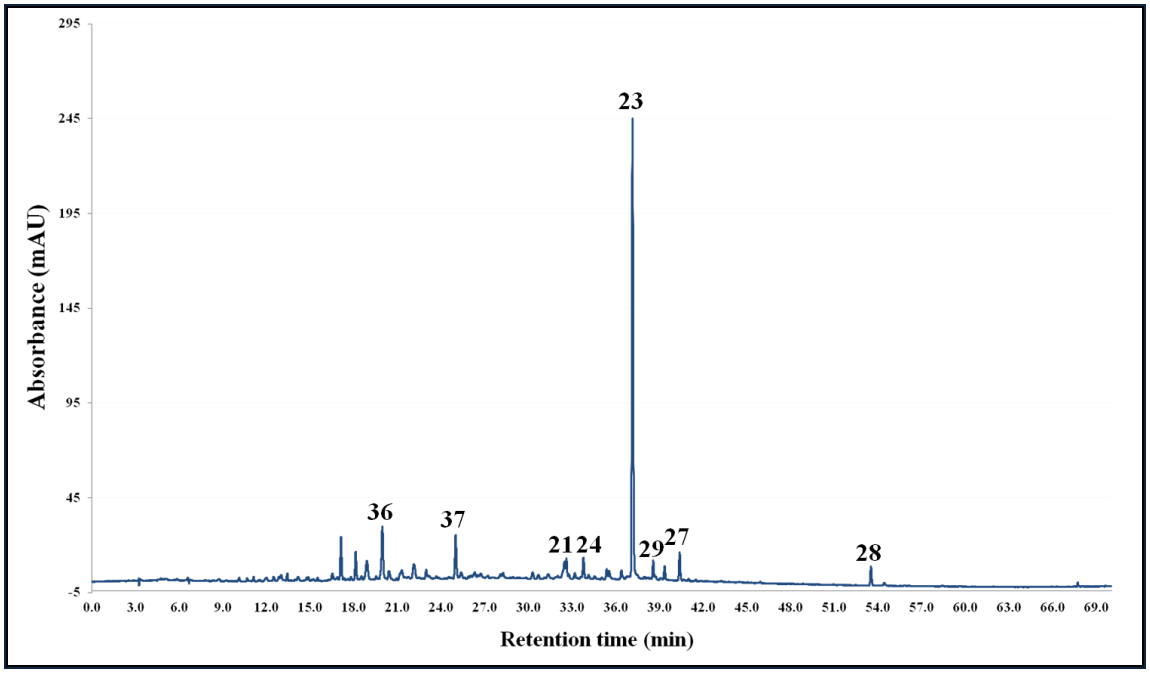


Figure 16. HPLC-UV chromatogram of *C. colurna* bark methanolic extract (see the chromatographic method in section **4.6.1.**).

In the *C. colurna* extracts nineteen phenolic compounds were detected, among them four hydroxycinnamic acid derivatives (compounds **31-34**), nine flavon-3-ol derivatives (compounds **21-29,**), four diarylheptanoids (compounds **1, 2, 19, 20**) and two other phenolics (compounds **36** and **37**) (Table 6). According to chromatographic data the main compound of the extracts was quercetin-3-*O*-rhamnoside (**23**), except for the leaves methanolic extract, where the most abundant constituent was characterised as kaempferol-3-*O*-glucuronide (**29**) (Figures 13-16.).

In the ethyl acetate extract of the leaves a caffeic acid derivative: 1-caffeoylquinic acid (**33**)**,** catechin/epicatechin (**36**), a quinic acid derivative (**32**), eight flavonol derivatives: myricetin-3-*O*-rhamnoside (**21**), quercetin-3-*O*-rhamnoside (**23**), quercetin-3-*O*-hexoside (**24**), quercetin-3-*O*-glucuronide (**25**), kaempferol-3-*O*-rhamnoside (**27**), kaempferol-3-*O*-glucuronide (**29**), kaempferol (**26**), kaempferol-(di)desoxyhexoside (**28**) two diarylheptanoid aglycones, compound **19** and hirsutenone (**1**) were characterised. In the methanolic extract of *C. colurna* leaves two caffeic acid derivatives 1-caffeoylquinic acid (**33**) and 1,3-dicaffeoylquinic acid (**34**), six flavonol derivatives also revealed in the ethyl acetate extract (compounds **22-24, 26-28**) and three other flavonols: myricetin-3-*O*-hexoside (**22**), quercetin-3-*O*-glucuronide (**25**) and kaempferol-glucuronide (**29**) were characterised.

In the extracts of *C. colurna* bark a caffeoyl-hexoside (**31**), two flavan derivatives: catechin/epicatechin (**36**) and a procyanidin dimer (**37**) seven flavonols (compounds **21, 23, 24, 25, 27, 28, 29**) a diarylheptanoid-glycoside oregonin (**2**) and a diarylheptanoid aglycone (**20**) were characterised. Compounds **35** and **25** revealed only in the methanolic, while **20** just in the ethyl acetate extract.

Myricetin-3-*O*-rhamnoside (**21**) quercetin-3-*O*-hexoside (**24**) quercetin-3-*O*-rhamnoside (**23**) and kaempferol-3-*O*-rhamnoside (**27**) were present in all the examined *C. colurna* extracts (Riethmüller et al. 2014).

Table 6. Phenolic compounds detected in the *C. colurna* extracts (Note that the results of HPLC-DAD-QMS, HPLC-DAD-TOF-MS and HPLC-DAD-MS/MS experiments are combined.)

t_R (min)	$[M-H]^-$ m/z calculated	$[M-H]^-$ m/z experimental	Error (ppm)	Molecular formula	Fragment ions	Compound	No.	LE	LM	BE	BM
18.1	353.0878	353.0895	-4.55	$C_{16}H_{18}O_9$	191,179,173,135, 93	**1-Caffeoylquinic acid**	**(33)**	x	x		
19.9	289.0718	289.0713	1.62	$C_{15}H_{14}O_6$	245,179,161,137, 123	**Catechin/epicatechin**	**(36)**	x		x	(x)
22.0	341.0878	341.0895	-4.98	$C_{15}H_{18}O_9$	179,143,119,89, 59	**Caffeoyl-hexoside**	**(31)**				x
22.4	515.1195	515.1205	-2.07	$C_{25}H_{24}O_{12}$	353,191,179	**1,3-Dicaffeoylquinic acid**	**(34)**		x		
24.9	577.1351	577.1339	2.08	$C_{30}H_{26}O_{12}$	451,407,339,289, 161,125	**Procyanidin dimer**	**(37)**			x	x
29.4	477.1766	477.1779	-2.65	$C_{24}H_{30}O_{10}$	417,335,327,205, 139,125	**Oregonin**	**(2)**			x	x
31.5	479.0831	479.0845	-2.81	$C_{21}H_{20}O_{13}$	317,316,287,271	**Myricetin-3-*O*-hexoside**	**(22)**		x		
31.5	393.1766	393.1749	4.31	$C_{17}H_{30}O_{10}$	191,149,113,89	**Quinic acid derivative**	**(32)**	x			
32.6	463.0882	463.0863	4.18	$C_{21}H_{20}O_{12}$	317,316,287,271, 211,179,147	**Myricetin-3-*O*-rhamnoside**	**(21)**	x	x	x	x
33.7	463.0882	463.0896	-3.03	$C_{21}H_{20}O_{12}$	301,300,271,255, 201,179,151	**Quercetin-3-*O*-hexoside**	**(24)**	x	x	x	x
35.3	477.0675	477.0692	-3.62	$C_{21}H_{18}O_{13}$	301,300,271,255, 211,179,151	**Quercetin-3-*O*-glucuronide**	**(25)**	x	x		x
36.4	343.1187	343.1183	1.03	$C_{19}H_{20}O_6$	283,270,211,125,	**Diarylheptanoid aglycone4**	**(19)**	x		x	
37.1	447.0933	447.0912	4.71	$C_{21}H_{20}O_{11}$	301,300,285,271, 255,179,151	**Quercetin-3-*O*-rhamnoside**	**(23)**	x	x	x	x
37.9	461.0725	461.0737	-2.48	$C_{21}H_{18}O_{12}$	285,284,255,227	**Kaempferol-glucuronide**	**(29)**	x	x		x
40.4	431.0984	431.0971	2.93	$C_{21}H_{20}O_{10}$	285,284,257,227	**Kaempferol-3-*O*-rhamnoside**	**(27)**	x	x	x	x
44.4	309.1132	309.1125	2.50	$C_{19}H_{18}O_4$	211,193,97,69	**Diarylheptanoid aglycone5**	**(20)**			x	
46.4	327.1238	327.1228	3.04	$C_{19}H_{20}O_5$	289,254,239,205	**Hirsutenone**	**(1)**	x			
49.5	285.1700	285.1707	2.74	$C_{15}H_{10}O_5$	255,227	**Kaempferol**	**(26)**	x	x		
53.5	577.1351	577.1335	2.8	$C_{30}H_{26}O_{12}$	285,284,255,227	**Kaempferol-(di)desoxyhexoside**	**(28)**	x	x	x	x

LE: leaves ethyl acetate extract, LM: leaves methanolic extract, BE: bark ethyl acetate extract BM: bark methanolic extract

5.3.3. *Corylus maxima* Mill.

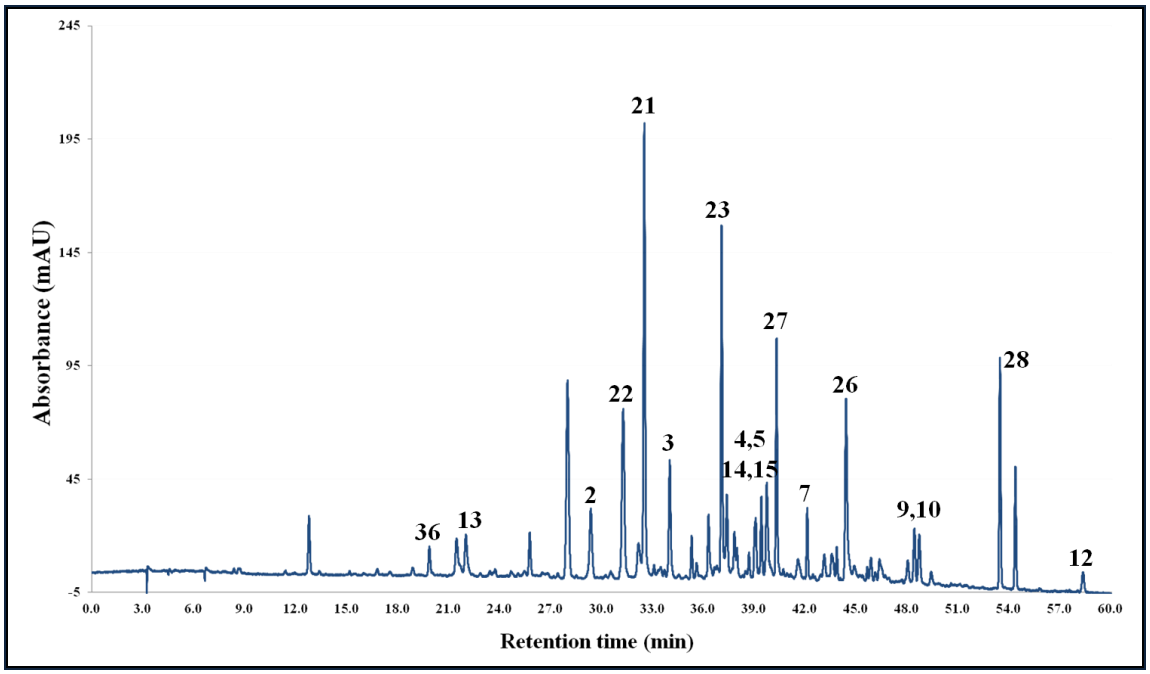


Figure 17. HPLC-UV chromatogram of *C. maxima* leaves ethyl acetate extract (see the chromatographic method in section **4.6.1.**).

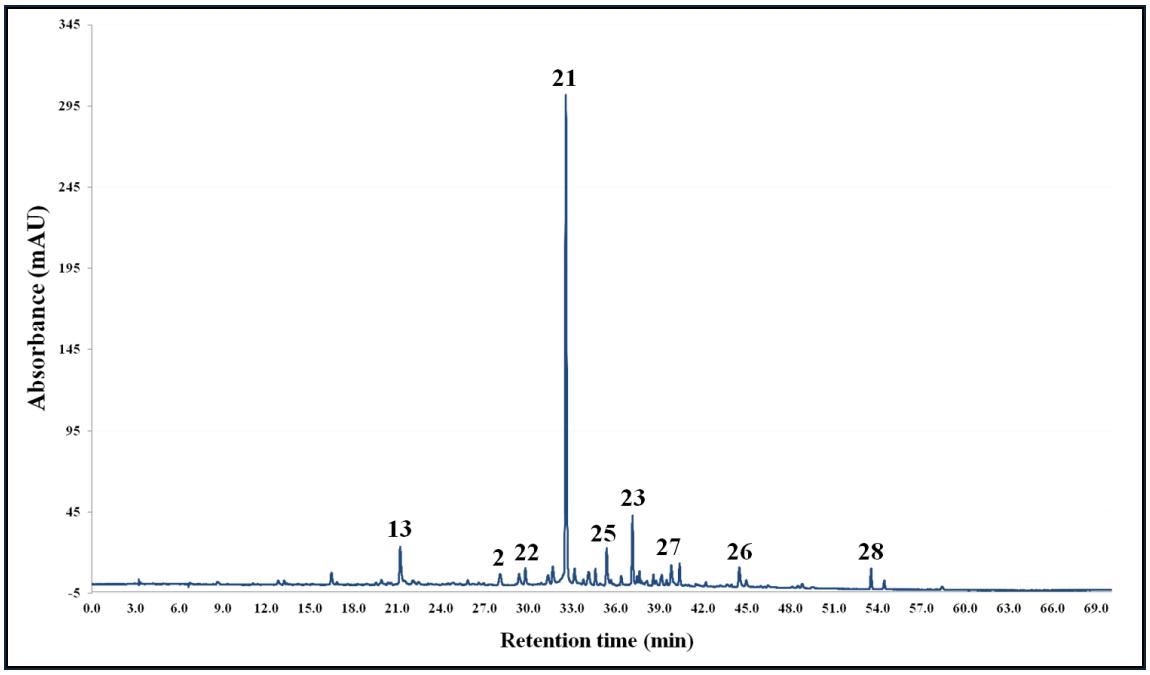


Figure 18. HPLC-UV chromatogram of *C. maxima* leaves methanolic extract (see the chromatographic method in section **4.6.1.**).

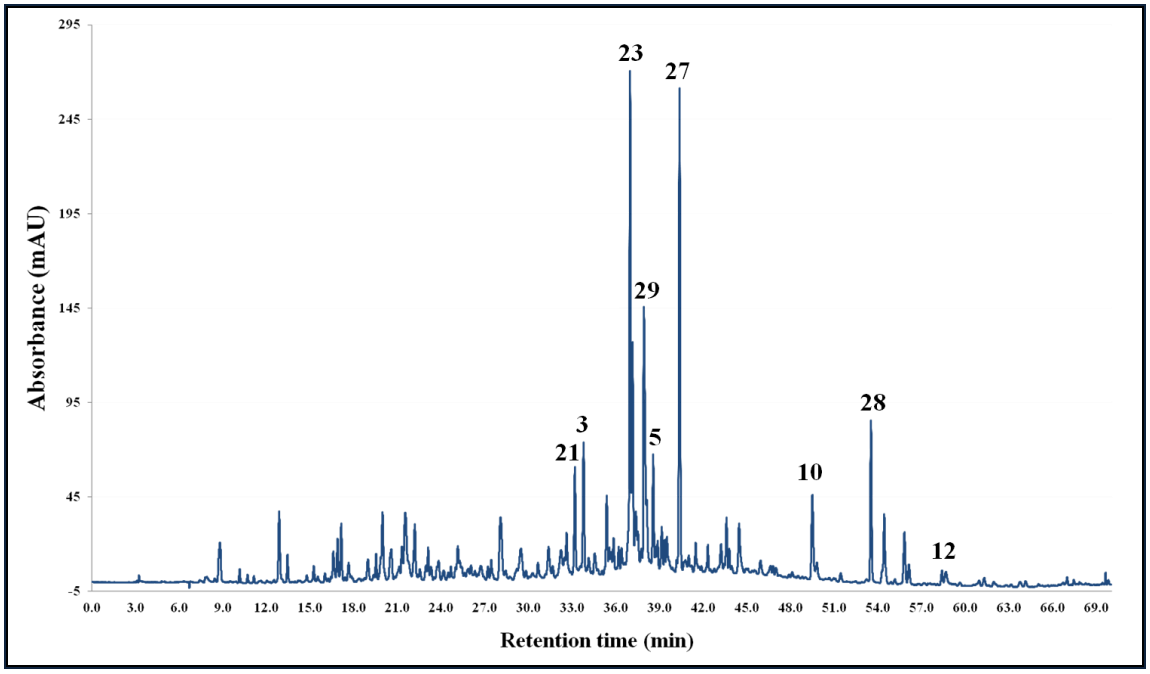


Figure 19. HPLC-UV chromatogram of *C. maxima* bark ethyl acetate extract (see the chromatographic method in section **4.6.1.**).

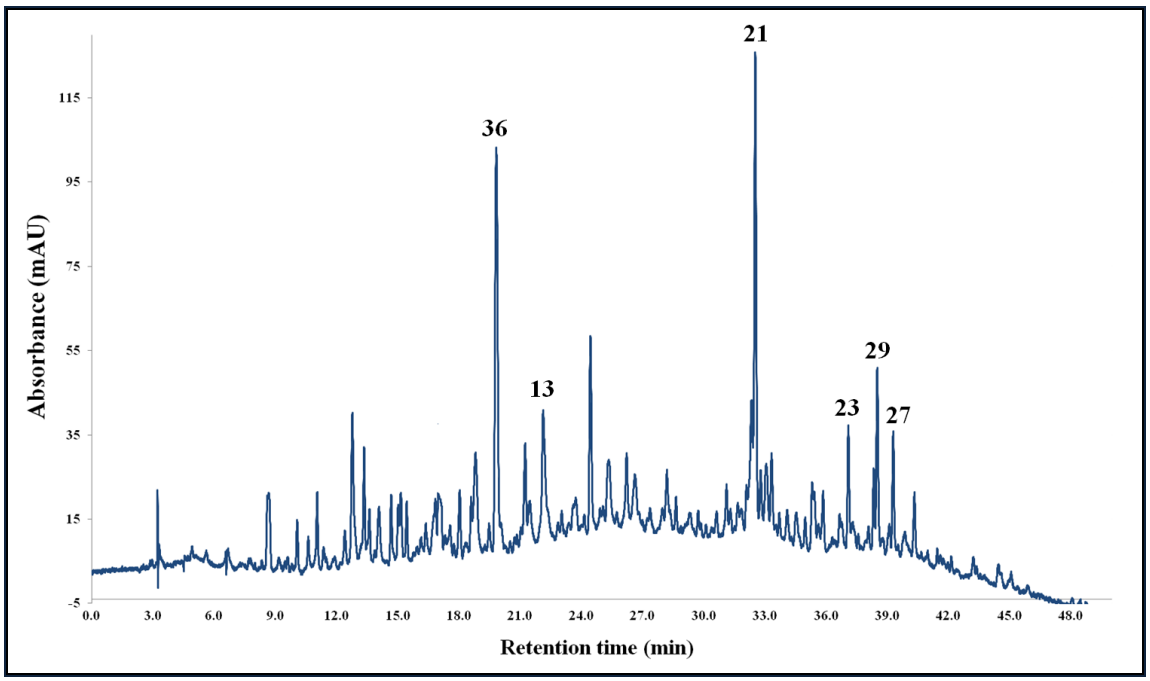


Figure 20. HPLC-UV chromatogram of *C. maxima* bark methanolic extract (see the chromatographic method in section **4.6.1.**).

Altogether twenty-two phenolics were characterised in the *C. maxima* extracts: one flavan derivative (**36**), seven flavonol derivatives (compounds **21, 22, 23, 26, 27, 29**) and fourteen diarylheptanoids (compounds **1-5, 7-15**) (Table 7, Figures 17-20).

In the ethyl acetate extract of the leaves one flavan derivative: catechin/epicatechin (**36**), five flavonol derivatives: myricetin-3-*O*-rhamnoside (**21**), quercetin-3-*O*-rhamnoside (**23**), kaempferol-3-*O*-rhamnoside (**27**), kaempferol (**26**) and kaempferol-(di)desoxyhexoside (**28**) and twelve diarylheptanoids: hirsutanolol-hexoside (**3**), oregonin (**2**), hirsutanolol (**13**), 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-one-5-*O*-pentoside (**4**), 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-one-5-*O*-pentoside (**5**), 5-hydroxy-1-(4-dydroxyphenyl)-7-(3,4-dihydroxyphenyl)-heptan-3-one (**14**), 5-hydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-one (**15**), platyphyllonol-pentoside (**7**), hirsutenone (**1**), 3-hydroxy-1,7-bis-(3,4-dihydroxyphenyl)-heptan (**9**), 3-hydroxy-1,7-bis-(3,4-dihydroxyphenyl)-hepten (**10**), 1,7-bis-(4-hydroxyphenyl)-4,6-heptadien-3-one (**12**) were characterised. In the methanolic extract of the leaves one flavan derivative (**36**), a flavonol-glycoside: myricetin-3-*O*-hexoside (**22**), five other flavonoids and twelve diarylheptanoids, also revealed in the ethyl acetate extract **21, 23, 27, 26, 28** and **1-5, 7, 9, 10, 12, 13, 14,** respectively, and one additional diarylheptanoid compound: 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-hept-4-en-3-one (**11**) were characterised.

In the extracts of *C. maxima* bark seven flavonol-glycosides (**21, 22, 23, 25, 27, 29**) and eleven diarylheptanoids (**1-5, 9, 10, 11, 13**) were detected. The flavonoids mentioned above were presented in both the extracts, while compounds **1, 3** only in the ethyl acetate, compounds **2** and **13** and **25** just in the methanolic extract.

According to the UV chromatograms, the main compounds of the extracts were myricetin-3-*O*-rhamnoside (**21**) quercetin-3-*O*-rhamnoside (**23**). The presence of oregonin (**2**) myricetin-3-*O*-rhamnoside (**21**), 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-one-5-*O*-pentoside (**4**), 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-one-5-*O*-pentoside (**5**), quercetin-3-*O*-rhamnoside (**23)** and kaempferol-3-*O*-rhamnoside (**27**) was found to be characteristic for both the leaves and bark extracts (Riethmüller et al. 2015).

Table 7. Phenolic compounds detected in the *C. maxima* extracts (Note that the results of HPLC-DAD-QMS, HPLC-DAD-TOF-MS and HPLC-DAD-MS/MS experiments are combined).

t_R (min)	$[M-H]^-$ m/z calculated	$[M-H]^-$ m/z experimental	Error (ppm)	Molecular formula	Fragment ions	Compound	No.	LE	LM	BE	BM
19.9	289.0713	289.0718	-4.0	$C_{15}H_{14}O_6$	245,205,179,161	**Catechin/epicatechin**	**(36)**	x	x		
22.2	345.1344	345.1327	2.20	$C_{19}H_{22}O_6$	205,179,165,121	**Hirsutanolol**	**(13)**	x	x		x
29.4	477.1779	477.1766	3.08	$C_{24}H_{30}O_{10}$	417,335,327,205	**Oregonin**	**(2)**	x	x	x	x
31.5	479.0845	479.0831	-2.00	$C_{21}H_{20}O_{13}$	317,316,287,271	**Myricetin-3-*O*-hexoside**	**(22)**	x	x	x	x
32.6	463.0863	463.0882	-2.65	$C_{21}H_{20}O_{12}$	317,316,287,271,179	**Myricetin-3-*O*-rhamnoside**	**(21)**	x	x	x	x
33.1	507.1872	507.1855	-4.58	$C_{25}H_{32}O_{11}$	327,205	**Hirsutanolol-hexoside**	**(3)**	x		x	
35.3	477.0675	477.0692	-3.62	$C_{21}H_{18}O_{13}$	301,300,271,255,211,179,151	**Quercetin-3-*O*-glucuronide**	**(25)**		x		x
37.1	447.0912	447.0933	-3.03	$C_{21}H_{20}O_{11}$	301,300,271,255,179	**Quercetin-3-*O*-rhamnoside**	**(23)**	x	x	x	x
38.0	461.0737	461.0725	1.13	$C_{21}H_{18}O_{12}$	285,284,255,227	**Kaempferol-3-*O*-glucuronide**	**(29)**			x	(X)
38.0	461.1817	461.1788	-2.31	$C_{24}H_{30}O_9$	311,205,189,121	**1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-heptan-3-one-5-*O*-pentoside**	**(4)**	x	x	x	x
38.2	461.1817	461.1788	4.31	$C_{24}H_{30}O_9$	311,205,189,121	**1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-one-5-*O*-pentoside**	**(5)**	x	x	x	x
38.5	329.1394	329.1378	-1.95	$C_{19}H_{22}O_5$	179,165, 63,149	**5-hydroxy-1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-heptan-3-one**	**(14)**	x	x		
38.5	329.1394	329.1378	4.23	$C_{19}H_{22}O_5$	179,165,163,149	**5-hydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-one**	**(15)**	x	x		
39.1	473.1817	473.1797	-3.12	$C_{25}H_{30}O_9$	293,89	**5-hydroxy-1,7-bis-(4-hydroxyphenyl)-hept-6-en-3-one-hexoside**	**(6)**		x		
40.4	431.0971	431.0984	2.69	$C_{21}H_{20}O_{10}$	285,284,255,227	**Kaempferol-3-*O*-rhamnoside**	**(27)**	x	x	x	(X)
42.2	445.1868	445.1851	-2.68	$C_{24}H_{30}O_8$	295,189,83	**Platyphyllonol-pentoside**	**(7)**	x	x	x	x
44.4	285.1707	285.1700	2.45	$C_{15}H_{10}O_5$	255,227	**Kaempferol**	**(26)**	x	(X)		
44.6	311.1289	311.1283	1.5	$C_{19}H_{20}O_4$	225,211,205,189	**1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-hept-6-en-3-one**	**(11)**		x	x	x

46.4	327.1228	327.1238	4.71	$C_{19}H_{20}O_5$	289,245,211,205	**Hirsutenone**	**(1)**	x	x	x	
48.7	331.1151	331.1545	3.32	$C_{19}H_{24}O_5$	303,193,121,108	**3-hydroxy-1,7-bis-(3,4-dihydroxyphenyl)-heptan**	**(9)**	x	x	x	x
48.8	329.1394	329.1383	2.84	$C_{19}H_{22}O_5$	207,109	**3-hydroxy-1,7-bis-(3,4-dihydroxyphenyl)-hepten**	**(10)**	x	x	x	x
53.5	577.1335	577.1351	2.8	$C_{30}H_{26}O_{12}$	285,284,255,227	**Kaempferol-(di)desoxyhexoside**	**(28)**	x	x		
58.5	293.1183	293.1172	-1.52	$C_{19}H_{18}O_3$	251,225,211,83	**1,7-bis-(4-hydroxyphenyl)-4,6-heptadien-3-one**	**(12)**	x	(x)	x	x

LE: leaves ethyl acetate extract, LM: leaves methanolic extract, BE: bark ethyl acetate extract BM: bark methanolic extract

5.4. Quantitative analyses by HPLC-MS/MS

According to the HPLC-DAD-ESI-MS results, the two main flavonoids of the extracts were myricetin-3-*O*-rhamnoside (**21**) and quercetin-3-*O*-rhamnoside (**23**), while the two most abundant diarylheptanoid compounds were oregonin (**2**) and hirsutenone (**1**). For the quantitative analysis of these constituents a HPLC-ESI-MS/MS method was developed, using MRM (multiple reaction monitoring) mode (see section **4.7.**). The HPLC method differed from that used for the qualitative studies (see section **6.3.**) in order shorten the analysis time. This HPLC method was completely appropriate for the determination of these constituents; base-line separation could be achieved in the case of all the four compounds. The methods were validated for linearity, intra- and inter-day precision and accuracy. It could have been interesting to gain information about the quantity of kaempferol-3-*O*-rhamnoside (**27**) in the extracts as well, but due to the lack of commercially available authentic standard, it was not feasible.

5.4.1. Method validation

The quantitative methods provided linear responses for all standards within the investigated range (Table 8). Retention time repeatability was suitable for all compounds, relative standard deviation was <0.15% (n = 6). Specificity of the methods was checked by injecting pure solvent. No co-elution was observed at the retention time of the analytes. Precision of the methods was tested by performing intra- and inter-day evaluation of solutions containing the target analyte in three concentrations (low, mid and high values of the calibration range), precision and accuracy tests were performed

in triplicate. The relative standard deviation for intra- and inter-day precision was <10% for all quantitative methods, while intra- and inter-day accuracy ranged from 91.2% to 108.3% (Table 9).

Table 8. Regression, LOQ and LOD of the MRM quantitative methods (standard solutions were prepared in triplicates and injected once)

Standard	Regression equation	r^2	Regression range (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)
Myricetin-3-*O*-rhamnoside	y=1330.4x+1062.5	0.9999	0.1-300	0.020	0.067
Quercetin-3-*O*-rhamnoside	y=1188.6x+2036	0.9996	0.1-300	0.010	0.033
Oregonin	y = 2293.9x + 2405	0.9999	0.1-100	0.005	0.017
Hirsutenone	y=332.82x+13.11	0.9977	0.01-50	0.002	0.007

Table 9. Method validation: Precision and accuracy of the quantitative methods

Nominal conc. (µg/ml)	Precision (RSD%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
Myricetin-3-*O*-rhamnoside				
1	5.93	6.85	95.6	97.8
50	2.15	5.11	102.3	99.2
300	3.75	5.85	98.5	102.6
Quercetin-3-*O*-rhamnoside				
1	5.42	6.28	103.2	102.7
50	2.61	3.84	99.8	97.4
300	3.22	4.91	98.3	100.4
Oregonin				
0.1	3.15	4.24	105.3	108.3
10	0.64	1.02	98.5	99.2
100	1.15	2.21	100.3	103.4
Hirsutenone				
0.01	8.41	8.73	92.5	91.6
1	2.30	3.84	98.7	97.3
50	4.90	5.60	94.1	95.6

5.4.2. Quantitative results

Quantity of myricetin-3-*O*-rhamnoside, quercetin-3-*O*-rhamnoside, hirsutenone and oregonin were determined by HPLC-MS/MS experiments using MRM (multiple reaction monitoring). Linear regression analyses were performed by using external calibration (Table 8). The quantifier and qualifier ions were designated by the evaluation of the mass spectra of the compounds. The qualifier/quantifier ion ratio remained within the ± 20% range of the determined value for each analyte (see sections **4.7.1.** and **4.7.2.**). Results of the quantitative analyses are presented in Table 10 as mean and standard deviation of three parallel measurements.

Table 10. Results of the quantitative analyses

	Quantity in the extracts (mean±SD, n=3) (µg/mg extract)			
	Myricetin-3-*O*-rhamnoside	**Quercetin-3-*O*-rhamnoside**	**Oregonin**	**Hirsutenone**
***C. avellana* leaves EtOAc**	37.70±0.97	34.94±0.87	n.d.	2.08±0.03
***C. avellana* leaves MeOH**	72.64±0.13	16.94±0.26	n.d.	0.33±0.01
***C. avellana* bark EtOAc**	13.06±0.81	28.30±0.85	n.d.	n.d.
***C. avellana* bark MeOH**	2.42±0.08	15.52±0.74	n.d.	n.d.
***C. colurna* leaves EtOAc**	16.80±0.22	89.65±0.56	n.d.	n.q.
***C. colurna* leaves MeOH**	5.89±0.09	41.50±0.38	n.d.	n.d.
***C. colurna* bark EtOAc**	3.89±0.26	39.20±0.34	3.1±0.05	n.d.
***C. colurna* bark MeOH**	1.65±0.19	7.86±0.11	2.1±0.03	n.d.
***C. maxima* leaves EtOAc**	16.90±0.09	8.20±0.02	3.40±0.04	8.39±0.01
***C. maxima* leaves MeOH**	30.10±0.80	19.80±0.75	2.21±0.21	0.59±0.05
***C. maxima* bark EtOAc**	15.86±0.22	38.52±0.02	1.34±0.02	0.54±0.00
***C. maxima* bark MeOH**	15.50±0.02	4.90±0.20	0.31±0.02	0.10±0.00

n.d.=not detected; n.q.=not quantified

5.5. HPLC-based DPPH scavenging assay

After spiking the *Corylus* samples with the DPPH radical solution (see section **4.8.**), the decrease in the peak areas of the main compounds was examined. The chromatograms of *C. colurna* bark ethyl acetate extract are presented in Figures 21 and 22 (the latter presents the magnification of the original chromatograms for better recognition of the minor constituents) as an example.

The results of the HPLC based DPPH scavenger activity analyses are presented in Tables A2-13 in section **13.2**. ΔArea was calculated according to eq. 1 and 2; and the ΔArea ratio according to eq 3.

$$\Delta\text{Area} = \text{peak area}_1 - \text{peak area}_2 \quad \text{(eq. 1)}$$

$$\text{Decrease in the peak area (\%)} = (\Delta\text{Area}/\ \text{peak area}_1)\cdot 100\% \quad \text{(eq. 2)}$$

$$\Delta\text{Area ratio} = (\Delta\text{Area}/\Sigma\ \Delta\text{Area})\cdot 100\% \quad \text{(eq. 3)}$$

where ΔArea (mAu·s) is the change in the peak area; peak area$_1$ (mAu·s) is the area of the compound's chromatographic peak in the control sample; peak area$_2$ (mAu·s) is the area of the compound's chromatographic peak in the DPPH spiked sample.

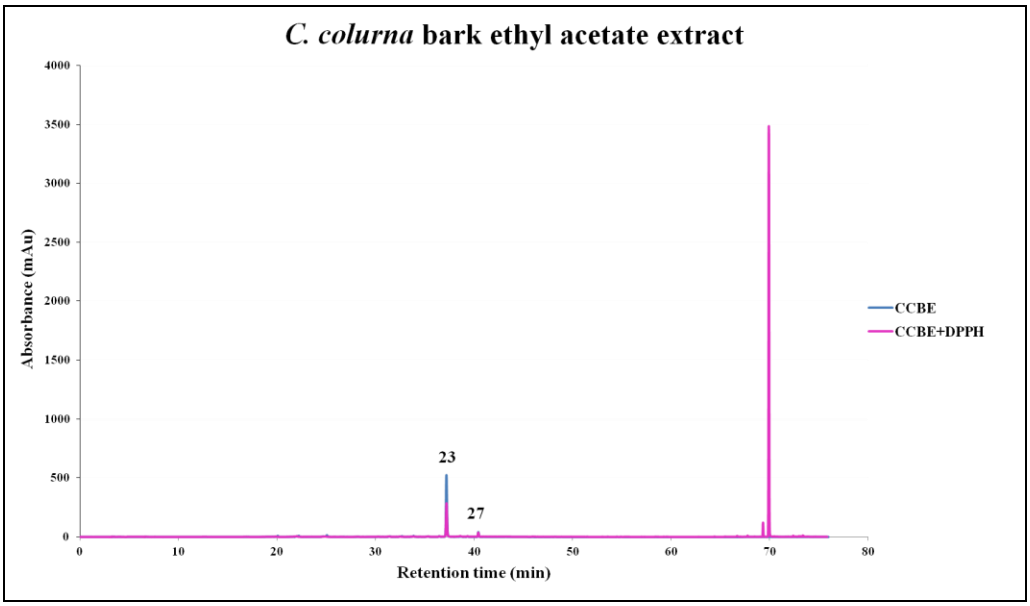


Figure 21. HPLC-UV chromatograms of the control sample and the sample after spiking with DPPH of *C. colurna* bark ethyl acetate extract (CCBE) **(**see section **4.8.**)

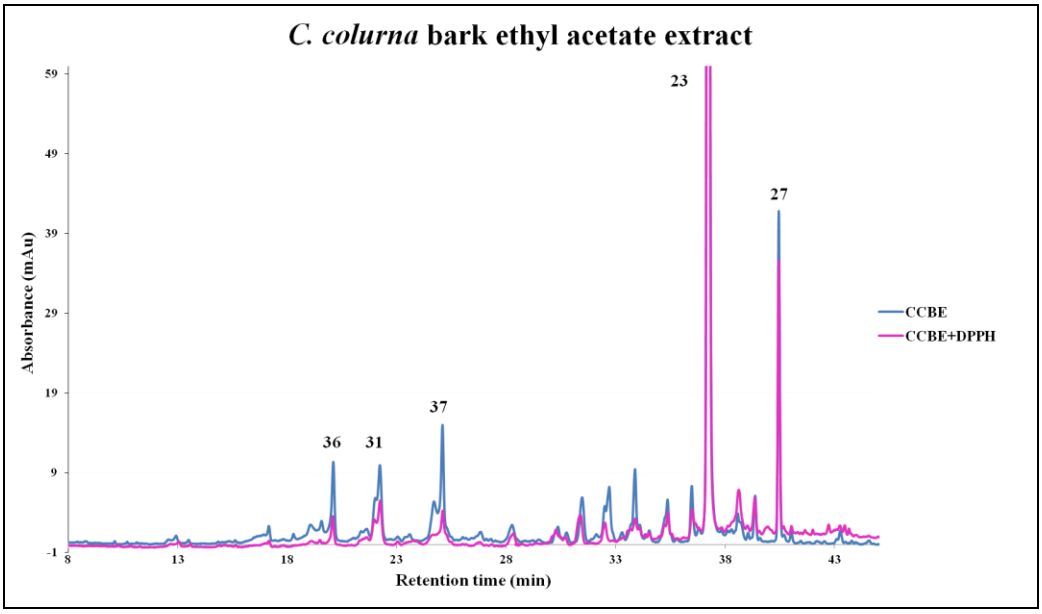


Figure 22. Enlarged chromatograms of the control sample and the sample after spiking with DPPH of *C. colurna* bark ethyl acetate extract (CCBE) **(**see section **4.8.)** Compound **23**: quercetin-3-*O*-rhamnoside, **27**: kaempferol-3-*O*-rhamnoside, **31**: caffeoyl-hexoside, **36**: catcechin/epicatechin, **37**: procyanidin dimer

6. DISCUSSION

6.1. Quantitative phytochemical analyses

Based on the results of the quantitative determination of phenolics in the *Corylus* crude drugs (Figure 23, Table 10) by spectrometric methods described in Ph. Hg. VIII. (2003), it could be concluded that all the crude drugs contained notable amounts of polyphenol compounds, with *C. maxima* bark being the richest in these constituents (3.63±0.27g total phenolics in 100g crude drug). *C. avellana* leaves and bark were found to contain high quantities of both tannins and flavonoid derivatives. In the leaves tannins (1.37±0.05 g/100g crude drug), while in the bark flavonoids (1.54±0.05 g/100g crude drug) were presented in higher amounts. In the case of *C. colurna* samples, the opposite was observed; the leaves were found to be richer in flavonoids (0.49±0.13 g/100g crude drug), than in tannins (0.38±0.01 g/100g crude drug); while in the bark tannins (1.33±0.37 g/100g crude drug) were presented in higher quantity compared with flavonoids (0.43±0.05 g/100g crude drug). In *C. maxima* leaves the lowest amount of tannins was measured among all the crude drugs, both the leaves and bark were found to be richer in flavonoids (0.96±0.13 g/100g crude drug in the leaves and 0.82±0.09 g/100g crude drug in the bark), than in tannin derivatives (0.10±0.01 g/100g crude drug in the leaves and 0.51±0.06 g/100g crude drug in the bark).

It has to be noted that the plant samples of the three species were obtained from different places in Hungary and also at different times of the year; thus considering that both these factors have an influence on the quantity of phenolic compounds in the crude drugs, some caution must be taken when comparing data regarding different species.

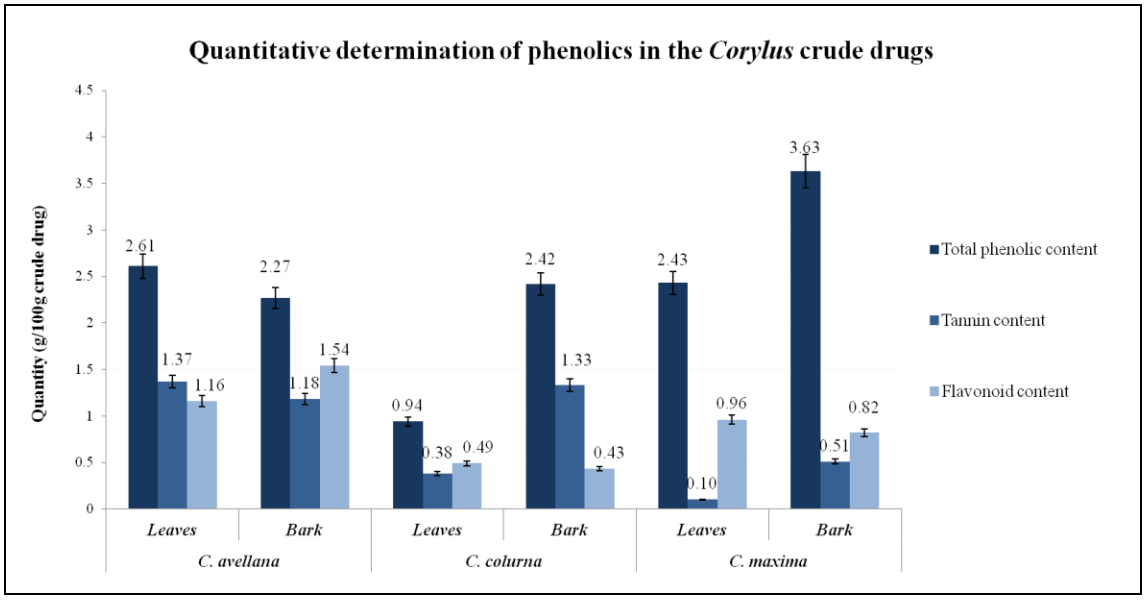


Figure 23. Quantitative determination of phenolics in the *Corylus* crude drugs

6.2. Antioxidant activity assays

The antioxidant activity of plant phenolics is widely reported, besides, the extracts of *C. avellana* and a natural mixture of flavonoids isolated from *C. colurna* have been shown to posses potent antioxidant activity (Delgado et al. 2010, Benov and Georgiev 1994). Several mechanisms have been proved to play role in the antioxidant effect of polyphenols that include free radical scavenging (ROS or RNS); suppressing ROS/RNS formation by enzyme inhibition or by chelating of trace elements; and also the enhancing of the antioxidant defence system (Cotelle 2001). The first effect mentioned above, the deactivation of free radicals, can occur by two main mechanisms, hydrogen atom transfer (HAT) and single electron transfer (SET) reactions. The dominant mechanism is determined by the structure and solubility of the antioxidant as well as the method of testing (Prior et al. 2005). The efficacy of the antioxidants is mainly determined by the bond dissociation energy (BDE) of their reactive functional group and the ionisation potential (IP).

Since the interest in antioxidants is progressively increasing, several methods have been developed and improved for the determination of free radical scavenging activity (Magalhaes et al. 2008, Prior et al. 2005). The HAT-based methods measure the ability of the antioxidant to quench free radicals by the donation of hydrogen. The reactivity

present in these assays mainly depends on the BDE of the H-donating group in the molecules (Wright et al. 2001, Huang et al 2005). The SET-based methods determine the ability of an antioxidant to transfer an electron to reduce radicals. This latter mainly depends on the IP of the antioxidant investigated. The *in vitro* tests using DPPH and ABTS as free radicals were chosen for the determination of the antioxidant activity of the *Corylus* extracts prepared with ethyl acetate and methanol. Although the DPPH and ABTS assays are usually classified as SET reactions these two radicals can also be neutralised by H atom transfer, with the latter occurring as a marginal reaction pathway (Ou et al 2005). Although the DPPH assay has been developed in 1958 (Blois 1958), it still has widespread application (Kedare et al. 2011, Thaipong et al. 2006, Brand-Williams et al. 1995). The main withdrawal of the method is that steric accessibility is considered one of the major determinants of the reaction, meaning that smaller molecules have better access to the radical site, thus show better scavenger capacity in the test (Prior et al. 2005). The latter method, that utilises ABTS as stable free radical, was found to be inappropriate for the quantitative determination of the antioxidant capacity, although it was successfully utilised to provide a ranking order of different antioxidants (Miller et al. 1996). The main advantages of the two methods mentioned above include rapid reaction with the antioxidants (1-30 min) (Brand-Williams et al. 1995, Re et al. 1999); the fact that both work well in a methanol or ethanol solvent system, which is important considering the solubility of the phenolics examined in our study; furthermore both tests are operationally simple (Awika et al. 2003). Since our aim was the preliminary investigation of the scavenger capacity of the *Corylus* extracts as well as the comparison of their activity, both the tests were found to be suitable for our experiments.

Based on the results (Tables 3 and 4) it could be concluded that all the extracts possessed notable activity in both *in vitro* tests (with IC_{50} values not higher than 50 µg/ml). For reasons of clarity, the antiradical power ($1/IC_{50,}$ ml/µg) of the extracts is depicted in Figure 24: the larger the antiradical power, the more efficient the antioxidant.

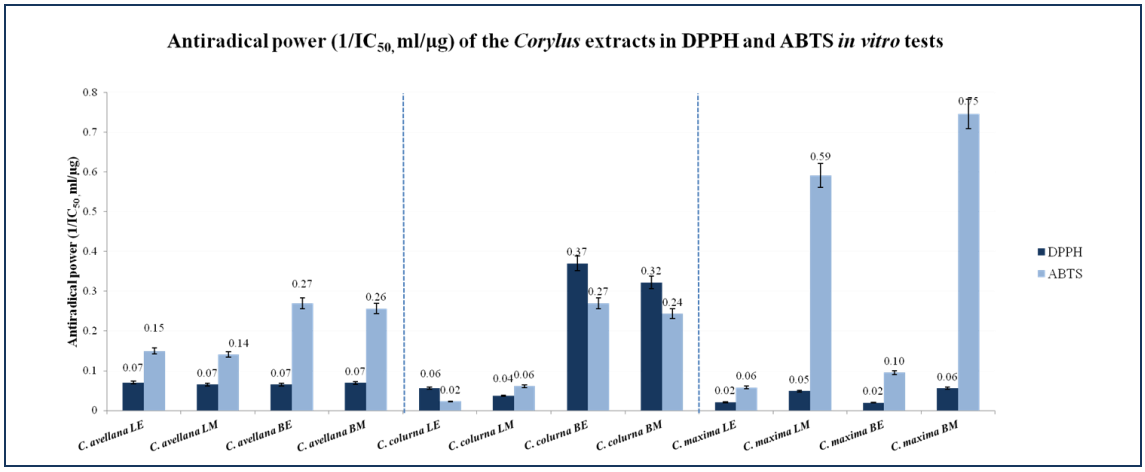


Figure 24. Antiradical power ($1/IC_{50,}$ ml/µg) of the *Corylus* extracts in the DPPH and ABTS *in vitro* tests

CALE: *C. avellana* leaves ethyl acetate extract, CALM: *C. avellana* leaves methanolic extract, CABE: *C. avellana* bark ethyl acetate extract, CABM: *C. avellana* bark methanolic extract; CCLE: *C. colurna* leaves ethyl acetate extract, CCLM: *C. colurna* leaves methanolic extract, CCBE: *C. colurna* bark ethyl acetate extract, CCBM: *C. colurna* bark methanolic extract; CMLE: *C. maxima* leaves ethyl acetate extract, CMLM: *C. maxima* leaves methanolic extract, CMBE: *C. maxima* bark ethyl acetate extract, CM BM: *C. maxima* bark methanolic extract.

The methanolic extracts of *C. maxima* leaves and bark showed significantly the highest scavenger capacity among the *Corylus* samples in the ABTS test. The bark samples acted noticeably stronger against ABTS free radical than the leaves, while the difference in the DPPH test was not significant. Both the leaves and bark methanolic extracts showed higher antioxidant activity than the ethyl acetate extracts in both *in vitro* tests.

The *C. colurna* bark extracts showed the highest scavenging capacity among the *Corylus* samples in the DPPH test, exceeding also the activity of all the standards except hirsutenone. The scavenger capacity of the bark extracts was found to be higher than that of the leaves in the ABTS test as well. The ethyl acetate extract of the leaves possessed more potent scavenger activity against DPPH free radical, while the methanolic extract acted stronger against ABTS. In case of the bark samples the difference between the activities of the ethyl acetate and methanolic extract was not explicit.

In case of *C. avellana* significant difference was not observed between the DPPH scavenging capacities of the extracts, while in the ABTS tests the bark samples showed

noticeably higher antioxidant activity than the leaves. Although neither in the latter case was significant difference found between the activities shown by the ethyl acetate and methanolic extracts of the bark or leaves.

It has to be noted that neither the DPPH nor the ABTS test can be considered physiological, since none of the two radicals occur in biological systems. Therefore, the results obtained by these methods cannot be extrapolated to *in vivo* data. However, the measured high *in vitro* scavenging capacity regarding the extracts indicated the presence of potential natural antioxidants, furthermore the fact that the measured antiradical activities did not show any trend, indicated notable differences in the phenolic profile of the extracts. Thus, characterisation of the phenolic fingerprint of the samples was found to be reasonable.

6.3. Characterisation of phenolics in the *Corylus* extracts by HPLC-MS

Discussion of the characterisation of phenolics detected in the *Corylus* extracts is based on structural groups not on plant species. Characterisation was based on results presented in Tables 5-7 (see section **5.3.**).

6.3.1. Diarylheptanoids

UV spectra with absorption maxima at 250-260 and 300-310 nm together with characteristic mass spectra indicated diarylheptanoid structures in the case of twenty compounds of the *Corylus* extracts. No previous literature data was found regarding diarylheptanoids in any of the investigated *Corylus* species, thus all the compounds described below are reported in these plants for the first time. From the accurate molecular mass and formula given by ESI-TOF, and fragmentation patterns acquired by collision-induced dissociation (CID) in ESI-MS/MS analyses compared to authentic standards and to literature data (Jiang et al. 2006) we were able to characterise the structures of fifteen diarylheptanoids, although the applied MS/MS method is not suitable for the accurate identification of the molecules where no matching standards were available.

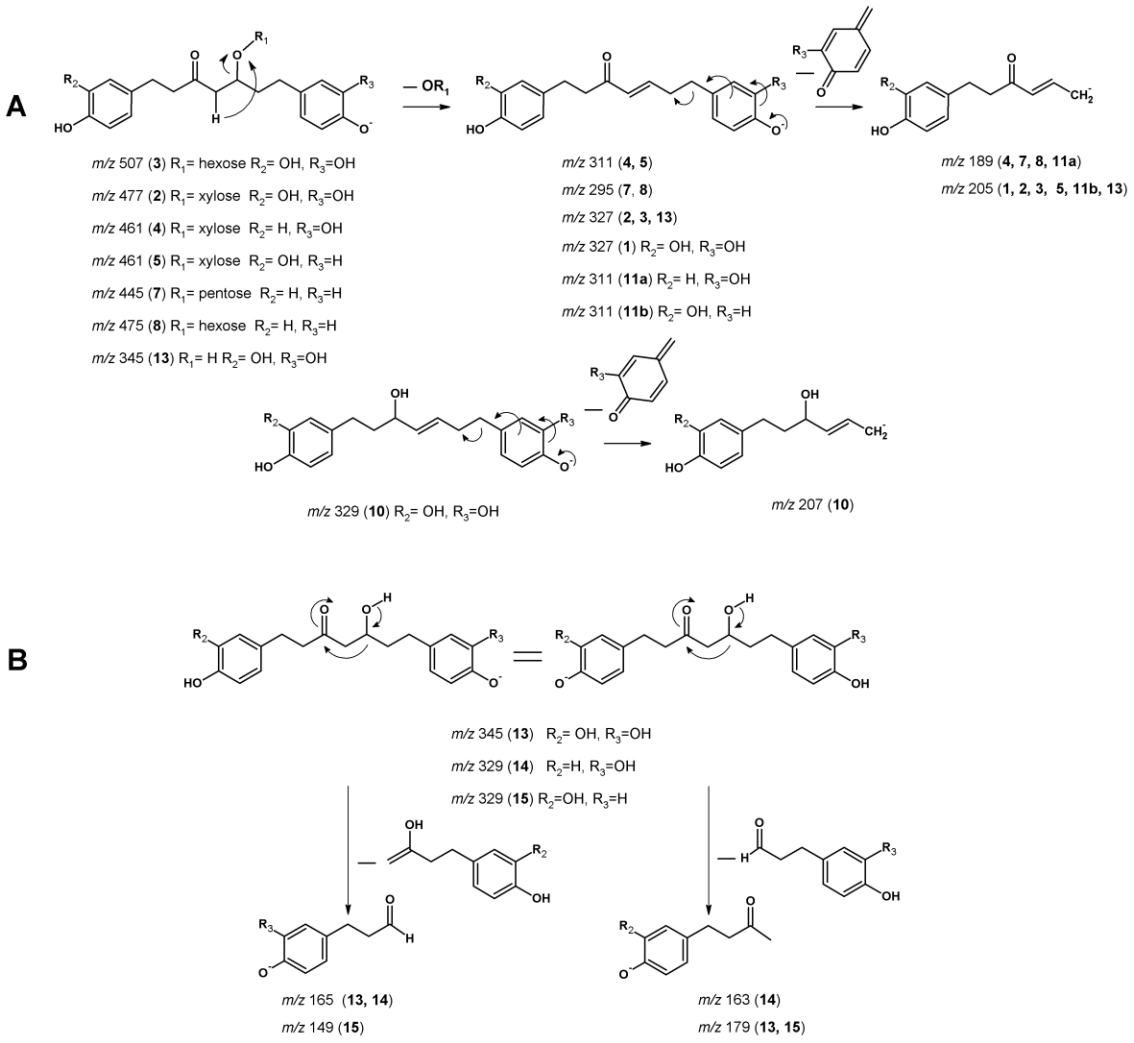


Figure 25. Assumed (-) ESI-MS/MS fragmentation of diarylheptanoids detected in the *Corylus* extracts.

Although the UV spectra and the molecular formulas of compounds **16-20** indicated diarylheptanoid aglycones, from the fragmentation pattern given by ESI-MS/MS analyses no appropriate conclusions could have been drawn about their structures. Among the fifteen compounds hirsutenone and oregonin were identified by comparing their chromatographic and spectrometric data to authentic standards. Since for other diarylheptanoid compounds no matching standards were available, tentative identification was based on comparison of their mass spectral data to hirsutenone and oregonin and other diarylheptanoids being present in the extracts. In general two fragmentation pathways (pathways **A** and **B**, see Fig. 25) were observed regarding these

compounds. In the case of compounds **1-12** the same trends in the CID were observed (**A**), while compounds **13-15,** where the presence of an unsubstituted hydroxyl and a keto group on the alkyl chains at positions 5 and 3, respectively, was assumed showed different fragmentation behaviour (**B**).

The molecular ion $[M-H]^-$ of **hirsutenone** (**1**) was detected at *m/z* 327.1228, the characteristic product ions at *m/z* 205 and 179. The ESI-TOF conjunction analysis and the molecular formula calculation pointed to the formula $C_{19}H_{20}O_5$. The (-) ESI-MS/MS fragmentation shown in Figure 26 was supposed based on literature data (Jiang et al. 2006). Identification of hirsutenone in the *Corylus* extracts was performed by comparison of chromatographic and mass spectrometric behaviour to those of an authentic standard, and also by spiking the sample solutions with the standard in two different chromatographic methods (see sections **4.6.1.** and **4.6.2.**).

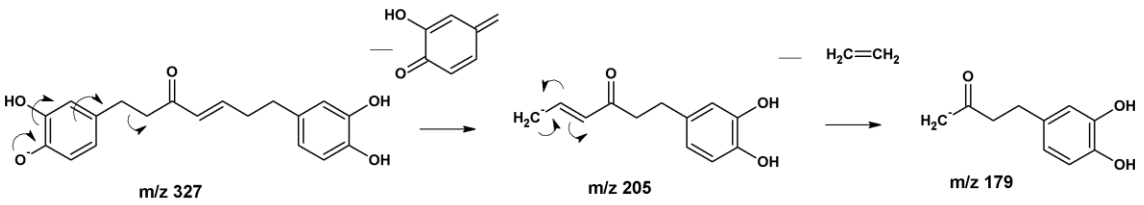


Figure 26. (-) ESI-MS/MS fragmentation of hirsutenone (**1**).

The molecular ion $[M-H]^-$ of **oregonin** (**2**) was detected at *m/z* 477.1779, exhibited characteristic fragment ions at *m/z* 327, 205 and 179. The ESI-TOF conjunction analysis and the molecular formula calculation pointed to the formula $C_{24}H_{30}O_{10}$. The neutral loss of 150 amu between *m/z* 477 and *m/z* 327 referred to a pentose sugar residue, while the aglycone fragment ion at *m/z* 327 and the characteristic fragment ions at *m/z* 205 and 179 indicated hirsutenone aglycone (Fig. 27). Identification of oregonin in the *Corylus* extracts was based on comparison of chromatographic and mass spectrometric behaviour to those of an authentic standard, and was also performed by spiking the sample solutions with the standard in two different chromatographic methods (see sections **4.6.1.** and **4.6.2.**).

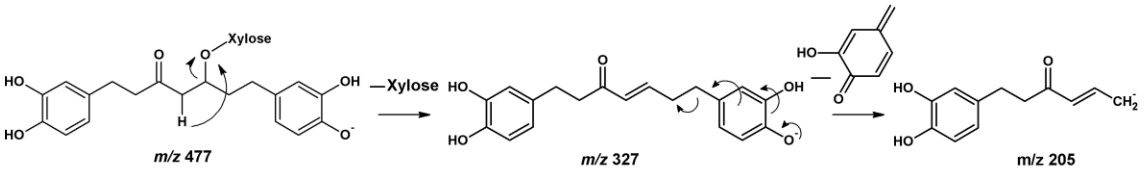


Figure 27. (-) ESI-MS/MS fragmentation of oregonin (**2**).

Compound **3** exhibited molecular ion at *m/z* 507.1855 and characteristic fragment ions at *m/z* 327 and 205. The ESI-TOF conjunction analysis and the molecular formula calculation pointed to the formula $C_{25}H_{32}O_{11.}$ The neutral loss of 180 amu indicated a hexose moiety, while the aglycone fragment at *m/z* 327 and the characteristic fragment ion at *m/z* 205 indicated hirsutenone aglycone. Therefore, similar fragmentation (Fig. 28) was assumed as in the case of oregonin and compound **3** was tentatively identified as **hirsutanolol-hexoside**.

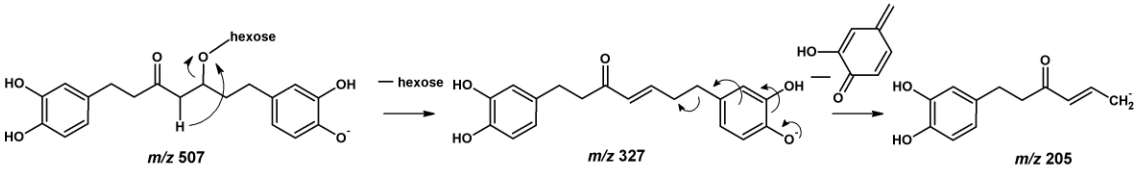


Figure 28. Assumed (-) ESI-MS/MS fragmentation of hirsutanolol-hexoside (**3**).

Compounds **4** and **5** exhibited molecular ions $[M-H]^-$ at *m/z* 461.1788 and characteristic fragment ions at *m/z* 311, 205 and 189. The ESI-TOF conjunction analysis and the molecular formula calculation pointed to the formula $C_{24}H_{30}O_9$ for both compounds, which differs from the molecular formula of oregonin with one oxygen atom. The neutral loss of 150 amu refers to a pentose sugar residue. In the aglycone fragment ions (*m/z* 311) a mass shift of 16 Da with reference to the molecular ion of hirsutenone (*m/z* 327) was observed, suggesting the absence of one oxygen atom in the aglycone molecules compared with hirsutenone. Based on the detection of the two other abundant product ions (*m/z* 205 and 189), the presence of only one hydroxyl group on one of the benzyl rings of the compounds and also the presence of a mixture of two structural isomers was supposed (Fig. 29). Compound **4** was tentatively identified as **1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-heptan-3-one-5-*O*-pentoside** and

compound **5** as **1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-one-5-*O*-pentoside**.

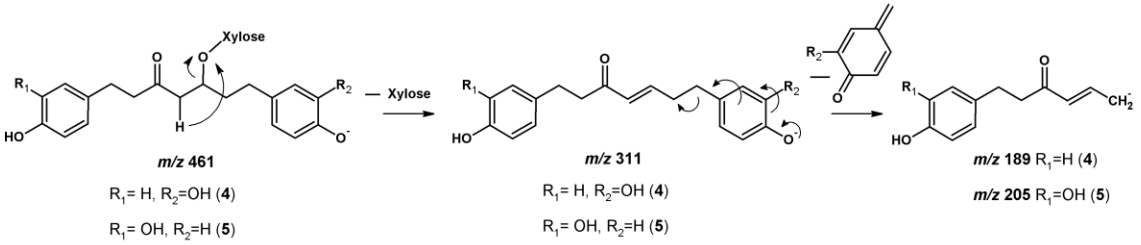


Figure 29. Assumed (-) ESI-MS/MS fragmentation of 1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-heptan-3-one-5-*O*-pentoside (**4**) and 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-one-5-*O*-pentoside (**5**).

UV spectrum of compound **6** pointed to diarylheptanoid structure. It exhibited molecular ion $[M-H]^-$ at *m/z* 473.1797 and an aglycone fragment ion at *m/z* 293. The ESI-TOF conjunction analysis and the molecular formula calculation corresponded to the formula $C_{25}H_{30}O_9$. The neutral loss of 180 amu indicated a hexose moiety. Based on the (-) ESI-MS/MS spectrum, the compound was characterised as **5-hydroxy-1,7-bis-(4-hydroxyphenyl)-6-hepten-3-one-hexoside**, which was also detected in aglycone form (**12**).

The molecular ion $[M-H]^-$ and characteristic product ions of **platyphyllonol-pentoside** (**7**) were detected at *m/z* 445.1868 and at *m/z* 295 and 189, respectively. The ESI-TOF conjunction analysis and the molecular formula calculation pointed to the formula $C_{24}H_{30}O_8$ which differed from the structure of oregonin ($C_{24}H_{30}O_{10}$) with two oxygen atoms. The neutral loss of 150 amu indicated a pentose sugar residue. In the aglycone fragment ion (*m/z* 295) a mass shift of 32 Da was observed with reference to the molecular ion of hirsutenone (*m/z* 327), while in the other characteristic product ion (*m/z* 189) a mass shift of 16 Da was detected with reference to the most intense product ion of hirsutenone (*m/z* 205). These observations suggested the presence of only one hydroxyl group on each benzyl rings (in the case of hirsutenone two hydroxyl groups are presented on both benzyl rings). Diarylheptanoids platyphyllon and platyphyllonol-glycosides had already been reported from Betulaceae plants (Novaković 2013),

therefore, the presence of the hydroxyl groups was supposed in position 4 of the benzyl rings and compound **7** was characterised as platyphyllonol-pentoside (Fig. 30).

The molecular ion $[M-H]^-$ and characteristic product ions of **platyphyllonol-hexoside** (**8**) were detected at *m/z* 475.1974 and at *m/z* 295 and 189, respectively. The ESI-TOF conjunction analysis and the molecular formula calculation pointed to the formula $C_{25}H_{32}O_9$. The neutral loss of 180 amu indicated a hexose sugar residue, while the aglycone fragment ion and the other characteristic product ions indicated platyphyllonol aglycone (Fig.30.).

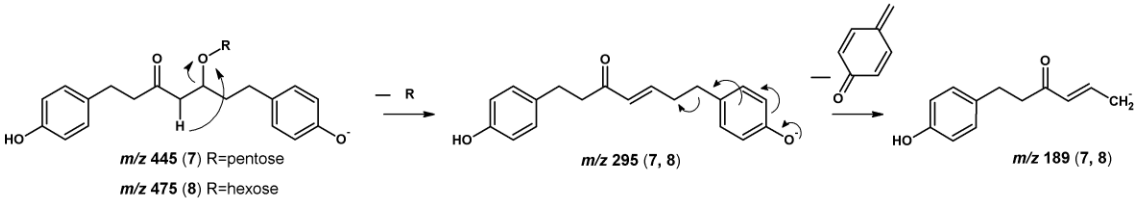


Figure 30. Assumed (-) ESI-MS/MS fragmentations of platyphyllonol-pentoside (**7**) and platyphyllonol-hexoside (**8**).

Compound **9** exhibited molecular ion $[M-H]^-$ at *m/z* 331.1545, while compound **10** at *m/z* 329.1383. In the molecular ion of compound **9** a shift of 4 Da was observed with reference to the molecular ion of hirsutenone (*m/z* 327), while the calculated molecular formulas ($C_{19}H_{24}O_5$ and $C_{19}H_{20}O_5$, respectively) showed a difference of 4 hydrogen atoms. Based on the (-) ESI-MS/MS spectra fragmentation shown in Figure 31 was assumed and compound **9** was tentatively identified as **3-hydroxy-1,7-bis-(3,4-dihydroxyphenyl)-heptan**. Both the exact masses and the molecular formulas of compounds **9** and **10** ($C_{19}H_{24}O_5$ and $C_{19}H_{22}O_5$, respectively) suggested the difference of two hydrogen atoms between the two compounds and possibly a presence of a double bond in the alkyl chain in case of compound **10.** In the molecular ion of compound **10** (*m/z* 329) a shift of 2 Da was observed with reference to the molecular ion of hirsutenone (*m/z* 327), while in the other characteristic product ion (*m/z* 207) also a mass shift of 2 Da was detected with reference to the most intense product ion of hirsutenone (*m/z* 205). Based on this data the presence of a hydroxyl group in position 5 and a double bond in the alkyl chain was supposed and compound **10** was tentatively

identified as **3-hydroxy-1,7-bis-(3,4-dihydroxyphenyl)-hepten** (Fig. 32.). Based on the molecular formulas and the (-) ESI-MS/MS fragmentation the possible structure 1,7-bis-(3,4-dihydroxyphenyl)-hepta-3-one for compound **10** cannot be excluded, but in that case the co-elution with compound **9** in the applied chromatographic system would be rather unlikely.

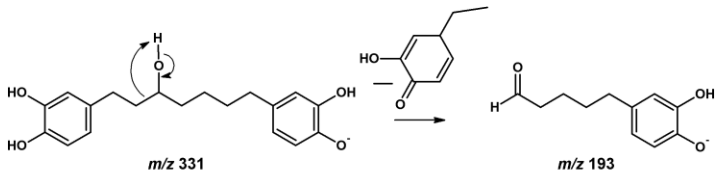


Figure 31. Assumed (-) ESI-MS/MS fragmentation of 3-hydroxy-1,7-bis-(3,4-dihydroxyphenyl)-heptan (**9**).

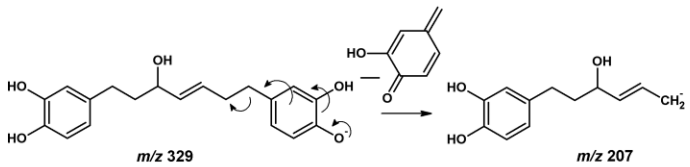


Figure 32. Assumed (-) ESI-MS/MS fragmentation of 3-hydroxy-1,7-bis-(3,4-dihydroxyphenyl)-hepten (**10**).

The molecular ion $[M-H]^-$ and characteristic fragment ions of compound **11** were detected at *m/z* 311.1283, and at *m/z* 205 and 189, respectively. The ESI-TOF conjunction analysis and the molecular formula calculation correspond to the formula $C_{19}H_{20}O_4$, which differed from that of hirsutenone ($C_{19}H_{20}O_5$) by one oxygen atom. The (-) ESI-MS/MS spectrum of the compound suggested the presence of only one hydroxyl group on one of the benzyl rings. Product ions at *m/z* 205 and 189 were produced by different neutral moiety losses, because either ring could be deprotonated during ionisation and hold the negative charge (Fig. 33.). Although it has to be noted that according to the (-) ESI-MS/MS spectrum the co-elution of two structural isomers 1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-hept-4-en-3-one (**11a**) and 1-(3,4-dihydroxyphenyl)-7-(4-hidroxyphenyl)-hept-4-en-3-one (**11b**) is also possible.

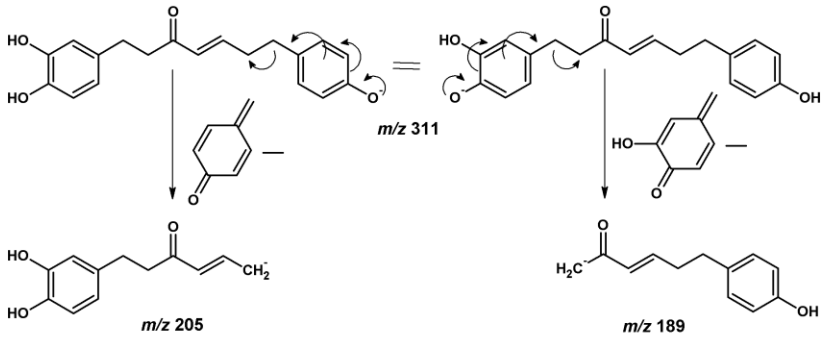


Figure 33. Assumed (-) ESI-MS/MS fragmentation of 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-hept-4-en-3-one (**11**).

The deprotonated molecular ion $[M-H]^-$ of **hirsutanolol** (**13**) was detected at *m/z* 345.1327. The ESI-TOF conjunction analysis and the molecular formula calculation pointed to the formula $C_{19}H_{22}O_6$, which differed from the formula of hirsutenone with two hydrogen and one oxygen atom. The characteristic fragment ions at *m/z* 205, 179 and 165 were also detected in the (-) ESI-MS/MS spectrum of the compound. The presence of a hydroxyl and a keto group on the alkyl chain at positions 5 and 3, respectively, was supposed and compound **13** was characterised as hirsutanolol. The detection of product ion at *m/z* 205 suggested fragmentation pathway **A,** while those at *m/z* 179 and 165 indicated that CID through pathway **B** also occurred, producing the mentioned ions via neutral moiety loss following a McLafferty rearrangement (Figure 34.) This latter was also observed in the case of compounds **14** and **15** (Fig. 35).

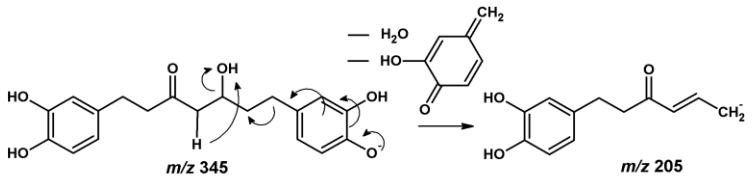


Figure 34. Assumed (-) ESI-MS/MS fragmentation of hirsutanolol (**13**).

Compounds **14** and **15** exhibited molecular ions $[M-H]^-$ at *m/z* 329.1339 and four abundant product ions at *m/z* 179, 165, 163 and 149. The ESI-TOF conjunction analysis and the molecule formula calculation pointed to the formula $C_{19}H_{22}O_5$. The UV spectra of the compounds corresponded to diarylheptanoid aglycone structure. The presence of a hydroxyl and a keto group on the alkyl chain at positions 5 and 3, respectively, was supposed, thus a loss of neutral moiety following a McLafferty rearrangement during

the (-) ESI-MS/MS analysis was assumed (Jiang et al, 2006). In case of compound **15** product ions at *m/z* 179 and 149 were produced by different neutral moiety losses, because either ring could be deprotonated and hold the negative charge (Fig. 35). The detection of product ions at *m/z* 165 and 163 supported the presence of a mixture of two isomers: **5-hydroxy-1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-heptan-3-one** (**14**) and **5-hydroxy-1-(3,4-dihydroxyfenil)-7-(4-hydroxyphenyl)-heptan-3-one** (**15**).

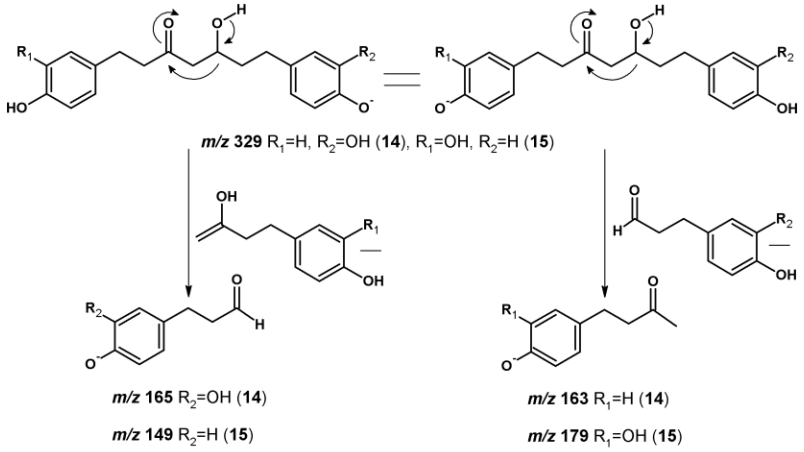


Figure 35. Assumed (-) ESI-MS/MS fragmentation of 5-hydroxy-1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-heptan-3-one (**14**) and 5-hydroxy-1-(3,4-dihydroxyfenil)-7-(4-hydroxyphenyl)-heptan-3-one (**15**).

6.3.2. Flavonoids

UV spectra with absorption maxima at 240-280 nm and 300-380 nm together with characteristic mass spectra indicated flavonoid structures in the case of nine compounds of the *Corylus* extracts (Fig. 36). From the accurate molecular mass and formula given by ESI-TOF, and fragmentation patterns acquired by collision-induced dissociation (CID) in ESI-MS/MS analyses compared to authentic standards and to literature data (Ablajan et al. 2013, Ablajan et al. 2006, Cuyckens and Claeys 2004, Fabre et al. 2001) it was possible to characterise the structures of the flavonoids, although the applied MS/MS method was not suitable for the accurate identification of the molecules where no matching standards were available. Myricetin-3-*O*-rhamnoside, quercetin-3-*O*-rhamnoside and kaempferol were identified by comparing their chromatographic and spectrometric data to authentic standards, while in the case of the other six flavonoid

compounds structural characterisation was carried out by comparison of the (-) ESI-MS/MS fragmentation to aglycone standards or to glycosides with the same aglycone but different sugar part, although it has to be noted that the unambiguous identification of the sugar moieties is not possible with the current MS/MS method. Besides this fact, according to literature data (Ablajan et al. 2006) the fragmentation behavior of flavonoid-*O*-glycosides strongly correlates with the glycosylation position. The cleavage of the glycosidic bond in deprotonated myricetin-, quercetin- and kaempferol-glycosides provide both radical aglycone ion ($[Y_0\text{-H}]^{-\bullet}$) and aglycone ion ($Y_0^-$) products. From the relative abundance of the aglycone ion it is possible to draw conclusions about the glycosilation position. Furthermore it has also been reported (Ablajan et al. 2013) that the type of the sugar moiety linked to the 3-*O* position has a certain effect on the formation and relative abundance on the $[Y_0\text{-H}]^{-\bullet}$ ion. Thus following optimization of the collision energy (CE) during the negative ion ESI-MS/MS analyses conclusions about both the glycosilation site and type of the sugar moiety in the flavonol-glycosides could have been drawn.

Since negative ion ESI-MS/MS characterisation of flavonoid aglycones and glycosides is well reported (Ablajan et al. 2013, Ablajan et al. 2006, Cuyckens and Claeys 2004, Fabre et al. 2001), explanation of fragmentation behavior is not discussed here in full details.

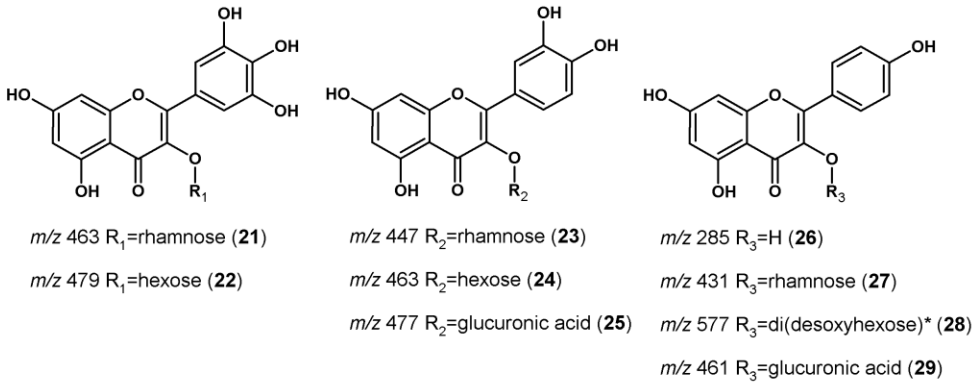


Figure 36. The flavonoid compounds of the *Corylus* extracts.

*in the case of compound **28** the accurate position of the R_3 group could not be identified

It has to be mentioned here that *C. avellana* leaves have already been reported to contain myricetin-3-*O*-rhamnoside, quercetin-3-*O*-rhamnoside, a quercetin-3-*O*-

hexoside and kaempferol-3-*O*-rhamnoside (Amaral et al. 2010), while no previous literature data was found about the presence of these compounds neither in the other two *Corylus* species nor in the bark of *C. avellana*.

Myricetin-3-*O*-rhamnoside (**21**) was identified in the extracts by comparison of chromatographic and mass spectrometric behaviour to those of an authentic standard, and also by spiking the sample solutions with the standard in two different chromatographic methods (see sections **4.6.1.** and **4.6.2.**). The compound provided deprotonated molecular ion $[M-H]^-$ at *m/z* 463.0871, aglycone fragments at *m/z* 316.0 ($[Y_0-H]^{-\bullet}$) and 316.9 (Y_0^-) and other characteristic product ions at *m/z* 287 and 271. The ESI-TOF conjunction analysis and the molecular formula calculation correspond to the formula $C_{21}H_{20}O_{12}$. The neutral loss of 146 amu confirmed the presence of a desoxyhexose sugar part. The product ion at *m/z* 316.0 ($[Y_0-H]^{-\bullet}$) was found to be relatively abundant in the (-) ESI-MS/MS spectra of both the compound and the reference standard. According to literature data (Ablajan et al. 2006), these observations strongly indicate the sugar moiety being located in 3-*O* position (in the case of flavonol-7-*O*-glycosides product ion $[Y_0-H]^{-\bullet}$ is not intense). These observations were applied for characterisation of the other myricetin-glycoside (compound **22**) in the extracts where no matching standard was available.

The molecular ion $[M-H]^-$ of **myricetin-3-*O*-hexoside** (**22**) was detected at *m/z* 479.0821, it exhibited characteristic fragment ions at *m/z* 316.0 ($[Y_0-H]^{-\bullet}$) and 316.9 (Y_0^-), 287 and 271. The ESI-TOF conjunction analysis and the molecular formula calculation corresponded to the formula $C_{21}H_{20}O_{13}$. The neutral loss of 162 amu pointed to a hexose sugar part. Based on the high relative intensity of the product ion at *m/z* 316.0 ($[Y_0-H]^{-\bullet}$) in the (-) ESI-MS/MS spectrum the hexose moiety was supposed to be in 3-*O* position. Since there is no literature data regarding the accurate structure of the hexose part in any of the *Corylus* species, it was not possible to draw any appropriate conclusions about it.

Quercetin-3-*O*-rhamnoside (**23**) was identified in the extracts by comparison of chromatographic and mass spectrometric behaviour to those of an authentic standard, and also by spiking the sample solutions with the standard in two different (see sections **4.6.1.** and **4.6.2.**). The compound provided deprotonated molecular ion $[M-H]^-$ at *m/z*

447.0919, aglycone fragments at *m/z* 299.9 ($[Y_0\text{-H}]^{-\bullet}$) and 301.0 ($Y_0^-$) and other characteristic product ions at *m/z* 271, 255, 179 and 151. The ESI-TOF conjunction analysis and the molecular formula calculation corresponded to the formula $C_{21}H_{20}O_{11}$. The neutral loss of 146 amu confirmed the presence of a desoxyhexose sugar part. Product ions at *m/z* 299.9 ($[Y_0\text{-H}]^{-\bullet}$), 271 and 255 were present in high abundances in the (-) ESI-MS/MS spectra of both the compound and the reference standard. According to literature data (Ablajan et al., 2006), these strongly indicated the sugar moiety being located in 3-*O* position (in the case of quercetin-7-*O*-glycosides product ion (Y_0^-) is not intense, while fragments *m/z* 271 and 255 are not presented in the (-) ESI-MS/MS spectrum). These observations were used for the characterisation of the quercetin-glycosides in the extracts where no matching standards were available.

Quercetin-3-*O*-hexoside (**24**) provided molecular ion $[M\text{-H}]^-$ at *m/z* 463.0871, aglycone fragments at *m/z* 301.0 ($[Y_0\text{-H}]^{-\bullet}$), 299.9 ($Y_0^-$) and other pronounced fragments at *m/z* 271, 255 and 151. The ESI-TOF conjunction analysis and the molecular formula calculation pointed to the formula $C_{21}H_{20}O_{12}$. Although both the exact mass and the molecular formula was found to be the same as in the case of myricetin-3-*O*-rhamnoside, the CID of the two compounds showed clear differences. The neutral loss of 162 amu from the molecular ion (*m/z* 463.1-> *m/z* 301.0) pointed to a hexose sugar part, while the characteristic product ions at *m/z* 301.0 ($[Y_0\text{-H}]^{-\bullet}$), 299.9 ($Y_0^-$), 271, 255 and 151 indicated quercetin aglycone. Based on the high relative intensity of the product ion at *m/z* 301.0 ($[Y_0\text{-H}]^{-\bullet}$) in the (-) ESI-MS/MS spectrum the hexose moiety was supposed to be in 3-*O* position. Since there is no literature data regarding the accurate structure of the hexose part in any of the *Corylus* species, it was not possible to draw any appropriate conclusions about it.

Quercetin-3-*O*-glucuronide (25) exhibited deprotonated molecular ion $[M\text{-H}]^-$ at *m/z* 477.0692, aglycone fragments at *m/z* 299.9 ($[Y_0\text{-H}]^{-\bullet}$) and 301.0 ($Y_0^-$) and other characteristic product ions at *m/z* 271, 255, 179 and 151. The ESI-TOF conjunction analysis and the molecular formula calculation pointed to the formula $C_{21}H_{18}O_{13}$. The neutral loss of 176 amu and the relatively high intensity of the product ion $[Y_0\text{-H}]^{-\bullet}$ suggested the presence of a glucuronide moiety in the 3-*O* position.

Kaempferol (**26**) was identified in the extracts by comparison of chromatographic and mass spectrometric behaviour to those of an authentic standard, and also by spiking the sample solutions with the standard in two different chromatographic methods (see sections **4.6.1.** and **4.6.2.**). The molecular ion of kaempferol was detected at *m/z* 285.1700, the characteristic product ions at *m/z* 255 and 227.

Compounds **27**, **28** and **29** exhibited the same aglycone fragment ion (*m/z* 285) and also the characteristic product ions at *m/z* 255 and 227 as kaempferol standard. In the MS spectrum of compound **27** the neutral loss of 146 amu pointed to a desoxyhexose moiety, in the case of compound **28** the neutral loss of 292 amu might indicate a di(desoxyhexose) sugar part, while in case of compound **29** the neutral loss of 176 amu suggested the presence of a glucuronide moiety. Therefore, compound **27** was tentatively identified as kaempferol-3-*O*-rhamnoside, compound **28** as kaempferol-di(desoxyhexoside) and compound **29 as** kaempferol-3-*O*-glucuronide. In case of compound **27** the characterisation of the sugar part was based on literature data, kaempferol-3-*O*-rhamnoside had been previously reported from hazelnut leaves (Amaral et al. 2010).

6.3.3. Other compounds

Although the applied chromatographic method was optimised for the investigation of diarylheptanoid and flavonoid derivatives in the *Corylus* extracts, it also allowed the characterisation of some compounds belonging to other structural groups, such as flavan derivatives (**32, 33**) and hydroxycinnamic acid derivatives (**30, 31, 34-37**) (Fig. 37).

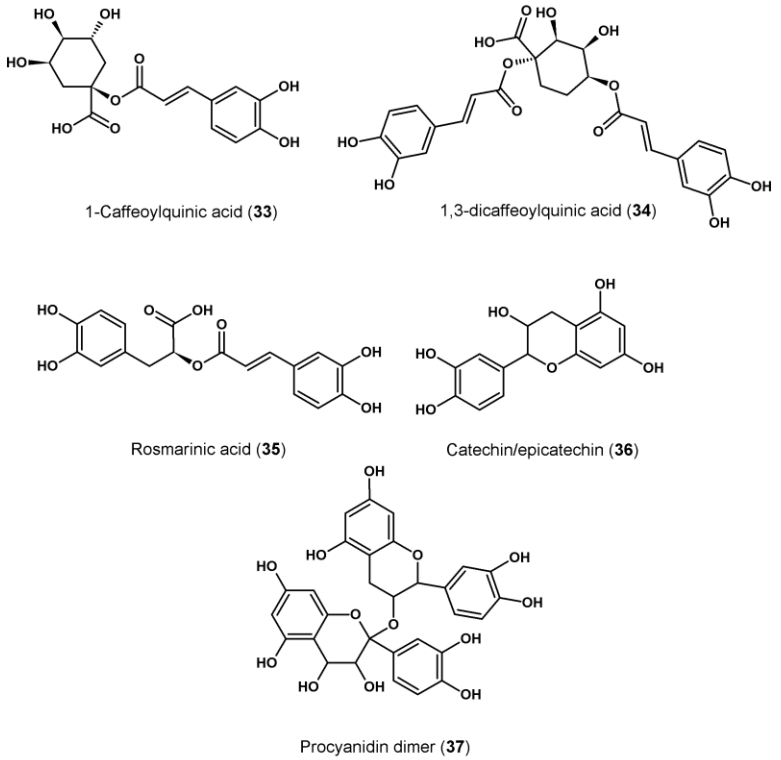


Figure 37. Structures of the hydroxycinnamic acid and flavan derivatives detected in the *Corylus* extracts.

Caffeic acid derivative (**30**) exhibited molecular ion $[M-H]^-$ at *m/z* 377.0885 and fragment ions at *m/z* 341, 215, 179 and 161. The ESI-TOF conjunction analysis and the molecular formula calculation pointed to the formula $C_{18}H_{18}O_9$. The compound was tentatively identified as a caffeic acid derivative based on comparison of its (-) ESI-MS/MS spectrum to that of caffeic acid authentic standard. Product ions at *m/z* 179 and 161 were presented in both the mentioned MS spectra, but no further conclusions could have been drawn about the accurate structure of the compound.

Caffeoyl-hexoside (**31**) provided molecular ion $[M-H]^-$ at *m/z* 341.0895 and fragment ions at *m/z* 179, 161, 143, 119, 89 and 59. The conjunction analysis of ESI-TOF and the molecular formula calculation suggested the formula $C_{15}H_{18}O_9$. The (-) ESI-MS/MS spectrum of the compound was compared to that of caffeic acid authentic standard. The neutral loss of 162 amu between *m/z* 341 and *m/z* 179 indicated a hexose sugar residue, while the other characteristic product ions a caffeic acid moiety, therefore, the compound was characterised as a caffeoyl-hexoside.

Quinic acid derivative (**32**) provided molecular ion $[M-H]^-$ and fragment ions at *m/z* 393.1749 and at *m/z* 191, 149, 113 and 89, respectively. The presence of fragment ion at *m/z* 191 in the (-) ESI-MS/MS spectrum might indicate a quinic acid moiety, but in the lack of matching standard and neither based on literature data it was not possible to draw conclusions about the accurate structure of the compound.

1-caffeoylquinic acid (**33**) exhibited molecular ion $[M-H]^-$ at *m/z* 353.0895 and fragment ions at *m/z* 191, 179, 173, 135 and 93. The ESI-TOF conjunction analysis and the molecular formula calculation pointed to the formula $C_{16}H_{18}O_9$. The peak at *m/z* 191 was found to be the base peak in the (-) ESI-MS/MS spectrum, while peaks at *m/z* 179, and 135 were relatively weak (<5% base peak) but still detectable. By comparison of these results to literature data (Nandutu et al. 2007) compound **33** was tentatively identified as 1-caffeoylquinic acid.

The molecular ion $[M-H]^-$ and product ions of **1,3-dicaffeoylquinic acid** (**34**) were detected at *m/z* 515.1195 and at *m/z* 353, 191 and 179, respectively. The peak at *m/z* 353 was the most intense in the (-) ESI-MS/MS spectrum, peak at *m/z* 191 was relatively intense, while peak at *m/z* 179 was comparatively weak but still detectable. Characterisation of the compound was based on comparison of its (-) ESI-MS/MS spectrum to literature data (Nandutu et al. 2007).

Rosmarinic acid (**35**) provided molecular ion $[M-H]^-$ at *m/z* 359.1126 and product ions at *m/z* 329, 269, 191 and 161. Identification of rosmarinic acid in the extracts was based on comparison of chromatographic and mass spectrometric behaviour to those of an authentic standard, and was also performed by spiking the sample solutions with the standard in two different chromatographic methods (see sections **4.6.1.** and **4.6.2.**).

The deprotonated molecular ion $[M-H]^-$ and characteristic fragment ions of **catechin/epicatechin** (**36**) were detected at *m/z* 289.0713 and at *m/z* 245, 205, 179, 161 and 137, respectively. Based on its UV, (-) ESI-MS/MS spectrum and molecular formula given by ESI-TOF ($C_{15}H_{14}O_6$) the compound was characterised as catechin or

epicatechin, although by the current method it was not possible to draw any further conclusions about its accurate structure.

Procyanidin dimer (**37**) exhibited molecular ion $[M-H]^-$ and fragment ions at at *m/z* 577.1339 and at *m/z* 451, 407, 339, 289, 161, and 125, respectively. The conjunction analysis of ESI-TOF and the molecular formula calculation suggested the formula $C_{30}H_{26}O_{12}$. Based on the comparison of UV and MS spectrum to literature data (Nandutu et al. 2007) we presumably identified the compound as a procyanidin dimer.

6.3.4. Comparison of the phenolic profile of the *Corylus* extracts

The HPLC-DAD-ESI-MS analyses revealed that the main compounds of all the *Corylus* extracts examined were flavonoid derivatives, except for the methanolic extract of *C. avellana* bark, in which a caffeic acid (**30**) derivative was found to be predominant. According to the HPLC chromatograms, in both the ethyl acetate and methanolic extracts of *C. avellana* leaves myricetin-3-*O*-rhamnoside (**21**) was the most abundant, while in the ethyl acetate extract of the bark the dominance of quercetin- (**23**) and kaempferol-3-*O*-rhamnosides (**27**) was observed. In all the *C. colurna* samples quercetin-3-*O*-rhamnoside (**23**), while in the *C. maxima* extracts myricetin-3-*O*-rhamnoside (**21**) were identified as the main compounds. The presence of myricetin- quercetin- and kampferol-3-*O*-rhamnoside was proved in all the extracts of the three *Corylus* species.

As it can clearly be seen in Figure 38, the greatest diversity regarding the structures of the detected diarylheptanoid compounds was observed in the case of the *C. maxima* extracts. Both in the leaves and bark extracts numerous structurally different diarylheptanoids were characterised (**1-7, 9, 11-15**). In the leaves of *C. avellana* also several diarylheptanoids were detected (**1, 3, 6, 8, 12, 14-18**), however in the bark extracts these compounds were not present. The *C. colurna* samples contained few diarylheptanoid compounds (**1, 2, 19, 20**) compared with the latter two.

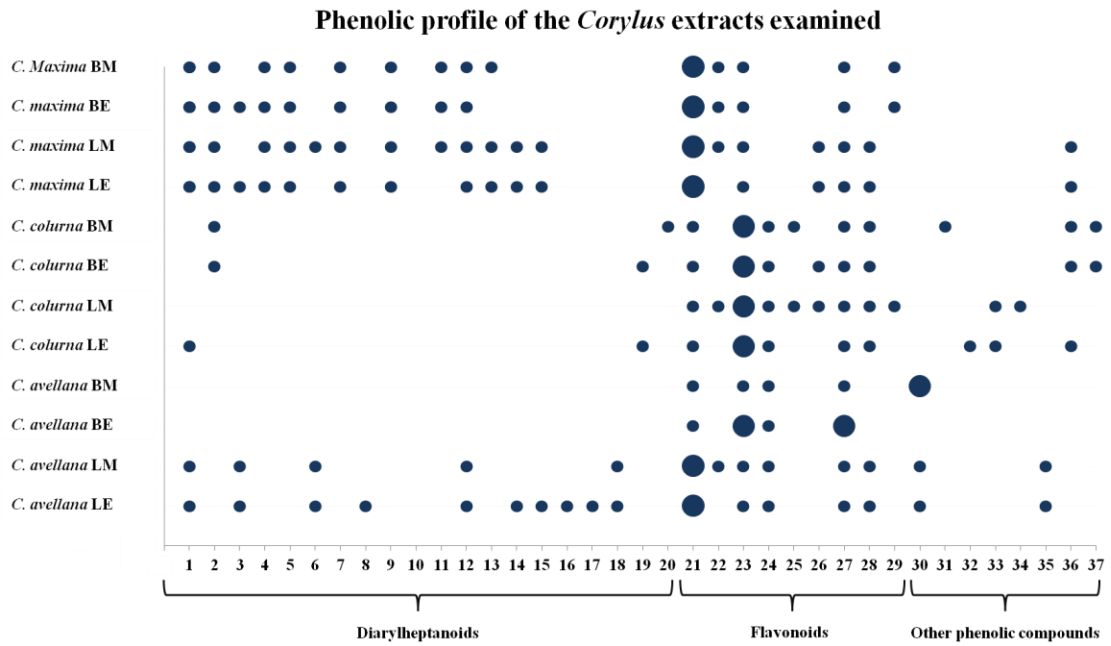


Figure 38. Phenolic profile of the *Corylus* extracts examined.

LE: Leaves ethyl acetate extract, LM: leaves methanolic extract, BE: bark ethyl acetate extract, BM bark methanolic extract. See compound numbering above and in Tables 5-7.

The results of the qualitative analyses by HPLC-DAD clearly indicated that in the cases of *C. avellana* and *C. colurna* the leaves extracts were shown to contain much higher number of phenolic compounds. For example, in the HPLC-DAD chromatogram of *C. colurna* leaves ethyl acetate and methanolic extracts circa 70 and 30 compound peaks were detected, respectively, while in the bark extracts these numbers were merely 11 and 20, respectively (Figures 9-20).

The HPLC-DAD-ESI-MS analyses also revealed that the ethyl acetate extracts showed higher chemical diversity regarding phenolic compounds compared with the methanolic extracts. In the case of all ethyl acetate samples significantly higher number of compound peaks could have been detected (Figures 9-20). This phenomenon was the most explicit in the case of *C. avellana* bark extracts, where in the ethyl acetate extracts circa 50, while in the methanolic extract merely 7 peaks could have been separated. Furthermore, it has also been observed that diarylheptanoids showed better solubility in ethyl acetate, thus it can be the appropriate solvent of choice for the enrichment of the *Corylus* extracts in these compounds.

6.4. Quantitative analyses by HPLC-MS/MS

The results of the quantitative analyses by HPLC-MS/MS clearly indicated that myricetin-3-*O*-rhamnoside (**21**) and quercetin-3-*O*-rhamnoside (**23**) were present in the extracts in much higher amounts than the investigated two diarylheptanoids, namely hirsutenone (**1**) and oregonin (**2**) (Fig. 39). Therefore, the quantity of the latter two compounds is shown separately in Figure 40, in order to make it easier to compare their content in the extracts.

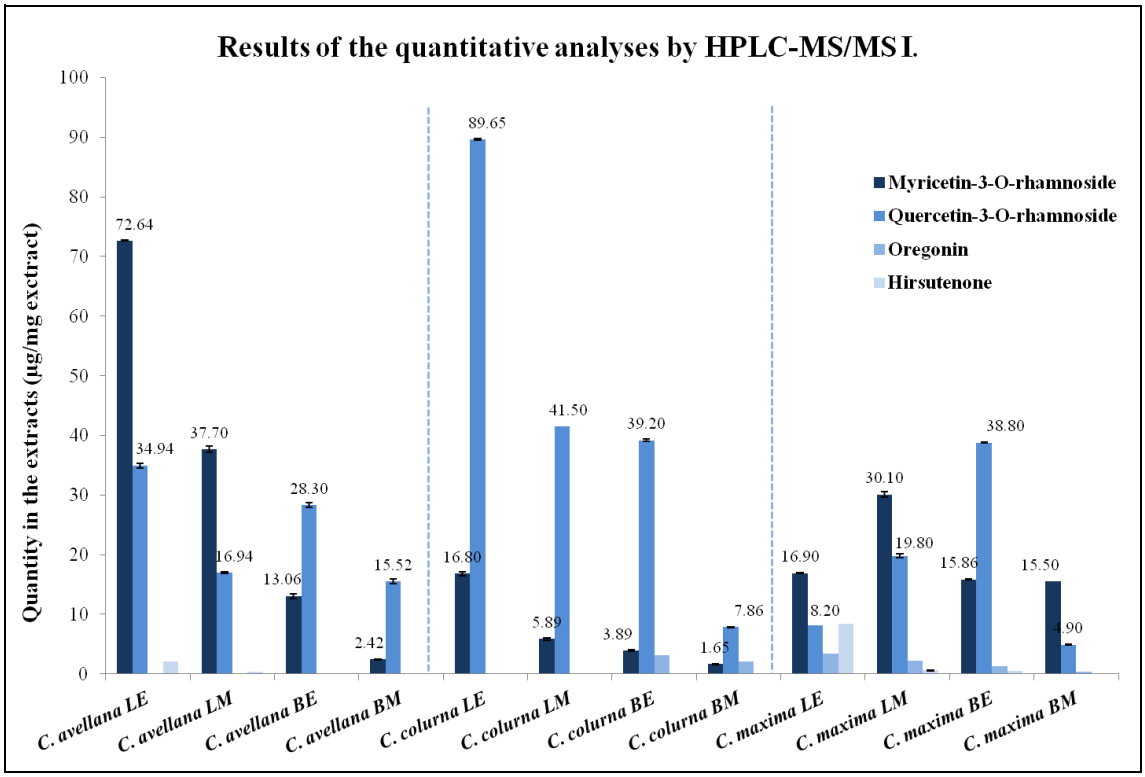


Figure 39. Results of the quantitative analyses by HPLC-MS/MS I.

LE: Leaves ethyl acetate extract, LM: leaves methanolic extract, BE: bark ethyl acetate extract, BM bark methanolic extract.

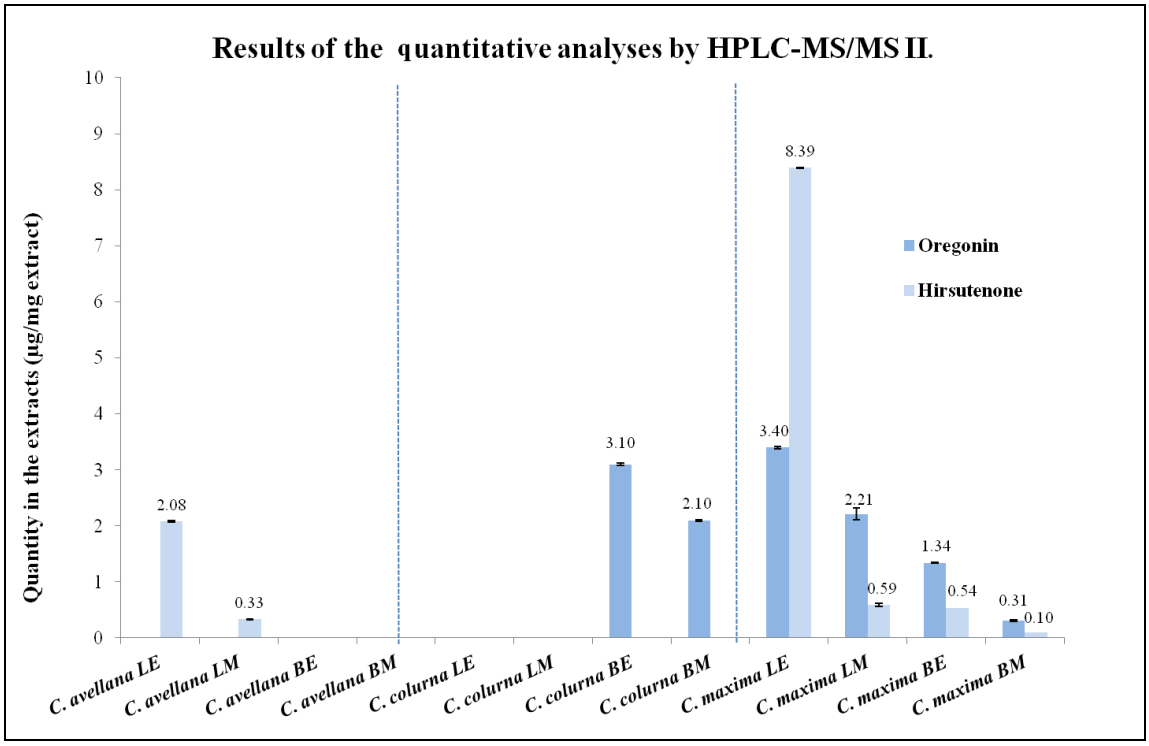


Figure 40. Results of the quantitative analyses by HPLC-MS/MS II.

It has to be noted that although the plant samples were collected during the same period of the year (August-September), late after the flowering stage, the plants were grown at different locations within Hungary, and the sample collection occurred in three consecutive years (2010-2012.). These parameters might influence the amount of certain secondary metabolites in the plants. Although the fact that flavonol-glycosides are present as main compounds in the *Corylus* extracts is unlikely to change with geographical or seasonal variation. This assumption is supported by the study carried out by Amaral et al., who investigated the influence of cultivar, geographical origin and ripening stage on the phenolic composition of *C. avellana* leaves (Amaral et al., 2010). The composition of 93 samples (19 cultivars collected along three crop years in two geographical locations) was studied. Besides, a seasonal pattern variation study was also performed on the phenolic composition of four cultivars under the same agricultural, geographical and climatic conditions from May to September. The main compound of all the samples was proved to be myricetin-3-*O*-rhamnoside (**21**), its content ranged from 1.79±0.004 to 21.68±0.76 µg/mg dry sample, while the quercetin-3-*O*-rhamnoside (**23**) content from 0.05±0.001 to 5.17±0.17 µg/mg dry sample. The authors concluded

that the total phenolic content in *C. avellana* leaves was mainly affected by the year of collection, and slightly by genetic variations. On the contrary, geographical location was proved not to have significant effect. It could have also been observed that although quantitative differences regarding the single compounds existed, the ratio among the flavonoid derivatives was relatively constant.

In our studies both myricetin-3-*O*-rhamnoside (**21**) and quercetin-3-*O*-rhamnoside (**23**) were present in all the extracts in amounts above the LOQ of the applied HPLC-MS/MS method. *C. colurna* leaves ethyl acetate extract was found to be the richest in quercetin-3-*O*-rhamnoside (**23**), while *C. avellana* leaves ethyl acetate extract in myricetin-3-*O*-rhamnoside (**21**) (see Table 10 in section **5.4.2.**). The ethyl acetate extracts were richer in both the flavonol-glycosides, except for the *C. maxima* extracts where this trend was unequivocal, moreover, in most cases the opposite was observed.

Regarding oregonin (**2**) and hirsutenone (**1**) the results (Table 10) showed that the quantity of the two compounds was below the LOQ of the applied HPLC-MS/MS method both in *C. avellana* bark and in *C. colurna* leaves extracts. Similarly, oregonin (**2**) was not detected in the leaves of *C. avellana*, while hirsutenone (**1**) in the bark of *C. colurna*. In the *C. maxima* extracts it was possible to quantify both oregonin (**2**) and hirsutenone (**1**). The highest quantities of the two diarylheptanoids were measured in *C. maxima* leaves ethyl acetate extract; this was found to be richer in hirsutenone (**1**), while the other three contained higher amounts of oregonin (**2**).

In general it could have been observed that in case of all the samples, the ethyl acetate extract contained the two investigated diarylheptanoids in higher amounts compared with the corresponding methanolic extract. This observation was in accordance with the results of the qualitative analyses (see section **5.3.**), that showed that greater structural diversity regarding diarylheptanoid compounds could be observed in the ethyl acetate extracts. These results together confirm that diarylheptanoids show better solubility in ethyl acetate, thus it can be the appropriate solvent of choice for the enrichment of the *Corylus* extracts in these compounds.

Literature data reporting high antioxidant activity regarding not only flavonoids but also diarylheptanoids, and the fact that the former were present in the extracts in notable amounts, made the correlation of the myricetin-3-*O*-rhamnoside (**21**) and quercetin-3-*O*-rhamnoside (**23**) content with the DPPH and ABTS scavenging capacity reasonable.

6.5. HPLC-based DPPH scavenging assay

Our qualitative and quantitative results supported the assumption that the phenolic compounds played an important role in the high antioxidant activity of the *Corylus* extracts. The dominance of flavonol-3-*O*-glycosides was observed in the case of all the examined samples, while diarylheptanoids were present in the extracts as minor constituents. Therefore, only the correlation between the content of the two main flavonoid derivatives, namely myricetin-3-*O*-rhamnoside (**21**) and quercetin-3-*O*-rhamnoside (**23**) in the extracts (see section **5.4.2.**), and the antiradical power presented in the DPPH and ABTS *in vitro* tests (see section **5.2.**) was investigated by plotting the $1/IC_{50}$ data as the function of the corresponding compound concentrations. As it can clearly be seen in Figure 41, no correlation has been found neither with the myricetin-3-*O*-rhamnoside (**21**) nor with the quercetin-3-*O*-rhamnoside (**23**) content of the samples, nor with the sum of the two.

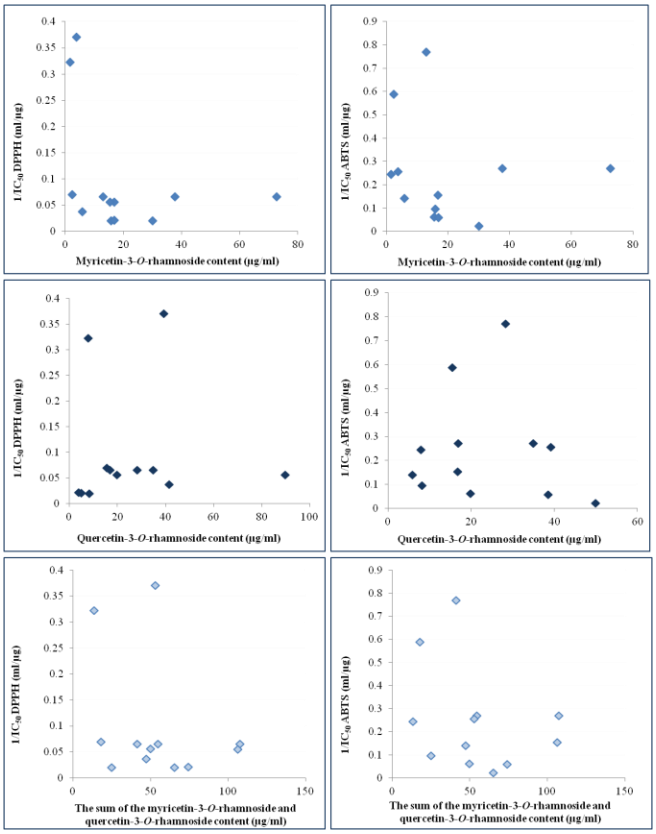


Figure 41. Antiradical power ($1/IC_{50}$ DPPH and ABTS) vs. the myricetin-3-*O*-rhamnoside and quercetin-3-*O*-rhamnoside content.

The interpretation of the antioxidant activity of complex mixtures of natural products, such as plant extracts, is rather limited: synergistic, antagonistic or additive interactions in redox and/or radical reactions can occur (Choe et al 2009, Decker et al. 2002). The observation that no correlation has been found between the antiradical power and the quantity of the main flavonoids in the extracts let us conclude that the scavenger capacity of the major constituents was influenced by the minor compounds, e.g. diarylheptanoids and caffeic acid derivatives. Therefore, examination of the contribution of certain compounds to the total antioxidant activity of the extracts was found to be reasonable. Coupling the DPPH assay to chromatographic separation was found to be most appropriate method of choice for this purpose.

The first such method was developed in 1967 (Glavind 1967): after TLC separation of the compounds, the radical scavengers were identified by spraying the TLC plate with the DPPH solution. Evidently, by the use of this method, achievement of high resolution between the antioxidant compounds is not always feasible. Therefore, HPLC-based DPPH assays are the favourable choice when investigating plant extracts containing multiple components. Such approaches have also been developed in order to identify the radical scavenger constituents in plant extracts by monitoring the decrease in the chromatographic peak areas of certain compounds after reaction with DPHH free radical. It has been demonstrated that the peak areas of constituents presenting antioxidant activity significantly decreased, whereas no change was observed regarding the peaks of compounds without scavenging activity (Könczöl et al. 2012, Tang et al. 2008). Similar approach was utilised for the identification of the antioxidant constituents in the *Corylus* extracts. For this purpose a HPLC-MS method coupled with DPPH free radical scavenging assay was developed (for the methods see section **4.8.**, for the results, section **13.2.**).

Regarding the decrease in the peak areas of the three main flavonoid compounds the following trend was observed: myricetin-3-*O*-rhamnoside (**21**) > quercetin-3-*O*-rhamnoside (**23**) > kaempferol-3-*O*-rhamnoside (**27**)**.** For the calculations applied see equations 1-3. in section **5.5**.

The decrease in the peak area of myricetin-3-*O*-rhamnoside **(21)** varied between 81.52 % and 100.0% among the different extracts. The two lowest values were observed

in the case of *C. colurna* leaves ethyl acetate extract (87.90%) and *C. maxima* bark methanolic extract (81.52%), in all the other extracts the decrease in the peak area was more than 95.20%. The observed peak area changes of quercetin-3-*O*-rhamnoside **(23)** varied between 29.77% and 100%. High values were measured in the case of the leaves methanolic extracts and also in *C. maxima* leaves ethyl acetate extract. The explanation of this phenomenon might be the low quercetin-3-*O*-rhamnoside content in these extracts compared with the others; hence the amount of DPPH utilized was enough to react with all the quercetin-3-*O*-rhamnoside molecules present in the samples. The average decrease in the peak areas regarding the other eight extracts was 45.05±10.50%, significantly lower than in the case of myricetin-3-*O*-rhamnoside (**21**). Regarding kaempferol-3-*O*-rhamnoside (**27**), the average value of the decrease in the peak areas was only 7.40±5.18%, significantly lower than that of myricetin-3-*O*-rhamnoside (**21**) and quercetin-3-*O*-rhamnoside (**23**), respectively (see section **13.2.**). The enlarged chromatograms of *C. avellana* leaves methanolic extracts are present in Figure 42 as an example.

These results are in good agreement with literature data. According to previous reports the major factors that determine the free radical scavenging activity of flavonoids are the number of free hydroxyl groups in the molecule; the ortho-dihydroxy structure on the B ring; the 2,3 double bond with a 4-oxo function in the C ring, which is responsible for electron delocalisation from the B ring; the 3- an 5-hydroxyl groups with the 4-oxo function in A and C rings and the 3-hydroxyl group (Dai et al. 2010). Since myricetin-3-*O*-rhamnoside (**21**), quercetin-3-*O*-rhamnoside (**23**) and kaempferol-3-*O*-rhamnoside (**27**) differ merely in the hydroxylation of the B ring, only the first two structural features contribute to the difference in their DPPH scavenging activity (Fig. 42).

In general, the rate of the radical quenching reaction of polyphenolic compounds is mainly determined by the BDE of the phenolic O-H bound. Electron donating groups neighboring the reactive hydroxyl function lower the BDE(O-H), while electron-withdrawing groups produce the opposite outcome (Amorati et al. 2012). This phenomenon depends on the ability of the above mentioned groups to stabilise the phenoxyl radical formed after H-atom abstraction. Intramolecular H-bonding that involves either the reactive hydroxyl groups or a remote hydroxyl function also influences the antioxidant effect. The phenolic hydroxyl function acts as H-bond donor

and acceptor as well, therefore *ortho* hydroxyl groups are able to stabilize the phenol and to larger extent, the phenoxyl radical (Amorati et al. 2012). Accordingly, myricetin-3-*O*-rhamnoside (**21**) and quercetin-3-*O*-rhamnoside (**23**) that bear pyrogallol and catechol moieties, respectively, presented enhanced radical quenching activity, with myricetin-3-*O*-rhamnoside (**21**) being the most potent antioxidant.

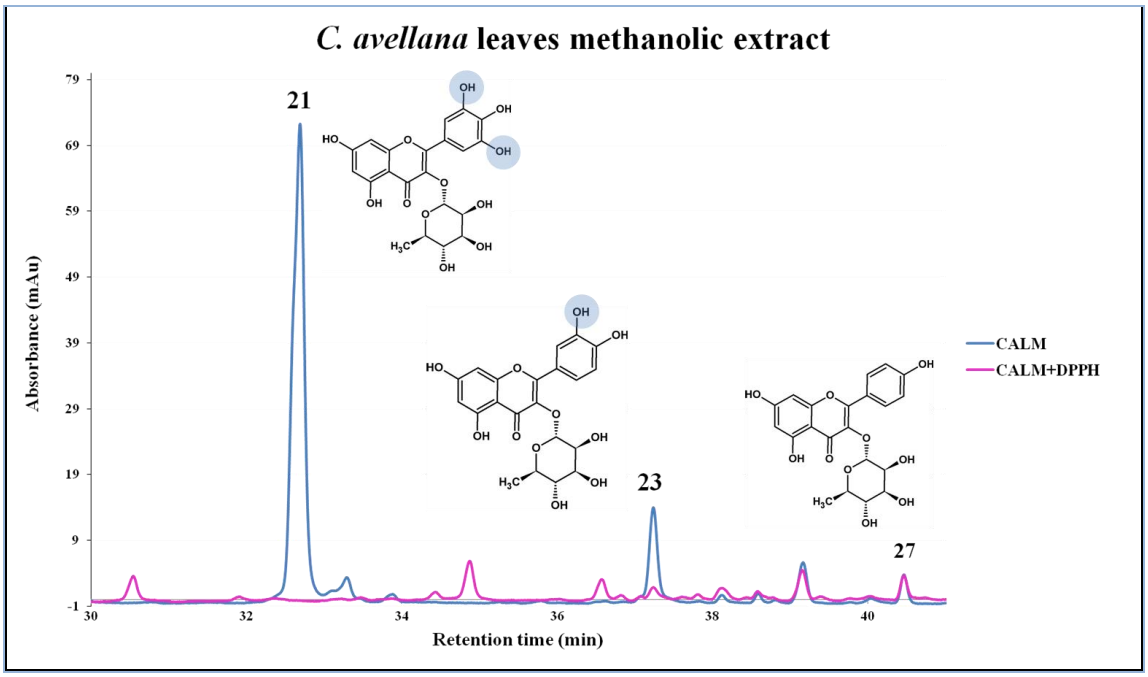


Figure 42. Chromatograms of the control sample and the sample after spiking with DPPH of *C. avellana* leaves methanolic extract (CALM) (see the method section **4.8.**)

It also has to be mentioned that the number of free hydroxyl groups in the molecules increases in the following order: kaempferol-3-*O*-rhamnoside (**27**) < quercetin-3-*O*-rhamnoside (**23**) < myricetin-3-*O*-rhamnoside (**21**) and so does the antioxidant activity. The positive effect of the catechol moiety on the radical scavenger activity of diarylheptanoids has also been proved: oregonin (**2**), 1,7-bis-(3,4-dihydroxyphenyl)-3-hydroxyheptane-5-*O*-β-D-xylopiranoside, platyphylloside and curcumin were examined in the DPPH assay (Ponomarenko et al. 2014). Oregonin (**2**) and 1,7-bis (3,4-dihydroxyphenyl)-3-hydroxyheptane-5-*O*-β-D-xylopiranoside presented very similar activity, both appeared significantly more potent than trolox and curcumin, while no scavenger activity was measured regarding platyphylloside. BDE(O-H) calculations fully supported these results. The lowest BDE(O-H)s were obtained for oregonin and

1,7-bis-(3,4-dihydroxyphenyl)-3-hydroxyheptane-5-*O*-β-D-xylopiranoside with the BDEs of the 4- and 4'-hydroxyl groups being slightly lower than those of the 3- and 3'-OH functions. Besides, the BDEs for curcumin and platyphylloside were much higher due to the lack of the catechol group that made them less active DPPH scavengers.

The analyses of the results obtained in the DPPH assay performed on the cyclic diarylheptanoids of *Myrica rubra* allowed the authors to draw conclusions about diarylheptanoid antioxidant action: a hydroxyl group at C-11 position instead of carbonyl does not improve the activity; an extra hydroxyl group at carbon C-5 is also irrelevant; on the other hand the loss of a methyl group causes a strong increase in the antioxidant effect; the presence of a sugar moiety, as well as its type and localisation also interfere the antioxidant activity (Silva et al. 2015).

In our experiments the decrease in the peak areas of diarylheptanoid aglycones, bearing catechol function, namely hirsutanolol (**13**) and 3-hydroxy-1,7-bis-(3,4-dihydroxypheyl)-hepten (**10**), was the most explicit. It has to be mentioned here again that steric accessibility is considered one of the major determinants of the DPPH quenching reaction, meaning that smaller molecules, e.g. aglycones have better access to the radical site, thus show better scavenger capacity in the test compared with their glycoside counterparts. It also has to be noted that the major part of diarylheptanoids was present as minor compounds in the extracts, thus detection of the decrease in their peak areas was not feasible in every case.

Based on our results (Tables A2-13 in section **13.2.**) the contribution of certain compounds to the total antioxidant activity could have also been estimated by using the ΔArea ratio value (see eq 3 in section **5.5.**). Evidently, these calculations can be used only if the stochiometry of the DPPH scavenging of the main compounds is similar. Fortunately, the flavonol-3-*O*-glycosides and also the previously mentioned diarylheptanoids are able to scavenge the DPPH free radical in the ratio of 1:2-3 (Ponomarenko et al. 2014, Siasos et al. 2013, Saito et al. 2005). Obviously, regarding the 'other', not identified compounds no such data exists, thus the contribution of these constituents to the total antioxidant activity should be considered merely estimation.

In general, the greatest diversity could have been observed in the leaves ethyl acetate extracts regarding the compounds that were shown to play role in the DPPH scavenging activity. In *C. avellana* ethyl acetate extract myricetin-3-*O*-rhamnoside (**21**) was

dominant, in the other two ethyl acetate extracts no compounds could have been identified as the main constituent responsible for the antioxidant effect. In *C. avellana* and *C. maxima* leaves methanolic extracts also myricetin-3-*O*-rhamnoside (**21**) played the most significant role in the free radical scavenging activity.

As it was reported already in section **5.3.**, the bark extracts in general were found to be less diverse regarding different components compared with the leaves extracts. Thus, it was perspicuous that the antioxidant compounds did not show as much diversity as in the leaves. The *C. avellana* and *C. colurna* bark extracts showed similar pattern: quercetin-3-*O*-rhamnoside (**23**) was proved to be dominant.

Results are presented in Figures 43-45. It has to be noted that the percentages reported here can not be considered as exact results, rather estimations.

In *C. avellana* leaves ethyl acetate extract relatively high amounts of myricetin-3-*O*-rhamnoside (**21**) and quercetin-3-*O*-rhamnoside (**23**) were determined (see section **5.4.2.**). According to the HPLC-based results the contribution of the two compounds to the DPPH scavenger activity was nearly 80% that resulted in moderate to high DPPH activity of the extract (see section **5.2.**). Similar results were obtained regarding the methanolic extract of the leaves; moderate to high content of the two previously mentioned flavonoids with nearly 90% contribution to the scavenger capacity determined moderate to high antioxidant activity for the whole extract. In the bark extracts quercetin-3-*O*-rhamnoside (**23**) was proved to be dominant regarding the DPPH scavenging effect, moderate amount of this compound led to moderate antioxidant activity.

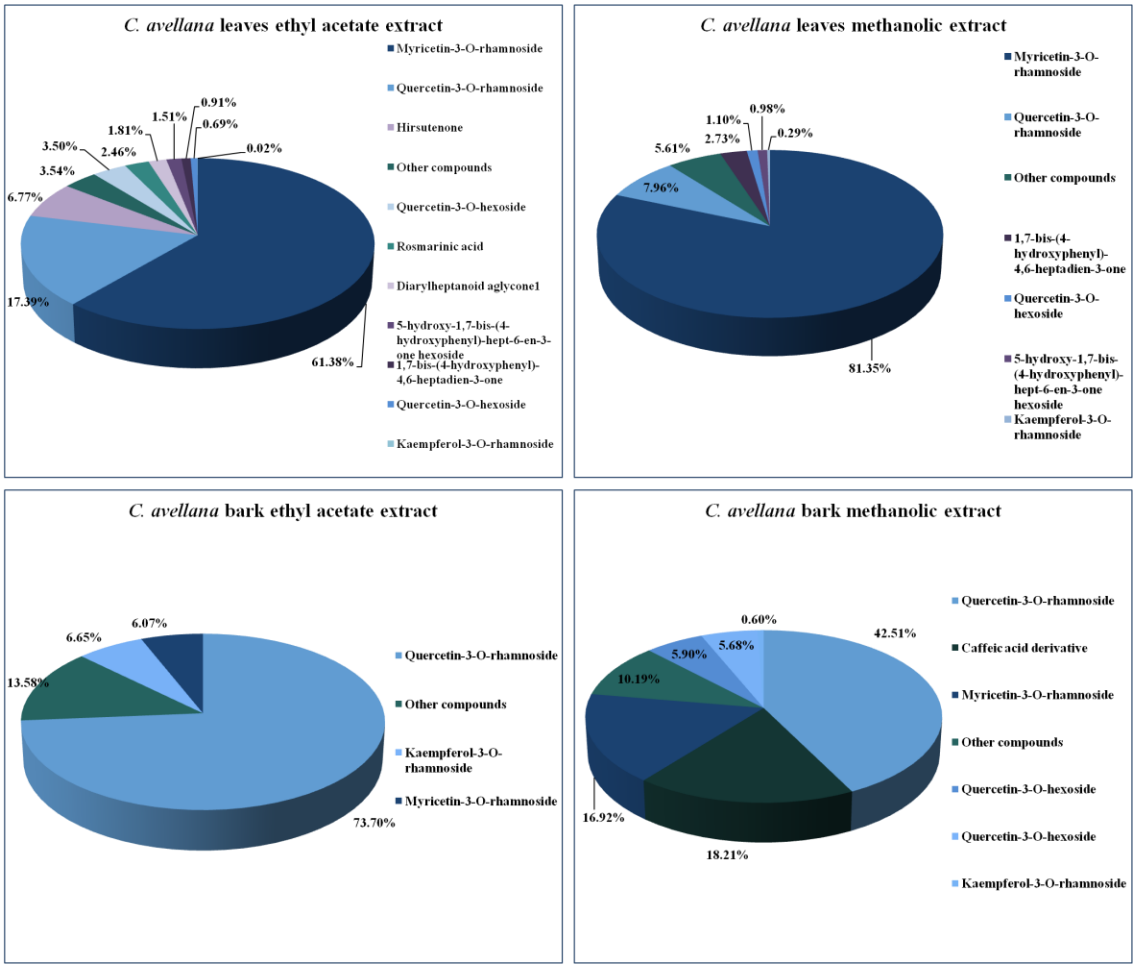


Figure 43. Contribution of the main compounds detected in the *C. avellana* extracts to the DPPH scavenger activity.

In the *C. colurna* leaves ethyl acetate extract the highest quercetin-3-*O*-rhamnoside (**23**) content was measured among the investigated samples, although the contribution of the compound to the antioxidant activity was relatively low. Furthermore, only moderate scavenger capacity was measured regarding the whole extract. These results might indicate antagonistic interaction between the antioxidant components. Similar conclusion could have been drawn regarding the methanolic extract. The highest DPPH scavenging activity was obtained in the case of the *C. colurna* bark extracts. Quercetin-3-*O*-rhamnoside (**23**) was found to be predominant in the antioxidant effect, which was present in high amounts in the ethyl acetate extract. It was also observed that the methanolic extract possessed lower scavenging activity compared with the ethyl acetate extract in accordance with the significant difference in their quercetin-3-*O*-rhamnoside

(**23**) content. Although, it has also to be mentioned that in the methanolic extract kaempferol-3-*O*-glucuronide, the main compound, was found to contribute mostly to the scavenger capacity.

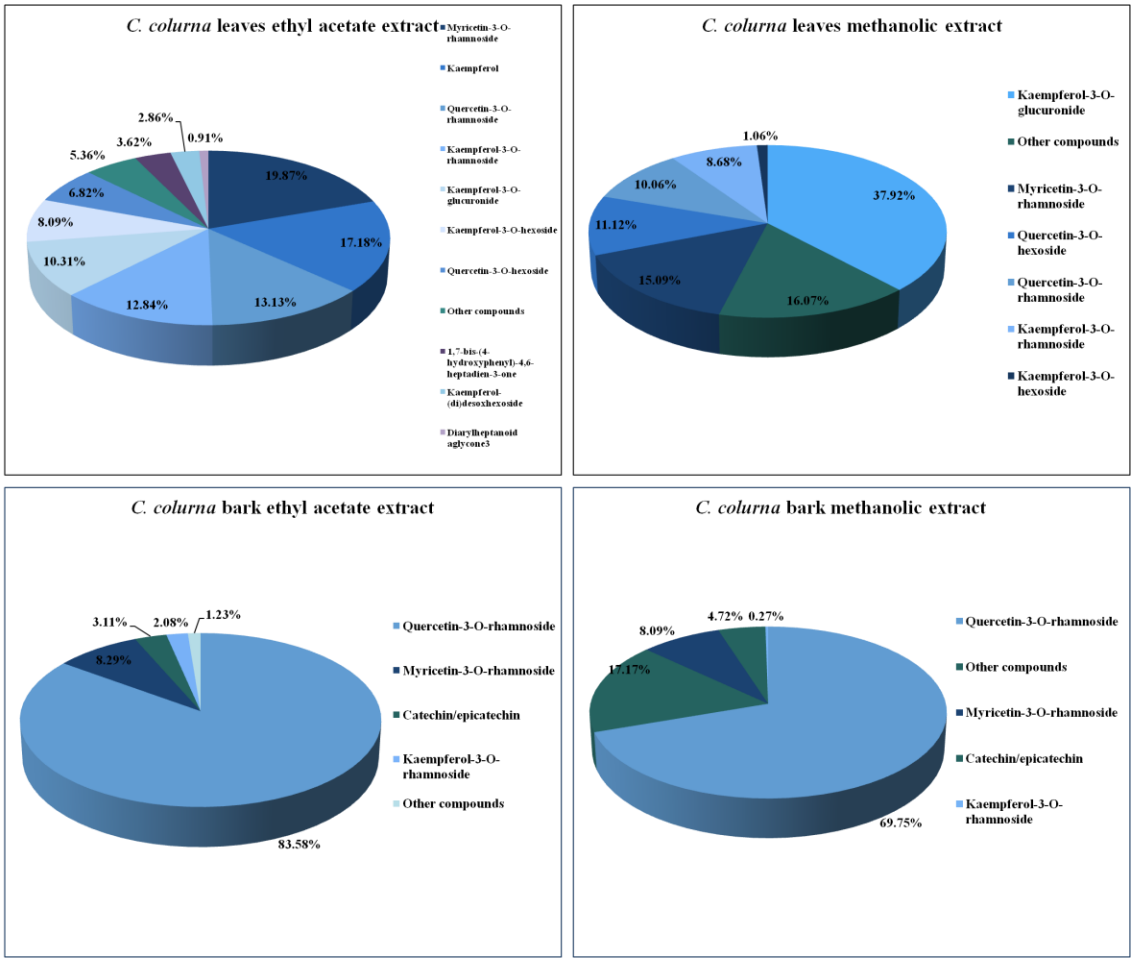


Figure 44. Contribution of the main compounds detected in the *C. colurna* extracts to the DPPH scavenger activity.

In the *C. maxima* leaves extracts moderate amounts of myricetin-3-*O*-rhamnoside (**21**) and quercetin-3-*O*-rhamnoside (**23**) were determined, although the extracts showed low DPPH scavenging activity. This can be contributed to the large diversity of these extracts regarding antioxidant compounds that makes several interactions possible. In the bark extracts also moderate amounts of the two flavonoids were measured, neither their contribution to the DPPH scavenger capacity was explicit, which led to moderate or low antioxidant activity regarding the whole extract.

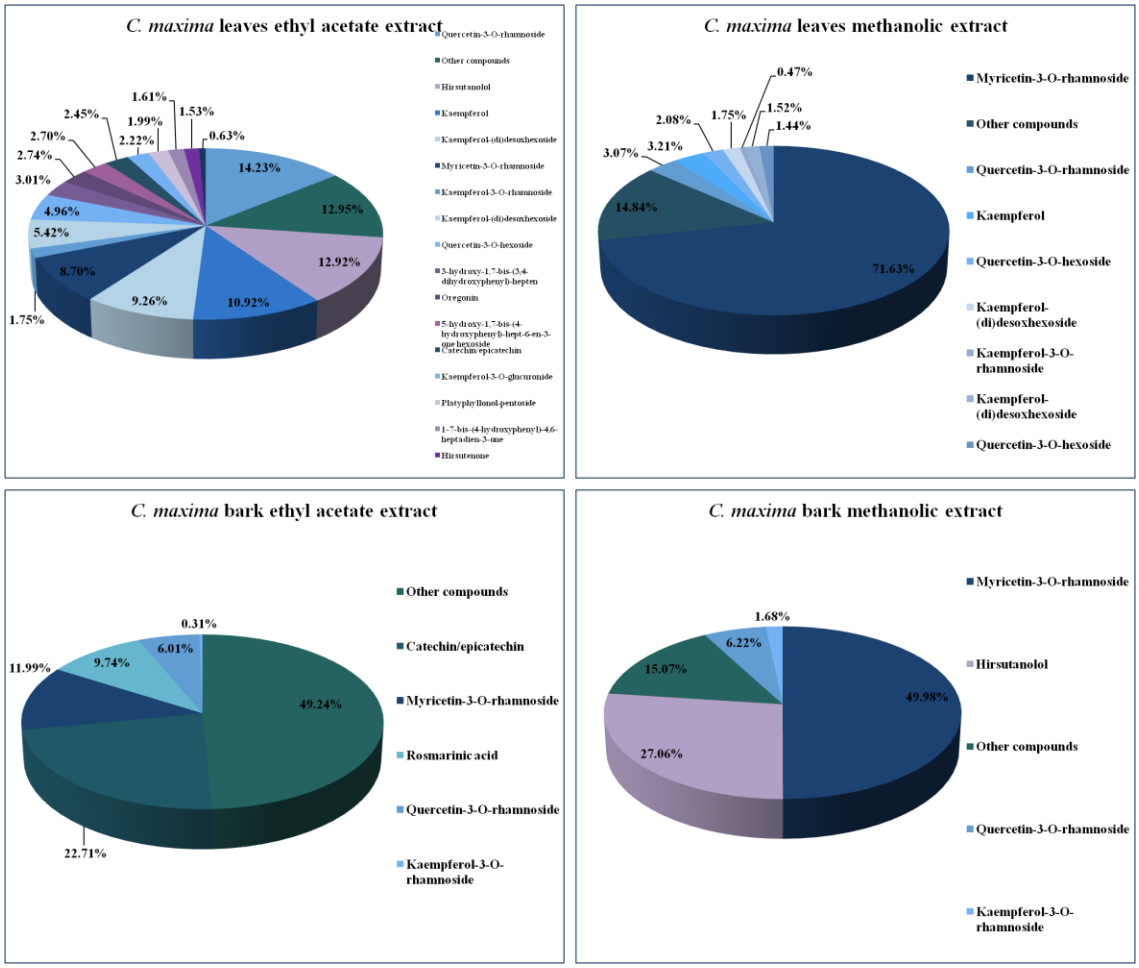


Figure 45. Contribution of the main compounds detected in the *C. maxima* extracts to the DPPH scavenger activity.

It could have been generally concluded that the correlation between the amount of the main antioxidant compounds and the scavenger capacity of the extracts is not always equivocal and let us assume the presence of different interactions among the constituents. Our results could be successfully utilised in further studies aiming the enhancement of the antioxidant capacity of the extracts by the preparation of fractions containing the most potent antioxidant compounds.

7. CONCLUSIONS

1. Total phenolic, flavonoid and tannin contents of the *Corylus* crude drugs were determined according to the spectroscopic methods described in Ph. Hg. VIII. Based on our results it could be concluded that all the crude drugs contained notable amounts of polyphenol compounds, with *C. maxima* bark being the richest in these constituents.

2. Since plant phenolics are widely reported to act as antioxidants, and the *Corylus* crude drugs were proved to be rich in these constituents, determination of the *in vitro* radical scavenging activity of the ethyl acetate and methanolic extracts was found to be reasonable. Two *in vitro* tests using DPPH and ABTS as free radicals were utilised. Our results indicated that all the extracts possessed notable activity in both *in vitro* tests compared to well-know antioxidant standards. Comparison of the results of the different extracts to each other revealed that no trend occurred regarding the scavenger capacity. These observations let us conclude that there were differences in the phenolic profile of the extracts studied.

3. HPLC-ESI-MS methods were developed for the characterisation of phenolics in the *Corylus* extracts that provided uniform platform for the investigation of both flavonoid and diarylheptanoid-type compounds. For the characterisation of the compounds UV spectral data, obtained by LC-DAD, accurate molecular mass and formula, acquired by LC-ESI-TOF and fragmentation pattern, given by LC-ESI-MS/MS analyses were compared to those of authentic standards and to literature data. Altogether twenty diarylheptanoids (**1-20**), nine flavonoid derivatives (**21-29**) and eight other compounds, mainly caffeic acid derivatives, (**30-37**) were characterised in the *Corylus* extracts. The presence of myricetin- (**21**) quercetin- (**23**) and kampferol-3-*O*-rhamnoside (**27**) was proved in all the extracts of the three *Corylus* species, but the obtained HPLC-fingerprints were different. Therefore, it could be concluded that the developed HPLC-DAD-ESI-MS methods can be successfully utilised for the identification and differentiation of the *Corylus* extracts obtained from the three species. One of the novel

findings of our qualitative studies was the characterisation of diarylheptanoids, since no previous paper had reported the presence of these compounds in *Corylus* species. Further studies, including isolation and identification of these constituents by NMR spectroscopy, would be worthy to perform. In addition to the the former results, the phytochemical investigation of *C. maxima* and *C. colurna* and the characterisation of their phenolic compounds have been reported in this study for the first time. Results of the detailed investigation of the phenolic profile of the *Corylus* species might explain their previously reported antioxidant, anti-inflammatory, anti-microbial and hepatoprotective effects. Besides, comparison of the phenolic profile of each *Corylus* species to other members of the Betaulaceae family would be interesting from the chemotaxonomic point of view as well.

4. For the determination of the two main flavonoid compounds, namely myricetin-3-*O*-rhamnoside (**21**) and quercetin-3-*O*-rhamnoside (**23**), and the two most abundant diarylheptanoids, hirsutenone (**1**) and oregonin (**2**), a HPLC-ESI-MS/MS method using MRM (multiple reaction monitoring) mode was developed and validated. The method was successfully utilised for the quantitative analyses of the *Corylus* samples, and might be useful in future studies aiming the quality control of different *Corylus* extracts used in medication. In addition, this quantitative method would be applicable for the investigation of the influence of cultivar, geographical origin, seasonal variations and ripening stage on the yield of the compounds with relevant biological activity.

5. Our qualitative and quantitative results supported the presumption that the phenolic compounds played significant role in the high antioxidant activity of the *Corylus* extracts. The dominance of flavonol-3-*O*-glycosides was observed in the case of all the examined samples, while diarylheptanoids were present as minor constituents. Literature data reporting high antioxidant activity regarding not only flavonoids but also diarylheptanoids, and the fact that no correlation has been found between the antiradical power and the quantity of the main

flavonoids in the extracts let us conclude that the scavenger capacity of the major constituents was influenced by the minor compounds, e.g. diarylheptanoids. Therefore, a high-performance liquid chromatographic-mass spectrometric method coupled with DPPH free radical scavenging assay was developed in order to gain information about the contribution of certain constituents to the total antioxidant activity. Based on our results we could conclude that in some cases the quantity of the main flavonol-3-*O*-glycosides in the extracts strongly affected the scavenger capacity, but in other cases, where several compounds played role in the antioxidant effect, the contribution of the main compounds was not equivocal. Thus, further studies aiming the clarification of the interactions between the antioxidant compounds (synergism, antagonism or additive effect) are needed, in order to investigate the possibility of the preparation of fractions with evaluated antioxidant effect from the extracts.

8. SUMMARY

Identification of the major and minor bioactive constituents of traditionally used medicinal plants is a clearly promising way of discovering novel lead compounds for pharmaceutical researches. Based on the fact that plant extracts are complex matrices with numerous different constituents, the development of sophisticated analytical methods is a crucial point in the analysis of phytotherapeutics.

The primary aim of our study was the phytochemical evaluation of the *Corylus* (Betulaceae) species native to Hungary: *Corylus avellana* L, *Corylus colurna* L. and *Corylus maxima* Mill. Despite the long-term use of these plants in traditional medicine, their phytochemical exploration is incomplete.

The total phenolic, flavonoid, and tannin contents of the bark and leaves crude drugs of the three Corylus species were determined by spectroscopic methods. All the plant samples were found to be rich in phenolic compounds.

Evaluation of *in vitro* scavenging activity of the *Corylus* extracts prepared with semi-polar solvents was chosen as the starting point for further analyses. Our results indicated that all the extracts possessed notable activity in both the DPPH and ABTS tests.

For the qualitative analysis of the phenolic composition of the *Corylus* extracts, as well as for the structural characterisation of the detected compounds, HPLC-DAD, HPLC-ESI-TOF and HPLC-ESI-MS/MS methods were developed. Altogether twenty diarylheptanoids, nine flavonoid derivatives and eight other compounds, mainly caffeic and quinic acid derivatives, were characterised. The presence of myricetin- quercetin- and kampferol-3-*O*-rhamnoside has been proved in all the extracts of the three *Corylus* species, but the obtained HPLC-fingerprints were different.

A HPLC-ESI-MS/MS method for the quantitative determination of the main phenolic compounds in the *Corylus* extracts was developed and validated.

A HPLC-DAD-ESI-MS method coupled with DPPH free radical scavenging assay was developed and successfully utilised for the investigation of the contribution of certain compounds to the total radical scavenging activity.

The developed HPLC-DAD-ESI-MS methods were found to be highly sensitive and selective for the successful utilisation in both the qualitative and quantitative analyses of the *Corylus* extracts examined.

9. ÖSSZEFOGLALÁS

A tradicionális gyógyászatban hosszú ideje alkalmazott gyógynövények hatóanyagainak azonosítása ma is jelentős szerepet játszik a gyógyszerkutatás korai fázisában. A növényi kivonatok vizsgálata azonban komplexitásukból adódóan kihívást jelentő feladat, amely szofisztikált analitikai módszerek fejlesztését igényli.

Kutatásunk elsődleges célja a Betulaceae család mogyoró nemzetségének Kárpát-medencében fellelhető fajai, a *Corylus avellana* L., a *Corylus colurna* L. és a *Corylus maxima* Mill. fenoloid-profiljának jellemzése volt. E növényeket a népgyógyászatban hosszú ideje alkalmazzák, fitokémiai jellemzőik azonban kevéssé feltártak.

Vizsgálataink kiindulópontját a *Corylus* levél- és kéregdrogok összes fenoloid, flavonoid és tannin tartalmának a Ph. Hg. VIII. spektroszkópiás módszereivel történő meghatározása képezte. Minden minta esetén magas fenoloid tartalom volt kimutatható.

A drogokból etil-acetáttal és metanollal készült kivonatok szabadgyökfogó aktivitását DPPH és ABTS *in vitro* tesztrendszerekben vizsgáltuk. Eredményeink alapján elmondható, hogy a *Corylus* kivonatok kiemelkedő antioxidáns hatással rendelkeznek.

Ezen kivonatok fenoloid-profilját HPLC-DAD-ESI-TOF-MS és HPLC-DAD-ESI-MS/MS módszerekkel vizsgáltuk. Összesen harminchét különböző fenoloid komponenst detektáltunk, köztük húsz diarilheptanoidot, kilenc flavonol-származékot, valamint nyolc egyéb polifenol komponenst. Új eredményként tartható számon többek között, hogy elsőként írtunk le e *Corylus* fajokban diarilheptanoidokat, emellett elsőként vizsgáltuk a török és csöves mogyoró fenoloid-összetételét.

A kivonatokra legjellemzőbb fenoloidok mennyiségi meghatározása céljából HPLC-ESI-MS/MS módszert fejlesztettünk és validáltunk.

Sikeresen alkalmaztuk az off-line módon kapcsolt DPPH-HPLC módszert az extraktumok antioxidáns hatásához hozzájáruló komponensek azonosítására.

Az általunk fejlesztett HPLC-DAD-ESI-MS módszerek megfelelően szelektív és érzékeny eszközt biztosítanak a mogyoró kivonatok komponenseinek minőségi és mennyiségi jellemzésére. Eredményeink mind farmakognóziai és fitoterápiás, mind kemotaxonómiai szempontból értékesek lehetnek, emellett további vizsgálatok kiindulópontjául szolgálhatnak.

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11. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

11.1. Publications related to the thesis

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Riethmüller E, Tóth G, Alberti Á, Végh K, Burlini I, Könczöl Á, Balogh GyT, Kéry Á. (2015) First characterisation of flavonoid- and diarylheptanoid-type antioxidant phenolics in *Corylus maxima* by HPLC-DAD-ESI-MS. J Pharm Biomed Anal, 107: 159-167

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11.2. Further scientific publications

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12. ACKNOWLEDGEMENTS

I wish to thank

Dr. Szabolcs Béni, Ph.D. director of the Department of Pharmacognosy, who has provided me the opportunity to complete my doctoral studies in the department.

Dr. Anna Blázovics, D.Sc. former director of the Department of Pharmacognosy, who has given me the opportunity to take part in the analytical projects of the Department.

Dr. Ágnes Kéry, Ph.D. my supervisor, for her patient guidance during my research work as a student researcher and for her optimist and kind support during my Ph.D. student years. I especially acknowledge her for evolving my scientific thinking and for maintaining my affection for medicinal plants.

Dr. György T. Balogh, Ph.D. head of the Compound Profiling laboratory, who encouraged me to carry out researches beside my daily routine work.

Dr. Csaba Séllei head of Thököly Pharmacy for providing me the opportunity to work as a pharmacist, thus the chance for me to make a living during my Ph.D. fellow years.

Dr. Ágnes Alberti-Dér, Ph.D. my first Jedi master, for introducing me into the world of scientific researches.

Dr. Gergő Tóth, Ph.D. for his indispensable help with the HPLC-MS experiments and with our publications.

Dr. Árpád Könczöl, Ph.D. my second Jedi master, for his vast help with my researches and thesis, and also for guiding me deeper into he world of science.

Dr. Krisztina Perjéssy-Végh and **Anna Káncz** collegues and friends for the delightsome scientific and less scientific conversations inside and outside the laboratory.

Ilaria Burlini, Mirella Sonati, Dorottya Szakál and **Francesco Ortolano** undergraduate students for their help with the simple experiments included in the thesis.

Anna Kriston, who shared her know-how of laboratory work with me.

All my collegues at the Department of Pharmacognosy and the Compound Profiling Laboratory who helped me during my Ph.D. studies and from whom I have learnt a lot.

Finally I wish to thank **my husband**'s, **friend**s' and **family**'s support, patience and faith in my work

13. APPENDIX

13.1. Diseases of different body systems taking effects from curcumin

Table A1. Diseases of different body systems taking effects from curcumin (based on Noorafshan and Ahkani-Esfahani 2013, see the references below the table R1-R41)

Body system	Diseases	Mechanism of action	Ref.	Human trials
Nervous system	Alzheimer's disease	Antioxidant effect, alteration of the effect of aluminium, elimination of Aβ molecules and phosphorylated tau protein, upregulation of BDNF-ERK signaling, inhibition of appoptosin-induced apoptosis via upregulating heme oxygenase-1 expression, anti-acethylcolin esterase activity	R1	Yes
	Parkinson's disease	Protection of dopaminergic neurons from neuronal degeneration via antioxidant effect, anti-inflammatory effect and anti-apoptotic effect	R2	Yes
	Brain tumour	Blocking of brain tumour formation, removement of brain tumour cells; activation of caspase 3/7, suprresion of Cyclin D1, NF-κB, Bcl-$X_{L,}$ Akt and VEGF	R3	No
	Multiple sclerosis	Beneficially affects astrocyte population in CNS neuroinflammatory environment lean to anti-inflammatory response and help to components in respects of CNS repair	R4	No
	Neuro-degenerative diseases	Lowers the cholesterol level in the brain by acting as an LXR agonist and by deactivation of the ABCA1 promoter, effect on brain insuline recepors, antioxidant effect, anti-inflammatory effect,anti-apoptotic effect	R5	Yes
	Neuro-inflammatory diseases	Anti-inflammatory effect, antioxidant effect	R6	Yes

	Traumatic brain injury	Elevation of the levels of AMP-activated protein kinase, ubiquitous mitochondrial creatine kinase, cythocrome-c ocidase 2.	R7	No
	Ischemia	Antioxidative effect, anti-inflammatory effect	R8	Yes
	Depression	Increment of brain-derived neurothropic factors	R9	Yes
Respiratory system	Lung cancer	Reduction of apoptosis by the suppresion off the STAT3 pathway, suppression of MMP-2 and MMP-9, imrovement of antioxidant defenses via upregulation of heme oxygenase-1 expression	R10	No
	Inflammatory lung diseases	Anti-inflammatory effect by suppressing IL-8 release	R11	Yes
	Pneumonia	Antibacterial effect by blocking bacterial growth, adherence, invasion and pro-inflammatory activation by suppressing IL-8 release	R12	No
Cardiovascular system	Cardio-myopathy	Downregulation of NOS by activation of NF-κB and proien-1	R13	No
	Oxidative heart injury	Antioxidative effect, Anti-inflammatory effect	R14	Yes
	Cardiac hypertrophy	Inhibition of p300-HAT, anti-inlfammatory activity	R15	No
	Myocardial infarction	Inhibition of p300-HAT, anti-inlfammatory activity, antioxidant activity	R16	Yes
Urinary system	Renal tubular fibrosis	Attenuation of TGF-b1-induced epithethelial mesenchymal transition in renal tubular cells via the downregulation of snail-1 and also affecting ILs and MMPs	R17	No
	Inflammatory kidney diseases	Antioxidative effect, Anti-inflammatory effect	R18	Yes
	Oxidative kidney injury	Antioxidative effect, anti-inflammatory effect	R19	Yes
	Ischemic kidney damage	Antioxidative effect, anti-inflammatory effect via upregulation of Mn-superoxide dismutase	R20	No

Reproductive system	Toxin induced oxidative damage	Amelioration of the testorsteron synthesis inhibitor effects of metronidazol by scaveging free radicals and the enchancement of serum level testosterone, attenuates DBP-induced testicular damages by stopping peroxidative alteration in the sperm, protection of the Leydig cells from the damage caused by chronic alcohol administration	R21	No
	Tumours of reproductive system	Reduction of prostate tumour volume, MMP-2 and MMP-9 activity, potent radiosenzitilizer in prostate cancer treatment, growth-inihibitory effect on human ovary cancer by the downregulation of p53 and Bcl-2 and also by apoptosis-inducing effect	R22	Yes
	Dysmenorrhea	Antagonism of oxytocin-induced contraction in the uterus	R23	No
Digestive and hepato-biliary system	Tumours	Anti-inflammatory effect, anigiogenesis inhibition, alteration of metastases of tumours	R24	Yes
	GI ulcers	Antioxidant effect, anti-inflammatory effect	R25	Yes
	Inflammatory bowel disease	Antioxidant effect, anti-inflammatory effect	R26	Yes
	Pancreatitis	Antioxidant effect, anti-inflammatory effect	R27	No
	Hepatitis	Antioxidant effect, anti-inflammatory effect	R28	No
	Cirrhosis	Antioxidant effect, anti-inflammatory effect	R29	No
	Hepatic injuries	Antioxidant effect, anti-inflammatory effect	R30	No
	Oxidative damage	Antioxidant effect, anti-inflammatory effect	R31	Yes
Musculoskeletal system and Skin diseases	Inflammatory diseases	Antioxidant effect, anti-inflammatory effect (mainly COX-2 inhibition)	R32	Yes
	Oxidative stress	Antioxidant effect, anti-inflammatory effect	R33	
	Ischemic damage	Antioxidant effect, anti-inflammatory effect, decrement of TNF-α and IL-1β levels	R34	

	Insulin resistance	Increment of the oxidation of fatty acid and glucose trough up-regulation pf phosphorylated AMP-activated protein kinase, CD36 and carnitine palmitoyl transferase 1, and down-regulating the expression of pyruvate-dehydrogenase 4 and phosphorylated-glycogene-dynthase	R35	Yes
	Tumours	Anti-inflammatory effect, anigiogenesis inhibition, alteration of metastases of tumours	R36	Yes
	Skin wound	Antioxidant effect, anti-inflammatory effect, induction of collagen synthesis and vascularization	R37	Yes
	Psoriasis	not evaluated	R38	Yes
	Oxidative skin injury	Antioxidant effect	R39	Yes
Endocrine system	Diabetes	Anti-hyperglycemic and hypo-cholesteromelic effects in type 2 diabetes as well as protective effect against pancreatic injury by its antioxidant and anti-inflammatory impact, amelioration of diabetic neuropathy, ratinopathy, nephropathy, and cardiomyopathy	R40	Yes
	Endocrine tumours	Anti-inflammatory effect, anigiogenesis inhibition, alteration of metastases of tumours	R41	No

R1: Ghosh et al. 2015, Zhang et al. 2015, Zheng et al. 2015, Mutsuga et al 2012, Kakkar et al. 2011.
R2: Fu et al. 2015.
R3: Purkayastha et al. 2009.
R4: Seyedzadeh et al. 2014.
R5: Tien et al. 2012.
R6: Noorafshan and Esfahani 2012.
R7: Sharma et al. 2009.
R8: Liu et al. 2012.
R9: Huangha et al. 2011.
R10: Alexandrow et al. 2012, Deryugina et al. 2011, Lee et al. 2010.
R11: Noorafshan and Esfahani 2012
R12: Lüer et al. 2011.
R13: Farhangkoee et al. 2003.
R14: Noorafshan and Esfahani 2012.
R15: Li et al. 2008, Morimoto et al. 2008.
R 16: Morimoto et al. 2008.
R17: Noorafshan and Esfahani 2012.
R18: Yousef et al. 2010.
R19: Yousef et al. 2010.
R20: Shahed et al 2001.
R21: El-Nahash et al. 2004.
R22: Nonn et al. 2007.

R23: El-Sayed 2008.
R24: S. Darvesh et al. 2012.
R25: Taylor et al. 2011.
R26: Taylor et al. 2011.
R27: Ali et al. 2012.
R28: Thapa et al. 2007, Messner et al. 2008.
R29: Thapa et al. 2007, Messner et al. 2008.
R30: Thapa et al. 2007, Messner et al. 2008.
R31: Thapa et al. 2007, Messner et al. 2008.
R32: Avci et al. 2012.
R33: Vazeille et al. 2012.
R34: Avci et al. 2012.
R35: Poylin et al. 2008.
R36: Davis et al. 2007.
R37: Maheshwari et al. 2006.
R38: Kurd et al. 2008.
R39: Maheshwari et al. 2006.
R40: Ghosh et al. 2015.
R41: Hong et al. 2006.

13.2. Results of the HPLC-based DPPH assay

Table A2. Results of HPLC-based DPPH scavenger activity analysis of *C. avellana* leaves ethyl acetate extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Myricetin-3-*O*-rhamnoside	1475.00	0.00	1,475.00	100.00%	61.38%
Quercetin-3-*O*-rhamnoside	1404.00	986.00	418.00	29.77%	17.39%
Hirsutenone	268.80	106.00	162.80	60.57%	6.77%
Other compounds	1227.00	1142.00	85.00	6.93%	3.54%
Quercetin-3-*O*-hexoside	84.14	0.00	84.14	100.00%	3.50%
Rosmarinic acid	445.00	386.00	59.00	13.26%	2.46%
Diarylheptanoid aglycone1	69.40	25.79	43.61	62.84%	1.81%
5-hydroxy-1,7-bis-(4-hydroxyphenyl)-hept-6-en-3-one hexoside	1091.40	1055.00	36.40	3.34%	1.51%
1,7-bis-(4-hydroxyphenyl)-4,6-heptadien-3-one	388.30	366.40	21.90	5.64%	0.91%
Quercetin-3-*O*-hexoside	16.60	0.00	16.60	100.00%	0.69%
Kaempferol-3-*O*-rhamnoside	569.30	568.70	0.60	0.11%	0.02%

Table A3. Results of HPLC-based DPPH scavenger activity analysis of *C. avellana* leaves methanolic extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Myricetin-3-*O*-rhamnoside	712.00	0.00	712.00	100.00%	80.73%
Quercetin-3-*O*-rhamnoside	89.90	13.60	76.30	77.45%	8.65%
Other compounds	80.00	30.90	49.10	61.38%	5.57%
1,7-bis-(4-hydroxyphenyl)-4,6-heptadien-3-one	122.70	98.84	23.86	19.45%	2.71%
Quercetin-3-*O*-hexoside	9.60	0.00	9.60	100.00%	1.09%
5-hydroxy-1,7-bis-(4-hydroxyphenyl)-hept-8-en-3-one hexoside	34.30	25.74	8.56	24.96%	0.97%
Kaempferol-3-*O*-rhamnoside	25.30	22.78	2.52	9.96%	0.29%

Table A4. Results of HPLC-based DPPH scavenger activity analysis of *C. avellana* bark ethyl acetate extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Quercetin-3-*O*-rhamnoside	1413.00	648.00	765.00	54.14%	73.70%
Other compounds	141.00	0.00	141.00	100.00%	13.58%
Kaempferol-3-*O*-rhamnoside	1223.00	1154.00	69.00	5.64%	6.65%
Myricetin-3-*O*-rhamnoside	63.00	0.00	63.00	100.00%	6.07%

Table A5. Results of HPLC-based DPPH scavenger activity analysis of *C. avellana* bark methanolic extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Quercetin-3-*O*-rhamnoside	183.83	82.00	101.83	55.39%	42.51%
Caffeic acid derivative	43.62	0.00	43.62	100.00%	18.21%
Myricetin-3-*O*-rhamnoside	40.54	0.00	40.54	100.00%	16.92%

Other compounds	24.40	0.00	24.40	100.00%	10.19%
Quercetin-3-*O*-hexoside	22.73	8.60	14.13	62.16%	5.90%
Quercetin-3-*O*-hexoside	17.10	3.50	13.60	79.53%	5.68%
Kaempferol-3-*O*-rhamnoside	55.54	54.10	1.44	2.58%	0.60%

Table A6. Results of HPLC-based DPPH scavenger activity analysis of *C. colurna* leaves ethyl acetate extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Myricetin-3-*O*-rhamnoside	157.00	19.00	138.00	87.90%	19.87%
Kaempferol	119.35	0.00	119.35	100.00%	17.18%
Quercetin-3-*O*-rhamnoside	280.70	196.47	84.23	30.01%	13.13%
Kaempferol-3-*O*-rhamnoside	755.33	666.10	89.23	11.81%	12.84%
Kaempferol-3-*O*-glucuronide	592.48	520.87	71.61	12.09%	11.55%
Kaempferol-3-*O*-hexoside	747.00	690.78	56.22	7.53%	9.06%
Quercetin-3-*O*-hexoside	171.40	124.00	47.40	27.65%	7.64%
1,7-bis-(4-hydroxyphenyl)-4,6-heptadien-3-one	248.00	222.82	25.18	27.89%	4.06%
Other compounds	133.60	96.34	37.26	10.15%	6.01%
Kaempferol-(di)desoxhexoside	61.42	41.58	19.84	32.30%	3.20%
Diarylheptanoid aglycone3	129.11	122.76	6.35	4.92%	1.02%

Table A7. Results of HPLC-based DPPH scavenger activity analysis of *C. colurna* leaves methanolic extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Kaempferol-3-*O*-glucuronide	592.48	520.87	71.61	12.09%	37.92%
Other compounds	61.20	30.85	30.35	49.59%	16.07%
Myricetin-3-*O*-rhamnoside	28.50	0.00	28.50	100.00%	15.09%
Quercetin-3-*O*-hexoside	21.00	0.00	21.00	100.00%	11.12%
Quercetin-3-*O*-rhamnoside	19.00	0.00	19.00	100.00%	10.06%

Kaempferol-3-*O*-rhamnoside	16.40	0.00	16.40	100.00%	8.68%
Kaempferol-3-*O*-hexoside	37.00	35.00	2.00	5.41%	1.06%

Table A8. Results of HPLC-based DPPH scavenger activity analysis of *C. colurna* bark ethyl acetate extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Quercetin-3-*O*-rhamnoside	3562.98	2031.11	1531.87	42.99%	83.58%
Myricetin-3-*O*-rhamnoside	152.00	0.00	152.00	100.00%	8.29%
Catechin/epicatechin	57.03	0.00	57.03	100.00%	3.11%
Other compounds	162.70	108.90	53.80	16.01%	2.08%
Kaempferol-3-*O*-rhamnoside	238.35	200.20	38.15	33.07%	1.23%

Table A9. Results of HPLC-based DPPH scavenger activity analysis of *C. colurna* bark methanolic extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Quercetin-3-*O*-rhamnoside	494.40	231.58	262.82	53.16%	69.75%
Other compounds	84.80	20.10	64.70	76.30%	17.17%
Myricetin-3-*O*-rhamnoside	31.00	0.50	30.50	98.39%	8.09%
Catechin/epicatechin	31.90	14.13	17.77	55.71%	4.72%
Kaempferol-3-*O*-rhamnoside	22.00	20.97	1.03	4.68%	0.27%

Table A10. Results of HPLC-based DPPH scavenger activity analysis of *C. maxima* leaves ethyl acetate extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Quercetin-3-*O*-rhamnoside	879.70	18.85	860.85	95.54%	13.80%
Other compounds	1694.30	929.30	765.00	45.15%	12.26%
Hirsutanolol	852.90	89.83	763.07	89.47%	12.23%

Kaempferol	645.30	0.00	645.30	100.00%	10.34%
Kaempferol-(di)desoxhexoside	562.10	14.80	547.30	97.37%	8.77%
Myricetin-3-*O*-rhamnoside	539.50	25.40	514.10	95.29%	8.24%
Kaempferol-3-*O*-rhamnoside	581.60	478.00	103.60	17.81%	1.75%
Kaempferol-(di)desoxhexoside	341.00	21.00	320.00	93.84%	5.13%
Quercetin-3-*O*-hexoside	336.90	43.81	293.10	87.00%	4.70%
3-hydroxy-1,7-bis-(3,4-dihydroxyphenyl)-hepten	177.80	0.00	177.80	100.00%	2.85%
Oregonin	279.90	118.16	161.74	57.79%	2.59%
5-hydroxy-1,7-bis-(4-hydroxyphenyl)-hept-6-en-3-one hexoside	169.10	9.70	159.40	94.26%	2.56%
Catechin/epicatechin	153.12	8.27	144.85	94.60%	2.32%
Kaempferol-3-*O*-glucuronide	190.40	59.07	131.33	68.98%	2.11%
Platyphyllonol-pentoside	164.70	47.30	117.40	71.28%	1.88%
1-7-bis-(4-hydroxyphenyl)-4,6-heptadien-3-one	95.10	0.00	95.10	100.00%	1.52%
Hirsutenone	90.30	0.00	90.30	100.00%	1.45%
Rosmarinic acid	49.80	12.48	37.32	74.94%	0.60%

Table A11. Results of HPLC-based DPPH scavenger activity analysis of *C. maxima* leaves methanolic extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Myricetin-3-*O*-rhamnoside	667.80	0.00	667.80	100.00%	67.83%
Other compounds	235.80	97.45	138.35	58.67%	14.05%
Quercetin-3-*O*-rhamnoside	84.10	55.50	28.60	34.01%	3.07%
Kaempferol	29.94	0.00	29.94	100.00%	3.04%
Quercetin-3-*O*-hexoside	19.42	0.00	19.42	100.00%	1.97%
Kaempferol-(di)desoxhexoside	16.30	0.00	16.30	100.00%	1.66%
Kaempferol-3-*O*-rhamnoside	23.30	18.90	4.40	18.88%	0.47%

Kaempferol-(di)desoxhexoside	14.15	0.00	14.15	100.00%	1.44%
Quercetin-3-*O*-hexoside	26.15	12.76	13.39	51.20%	1.36%

Table A12. Results of HPLC-based DPPH scavenger activity analysis of *C. maxima* bark ethyl acetate extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Other compounds	429.20	269.40	159.80	37.23%	49.24%
Catechin/epicatechin	73.70	0.00	73.70	100.00%	22.71%
Myricetin-3-*O*-rhamnoside	38.90	0.00	38.90	100.00%	11.99%
Rosmarinic acid	121.70	90.10	31.60	25.97%	9.74%
Quercetin-3-*O*-rhamnoside	37.50	18.00	19.50	52.00%	6.01%
Kaempferol-3-*O*-rhamnoside	11.90	10.90	1.00	8.40%	0.31%

Table A13. Results of HPLC-based DPPH scavenger activity analysis of *C. maxima* bark methanolic extract

Compound	Peak area$_1$ (mAu·s)	Peak area$_2$ (mAu·s)	ΔArea (mAu·s)	ΔArea (%)	ΔArea ratio (%)
Myricetin-3-*O*-rhamnoside	163.60	30.23	133.37	81.52%	49.98%
Hirsutanolol	119.50	47.30	72.2	60.42%	32.47%
Other compounds	65.20	25.00	40.2	61.66%	18.08%
Quercetin-3-*O*-rhamnoside	34.40	17.80	16.6	48.26%	7.47%
Kaempferol-3-*O*-rhamnoside	26.70	22.23	4.47	16.74%	1.68%