Phenylethanoid glycosides in selected species of *Fraxinus, Plantago,* and *Forsythia* plants

Ph.D. thesis booklet

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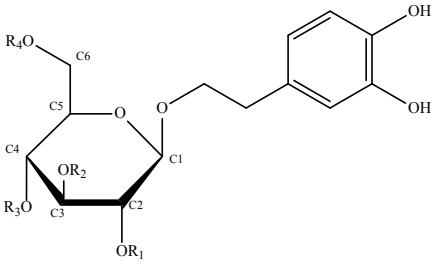
1 Introduction

Cinnamic acid and its derivatives are a group of phenolic acids, usually found in form of esters or glucosides throughout the plant kingdom. They are characterized by C6-C3 entities, i.e., a chain of three carbon units, which is attached to a phenolic ring. Esters of caffeic and quinic acids are known as caffeoylquinic or chlorogenic acids. However, the term chlorogenic acid is also used to describe 5-caffeoylquinic acid (5-CQA), which is the most abundant caffeoylquinic acid, following the IUPAC nomenclature. Structurally and biosynthetically, cinnamic acid and related compounds derive from the aromatic acids L-phenylalanine and L-tyrosine, supplied by the endogenous shikimate pathway. As C6-C3 compounds, they provide building blocks for more complex secondary metabolites, such as phenylethanoid glycosides (PhEGs), lignans, coumarins and flavonoids

PhEGs are widely distributed water-soluble natural compounds consisting of a phenylethyl alcohol moiety, which is connected to a central β-D-glucopyranose or β-D-allopyranose via a glycosidic bond (Figure 1). Usually, this molecular skeleton is complemented by further substituents such as aromatic acids and various sugars via ester and glycosidic bonds to the central glucose residue, respectively.



R_1	R_2	R_3	R_4	
-H	Rhamnosyl	*(E)*-caffeoyl	-H	Acteoside
-H	Rhamnosyl	-H	*(E)*-caffeoyl	Isoacteoside
-H	-H	*(E)*-caffeoyl	-H	Desrhamnosylacteoside
-H	-H	-H	*(E)*-caffeoyl	Desrhamnosylisoacteoside
-H	Glucosyl	*(E)*-caffeoyl	-H	Plantamajoside
-H	Glucosyl	-H	*(E)*-caffeoyl	Isoplantamajoside
-H	-H	*(E)*-caffeoyl	Rhamnosyl	Forsythoside A
-H	*(E)*-caffeoyl	-H	Rhamnosyl	Forsythoside I
(E)-caffeoyl	-H	-H	Rhamnosyl	Forsythoside H

Figure 1: General structure of PhEGs investigated in this thesis.

Several PhEGs have been suggested as chemotaxonomic markers, such as acteoside (AO)

for the order Lamiales and plantamajoside (PM) for the genus *Plantago.*

PhEGs may be promising medicinal agents, as all PhEGs investigated in this thesis have significant antioxidant and antiproliferative effects. AO and isoacteoside (IsAO) also exhibited neuroprotective, immune-enhancing and xanthin oxidase inhibitory activities. Cichoriin expressed hepatoprotective and antibacterial effects. Antibacterial effects have also been described for plantamajoside (PM) and forsythoside A (FA). The wound healing activity of PM and anti-inflammatory activity of AO, IsAO, and FA were confirmed. The remarkable antiviral activity of FA against influenza and avian bronchitis viruses, and that of AO against the respiratory syncytial virus and Dengue virus 2 has also been confirmed, highlighting the relevance of testing all PhEGs for their antiviral potency against other viruses such as SARS-CoV-2. The bioactivity of the minor PhEGs isoplantamajoside (IsoPM),

forsythoside H (FH), and forsythoside I (FI) has been less frequently studied due to their limited availability. The antioxidant properties of these metabolites as well as the antibacterial effects of FH and FI, and the antihypertensive and cardioprotective effects of IsoPM, have already been confirmed. However, prior to performing further efficacy studies with these PhEGs, their non-toxicity against normal cell lines should also be investigated.

The inflorescences of *Fraxinus* trees frequently contain abnormal outgrowths of plant tissue, known as galls. These may be formed by the phytophagous mite *Aceria fraxinivora,* which attacks the inflorescences, resulting in sizable, irregular deformities formed mainly from the flower stalks.

2 Objectives

The main objective of this thesis was the determination of the main secondary metabolites (SMs), especially the PhEG-type compounds in *Fraxinus, Plantago* and *Forsythia* tissues, which have not (or not extensively) been analyzed before. Namely, the analysis of 1) the galls of three European *Fraxinus* species, 2) the underground parts of wild-grown *Plantago* species, commonly found in Hungary, and 3) the leaves and separated fruit parts of *Forsythia* species, was planned. Based on this, our aims were to:

1) Determine the main SMs in the galls of *Fr. angustifolia, Fr. excelsior* and *Fr. ornus*,
2) Determine the PhEG composition in the roots and rhizomes of wild-grown *P. lanceolata, P. major* and *P. media*,
3) Compare the PhEG profiles of *Forsythia* leaves (represented by *Fo.* × *intermedia, Fo. europaea* and *Fo. suspensa*), and track

changes in the phytochemical composition of *Fo. europaea* and *Fo. suspensa* fruit parts (i.e., fruit wall and seed) during their ripening process,

4) Develop an ultra high performance liquid chromatography-mass spectrometry (UHPLC-MS) method to achieve base line separation of related PhEG-type compounds being present next to each other,
5) Increase the yield of minor PhEGs (IsAO, DeAO, DeIsAO, IsoPM, FI, FH) by optimizing heat treatments of AO, PM, and FA, allowing the isolation of minor PhEGs by one-step preparative HPLC,
6) Study conversion processes of AO, PM, and FA during their heat treatments resulting in the formation of corresponding minor PhEGs and interpret the isomerization processes of PhEGs induced by heat

treatments at the atomic level, using a computational method,

7) Analyze MS fragment profiles of PhEGs to determine diagnostic ions suitable for the distinction of regioisomeric pairs AO-IsAO, DeAO-DeIsAO, FA-FI-FH, and PM-IsoPM,
8) Confirm the real potential of selected *Fraxinus, Plantago* and *Forsythia* tissues for the high-yield isolation of PhEGs,
9) Determine the *in vitro* toxicity of isolated compounds against normal cells (Vero E6), aiming to confirm their safe use as natural medicines and allowing to test their antiviral potency.

3 Methods

Samples of *Fraxinus* galls, underground parts of *Plantago* species, and fruits and leaves of *Forsythia* species were collected from different locations in Hungary, pulverized and their methanolic extracts analyzed by a combination of UHPLC-HR-MS/MS and NMR spectroscopy. To increase the yield of rarely occurring PhEGs, DW and TFA treatments of the methanolic extracts were optimized and subsequently isolated by one-step preparative HPLC. UHPLC-MS quantification of compounds in the intact plant samples was performed by linear regression analyses of isolated PhEGs and standards, using an external standard method. To confirm quantitative results, qNMR analyses were performed, using an external standard method.

Computational modeling was performed to describe the isomerization processes and to confirm all structures residing at the minima on their PESs.

The in vitro cytostatic or cytotoxic effect of isolated PhEGs and coumarins was measured on Vero E6 cells of non-human primate origin. Additionally, the hemolytic activity of AO, IsAO, DeAO, DeIsAO, cichoriin, and aesculin was tested on human erythrocytes.

4 Results

During this work, 14 phenolic compounds were detected in the galls of *Fraxinus*, the underground parts of *Plantago*, as well as the fruits and leaves of *Forsythia* species. Even though a high number of compounds is described in the investigated species, we only identified 14 compounds since we focused on the presence of PhEGs. AO and IsAO were distributed throughout all investigated samples, while PM and IsoPM as well as FI and FH were limited to the genera *Plantago* and *Forsythia,* respectively. The presence of DeAO and DeIsAO was limited to galls of *Fraxinus* sp. Furthermore, *Fraxinus* galls were characterized by the presence of cichoriin and aesculin, as well as their methoxy derivatives, while rutin was limited to the fruits and leaves of *Forsythia* species. We detected 5-CQA in samples of *Fraxinus* and *Forsythia* species, however, it was not

ubiquitously present among the samples within each genus.

PhEGs could be separated from each other by one-step preparative HPLC. The UHPLC-DAD-UV separations of methanolic extracts showed similar metabolic profiles within each genus. However, the ratio of compounds was different for each species.

Compounds with identical elemental composition are indistinguishable from each other by HR-MS. Subsequent tandem mass spectra (MS/MS) of PhEGs characterized by the same molecular formula show comparable fragment ions after CID using an optimized fragmentation energy.

The quantitative UHPLC-MS analyses of 15 gall samples collected from five different habitats of *Fr. angustifolia, Fr. excelsior* and *Fr. ornus*, showed extraordinarily high amounts of AO (86.7 – 139.9 mg/g and 71.7 – 161.2 mg/g in galls of *Fr. angustifolia* and *Fr. excelsior*, respectively) and cichoriin (143.0 – 232.0 mg/g in galls of *Fr. ornus*),

which was confirmed by qNMR spectroscopy. The quantitative determination of PhEGs in 25 samples of underground parts of three wild-grown *Plantago* species from different habitats by UHPLC-MS revealed the high-yield accumulation of AO (53.0 – 99.7 mg/g) and PM (66.3 – 82.8 mg/g) in the several-year-old rhizome of *P. media* and *P. major*, respectively. Furthermore, we detected high amounts of IsAO (30.4 – 33.0 mg/g) and IsoPM (4.54 – 6.76 mg/g) in several-year-old roots of *P. media.* The main components in the leaves of *Fo. europaea* and *Fo. suspensa* were the PhEGs AO and FA, with their highest amounts being 73.9 mg/g and 96.0 mg/g, respectively. The leaves of *Fo.* × *intermedia* cultivars were characterized by the simultaneous presence of AO and FA, with FA amounts being higher than those of AO. The phytochemical composition of seed and fruit wall parts of *Fo. europaea* and *Fo. suspensa* fruits was followed as a function of ripening time, showing

time-dependent accumulation of AO and FA, respectively.

Isolated PhEGs and coumarins showed no cytostatic or cytotoxic on Vero E6 cells.

5 Conclusions

In this thesis, new sources of valuable PhEG-type metabolites were determined, allowing their high-yield isolation:

1) Analysis of the gall composition of the three European *Fraxinus* species performed for the first time confirmed an extraordinarily high amount of the PhEG AO (in the galls of *Fr. angustifolia* and *Fr. excelsior*) and that of the coumarin cichoriin (in the galls of *Fr. ornus*).
2) Tissue- and age-specific accumulation of the PhEGs PM and AO were analyzed and confirmed in the underground parts of widely distributed *Plantago* species (*P. lanceolata, P. major,* and *P. media*) for the first time. The several-year-old rhizome of *P. major* was determined as the most important source of PM known to date.

3) A comprehensive phytochemical study on the leaves and separated fruit parts during the fruit development of three *Forsythia* species (*Fo. europaea, Fo. suspensa* and various cultivars of *Fo.* × *intermedia*) revealed the unripe fruit wall of *Fo. suspensa* to be the optimum source of the PhEG FA, allowing its impurity-free, high-yield isolation.
4) In addition to the main PhEGs AO, PM and FA, optimized heat treatments of their optimum sources resulted in their characteristic conversions. This allowed the isolation of rarely occurring PhEG-type compounds IsAO, DeAO, DeIsAO, IsoPM, FH, and FI in the highest yield reported in the plant kingdom so far

As novelty in the analysis of PhEGs:

1) Molecular modeling was conducted to interpret the isomerization processes.

2) UHPLC-HR Orbitrap-MS/MS fragmentation studies of the closely related compounds confirmed characteristic differences between the ion intensities of key fragment ions. Thus, the regioisomeric pairs DeAO-DeIsAO and PM-IsoPM could be distinguished from each other. Forsythosides could not be differentiated from each other, however, they were distinguishable from the isomeric pair AO-IsAO.

As to the medicinal significance of the PhEGs (AO, IsAO, DeAO, DeIsAO, PM, IsoPM, FA, FH, and FI) and coumarins (aesculin and cichoriin):

1) they showed no cytostatic activity on Vero E6 cells, therefore supporting the safe use of these compounds as natural medicines and allowing to perform further *in vitro* profiling on different systems

(such as against intracellular pathogens). Thus, their antiviral potency (e.g., against SARS-CoV-2) can also be tested.

6 Bibliography of candidate's publications

Publications related to the thesis

1. Zürn M, Tóth G, Kraszni M, Sólyomváry A, Mucsi Z, Deme R, Rózsa B, Fodor B, Molnár-Perl I, Horváti K, Bősze Sz, Pályi B, Kis Z, Béni Sz, Noszál B, Boldizsár I (2019) **Galls of *Fraxinus* trees as new and abundant sources of valuable phenylethanoid and coumarin glycosides**. *Industrial Crops and Products*, **139**: 111517.
2. Zürn M, Tóth G, Ausbüttel T, Mucsi Z, Horváti K, Bősze Sz, Sütöri-Diószegi M, Pályi B, Kis Z, Noszál B, Boldizsár I (2021) **Tissue-Specific Accumulation and Isomerization of Valuable Phenylethanoid Glycosides from *Plantago* and *Forsythia* Plants**. *International Journal of Molecular Sciences*, **22**(8): 3880.

Publications unrelated to the thesis

1. Könye R, Tóth G, Sólyomváry A, Mervai Zs, Zürn M, Baghy K, Kovalszky I, Horváth P, Molnár-Perl I, Noszál B, Béni Sz, Boldizsar I (2018) **Chemodiversity of *Cirsium* fruits: Antiproliferative lignans, neolignans and sesquineolignans as chemotaxonomic markers**. *Fitoterapia*, **127**: 413-419.