CAFFEIC ACID-CONTAINING COMPOUNDS AND LIGNANS DERIVED FROM SPECIES OF THE GENERA *ANTHRISCUS, FORSYTHIA* AND *LINUM*

PhD thesis booklet

Tim Ausbüttel

Semmelweis University
Doctoral College
Pharmaceutical Sciences and Health Technologies Division

Supervisor:	Imre Boldizsár, Ph.D.
Official reviewers:	Ágnes M. Móricz, Ph.D., Arash Mirzahosseini, Ph.D.
Head of the Complex Exam Committee:	Éva Szökő, D.Sc.
Members of the Complex Exam Committee:	Anna Sólyomváry, Ph.D., Nóra Gampe, Ph.D.

Budapest
2026

1 INTRODUCTION

Caffeic acid-derived compounds (CPhEGs and CQAs) and lignans are plant derived phenolics with biological activity, sharing caffeic acid as a key precursor. They often co-occur in plants such as *Anthriscus*, *Forsythia*, and *Linum*, contributing to their medicinal properties. Structurally, CPhEGs are based on a glucose core, while CQAs contain a quinic acid core, both substituted with caffeic acid units. Lignans are formed by linking two caffeic acid-derived units and show high structural diversity due to differences in their chemical modifications. This results in important subclasses such as aryltetralin (AT), dibenzylbutyrolactone (DBL), and arylnaphthalene (AN) lignans.

Plants of the genera *Anthriscus* and *Forsythia* have long been used in traditional medicine, but only a few plant parts have been studied so far. Consequently, many tissues remain unexplored. Known compounds from *Anthriscus*, *Forsythia* and *Linum* exhibit broad spectrum of antiviral and antiproliferative properties, indicating that they may contain further undiscovered bioactive metabolites with promising pharmacological activities.

2 OBJECTIVES

1) Analyze MS fragment ions of isomeric CPhEGs to find diagnostic ions for differentiation (*F. suspensa*).
2) Determine AN lignans in roots/rhizomes of *L. austriacum* and *L. perenne*.
3) Monitor AN lignan accumulation over one vegetation cycle (*Linum* spp.).
4) Develop acidic/enzymatic treatments for conversion + minor compound enrichment (*Linum* spp.).
5) Define optimal tissues and methods for high-purity AN lignan isolation (*Linum* spp.).
6) Determine AT, DBL lignans + CQA profile in *Anthriscus* spp.; study accumulation.
7) Identify tissues with highest compound levels (*Anthriscus* spp.).
8) Evaluate *in vitro* cytostatic activity of compounds isolated from *Linum* and *Anthriscus* spp.
9) Assess *in vitro* antiviral activity (SARS-CoV-2) of compounds isolated from *Linum* and *Anthriscus* spp.

3 MATERIAL AND METHODS

Plant material included *F. suspensa* fruits (Hungary, July 2018), roots/rhizomes of *L. austriacum* and *L. perenne* (sampled four times in 2020), and various organs of *Anthriscus* spp. (early growth and flowering stages). All samples were collected from Hungarian locations, pooled from ≥10 plants per time point, and immediately lyophilized. The collected samples were immediately lyophilized on the day of collection, then100 mg plant material was extracted three times with methanol at 60 °C for 30 minutes, and then diluted for HPLC-UV-HR-MS analysis (*Anthriscus, Forsythia, Linum*).

3.1 *Linum* species

Plant tissues (100 mg) were incubated in water (30 min, RT) for endogenous enzymatic conversion, then lyophilized and extracted with methanol (3×, 60 °C) for HPLC-UV-HR-MS analysis. Concentrated extracts were used for isolation of linadiacin A by preparative HPLC. Linadiacin A and *L. austriacum* extracts were treated with TFA (0.2–2 M, 100 °C, 3–30 min), dried, and analyzed by HPLC-UV-HR-MS. Acid treatment enabled isolation of linadiacin B (3 min) and diphyllin/justicidin B (30 min).

3.2 *Anthriscus* species

Tissues were extracted with methanol for HPLC-UV-HR-MS/MS analysis and compound isolation. Analyses were performed by UHPLC-DAD–Orbitrap MS using standard settings and formic acid/acetonitrile mobile phases.

Analytical HPLC separations were performed using C18 columns with species-specific gradients and flow rates (0.3–0.4 mL/min) for *Forsythia*, *Linum*, and *Anthriscus*. Preparative HPLC (C18 columns) was used for compound isolation from *Linum* and *Anthriscus* using formic acid/acetonitrile gradients, with detection at 280 or 330 nm. Quantification was performed by external standard HPLC-UV (230–400 nm) with linear calibration ($r^2 > 0.999$), using isolated standards or reference compounds.

3.3 Bioactivity assessment and structure confirmation

Bioactivity was assessed *in vitro* (cytostatic, antiviral, antiadhesive) using cell-based assays, and compound structures were confirmed by NMR spectroscopy with collaborating research partners.

4 RESULTS

4.1 *Forsythia suspensa*

UHPLC-UV-HR-MS of unripe *F. suspensa* fruit wall revealed three major compounds, Forsythosides A, H and I, with FA most abundant, previously tentatively identified as isomeric CPhEGs (FI, FH, FA). MS/MS spectra (m/z 623, 20–40 eV CID) were generated and confirmed their structures, but did not allow discrimination between them.

4.2 *Linum* species

The roots and rhizomes of *L. austriacum* were analyzed by HPLC-UV-HR-MS/MS after no treatment, acid treatment (2 M TFA at 100 °C for 3–30 minutes) or enzymatic treatment (in water for 30 minutes), with homogenisation enabling enzymatic conversion. HPLC-UV-HR-MS/MS of *L. austriacum* roots/rhizomes revealed eight main compounds with consistent retention times, formulas, and MS/MS spectra, including diphyllin, justicidin B, and four glycosides, while two compounds (5, 6) showed unknown molecular formulas not previously reported in *Linum* species. A conversion study with calibrated quantification demonstrated that one of the new compounds (5) is unstable and undergoes quantitative conversion to justicidin B following acid or enzymatic treatment, as evidenced by corresponding increases of ~20–21 µmol/g. Acid treatment revealed an intermediate compound (6), indicating a two-step formation process. To confirm this pathway and obtain compound 6, acid treatments were performed on isolated compound 5 (0.2–2 M TFA, 100 °C, 3–30 min). Compound 5 was shown to convert rapidly to intermediate 6, and then to justicidin B, in a quantitative two-step process (approximately 1 µmol in total) with no degradation. NMR analysis identified compounds 5 and 6 as the new AN lignans linadiacin A and B. Acid treatment (2 M TFA, 100 °C) quantitatively hydrolyzed diphyllin glycosides (1–4) to diphyllin, with yields matching total glycoside content (~12.5–13.0 µmol/g). HPLC-UV-HR-MS of *Linum* roots/rhizomes over one year showed identical profiles

(compounds 1–8), with compound 5 dominant and best isolated from rhizomes due to higher ratios vs. glycoside 4, enabling high-purity isolation for further studies. A root sample rich in target compounds was acid-treated (2 M TFA, 3 or 30 min) to optimize formation of compound 6 or diphyllin/justicidin B, enabling their isolation by preparative HPLC for structural and bioactivity studies.

In order to elucidate the structure and assess the proposed conversion, MS/MS analysis of linadiacins A and B (± ionization, 10–30 eV CID) revealed characteristic fragmentation of deprotonated linadiacin A (m/z 773 → 585 → 423 → 381), indicating sequential loss of hydroxy-methylglutaryl and glucosyl moieties with decarboxylation. The intermediate ion m/z 423, corresponding to decarboxylated linadiacin B, led to a common fragmentation pathway for both compounds, confirming the presence of a malonyl unit. In positive ionization mode, linadiacins A and B showed a prominent fragment ion at m/z 365, likely corresponding to protonated justicidin B. This ion was also observed in MS1 spectra (14.4% and 42.7% relative intensities), indicating in-source fragmentation. MS/MS spectra of protonated justicidin B and the m/z 365 ion from linadiacins A and B were highly comparable, indicating that m/z 365 corresponds to justicidin B. This confirms a shared core structure convertible to justicidin B, while successive H_2O losses indicate four free hydroxyl groups in linadiacin A.

Isolated AN lignans (linadiacin A/B, diphyllin and justicidin B) exhibited low cytotoxicity in healthy Vero E6 cells. The previously unreported compounds linadiacin A and linadiacin B showed antiproliferative activity, particularly in EBC-1, A2058, and PC-3 cells, while diphyllin and justicidin B also exhibited antiproliferative effects against these cell lines, consistent with previous reports. Moreover, Linadiacin A, diphyllin and justicidin B displayed dose-dependent anti-SARS-CoV-2 activity, whereas linadiacin B was inactive.

4.3 *Anthriscus* species

4.3.1 Aryltetralin and dibenzylbutyrolactone lignans

Roots, leaves, and inflorescences of *A. caucalis*, *A. nitida*, and *A. sylvestris* were analyzed by HPLC-UV-HR-MS/MS. Compounds detected in the chromatogram at 12.5–17.0 min showed UV and HR-MS characteristics consistent with lignans such as desmethylyatein (DeMeYA), dimethylmatairesinol (DeMeMAT), deoxypodophyllotoxin (DPT), yatein (YA), kaerophyllin (KAP), anhydropodorhizol (AHP), and angeloyl podophyllotoxin (APT), in order of increasing retention time. For the first time, the occurrence of lignans was demonstrated in *A. nitida* and *A. caucalis*. With the exception of APT, which was not detected, all lignans previously identified in *A. sylvestris* were also found in *A. nitida*. Its congener, *A. caucalis* was confirmed as a new source of DPT, AHP, and YA. In addition, the DBL-type lignans DeMeYA and KAP were identified in *A. nitida*, establishing this species as the first producer of these compounds within the genus *Anthriscus*. Notably, DeMeYA has been proposed as a biosynthetic intermediate in the formation of AT-type lignans such as DPT; however, to date it has not been reported as an isolated metabolite in *Anthriscus* species. Lignan concentrations were detected in roots of *A. sylvestris* (1.41% DPT, 1.32% AHP, 0.91% APT, 0.26% YA) and *A. nitida* (1.09% KAP, 0.25% DeMeYA, 0.13% DiMeMAT), enabling efficient one-step preparative HPLC isolation. In contrast, the highest lignan content in *A. caucalis* was observed in inflorescences.

The AT-type lignans DPT and APT, together with the DBL-type lignan AHP, were evaluated for antiadhesive effects in HeLa and preosteoblast cells: DPT and APT showed antiadhesive activity at submicromolar concentrations in both cell types.

4.3.2 Malonyl-dicaffeoylquinic acids from *A. sylvestris*

In the HPLC–UV chromatogram of *A. sylvestris* leaf extract, two early-eluting peaks (7.3 and 7.6 min) were observed, indicating highly polar compounds relative to lignans (12.5–17.0 min). MS/MS analysis revealed co-elution at 7.6 min, which was resolved into two peaks (HP-2 and HP-3) by gradient modification, while the 7.3 min

peak remained a single component (HP-1). HR-MS spectra of HP-1–HP-3 indicated a common molecular formula ($C_{28}H_{25}O_{15}$), consistent with malonyl-dicaffeoylquinic acids previously tentatively reported in *A. cerefolium* without structural confirmation.

MDiCQAs were analyzed in inflorescence, leaf, and root tissues of *A. sylvestris* during flowering and at the beginning of the vegetation cycle (BVC). Tissue-specific differences were observed, with HP-2 and HP-3 accumulating predominantly in BVC leaves (2.08 and 1.91 mg/g, respectively), while HP-1 was also detected at high levels in inflorescences. Accordingly, the accumulation patterns of these MDiCQAs were organ- and development stage-dependent and early-spring leaf material enabled simultaneous isolation of all three MDiCQAs.

Among the MDiCQAs, only HP-1 showed cytostatic activity in several human cancer cell lines, but also reduced viability of non-tumorigenic Vero E6 cells, indicating no cancer-specific selectivity.

5 CONCLUSION

This work presents a comprehensive phytochemical and bioactivity-oriented investigation of *Forsythia suspensa*, *Linum austriacum*/*L. perenne*, and *Anthriscus* species, focusing on phenylpropanoid-derived metabolites. The integration of optimized extraction and conversion strategies with advanced analytical techniques provided key methodological and biological insights.

In *Linum* species, a previously unreported two-step conversion of linadiacin A to linadiacin B and subsequently to justicidin B was identified. Optimized acidic and enzymatic treatments enabled selective and quantitative preparation of lignans, supporting their isolation and evaluation. Diphyllin and justicidin B showed pronounced anti-SARS-CoV-2 activity with acceptable cytotoxicity, indicating potential as antiviral lead compounds.

In *Anthriscus* species, lignans were identified for the first time in *A. nitida* and *A. caucalis*, expanding the chemotaxonomic profile of the genus. Roots of *A. sylvestris* and *A. nitida* were confirmed as rich lignan sources, enabling efficient one-step isolation. Three malonyl-dicaffeoylquinic acids were structurally elucidated as new natural products, with organ- and development stage-dependent accumulation; early-spring leaves were identified as suitable material for isolation. A representative MDiCQA showed no cancer-specific activity, contributing to the assessment of their pharmacological relevance.

In *Forsythia suspensa*, MS/MS did not allow discrimination of isomeric caffeoyl phenylethanoid glycosides, indicating the need for complementary analytical approaches for reliable differentiation.

Overall, targeted tissue selection combined with controlled enzymatic and thermal conversions enhances access to bioactive natural products. When integrated with advanced analytical techniques, this approach enables the efficient isolation and evaluation of minor or unstable metabolites, expands chemotaxonomic insight, and supports the identification of antiviral and antiproliferative lead compounds.

6 BIBLIOGRAPHY OF CANDIDATE'S PUBLICATIONS

6.1 Publications related to the thesis

1. Péter B[1], **Ausbüttel T[1]**, Kanyo N, Joó P, Tóth K, Szittner Z, Szekacs I, Bősze S, Kovács GM, Boldizsár I, Horvath R. In situ monitoring of cell adhesion kinetics using label-free optical biosensors: Evaluating anticancer potential and selectivity of natural compounds. **VIEW**. **2025**;6(1):20240043.

2. Nazli A, Gáborová M, **Ausbüttel T**, Stipsicz B, Tóth G, Bősze S, Béni S, Boldizsár I. Malonyl-caffeoylquinic acids and malonyl-flavonoid-glucosides from three edible Apiaceae plants Anthriscus cerefolium, Anthriscus sylvestris, and Chaerophyllum bulbosum. **ACS Omega**. **2025**;10(1):235.

3. Tóth G, Horváti K, Kraszni M, **Ausbüttel T**, Pályi B, Kis Z, Mucsi Z, Kovács GM, Bősze S, Boldizsár I. Arylnaphthalene lignans with anti-SARS-CoV-2 and antiproliferative activities from the underground organs of Linum austriacum and Linum perenne. **J Nat Prod**. **2023**;86(8):1375–87.

4. Zürn M, Tóth G, **Ausbüttel T**, Mucsi Z, Horváti K, Bősze S, Pályi B, Kis Z, Noszál B, Boldizsár I. Tissue-specific accumulation and isomerization of valuable phenylethanoid glycosides from Plantago and Forsythia plants. **Int J Mol Sci**. **2021**;22(8):3880.