MASTERARBEIT | MASTER'S THESIS

Titel | Title

„Phytochemical comparison of *Galium sp.* and exploration of their anti-inflammatory properties”

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Magister pharmaciae (Mag. pharm.)

Wien | Vienna, 2026

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 605
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Pharmazie
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List of abbreviations

AA (acetic acid)

BuOH (n-butanol)

DAD (diode array detector)

DCM (dichloromethane)

ddH_20 (double-distilled water)

ELSD (evaporative Light Scattering Detector)

ESI (electrospray Ionisation)

EtOAc, Ea (ethyl acetate)

FA (formic acid)

H (n-hexane)

HEMWat (n-hexane/ethyl acetate/methanol/water)

HPCCC (high performance countercurrent chromatography)

HUVECs (*human umbilical vein endothelial cells*)

LL (lower layer)

LLE (liquid liquid extraction)

m/z (mass-to-charge ratio)

MeOH (methanol)

MS (mass spectrometry)

MW (molecular weight)

NPA (nature product reagent A)

PBMC lymphocytes (*peripheral blood mononuclear cells*)

PDA (photo diode array)

PEG400 (polyethylene glycol 400)

QDa (quadrupole dalton mass detector)

RT (retention time)

TLC (thin layer chromatography)

UL (upper layer)

UPLC (ultra performance liquid chromatography)

UV_{254} (ultraviolet 254 nm)

UV_{366} (ultraviolet 366 nm)

ABSTRACT

Representatives of the genus *Galium* L. have been traditionally used for its antioxidant, anti-inflammatory, immunomodulatory, antimicrobial, and wound healing properties in folk medicine. Despite their ethnopharmacological relevance, the phytochemical profile and pharmacological potential of Austrian *Galium* species remained underexplored. This work aimed to comparatively explore the chemical space of commonly used species, like *G. aparine, G. odoratum* and *G. album*, as well as the underexplored species *G. sylvaticum*. Methanolic extracts of aerial parts of the plants were subjected to liquid-liquid extraction (LLE) to obtain fractions with different chemical profile, separated by their polarity. Chromatographic methods, including thin-layer chromatography (TLC) and ultra-high-performance liquid chromatography (UHPLC-UV-ELSD/MS) enabled the annotation of the main secondary metabolites. Additionally, the EtOAc/BuOH fraction of *G. sylvaticum* was separated using high-performance countercurrent chromatography (HPCCC) for further phytochemical characterisation. The annotation of the generated fractions indicated similar constituents as reported in other *Galium* species, including flavonoids (e.g. rutin), phenols/hydroxycinnamic acids (e.g. chlorogenic acid) and iridoids (e.g. asperuloside) as main constituents. A preliminary biological evaluation using a HUVEC/PBMC cell adhesion assay showed potential anti-inflammatory properties of the generated *Galium* extracts and fractions.

Overall, this study provided insights into the chemical composition of selected *Galium* species native to Austria and uncovered several secondary metabolites of the underexplored species *G. sylvaticum*.

ZUSAMMENFASSUNG

Vertreter der Gattung *Galium* L. werden in der traditionellen Volksmedizin aufgrund ihrer antioxidativen, entzündungshemmenden, immunmodulatorischen und antimikrobiellen Eigenschaften, sowie zur Behandlung von Erkrankungen des Gastrointestinaltrakts und zur Wundheilung eingesetzten. Trotz der ethnopharmakologischen Bedeutung dieser Pflanzengattung blieben das phytochemische Profil und das pharmakologische Potenzial einiger österreichischer *Galium*-Arten bislang unzureichend untersucht. Ziel dieser Arbeit war es daher, die Inhaltsstoffe umfassend untersuchter Arten wie *G. aparine, G. odoratum* und *G. album* vergleichend zu analysieren und gleichzeitig besonderen Schwerpunkt auf die bislang weniger erforschte Art *G. sylvaticum* zu legen. Methanolische Extrakte der oberirdischen Pflanzenteile wurden einer Flüssig-Flüssig Verteilung (LLE) unterzogen, um Fraktionen mit unterschiedlichen, polaritätsabhängigen Stoffzusammensetzung zu erhalten. Chromatographische Methoden, darunter Dünnschichtchromatographie (TLC) und Ultra-Hochleistungsflüssigchromatographie (UHPLC-UV-ELSD/MS), dienten zur Erstellung der Annotation der enthaltenen Substanzen. Der Ethylacetat/n-Butanol Extrakt von *G. sylvaticum* wurde schließlich mittels Verteilungschromatographie (HPCCC) weiter fraktioniert, wodurch eine gezielte Anreicherung spezifischer Verbindungen erreicht werden konnten. Die Ergebnisse zeigten eine weitgehende Ähnlichkeit mit bekannten *Galium*-Arten, wobei Flavonoide (z.B. Rutin), Phenole/Hydroxyzimtsäure-Derivate (z.B. Chlorogensäure) und Iridoide (z.B. Asperulosid) als Hauptinhaltsstoffe identifiziert wurden. Mittels eines HUVEC/PBMC-Zelladhäsionsassays konnten im Rahmen dieser Arbeit potenzielle entzündungshemmende Eigenschaften der hergestellten *Galium*-Extrakte und Fraktionen gezeigt werden. Insgesamt lieferte diese Studie Einblicke in die chemische Zusammensetzung ausgewählter, in Österreich heimischer *Galium*-Arten und deckte mehrere Sekundärmetaboliten der bislang wenig erforschten Art *G. sylvaticum* auf.

1 INTRODUCTION

1.1 *Galium* species

1.1.1 Traditional and ethnopharmacological use of *Galium* species

Galium is a large genus of annual or perennial herbaceous plants in the family of Rubiaceae. They are very widespread in temperate climate zones of the northern & southern hemispheres. There are approximately 650 species of *Galium* around the world, mostly native and only introduced to some islands and were first published in Species Plantarum (1753) by Carl von Linné (Bremer & Manen, 2000). The scientific name of this species, as well as the German designation, Labkraut, both go back to the use as a coagulant in historic cheese production. „Gala" is the Greek word for milk and „Lab" which means rennet, a mixture of the enzymes chymosin and pepsin, originally obtained from the abomasum of young ruminants (Shakirullaha et al., 2009).

Aerial parts of *Galium* species have been traditionally used for its anti-cancer, antioxidant, anti-inflammatory, immunomodulatory, antimicrobial, cardioprotective effects, for gastrointestinal diseases and wound healing in folk medicine (Atmaca et al., 2016), (Ouasti et al., 2024).

In the course of the work, four selected *Galium* species were investigated, including *G. aparine* L., *G. odoratum* L., *G. album* L. and *G. sylvaticum* L., with special focus on the latter, since little is known about its phytochemical composition.

1.1.2 Phytochemical composition of aerial parts of *Galium* species

The aerial parts of various *Galium species* are rich in diverse secondary metabolites contributing collectively to their potential therapeutic properties. Major constitutes of the extracts include **iridoids**, e.g., monotropein, 10-desacetylasperulosidic acid asperulosid and asperulosidic acid; **hydroxycinnamic acid derivatives**, e.g., 3-*O*-caffeoylquinic, 5-*O*-caffeoylquinic, 3,4-*O*-dicaffeoylquinic, 3,5-*O*-dicaffeoylquinic, 4,5-O-dicaffeoylquinic acids and caffeic acid derivatives; **flavonoids**, e.g., rutin, hyperoside, apigenin, quercetin-3-*O*-rhamnoglucoside-7-*O*-glucoside, kaempferol, kaempferol-3-*O*-glucoside, neohesperidin, luteolin and luteolin-7-*O*-diglucoside; **phytosterols**, e.g., campesterol, sitosterol and stigmasterol; **triterpenoids**, e.g., oleanolic, ursolic, euscaphic and tormentic acids, betulin and lupeol; sesquiterpenoids, squalene, aromatic compounds and higher alkanes, fatty acids, chlorophylls, and carotenoids were described for extracts of cleavers herb (Ilina et al., 2019).

1.2 Preliminary data

The scientific interest in this plant genus, particularly the aforementioned *Galium* species, originated from observations made during a self-medication trial involving a patient diagnosed with psoriatic arthritis. The patient reported improvement and relief after the administration of self-made *G. aparine extracts*, which led to further inquiry into its pharmacological properties. Additionally, the study was influenced by knowledge of the documented traditional use of *Galium* species in the treatment of skin diseases. These reports led to promising preliminary investigations of anti-inflammatory properties conducted on methanolic *G. aparine* extracts by Mag. Dr. Benjamin Kirchweger at Ludwig-Maximilians-Universität (LMU) Munich (data not shown). The assay utilized in this study is a cell adhesion assay, designed to assess the interaction between HUVECs and PBMCs, specifically lymphocytes. A detailed description of the experiment can be found in chapter 4.

The connection of empirical observations and ethnobotanical evidence provided the impulse for a more systematic exploration of the bioactive compounds present in these species and their potential applications in inflammatory disorders.

1.3 Ethnopharmacological significance in wound healing

The VOLKSMED Database is a scientific ethnobotanical resource that documents the traditional use of medicinal plants in Austrian folk medicine. Its main purpose is to preserve this cultural and therapeutic knowledge, which is often passed down many generations, with the risk to fade. By collecting information on different species, indications, preparation methods and pharmacological effects the database sets up a valuable bridge between historical folk practices and modern pharmacological research.

To highlight the value of traditional knowledge in identifying potential new wound healing herbal remedies, the study of (Eichenauer et al., 2024), investigated both well-known taxa such as *Hypericum perforatum* L. and *Calendula officinalis* L. as well as underexplored remedies such as *Sambucus racemosa* L. or *Anthyllis vulneraria* L., which all promoted healing in acute or chronic wounds *in vitro* (Eichenauer et al., 2024). With *Galium aparine* also being included in this database, their phytochemical composition and documented role in European folk medicine, *Galium sp.* deserve renewed attention, suggesting possible antioxidant, anti-inflammatory and antimicrobial effects.

1.4 Aim of the work and workflow

The primary objective of this study was to conduct a comprehensive investigation and comparison of the phytochemical composition of extracts from different *Galium* species (*G. aparine*, *G. album*, *G. odoratum* and *G. sylvaticum*), with emphasis on the best possible candidate for their anti-inflammatory properties. Given the long-standing use of *Galium* species in traditional and ethnopharmacological practices this study aimed to provide a more detailed look in the phytochemical constituents of *G. sylvaticum*, an underexplored member of this genus. To accomplish this, phytochemical methods were employed, including extraction and fractionation using different chromatographic techniques and characterization of constituents, while the anti-inflammatory properties were evaluated using a HUVEC-PBMC cell adhesion assay (in collaboration with LMU Munich, Germany). Initially, several extracts were prepared from the herb of *G. aparine* using a range of solvents to determine the most promising method for the extraction and enrichment of secondary metabolites. The established extraction protocol was applied to ensure reproducibility and allow for comparative analyses across different species. Phytochemical profiling of these extracts was conducted using a combination of advanced chromatographic techniques, including, ultra-high-performance liquid chromatography (UHPLC) and high-performance countercurrent chromatography (HPCCC). These methods enabled the separation and identification of main secondary metabolites, such as flavonoids, iridoids and phenolic acids. Metabolite annotation was performed by comparison of spectroscopic data with literature, allowing a comparative phytochemical assessment among the analysed species. Overall, this research provides valuable insights into the phytochemical diversity and the eventual pharmacological potential of *Galium* species.

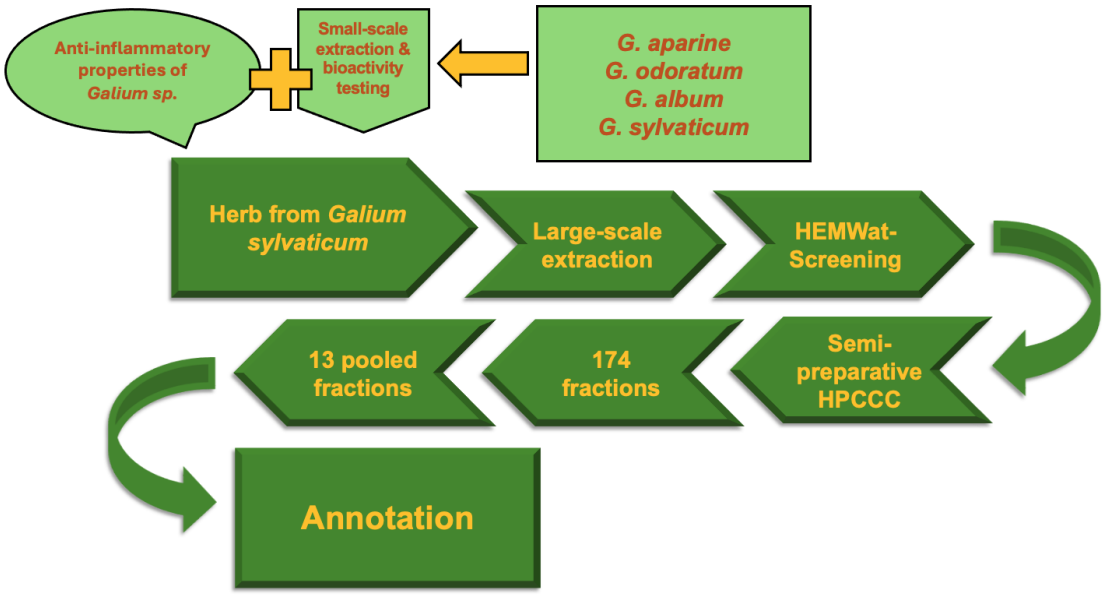

Figure 1: Workflow chart.

2 RESULTS AND DISCUSSION

2.1 Selection of the best extraction method using *Galium aparine*

One of the primary goals of this work was to elucidate and characterize the aerial parts of the underexplored species, *G. sylvaticum* that is native to Middle and South Europe (https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:750701-1). To identify the most promising extraction method to enrich the secondary metabolites, preliminary extraction experiments were first conducted using the well-known herb of *G. aparine*. In total, seven different extracts were prepared using solvents differing in polarity, including methanol (MeOH), ethanol (EtOH), ethyl acetate (EtOAc) and dichloromethane (DCM). A more detailed description of the extract generation can be found in chapter 6.3. The resulting extracts were analysed using UHPLC-UV-ELSD to assess their phytochemical profile.

The UHPLC-ELSD chromatograms of the prepared preliminary extracts did not provide insights into the chemical composition. Most chromatograms show a large peak with low RT (~1.80 min) caused by very hydrophilic constituents such as sugars (Figure 2). Additionally, extracts prepared with more apolar solvents also possess very lipophilic constituents with high RT (< 14.70 min) that were tentatively assigned to lipids (e.g. fatty acids). The obtained results showed that the extraction approaches lacked in sufficient selectivity and efficiency to extract the secondary metabolites known for *G. aparine* which were visible in the corresponding UV chromatograms of the extracts (RT 4.00 - 6.50 min) (Figure 3). This clearly indicated the necessity of a more specific approach to extract the secondary metabolites.

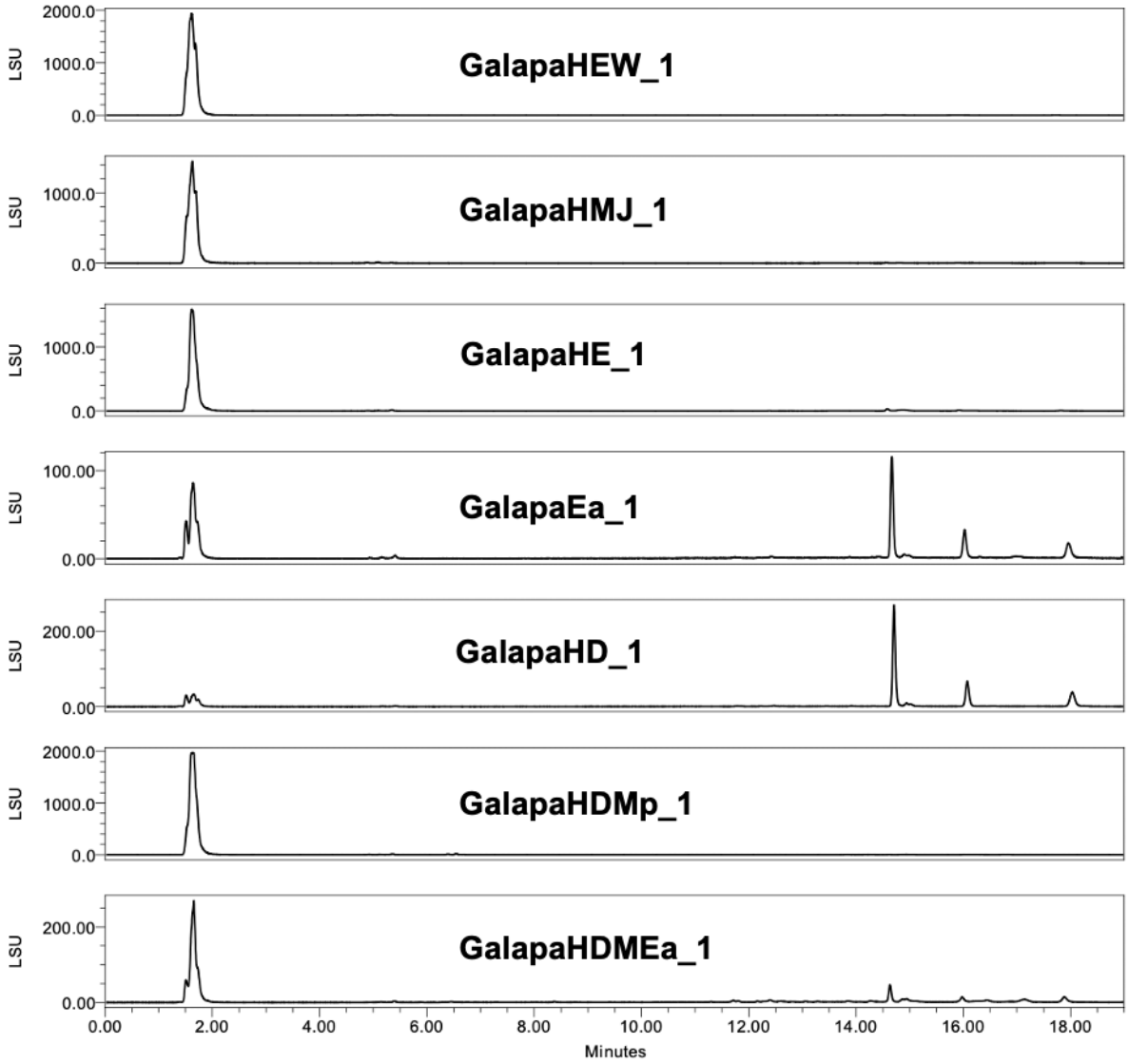

Figure 2: UHPLC-ELSD chromatograms of the preliminary *G. aparine* extracts.

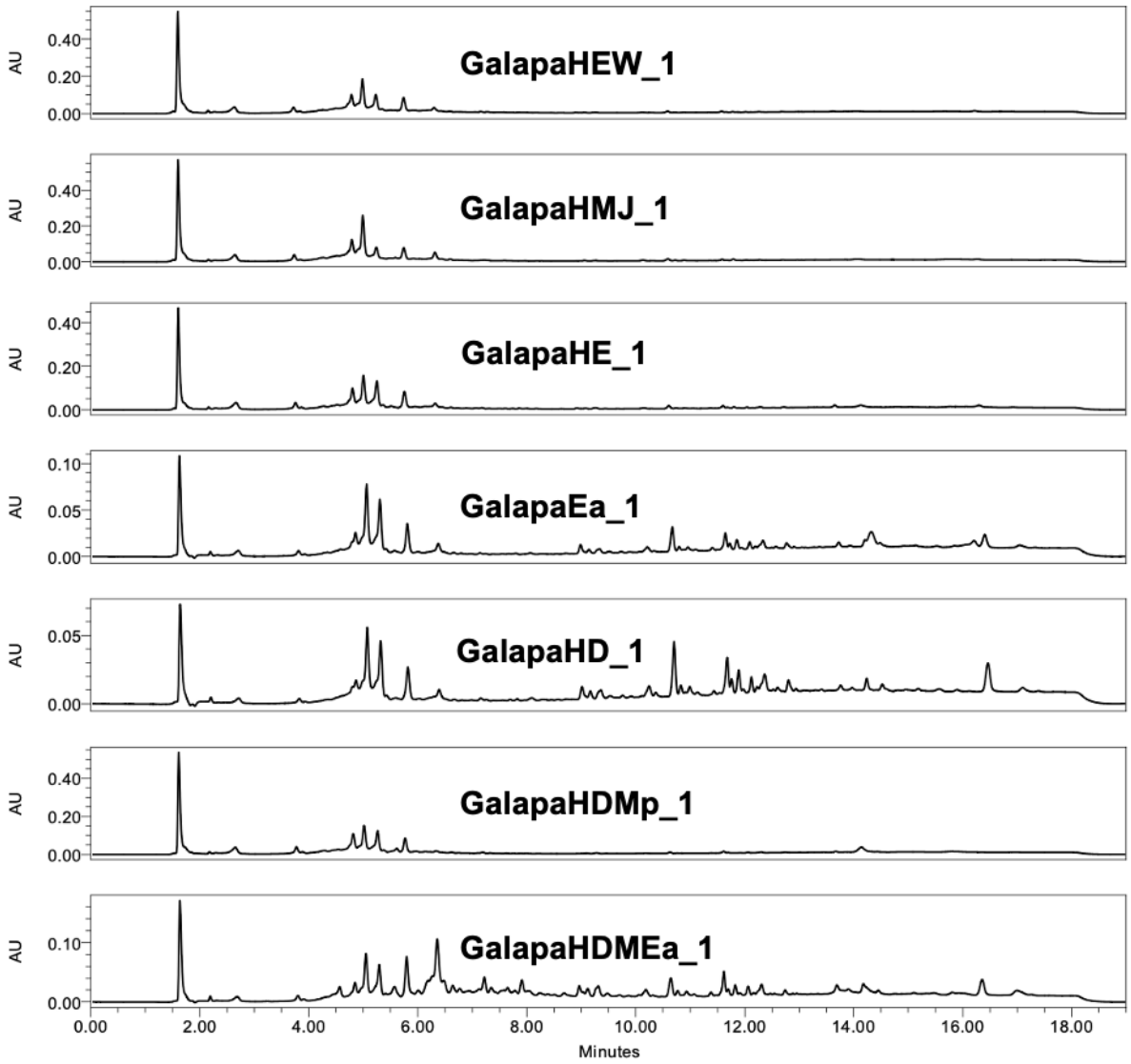

Figure 3: UHPLC-UV chromatograms (254 nm) of the preliminary *G. aparine* extracts.

2.2 Methanolic & liquid-liquid extraction of different *Galium* species

In a second approach, methanolic extracts were prepared from the herb of *G. aparine*. To achieve an enrichment of the secondary metabolites, a crude methanolic extract was subjected to liquid-liquid extraction (LLE). This procedure resulted in three fractions with different polarities. The DCM fraction predominantly contained non-polar constituents, the EtOAc/n-butanol (BuOH) fraction was enriched in the targeted secondary metabolites, and the aqueous fraction retained highly polar constituents. This procedure was repeated with the aerial parts of *G. album*, *G. sylvaticum* and *G. odoratum*. In total, 12 LLE fractions of four different species (i.e. three fractions for each species) were obtained. The methanolic crude extracts and LLE fractions of each species were subsequently analyzed by TLC and UHPLC with special focus on the EtOAc/BuOH fraction.

2.2.1 Comparison of *Galium* extracts via TLC

TLC analysis was carried out to analyse the extracts and fractions of *G. aparine*, *G. sylvaticum* and *G. odoratum* using a previously optimized solvent system (see chapter 6.4.1). The extract and fractions derived from each species were applied in parallel at a concentration of 5 mg/ml, together with reference substances, chlorogenic acid, rutin and coumarin, each with a concentration of 1 mg/ml (Figure 4, 5 and 6).

The plates were evaluated prior and after the derivatization using vanillin (1% in MeOH) and sulfuric acid (5% in MeOH) and were analysed under visible light as well as under UV light at a wavelength of 254 nm and 366 nm. Lipophilic constituents of the DCM fraction were detected near the solvent front with an R_f value > 0.6. The coumarin with an R_f value of 0,9, was more prominent in *G. odoratum*, which is also characteristic of the herb's scent. The constituents of the EtOAc/BuOH fraction were well distributed across the entire chromatogram. The hydrophilic and polar components from the aqueous fraction were found at lower R_f values (TAMAS et al., 2006).

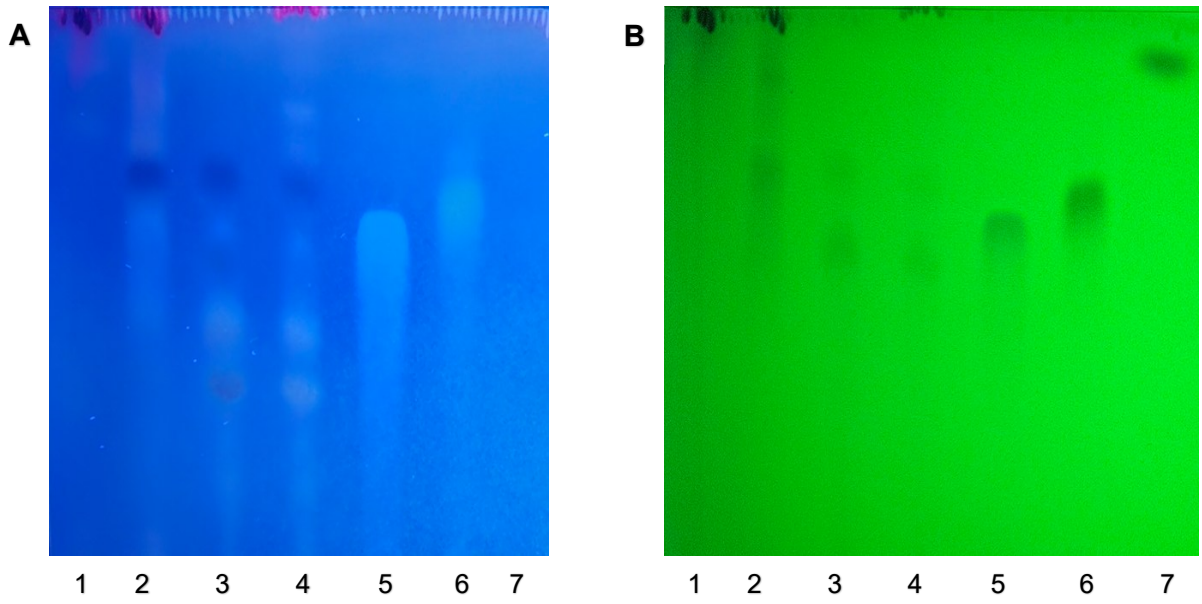

Figure 4: TLC of methanolic *G. aparine* extract and fractions. (1) DCM fraction, (2) EtOAc/BuOH fraction, (3) aqueous fraction, (4) methanolic crude extract, (5) chlorogenic acid, (6) rutin, (7) coumarin; detected at (A) UV_{366} after spraying agent, (B) UV_{254}.

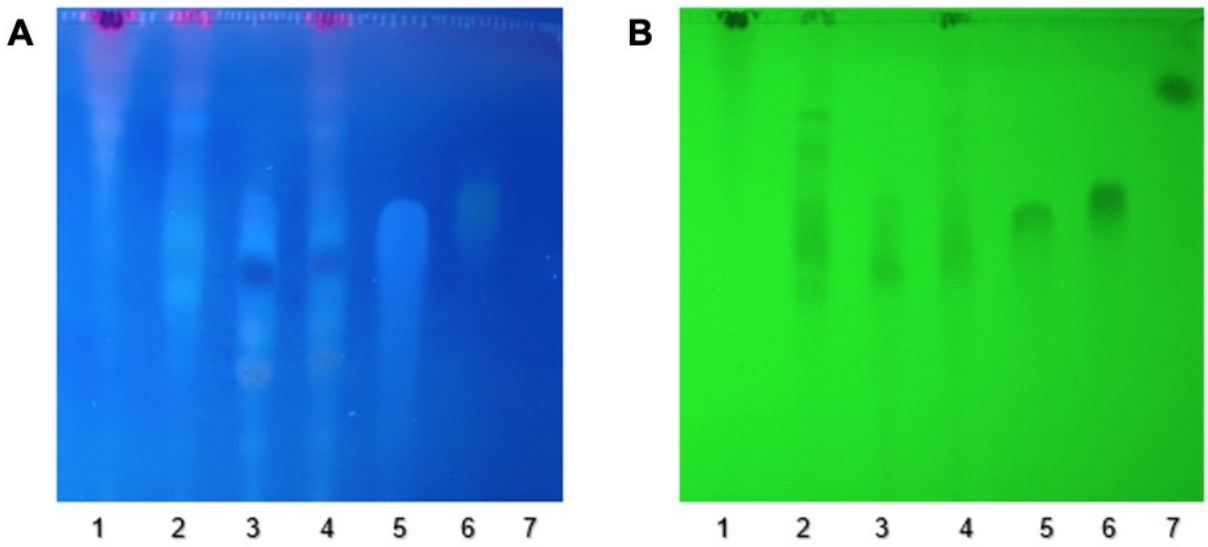

Figure 5: TLC of methanolic *G. sylvaticum* extract and fractions. (1) DCM fraction, (2) EtOAc/BuOH fraction, (3) aqueous fraction, (4) methanolic crude extract, (5) chlorogenic acid, (6) rutin, (7) coumarin; detected at (A) UV_{366} after spraying agent, (B) UV_{254}.

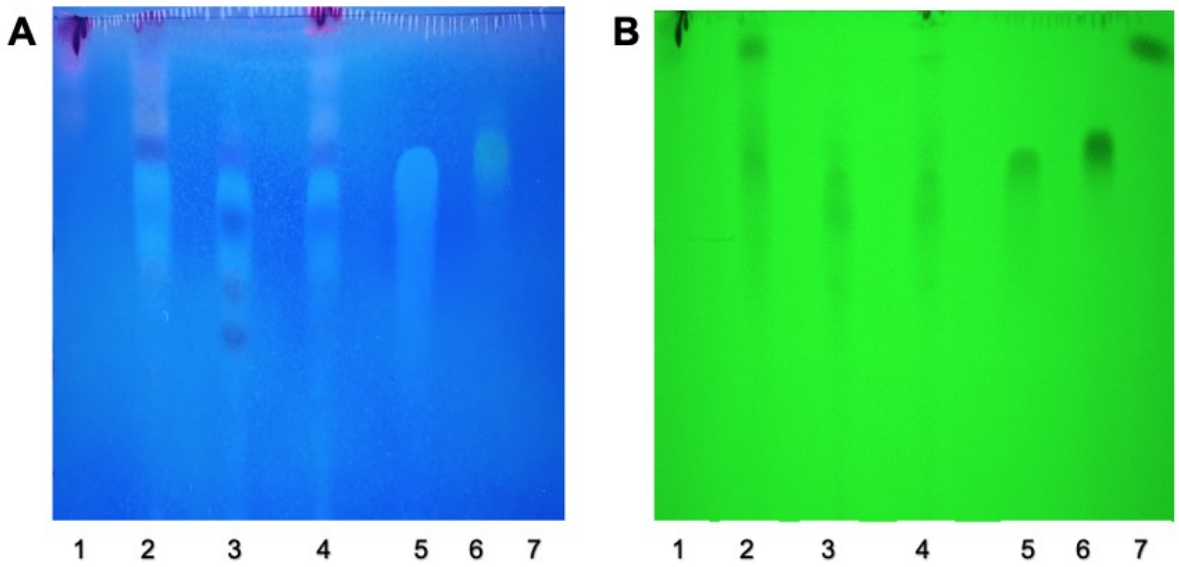

Figure 6: TLC of methanolic *G. odoratum* extract and fractions. (1) DCM fraction, (2) EtOAc/BuOH fraction, (3) aqueous fraction, (4) methanolic crude extract, (5) chlorogenic acid, (6) rutin, (7) coumarin; detected at (A) UV_{366} after spraying agent, (B) UV_{254}.

2.2.2 Comparison of *Galium* extracts via UHPLC

For all samples described above, UHPLC-UV-ELSD/MS analyses were performed (see chapter 6.4.2). The combination of UV- and MS-based spectroscopic data enabled a comprehensive characterization of the phytochemical profiles of the main constituents detected in the ELSD chromatograms.

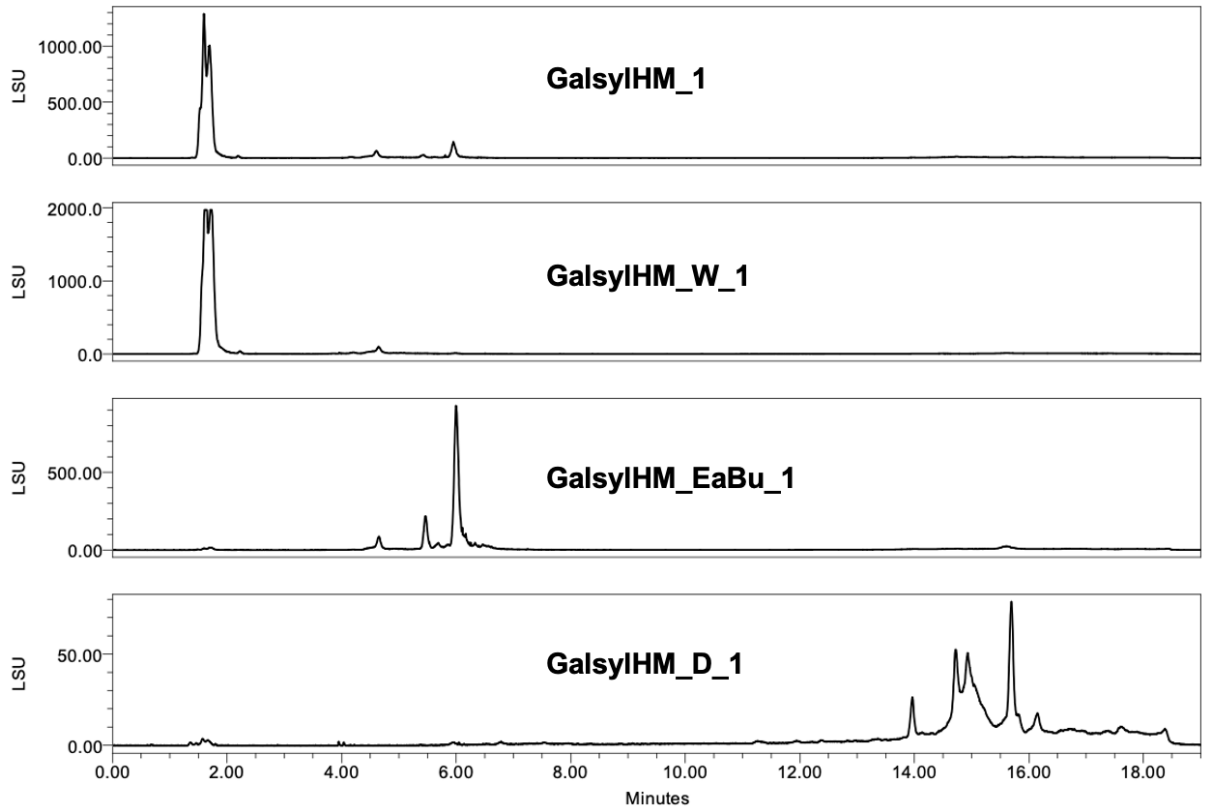

Figure 7: UHPLC-UV-ELSD chromatograms of *G. sylvaticum* extract and fractions; top to bottom crude extract, aqueous, EtOAc/BuOH and DCM fraction.

The UHPLC-ELSD chromatograms of *G. sylvaticum* fractions reflect the polarity-based separation of the constituents, with the non-polar constituents contained in the DCM fraction. The very polar constituents with limited chromatographic resolution were depleted into the aqueous phase. These peaks were most likely caused by primary metabolites such as sugars and small organic acids. The EtOAc/BuOH fraction showed several distinct peaks of secondary metabolites which were annotated based on the collected UV and MS data.

Comparison of the EtOAc/BuOH fractions of all *Galium* species (Figures 8 to 11) showed that *G. sylvaticum* had a similar secondary metabolite profile as the three other species, while there were still species-specific differences observed. Overall, the chemical profile of *G. sylvaticum* is comparable to the other anaylsed *Galium* species.

The EtOAc/BuOH fraction was selected for further phytochemical investigation (see chapter 3.2).

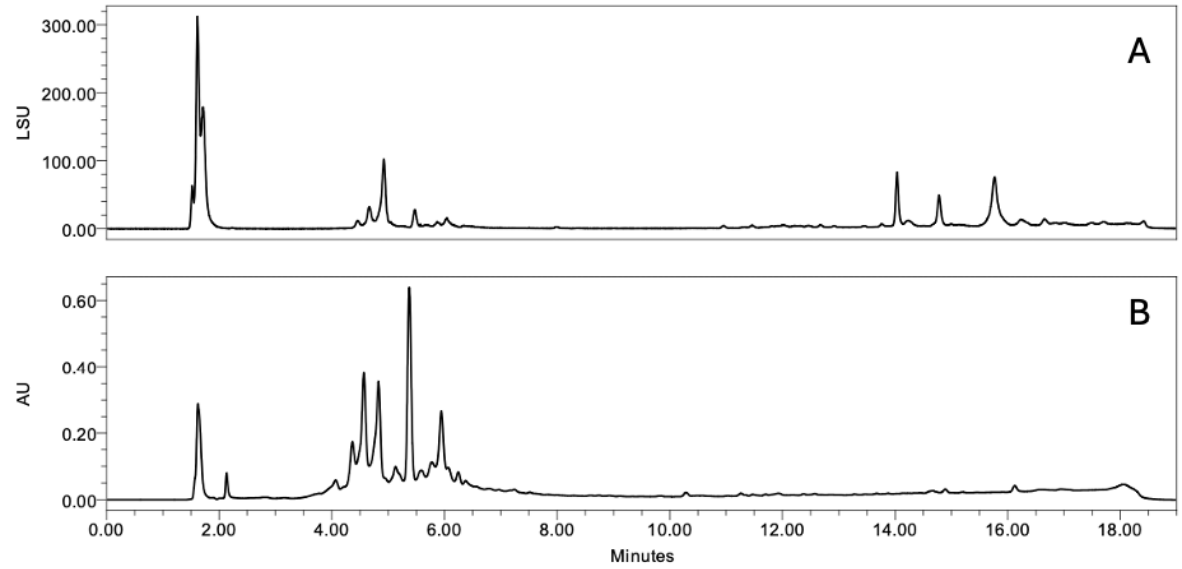

Figure 8: (A) UHPLC-ELSD and (B) UHPLC-UV (254 nm) chromatograms of the *G. aparine* EtOAc/BuOH fraction.

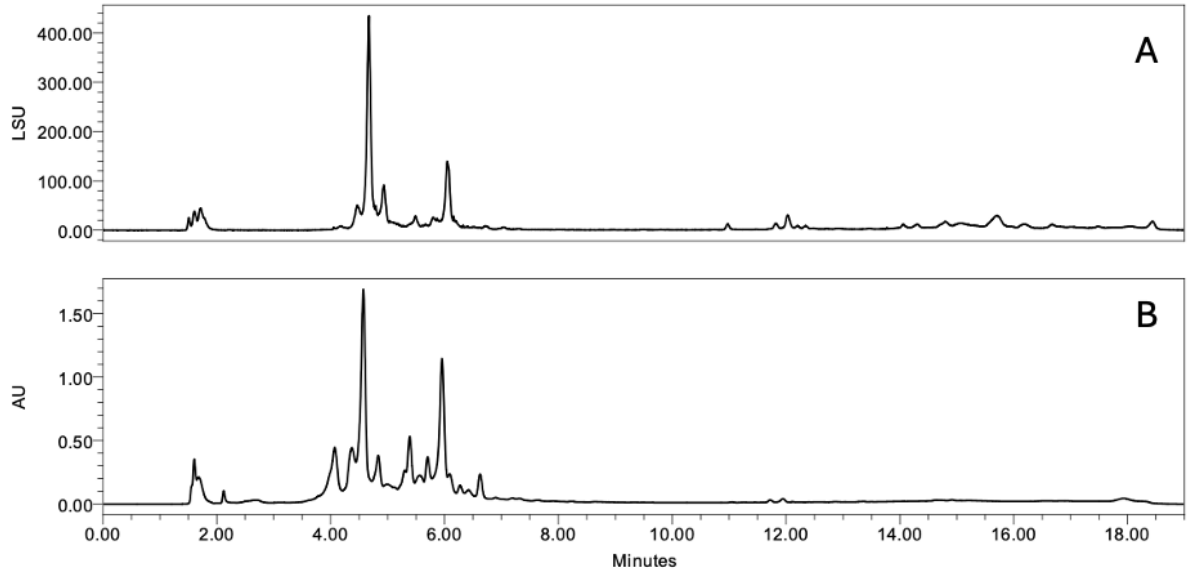

Figure 9: (A) UHPLC-ELSD and (B) UHPLC-UV (254 nm) chromatograms of the *G. odoratum* EtOAc/BuOH fraction.

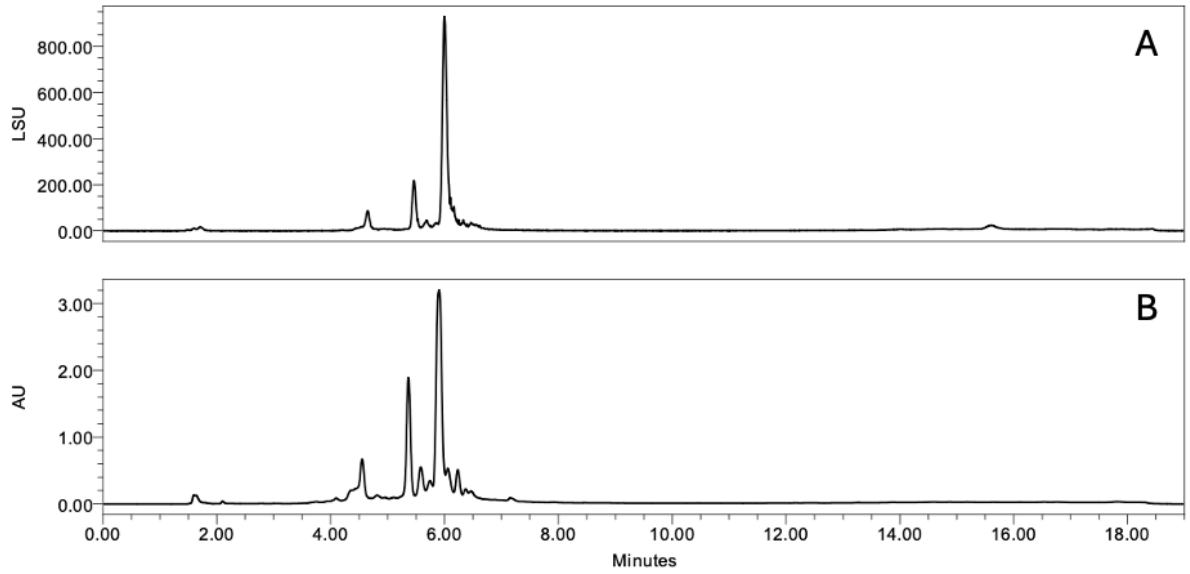

Figure 10: (A) UHPLC-ELSD and (B) UHPLC-UV (254 nm) chromatograms of the *G. sylvaticum* EtOAc/BuOH fraction.

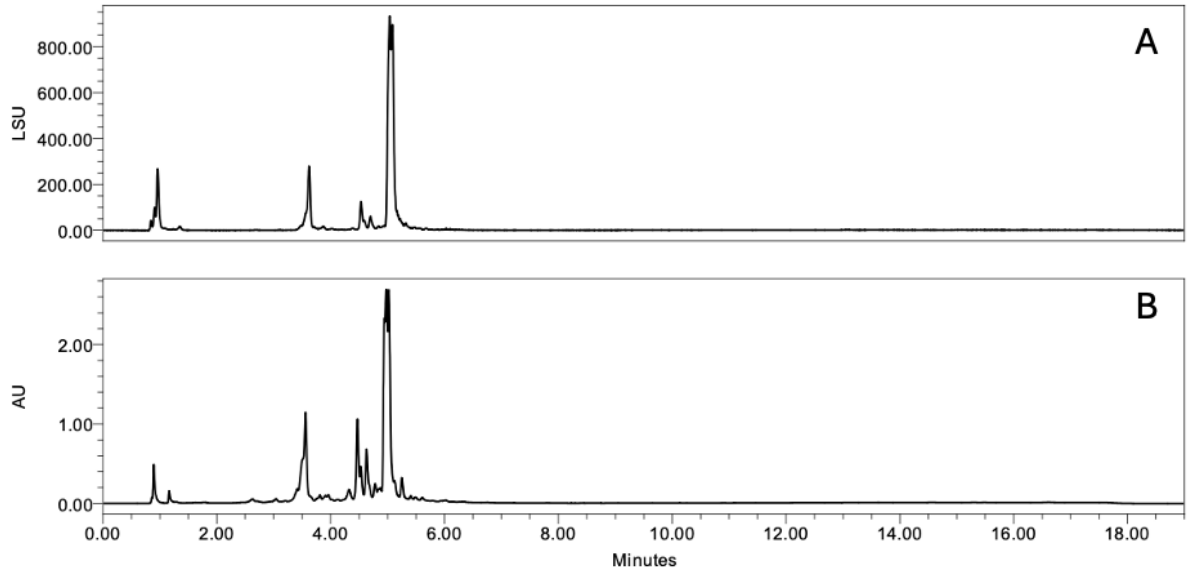

Figure 11: (A) UHPLC-ELSD and (B) UHPLC-UV (254 nm) chromatograms of the *G. album* EtOAc/BuOH fraction.

2.2.3 Annotation of secondary metabolites of EtOAc/BuOH fraction

The annotation of the secondary metabolites detected in the EtOAc/BuOH fractions of the *Galium* species was based on evaluating spectroscopic UV and MS data, which were compared with literature data (Table 1). As the fraction of *G. album* was measured not in the same sequence as the other fractions, larger RT shifts were observed. The annotation allowed a comparative assessment of the phytochemical composition of the different *Galium* species. It served as a foundation for subsequent metabolite identification and interpretation. In total, six different constituents could be identified. All four annotated species shared the presence of chlorogenic acid. The second most common substance was the iridoid glycoside asperuloside, which was detected in *G. aparine, G. odoratum* and *G. album*. The flavonoid rutin was identified in two of them (*G. aparine* and *G. sylvaticum*), while asperulosidic acid (*G. aparine*), a kaempferol-?-*O*-rutinoside isomer (*G. sylvaticum*) and a dicaffeoylquinic acid isomer (*G. odoratum*) were only annotated in one of the species as main constituent. Overall, the comparative annotation of the EtOAc/BuOH fractions revealed both shared and species-specific secondary metabolites. In contrast to *G. aparine, G. odoratum* and *G. album*, for which phytochemical data are relatively well documented, the composition of *G. sylvaticum* still remains largely unknown. Subsequent analyses were directed toward a more detailed characterization of the secondary metabolites present in the EtOAc/BuOH fraction of this species. The targeted approach addresses the gap in current literature and provides the base for a more comprehensive understanding of its constituents.

Table 1: Annotation of the EtOAc/BuOH fractions of *G. aparine* (yellow), *G. odoratum* (blue), *G. sylvaticum* (orange), and *G. album* (grey).

RT [min]	*m/z* positive mode	*m/z* negative mode	UV max [nm]	Estimated molecular weight [g/mol]	Proposed substance (substance class)	Literature	CAS
4,65	455,1 471,1	431,2		432	Asperulosidic acid (iridoid glycoside)	(Deliorman et al., 2001)	25368-11-0
4,86	377,1 393,1	353,1	326	354	Chlorogenic acid (polyphenol)	(Senio et al., 2018)	327-97-9
5,12	437,1 453,1	413,2		414	Asperuloside (iridoid glycoside)	(Trim, 1952)	14259-45-1
5,66	633,2 649,2	609,3	352	610	Rutin (flavanoid)	(Tzakou et al., 1994)	153-18-4
4,62	377,1 393,1	353,1	326	354	Chlorogenic acid (polyphenol)	(Senio et al., 2018)	327-97-9
5,03	437,1 453,1	413,3		414	Asperuloside (iridoid glycoside)	(Trim, 1952)	14259-45-1
6,16	539,1 555,1	515,2	327	516	dichaffeoylquinic acid isomer (polyphenol)	(Ilina et al., 2020)	2450-53-5
4,72	377,0 393,0	353,1	325	354	Chlorogenic acid (polyphenol)	(Senio et al., 2018)	327-97-9
5,50	633,2 649,2	609,3	352	610	Rutin (flavanoid)	(Tzakou et al., 1994)	153-18-4
5,72	617,1 633,1	593,1	256	594	Kaempferol-?-O-rutinoside (flavanoid)	(Tzakou et al., 1994)	17650-84-9
3,56	377,1 393,1	353,1	326	354	Chlorogenic acid (polyphenol)	(Senio et al., 2018)	327-97-9
5,16	437,1 453,1	413,3		414	Asperuloside (iridoid glycoside)	(Trim, 1952)	14259-45-1

3 Phytochemical investigation of *G. sylvaticum*

The UHPLC analysis of the EtOAc/BuOH fraction of G. sylvaticum showed that flavonoid glycosides and phenolic acids are among the main constituents of this underexplored species. To gain insights into the minor constituents, further phytochemical investigations were conducted. Therefore, a large-scale extraction followed by LLE was performed to obtain sufficient material for in-depth investigation.

To enable efficient monitoring during separation, TLC conditions were further optimized. Afterwards, a systematic HEMWat screening was conducted to identify suitable solvent systems for HPCCC fractionation. The EtOAc/BuOH fraction was subjected to semi-preparative HPCCC and the collected fractions were then pooled according to their TLC fingerprints. At last, the pooled fractions were analysed by TLC and UHPLC-UV-ELSD/MS, allowing the dereplication and tentative annotation of secondary metabolites of *G. sylvaticum*.

3.1 Large-scale extraction of *G. sylvaticum*

To obtain a sufficient yield of extract for subsequent analytical investigations, a large-scale extraction of *G. sylvaticum* herb was carried out (see chapter 6.3.3). After methanol extraction, LLE was performed to obtain three fractions (DCM, EtOAc/BuOH and aqueous fraction).

Table 2: Yields of *G. sylvaticum* fractions after large-scale extraction.

	label	yield [mg]
MeOH extract	GalsylHM_3	not determined
DCM fraction	GalsylHM_DCM_3	627,75
EtOAc/BuOH fraction	GalsylHM_EaBu_3	484,06
Aqueous fraction	GalylHM_W_3	3637,88

3.1.1 TLC system development and optimization

The aqueous, EtOAc/BuOH and DCM fraction were evaluated using different TLC mobile phases to optimize the chromatographic separation.

In total, three different TLC systems were tested, applying the aqueous, DCM, EtOAc fractions (5 mg/ml) and reference substances (rutin, chlorogenic acid, coumarin; 1 mg/ml). The first system consisted of EtOAc/FA/AA/MeOH (100:11:11:54; v/v/v/v) as the mobile phase and used natural product reagent A (NPR A)/PEG 400 as derivatization reagents.

The second system was composed of $CHCl_3$/MeOH/ddH_2O/FA (67:30:2:1; v/v/v/v) as its mobile phase and the TLC was derivatized with NPR A/PEG 400. The mobile phase of the last system consisted of EtOAc/FA/AA/H_2O (100:11:11:26; v/v/v/v) with NPR A/PEG 400 used for derivatization, which proved to be the most suitable for the analysed extracts. It provided the most efficient separation, showing clear bands and strong fluorescence. After derivatisation phenols appeared light blue, flavonoids were coloured in yellow or orange. For exact parameters see chapter 6.4.1.

Figure 12: TLC of *G. sylvaticum* (1) DCM, (2) EtOAc, (3) aqueous fractions using EtOAc:FA:AA:MeOH (100:11:11:54) as mobile phase and NRA/PEG400 as spraying agent visualized under UV_{366}.

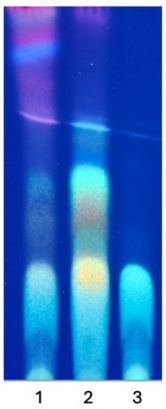

Figure 13: TLC of *G. sylvaticum* (1) DCM, (2) EtOAc, (3) aqueous fractions using $CHCl_3$:MeOH:ddH_2O:FA (67:30:2:1) as mobile phase and NRA/PEG400 as spraying agent visualized under UV_{366}.

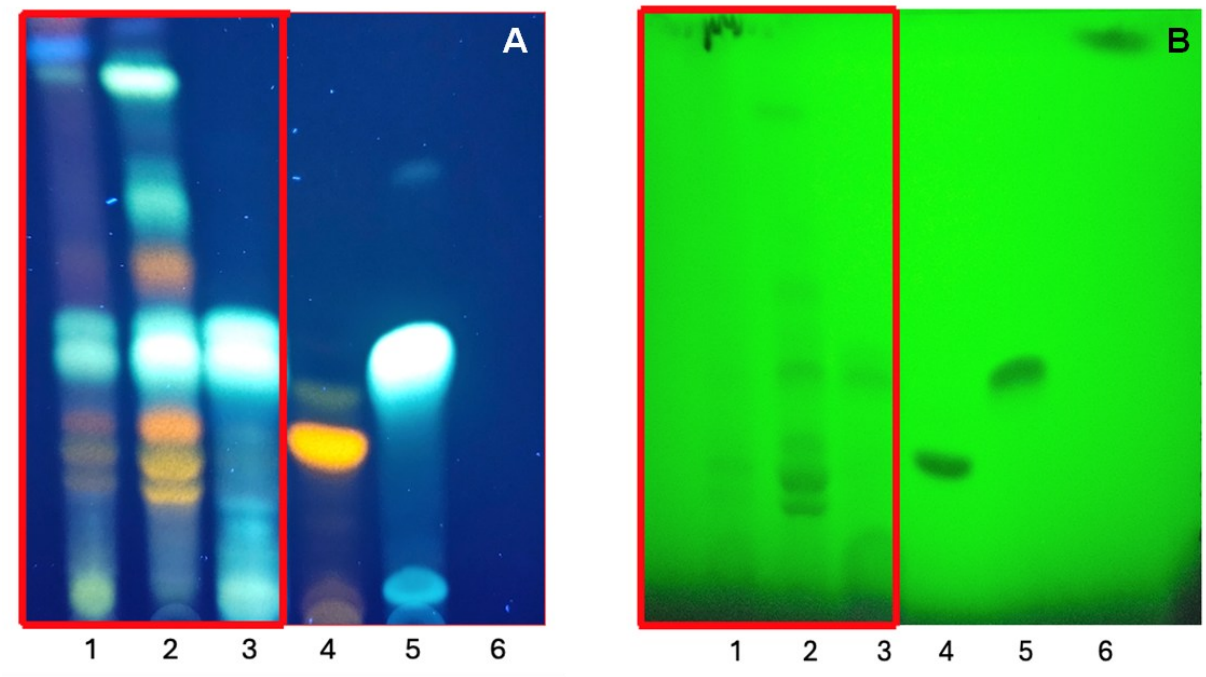

Figure 14: TLC of *G. sylvaticum* (1) DCM, (2) EtOAc, (3) aqueous fractions, as well as (4) rutin, (5) chlorogenic acid, (6) coumarin using EtOAc:FA:AA:ddH_2O (100:11:11:26) as mobile phase and NRA/PEG400 as spraying agent visualized under (A) UV_{366} and (B) UV_{254}.

3.2 HEMWat screening of *G. sylvaticum* EtOAc/BuOH fraction

For further phytochemical investigation of the secondary metabolites of *G. sylvaticum*, the EtOAc/BuOH fraction was selected for further fractionation. Based on the relative low yield of that fraction (484.06 mg), HPCCC was selected for chromatographic separation which is characterized by low sample loss since there are no adsorption effects with the stationary phase. Compared to common column chromatographic methods that are based on solid stationary phases, HPCCC uses two immiscible liquid phases which serve as stationary and mobile phase. Consequently, the first step toward an efficient fractionation of the *G. sylvaticum* EtOAc/BuOH fraction by HPCCC is the selection of an appropriate solvent system. TLC analysis was used as screening tool to evaluate a series of solvent systems with two immiscible phases composed of varying volume ratios of n-hexane, EtOAc, MeOH, BuOH and water, also called as HEMWat system. In total, eleven different solvent systems were tested, arranged in descending order with increase in polarity, meaning the lower the system number, the higher is the polarity. See Table 13 for exact composition.

Based on the TLC fingerprints, the distribution of the constituents within the organic, upper layer (UL) and the aqueous, lower layer (LL) in each solvent system was assessed. As depicted in seen in Figure 15, all detected metabolites were only mostly found in the LL of the systems 17, 15 and 13, before gradually migrating to the UL of more polar systems. As the metabolite with the highest Rf value (0,95) colored in blue was detected in higher amounts in the UL than in the LL of system no. 9, this solvent system was selected as initial solvent system for the HPCCC, applying the LL and UL as stationary and mobile phase, respectively. Moreover, the ULs of 7, 5 and 4 were selected as additional mobile phases and to achieve gradient elution of the metabolites. LL of system 9 formed the stationary phase.

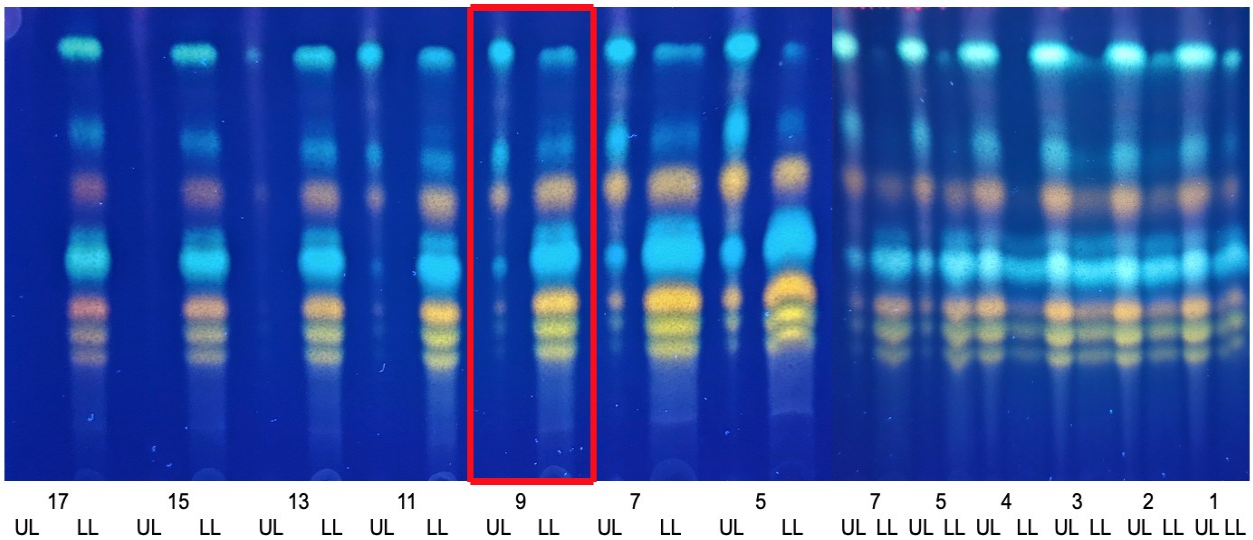

Figure 15: TLCs of the HEMWat screening of the EtOAc/BuOH fractions.

3.3 HPCCC fractionation and pooling

For the following semi-preparative HPCCC of the EtOAc/BuOH fraction of *G. sylvaticum*, the HEMWat solvent systems were used as determined by the previous solvent screening. A detailed description of HPCCC system preparation and parameters can be found in chapter 6.4.4.2 and 6.4.4.3, respectively.

In total, 174 fractions were collected during HPCCC separation and analysed by TLC. Fractions which showed similar TLC fingerprints were pooled, resulting in a total of 13 pooled fractions. The total yields and composition of the fractions are provided in Table 15. While some phenolic acids (Rf of 0,95 and 0,75) could be well separated into the first fractions (F2 and F3), most of the remaining metabolites eluted simultaneously with small variations of intensity. Particularly, the orange-coloured flavonoids (Rf of 0,30 – 0,45) eluted almost over the entire chromatographic run time. One cause might be the appearance of column bleeding (i.e. the co-elution of the stationary phase) as all metabolites were found in the collected fraction 16. Although the HPCCC separation might have suffered due to the column bleeding, the pooled fractions were further analysed by UHPLC-UV-ELSD/MS.

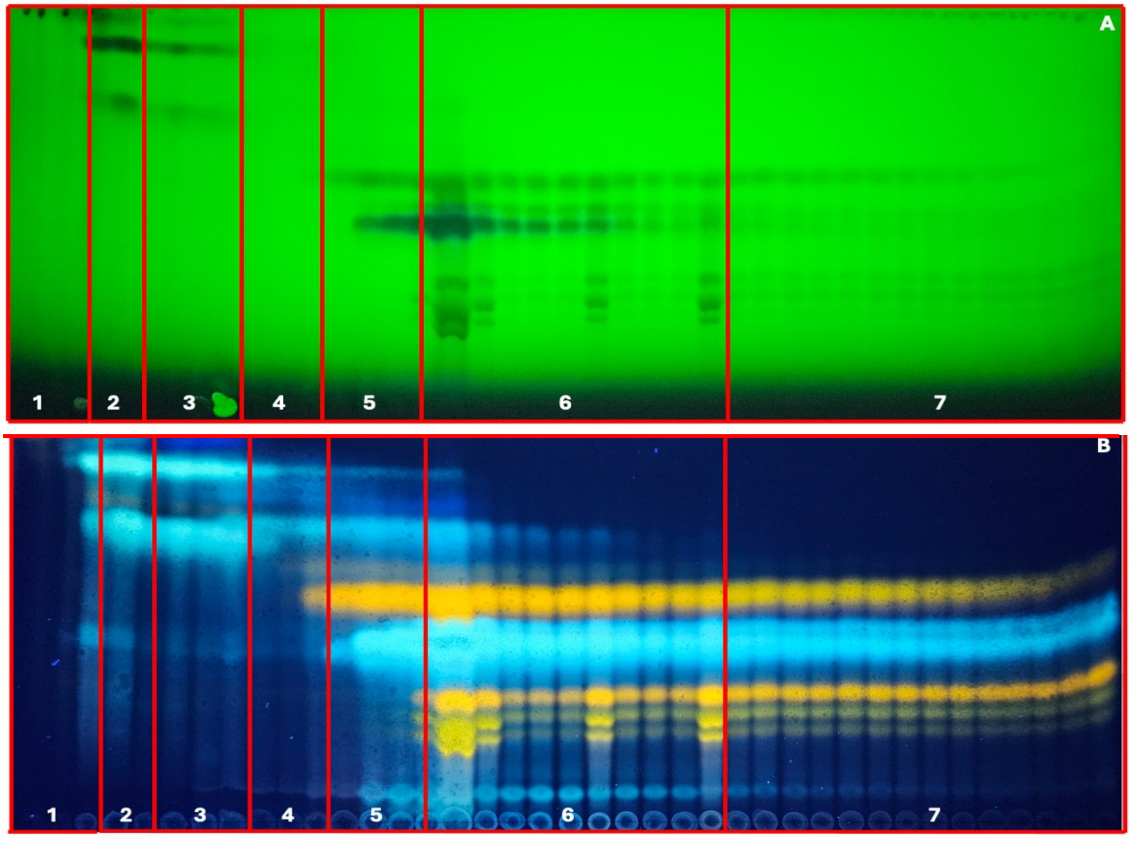

Figure 16: TLC of HPCCC fractions 1 to 40; (A) UV_{254}, (B) UV_{366} after derivatization with NRA/PEG400.

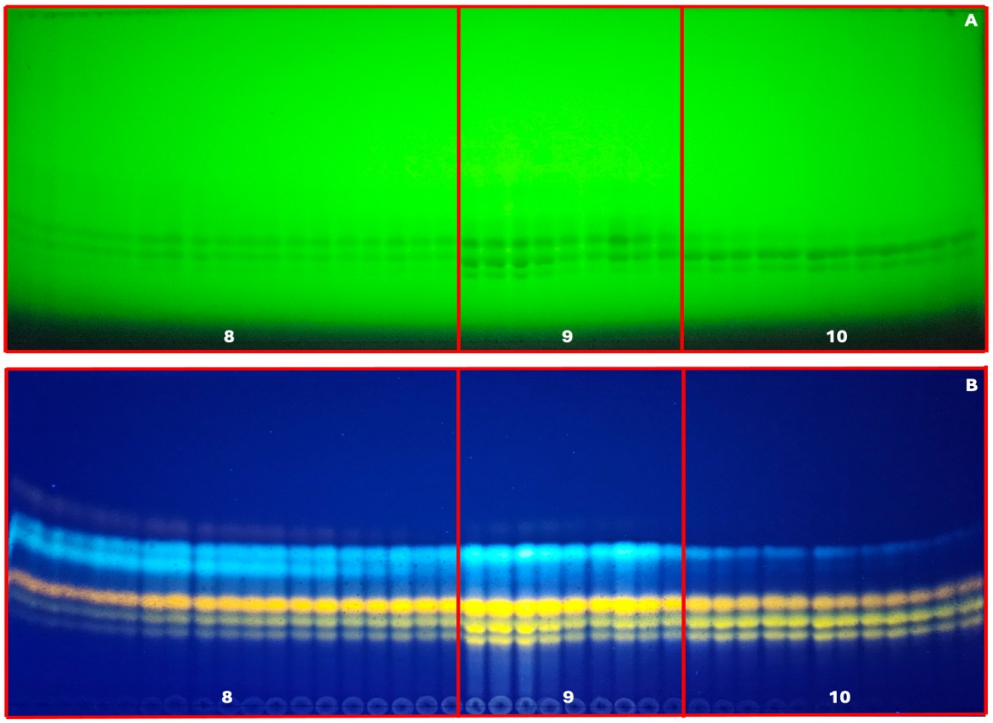

Figure 17: TLC of HPCCC fractions 41 to 80; (A) UV_{254}, (B) UV_{366} after derivatization with NRA/PEG400.

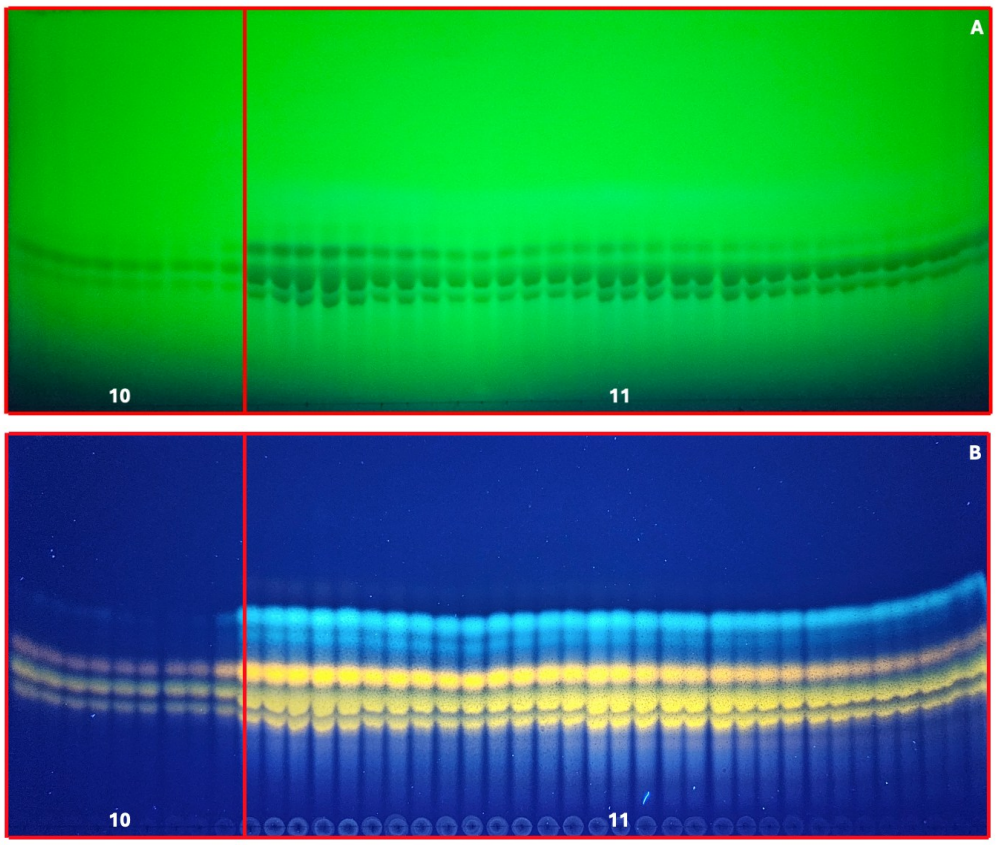

Figure 18: TLC of HPCCC fractions 81 to 120; (A) UV_{254}, (B) UV_{366} after derivatization with NRA/PEG400.

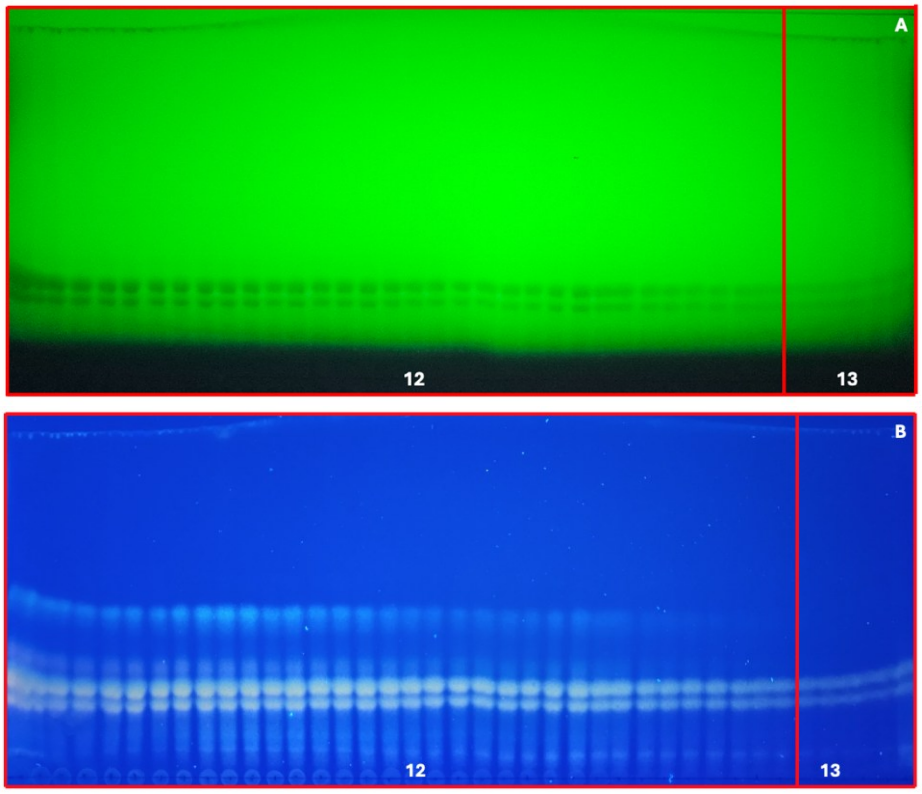

Figure 19: TLC of HPCCC fractions 121 to 160 of GalsylHM_EaBu_3; (A) UV_{254}, (B) UV_{366} after derivatization with NRA/PEG400.

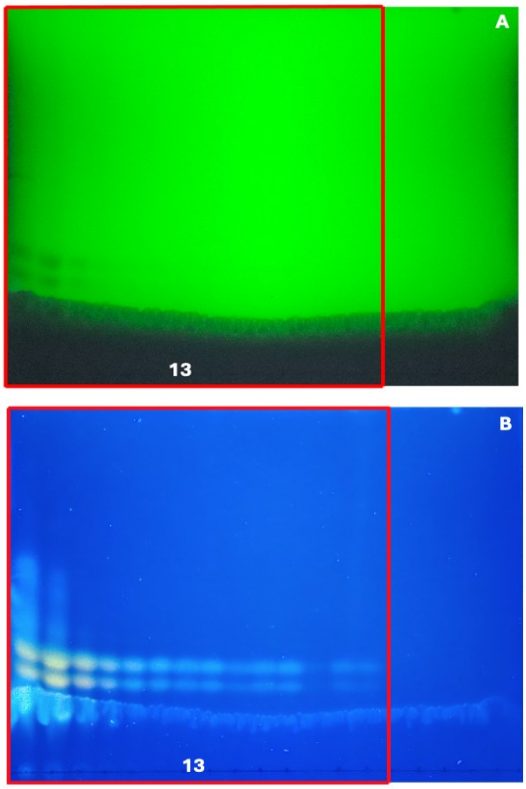

Figure 20: TLC of HPCCC fractions 160 to 174; (A) UV_{254}, (B) UV_{366} after derivatization with NRA/PEG400.

3.4 TLC analysis of pooled fractions

A collective TLC analysis of the 13 pooled HPCCC fractions was carried out. It confirmed the gradual elution and separation of constituents based on their polarity, as shown in Figure 21. Blue zones of pooled fractions 1 to 4 with a relatively high Rf values (approximately Rf 0,60 to 0,90) were tentatively attributed to apolar aromatic constituents, such phenolic acids, e.g. dicaffeoylquinic acid isomers. Shiny blue bands at intermediate Rf values (0,40 to 0,60) were consistently present across multiple pooled fractions and are indicative of phenolic acid derivatives, including chlorogenic acid. Prominent yellow and orange, fluorescent bands were detected at Rf values 0,20 and 0,60, characteristic of flavonoid glycosides, including rutin and quercetin and kaempferol glycosides, which are known constituents of *Galium sp.* (Tzakou et al., 1994), (Laanet et al., 2023). At the bottom of the solvent front additional yellow bands, with lower intensity, can be found suggesting the presence of more polar flavonoid glycosides.
Overall, the TLC analysis of the HPCCC fractionation demonstrated a partially successful fractionation of the EtOAc/BuOH fraction of *G. sylvaticum* and provided a qualitative overview of the phytochemical profile.

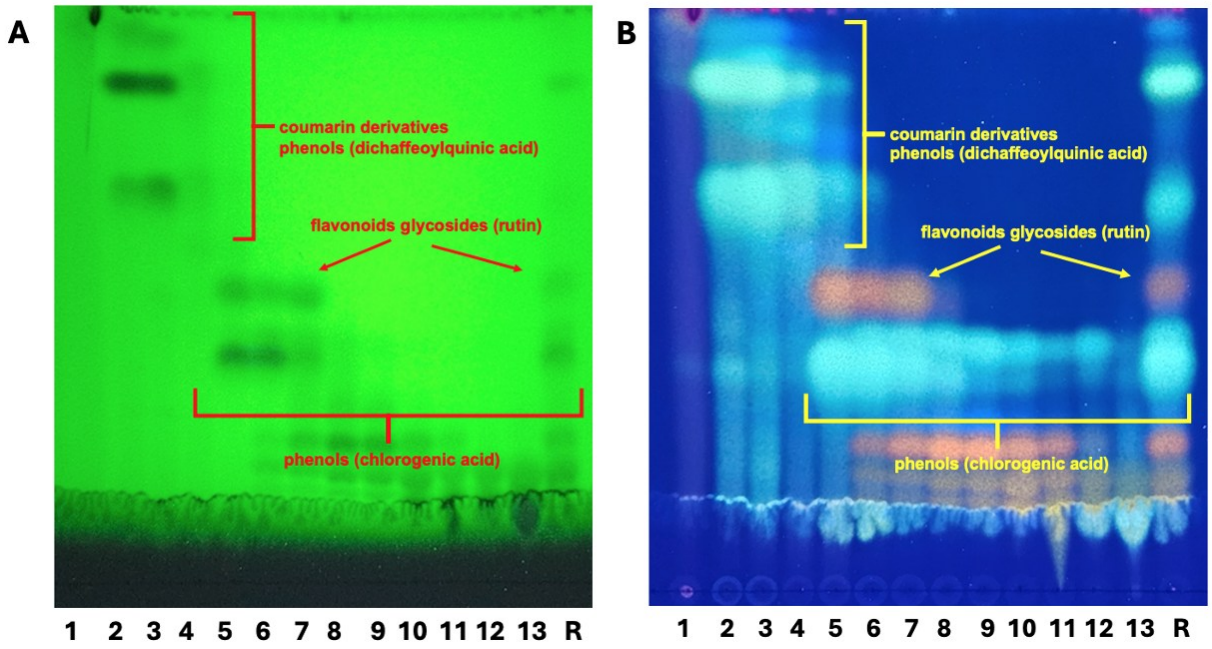

Figure 21: Collective TLC of fractions 1 - 13 and (R) the EtOAc/BuOH fraction (GalsylHM_EaBu_3); (A) UV_{254}, (B) UV_{366} after derivatization.

3.5 Dereplication of pooled fractions with UHPLC-UV-ELSD/MS

UHPLC-UV-ELSD/MS with an optimized method (see chapter 6.4.2) was carried out with all 13 pooled fractions (Figure 22 to 24), with the results supporting the collective TLC analysis (Laanet et al., 2023).

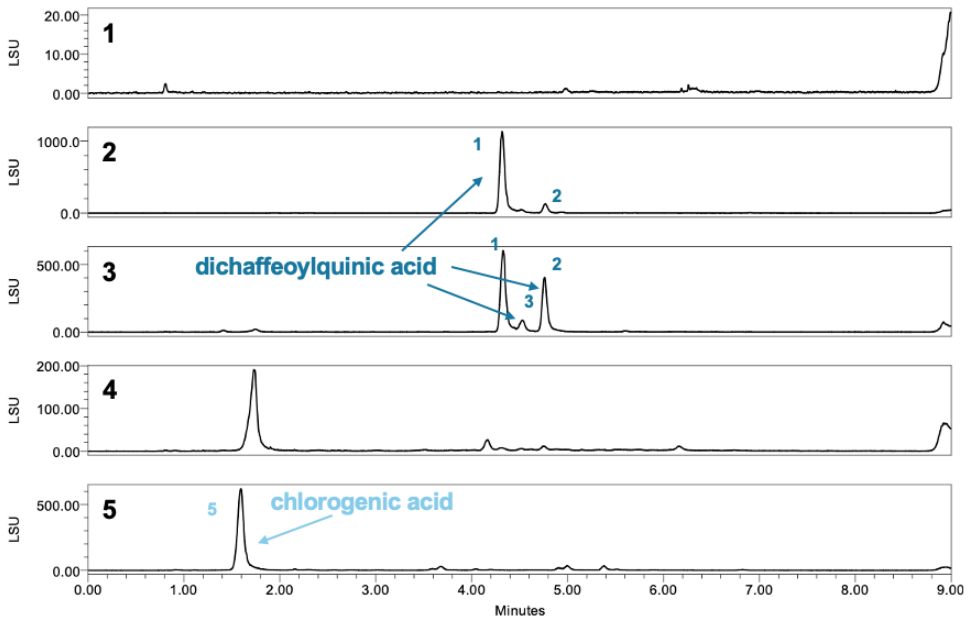

Figure 22: HPLC-ELSD chromatograms and annotation of putative compounds of the EtOAc/BuOH fraction of *G. sylvaticum*; pooled fractions 1 to 5.

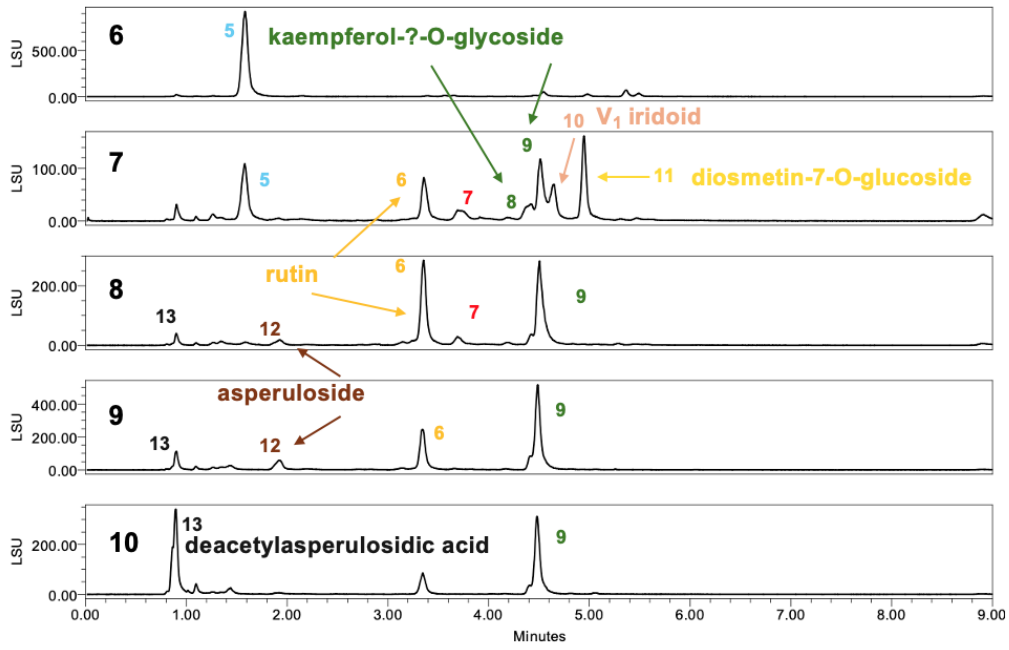

Figure 23: UHPLC-UV-ELSD detection and annotation of putative compounds of GalsylHM_EaBu_3; fractions 6 to 10.

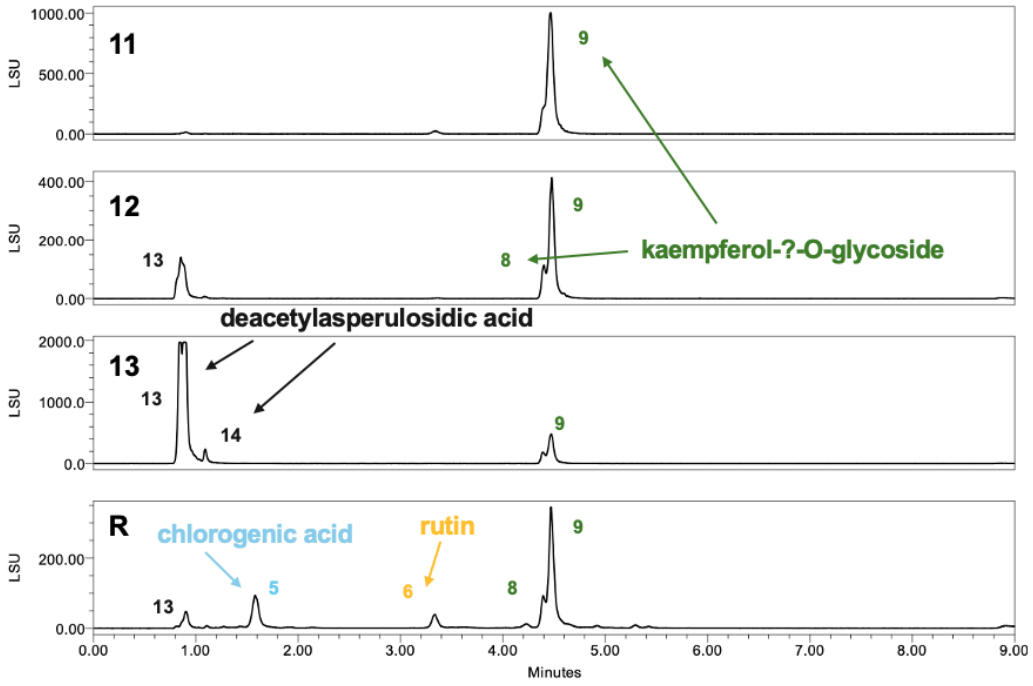

Figure 24: UHPLC-UV-ELSD detection and annotation of putative compounds of GalsylHM_EaBu_3; fractions 11 to 13 & (R) methanolic crude extract.

Table 3: Annotation of pooled fractions of the EtOAc/BuOH fraction from *G. sylvaticum* using UHPLC-UV-ELSD/MS.

Peak Nr.	Detected in Fs	RT [min]	*m/z* positive mode	*m/z* negative mode	UV max [nm]	Estimated molecular weight [g/mol]	Putative annotation (substance class)
1	2-4	4,32	539,1 555,1	515,2	296	516	Dichaffeoylquinic acid (Polyphenol)
2	2-4	4,76	539,1 555,1	515,2	327	516	Dichaffeoylquinic acid (Polyphenol))
3	2 & 3	4,53	539,1 555,1	515,2	324	516	Dichaffeoylquinic acid (Polyphenol)
4	4	1,73	383,1 399,1	359,1	281	360	Unknown
5	5-7 & R	1,58	377,1 393,1	353,1	319	354	Chlorogenic acid (Polyphenol)
6	7-9 & R	3,36	633,2 649,2	609,3	353	610	Rutin (Flavonoid)
7	7 & 8	3,69	545,1 561,1	521,1	344	520	Unknown
8	7 & 12 & R	4,42	617,2 633,2	593,1	346	594	Kaempferol-?-*O*-rutinoside isomer (Flavonoid)
9	7-13 & R	4,51	617,2 633,2	593,1	344	594	Kaempferol-?-*O*-rutinoside isomer (Flavonoid)

10	7	4,65	543,2 559,2	-	275	520	V_1 iridoid (Iridoid glycoside)
11	7	4,95	463,1	461,1	344	462	Diosmetin-7-*O*-glucoside isomer (Flavonoid)
12	8 & R	1,92	437,1 453,1	413,2	236	414	Asperuloside (Iridoid glycoside)
13	8 & 9 & 10 & 12 & 13 & R	0,82	413,1 429,1	389,2	240	390	Deactylasperulosidic acid (Iridoid glycoside)
14	1,09	1,09	413,1 429,1	389,2	234	390	Deactylasperulosidic acid (Iridoid glycoside)

A total of 14 peaks were annotated, with 11 of them annotated by comparison of RT, *m/z*, UV maximum and molecular weight with published literature (Table 3). An early eluting phenolic constituent, **chlorogenic acid** (4) was detected at RT 1,58 to 1,73 minutes. **Dichaffeoylquinic acids** (1-3) showed intermediate RT around 4-5 min. Variations in RT and UV absorption maxima suggested the presence of different isomers, a common feature of hydroxycinnamic acid derivatives. Flavonoid glycosides were detected predominantly at intermediate RT. **Rutin,** a glycoside of quercetin, was identified at compound 6.

Three other flavonoids were detected and were annotated as **kaempferol-?-O-rutinoside isomers** (8, 9) and **diosmetin-?-O-glucoside isomer** (11)**.** Additionally, two iridoid glycosides, **asperuloside** (12) and **deacetylasperulosidic acid** (13) were tentatively annotated in the final fraction, while two constituents (4, 7) remained unknown.

Based on the combined information from the different analytical approaches, it may be assumed that the phytochemical composition of *Ga. sylvaticum* is comparable to previously investigated and well-documented *Galium sp*., like *G. aparine, G. odoratum* or *G. album*.

4 Anti-inflammatory properties of *Galium* extracts

All biological testings were performed by Mag. Dr. Benjamin Kirchweger at the Ludwig Maximilian University of Munich, Germany. The methanolic extracts and LLE fractions of *G. aparine, G. odoratum* and *G. sylvaticum* were tested in a proliferation assay in human umbilical vein endothelial cells (HUVECs) and in a cell adhesion assay using HUVECs and lymphocytes from peripheral blood. HUVECs, which are endothelial cells derived from the umbilical vein, serve as a model for vascular endothelium, while lymphocytes belong to peripheral blood mononuclear cells (PBMCs) involved in inflammatory responses.

The proliferation assay shows the cell proliferation of HUVECs. An anti-proliferative effect might indicate an anti-angiogenic effect but might also be a result of cytotoxic effects. In the cell adhesion assay, lymphocytes are stimulated with tumour necrosis factor alpha (TNF-α) to induce an activated, pro-inflammatory state. The primary objective is to determine whether these stimulated lymphocytes adhere to the endothelial cell layer, thereby mimicking the process of immune cell extravasation into inflamed tissue, which is a key step in the inflammatory cascade. A reduction in lymphocyte adhesion to HUVECs suggests impaired leukocyte-endothelium interaction, which in turn may indicate a decrease in inflammatory activity. Such findings would provide evidence for a potential anti-inflammatory effect, as diminished adhesion is associated with reduced immune cell infiltration into affected tissues, a critical mechanism in inflammation-driven pathologies.

Briefly, HUVECs are cultured until a confluent layer covering the entire well was formed. In parallel, lymphocytes are cultured separately under appropriate conditions. HUVECs were stimulated with TNF-α to induce an inflammatory response. In addition, cells are treated with or without extract or fraction tested at three concentrations (10, 25 and 50 µg/mL).

This treatment aims to mimic an inflammatory environment and increase endothelial cell adhesiveness. Following 24 hours of treatment, lymphocytes are fluorescently labelled and subsequently added to the endothelial cell layer. Under inflammatory conditions, activated endothelial cells exhibit increased adhesion properties, facilitating lymphocyte binding. After the incubation period, non-adherent lymphocytes are removed by washing. The number of remaining adherent lymphocytes is quantified by measuring fluorescence intensity, indicating endothelial inflammation and the potential anti-inflammatory effect. For exact experimental design, see chapter 6.5.

Figure 25 shows the results from the proliferation assay in HUVECs, while Figure 26 provides the results from the cell adhesion assay. The DCM fractions of all species showed anti-proliferative effects (cell proliferation < 50% compared to control) at 50 µg/mL. However, these data must be interpreted with caution and in the context of reduced cell viability.

In particular, the DCM fractions led to unstable and non-confluent HUVEC-layer, indicating substantial cytotoxicity that may confound the interpretation of reduced lymphocyte adhesion. The extract and fractions derived from *G. aparine* were inactive in the performed assays. The discrepancy between these findings and the more promising effects observed in preliminary experiments cannot be conclusively explained. One possible reason being the degradation of an active constituent due to repeated freeze-thaw cycles during storage and handling. For *G. odoratum*, minor inhibitory effects were observed for the extract and DCM fraction, however, these effects were associated with cytotoxicity. The DCM extract of *G. sylvaticum* demonstrated clear cytotoxic effects. A weak reduction in lymphocyte adhesion without apparent cytotoxicity was observed only for the EtOAc/BuOH fraction of *G. sylvaticum*.

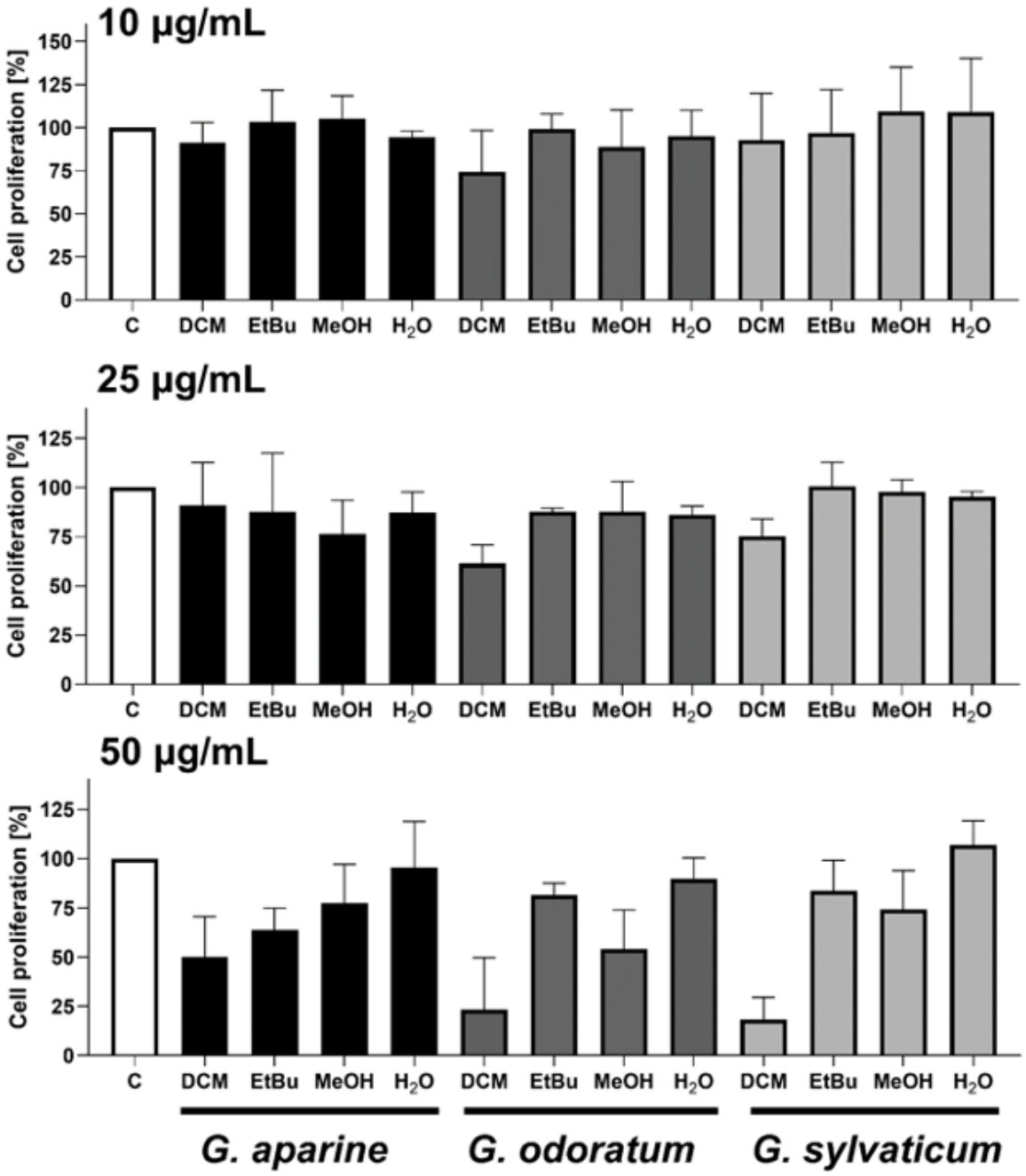

Figure 25: Effects of *Galium* extracts and fractions at indicated concentrations on HUVEC proliferation, n = 3. Data are depicted as % of mean ± SD of C (vehicle control, 0.1% DMSO).

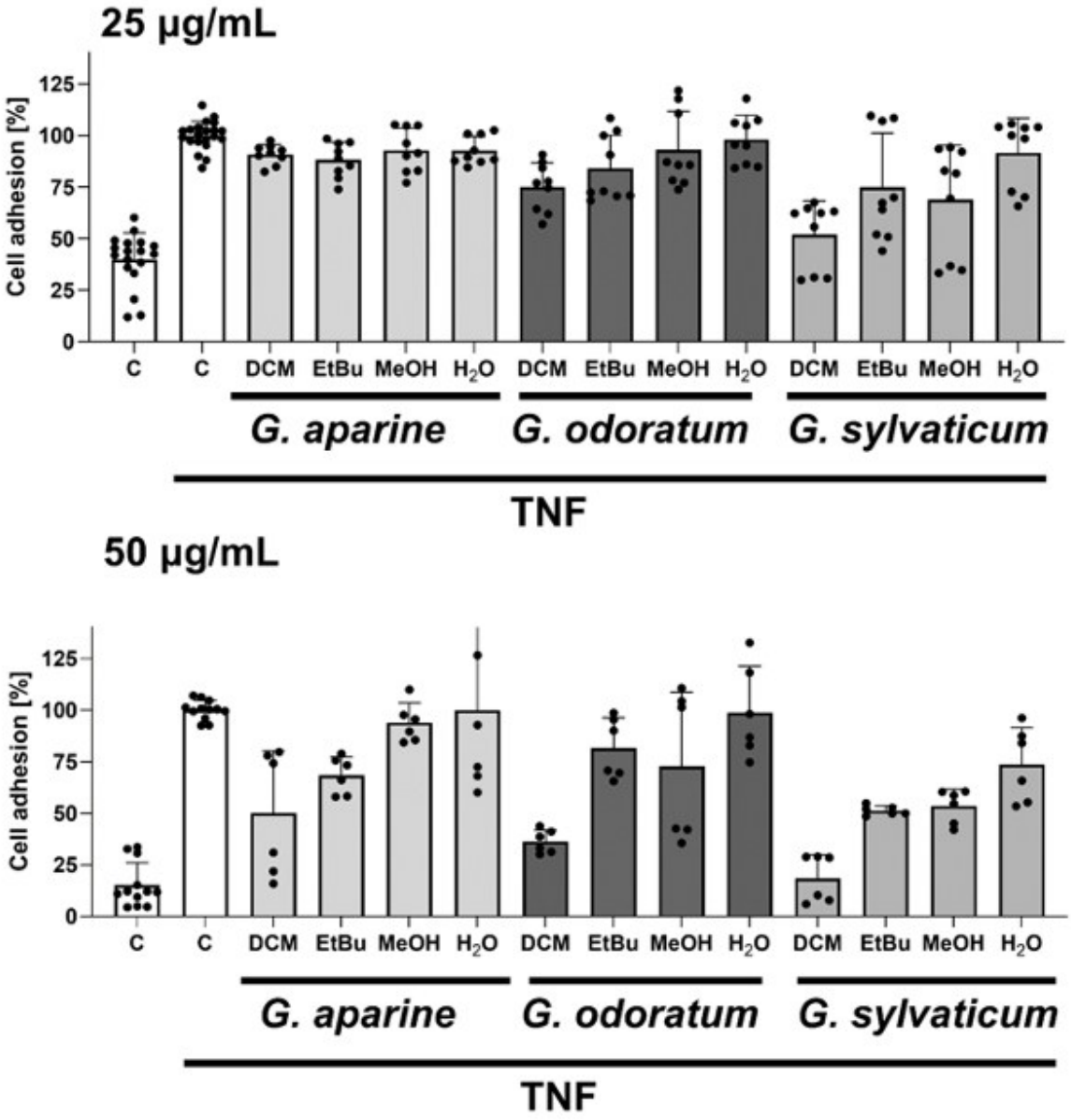

Figure 26: Effects of *Galium* extracts and fractions at indicated concentrations on HUVEC/PBMC lymphocyte cell adhesion. Data are depicted as % of TNF stimulated, vehicle control (C, 0.1% DMSO). Each point depicts one well replicate whereby three well replicates were performed per donor and independent experiment. N ≥ 2.

5 CONCLUSION AND OUTLOOK

The findings of this work contribute to a better understanding of the role of *Galium* species in ethnopharmacology and evaluated their anti-inflammatory properties.

Further, this study compared the phytochemical composition of the aerial parts of four *Galium* species, *G. aparine, G. odoratum, G. album* and *G. sylvaticum*. Therefore, an extraction protocol was developed which successfully enriched the secondary metabolites in the EtOAc/BuOH fraction of a methanolic extract. Based on TLC and UHPLC analyses, secondary metabolites of the four *Galium* species could be analysed and compared, showing similar chemical profiles with subtle differences. Compound classes identified across all species comprised phenolic acids, flavonoid and iridoid glycosides.

The implementation of HPCCC proved to be an effective chromatographic technique for the fractionation of the *G. sylvaticum* EtOAc/BuOH fraction, providing deeper insights into the chemical composition. Eventually, annotation of the 13 pooled fractions based on RT, MS and UV spectra indicated the occurence of dicaffeoylquinic acids, chlorogenic acid, iridoid glycosides, including asperuloside and deacetylasperulosidic acid, as well as rutin and kaempferol-?-O-rutinoside isomers. In conclusion, this work expands the phytochemical knowledge of *G. sylvaticum.*

Preliminary biological testings using a HUVEC proliferation assay and HUVEC/PBMC cell adhesion assay evaluated the anti-inflammatory potential of the prepared *Galium* extracts. In this experimental setting, none of the tested extracts and fractions showed promising anti-inflammatory properties. To evaluate the traditional wound-healing properties of *Galium* species, other *in vitro* assays such as a scratch assay on keratocytes might be a suitable approach.

6 MATERIAL AND METHODS

6.1 Literature research

For literature search, databases SciFinder® (scifinder.cas.org/), PubMed® (https://pubmed.ncbi.nlm.nih.gov/) and Google Scholar (https://scholar.google.com/), were used, including key words „galium", „anti-inflammatory", „skin diseases", „psoriasis", „wound healing", „constituents", „ethnopharmacological", „ethnobotanical", „traditional use" „TLC", „HPCCC", „phytochemical" & „annotation".

6.2 Plant material

The plant material used for further processing and production of the different extracts was either purchased from Kottas Pharma GmbH, Eitnergasse 8, 1230 Vienna, Austria or came from wild collections in Austria. In the case of *G. odoratum*, the used material came from Kottas Pharma GmbH. Product name Herba asperulae, charge number P24301282, tested according to Ph. Eur. Materials of *G. aparine* originate from wild collection of Univ.-Prof. Dr. Judith M. Rollinger labelled JR-20240623-A1, June 2024, located on Karmelweg, Innsbruck.
For extracts of *G. sylvaticum* a wild collection of Mag. Dr. Andreas Wasilewicz was used, collected in September 2024, at 1. Wiener Wasserleitungsweg close to Kaiserbrunn.

6.3 Extract generation and labelling

The small-scale extracts were labeled using a combination of the first three letters of the genus and species, followed by the initials of the organ (H for herb) and e.g. the abbreviation M for methanol extraction. Additionally, p is appended due to the use of polyamide gel solid-phase extraction, or the abbreviation of further solvents was used (e.g. Ea for ethyl acetate), indicating the performance of LLE. The number at the end of the label represents the number of the extract batch.

6.3.1 Extraction method screening

To understand which extracts can enrich most of the secondary metabolites, phytochemical experiments started with a couple of extractions with various solvents. Around 300 mg dried, ground plant material of *Galium aparine* were ultrasonicated for 20 minutes with 10 ml of either EtOH 96%, EtOH 70%, EtOAc or DCM (listed in Table 4). After centrifugation, extracts were then filtered through cotton wool, while the remaining plant material was extracted for a second time following the same procedure. Finally, the two eluates were pooled and evaporated to dryness (40°C). Reconstituted in MeOH, extracts (5 mg/ml) were analysed by UHPLC-UV-ELSD.

Additional extracts were prepared based on an adapted protocol from (Camp et al., 2012). This extraction method was used to remove disruptive compounds like fats and tannins and to increase the quantity of components with drug-like properties (Camp et al., 2012). Briefly, 300 mg of the dried, ground material was extracted twice with 10 ml of n-hexane on ultrasonic bath for 10 minutes for defatting. Afterwards the extract was decanted. The remaining plant material was extracted with 10 ml of DCM for 15 minutes on ultrasonic bath. The extract was filtered through a cotton wool and collected in a round-bottom flask. A second extraction of the same natural material was done with 10 ml of MeOH, also on a 15-minute ultrasonic bath. Once again filtered and transferred in a round-bottom flask. The MeOH and DCM extracts were pooled and evaporated to dryness (40°C) and reconstituted in 4 ml of methanol. For the removal of tannins, a solid-phase extraction (SPE) was performed by applying the extract to a 3 ml polyamide-packed cartridge. The cartridge contained CC-6 polyamide gel and a frit with 20 µM pore size. The extract was loaded onto the solid-phase and washed with 4 ml of MeOH twice. The eluate was reduced in volume, transferred to a pre-labeled, pre-weighted tube and finally brought to dryness (40°C) in the sample concentrator and desiccator. Reconstituted in MeOH, extracts (5 mg/ml) were analysed by UPLC-UV-ELSD.

For comparison, there was an alternative procedure of liquid-liquid extraction (LLE) performed for the removal of hydrophilic constituents and tannins, respectively. The model for the LLE process was the recent paper by Mag. Dr. Andreas Wasilewicz (Wasilewicz et al., 2025).

Combined DCM and MeOH extracts as prepared above were dissolved in 5 ml EtOAc and ddH_2O equally. The two phases were separated in a separation funnel. Followed by another LLE of the aqueous phase with 5 ml EtOAc. The two EtOAc phases were reduced in volume on rotary evaporator (40°C) and transferred in a pre-weighted vial to dry.

Table 4: Preliminary extracts.

label	solvent	drug used [mg]	extract yield [mg]	extract description
GalapaHE_1	EtOH 96%	301,25	33,07	Ethanolic (96%) extract of *G. aparine* herb
GalapaHEa_1	EtOAc	300,90	13,73	EtOAc extract of G. aparine herb
GalapaHD_1	DCM	300,80	8,97	DCM extract of G. aparine herb
GalapaHEW_1	EtOH 70%	300,73	69,42	Ethanolic (70%) extract of G. aparine herb
GalapaHH_1	n-hexane	302,32	31,56	n-Hexane extract of G. aparine herb
GalapaHDMEa_1	DCM, EtOAc	300,84	6,05	n-hexane defatted, DCM and MeOH extract of *G. aparine* herb; MeOH extract purified by LLE using EtOAc/BuOH/H_2O, organic phase combined with DCM extract
GalapaHDMp_1	DCM, MeOH	300,41	29,14	n-hexane-defatted, combined DCM MeOH of *G. aparine* herb, extract further purified by solid-phase extraction using polyamide gel

6.3.2 Preparation of analytical extracts

The production of the main extracts of each plant material for further processing went as follows: For the first step of the extraction, around 5 g of natural material was grounded. Subsequently, they were extracted with 170 ml of methanol in a 20-minute ultrasonic bath. The extract was then removed and filtered through filter paper, afterwards evaporated via rotary evaporator (40°C) to minimal volume and transferred to pre-weighted vials of 10 ml.
Yields of the larger scale extraction were 754,66 mg for *G. aparine*, 508,69 mg for *G. odoratum*, 955,64 mg for the extract of *G. sylvaticum* and 572,15 mg for *G. album/mollugo* (see Table 5). Approximately 400 mg of the dried methanolic extracts were then extracted via LLE in a separatory funnel once with a mixture of equal parts of 250 ml DCM and ddH_2O, followed by a second extraction of the remaining aqueous phase with 125 ml DCM. The aqueous phase then was extracted twice with a mixture of 125 ml EtOAc and n-Butanol in proportion 3:2. All three phases (i.e. DCM, EtOAc/BuOH and aqueous phase) were collected, evaporated (40°C) and dried in pre-weighted tubes.

Table 5: Yields of LLE fractions.

fraction	label	yield [mg]	extract description
methanol	GalapaHM_2	319,88	Methanolic extract of *G. aparine* herb
dichloromethane	GalapaHM_D_2	48,36	DCM fraction derived from GalapaHM_2 using LLE
ethyl acetate / butanol	GalapaHM_EaBu_2	33,48	EtOAc/BuOH fraction derived GalapaHM_2 using LLE
ddH_2O	GalapaHM_W_2	228,18	Aqueous fraction derived from GalapaHM_2 using LLE
methanol	GalodoHM_1	83,51	Methanolic extract of *G. odoratum* herb
dichloromethane	GalodoHM_D_1	80,09	DCM fraction derived from GalodoHM_1 using LLE
ethyl acetate / butanol	GalodoHM_EaBu_1	44,25	EtOAc/BuOH fraction derived GalodoHM_1 using LLE
ddH_2O	GalodoHM_W_1	182,68	Aqueous fraction derived from GalodoHM_1 using LLE
methanol	GalsylHM_1	258,34	Methanolic extract of *G. sylvaticum* herb
dichloromethane	GalsylHM_D_1	49,39	DCM fraction derived from GalsylHM_1 using LLE
ethyl acetate / butanol	GalsylHM_EaBu_1	35,76	EtOAc/BuOH fraction derived GalsylHM_1 using LLE
ddH_2O	GalsylHM_W_1	119,55	Aqueous fraction derived from GalsylHM_1 using LLE
methanol	GalmolHM_1	572,15	Methanolic extract of *G. album*
dichloromethane	GalmolHM_D_1	77,69	DCM fraction derived from GalmolHM_1 using LLE
ethyl acetate / butanol	GalmolHM_EaBu_1	53,78	EtOAc/BuOH fraction derived GalmolHM_1 using LLE
ddH_2O	GalmolHM_W_1	201,92	Aqueous fraction derived from GalmolHM_1 using LLE

6.3.3 Large-scale extraction of *G. sylvaticum* herb

Approximately 40,0 g of dried and grounded plant material was extracted twice with approximately 1,3 l of MeOH for a total of 48 h on an orbital shaker. The obtained methanolic extract was filtered and desiccated on the rotary evaporator (40°C). Next, LLE was performed three times with a mixture of 750 ml DCM and 750 ml ddH_2O, followed by another LLE with 750 ml EtOAc/BuOH (3:2) and the aqueous phase, separated three times as well.

6.4 Chromatographic methods

6.4.1 TLC systems for *Galium* sp. extracts

A preliminary TLC system was developed, with the parameters shown in table 6. Each sample had a concentration of 5 mg/ml, while reference substances chlorogenic acid, rutin, coumarin were measured at 1 mg/ml. TLC was performed with chamber saturation and filter paper. The TLC plate was analysed before and after derivatization with spraying reagents under daylight and ultraviolet light (UV_{254} and UV_{366}). After derivatization the plate was dried for five minutes at 100°C.

Table 6: TLC system parameters.

stationary phase	TLC silica gel 60 F_{254}
mobile phase	ethyl acetate:formic acid:acetic acid:MeOH 20 ml (100:11:11:54)
spraying reagent	vanillin 1% in methanol and sulfuric acid 5% in methanol
detection	UV_{254}, UV_{366}, VIS

A second, optimized TLC system was developed, including change of mobile phase & spraying agents (Table 7).

Table 7: TLC system optimized parameters.

stationary phase	TLC silica gel 60 F_{254}
mobile phase	ethyl acetate:formic acid:acetic acid:ddH_2O 20 ml (100:11:11:26)
spraying reagent	natural product reagent A 1% in methanol and polyethylene glycol 400 in 96% ethanol
detection	UV_{254}, UV_{366}, VIS

6.4.2 UHPLC systems and method settings for *Galium* sp. extracts

For UHPLC analysis of all fractions, 1 mg each of the prepared extract was dissolved in 200 µl methanol, resulting in a concentration of 5 mg/ml, while for pure reference substances chlorogenic acid, rutin and coumarin a solution with a concentration of 1 mg/ml was prepared. Different UHPLC methods were prepared used for the analysis of the methanolic crude extracts and LLE fractions, and the HPCCC fractions derived from GalsylHM_EaBu_3.

Table 8: UHPLC-UV-ELSD method for analysis of methanolic crude extracts and LLE fractions.

Instrument	Acquity UPLC H-Class System by Waters		
Column	Acquity BEH C18 (2.1 x 100mm, 1.7µm)		
Injection volume [µl]	3.0 (5 mg/ml)		
Flow rate [ml/min]	0.3		
Temperature [°C]	40		
PDA wavelength [nm]	205; 220; 254; 360		
ELSD [gain]	500		
Mobile phase A	H_2O + 0.1% formic acid		
Mobile phase B	ACN + 0.1% formic acid		
Temperature [°C]	40		
Gradient	time [min] 0 12 16 16,1 19	% A 95 2 2 95 95	% B 5 98 98 5 5

Table 9: UHPLC-UV-MS method for analysis of methanolic crude extracts and LLE fractions.

Instrument	Acquity UPLC H-Class System by Waters
Column	Acquity BEH C18 (2.1 x 100mm, 1.7µm)
Injection volume [µl]	3.0 (5 mg/ml)
Flow rate [ml/min]	0.3
Temperature [°C]	40
PDA wavelength [nm]	205; 220; 254; 360
ISM	H_2O/MeOH 9:1 + 0.1% formic acid flow rate 0,4 ml/min
QDa **mass range [Da]**	positive and negative mode 100-1200

Mobile phase A	H_2O + 0.1% formic acid		
Mobile phase B	ACN + 0.1% formic acid		
Temperature [°C]	40		
Gradient	time [min] 0 12 16 16,1 19	% A 95 2 2 95 95	% B 5 98 98 5 5
Cone voltage	positive scan 15 V negative scan 30 V		
Capilary voltage	positive scan 0,8 kV negative scan 0,8 kV		

Table 10: UHPLC-UV-ELSD method for analysis of HPCCC fractions derived from GalsylHM_EaBu_3.

Instrument	Acquity UPLC H-Class System by Waters		
Column	Acquity BEH C18 (2.1 x 100mm, 1.7µm)		
Injection volume [µl]	3.0 (5 mg/ml)		
Flow rate [ml/min]	0.3		
Temperature [°C]	40		
PDA wavelength [nm]	205; 220; 254; 360		
ELSD [gain]	500		
Mobile phase A	H_2O + 0.1% formic acid		
Mobile phase B	ACN + 0.1% formic acid		
Temperature [°C]	40		
Gradient	time [min] 0 4 7 7,3 9 9.3 11	% A 85 70 70 2 2 85 85	% B 15 30 30 98 98 15 15

Table 11: UHPLC-UV-ELSD method for analysis of HPCCC fractions derived from GalsylHM_EaBu_3.

Instrument	Acquity UPLC H-Class System by Waters		
Column	Acquity BEH C18 (2.1 x 100mm, 1.7µm)		
Injection volume [µl]	3.0 (5 mg/ml)		
Flow rate [ml/min]	0.3		
Temperature [°C]	40		
PDA wavelength [nm]	205; 220; 254; 360		
ISM	H_2O/MeOH 9:1 + 0.1% formic acid flow rate 0,4 ml/min		
QDa **mass range [Da]**	positive and negative mode 100-1200		
Mobile phase A	H_2O + 0.1% formic acid		
Mobile phase B	ACN + 0.1% formic acid		
Temperature [°C]	40		
Gradient	time [min] 0 4 7 7,3 9 9.3 11	% A 85 70 70 2 2 85 85	% B 15 30 30 98 98 15 15
Cone voltage	positive scan 15 V negative scan 30 V		
Capilary voltage	positive scan 0,8 kV negative scan 0,8 kV		

6.4.3 MS-based dereplication of constituents of *Galium* extracts

Based on the obtained MS spectra of the analysed extracts, dereplication of the potential constituents was performed. The peaks detected in the analysed extracts were compared to data collected from literature (https://scifinder-n.cas.org/) and were putatively annotated.

6.4.4 HPCCC of *G. sylvaticum* EtOAc/BuOH fraction

6.4.4.1 HEMWat system screening

For the HPCCC separation of GalsylHM_EaBu_3, HEMWat solvent systems were used. These solvent systems consist of two immiscible phases and different volume ratios of n-hexane, ethyl acetate, methanol, water and butanol. The polarity decreases in ascending order, meaning the lower the system numbers, the higher polarity.

For the selection of the most suitable systems for separation, HEMWat systems from numbers 17 to 1 were screened (exact composition in table 12). For that purpose, 1 mg of the extract was solved in 1 ml of each solvent system. The two phases, upper layer (UL) and lower layer (LL), were separated and analysed by TLC. After evaluation of the obtained TLC fingerprints, HEMWat systems 9, 7, 5 and 4 were selected for HPCCC.

Table 12: Composition of screened HEMWat systems in parts; apolar (Nr. 17) to polar (Nr.1).

Nr.	n-Hexane	EtOAc	MeOH	n-butanol	ddH_2O
17	1	1	1	0	1
15	2	3	2	0	3
13	2	5	2	0	5
11	1	4	1	0	4
9	1	6	1	0	6
7	1	19	1	0	19
5	0	4	0	1	5
4	0	3	0	2	5
3	0	2	0	3	5
2	0	1	0	4	5
1	0	0	0	5	5

6.4.4.2 Sample and solvent system preparation

In a semi-preparative HPCCC run of GalsylHM_EaBu_3, approx. 400 mg of the extract was dissolved in 5 ml of UL 9 and 5 ml of LL 9 using ultrasonication for 10 min followed by centrifugation for 10 min at 3500 rpm. The LL9 and all ULs used were prepared separately. Table 13 with the composition of each HEMWat system represents the respective volumes of each solvent used.

Table 13: Composition of used HEMWat systems in % (LL = lower layer, UL = upper layer).

Phase	n-Hexane	EtOAc	MeOH	n-butanol	ddH_2O
LL 9	0	8,37	13,01	0	78,62
UL 9	15,08	81,93	1,81	0	1,18
UL 7	5,11	92,05	0,76	0	2,08
UL 5	0	74,33	0	16,48	9,19
UL 4	0	52,23	0	33,78	13,99

6.4.4.3 HPCCC fractionation process

For HPCCC fractionation, the Dynamic Extractions HPCCC device was connected to an Interchim PuriFlash® 4250 and a cooling unit. The PuriFlash® 4250 instrument is equipped with a pump used for delivering the stationary and mobile phase. The combination with the Interchim instrument allows the use of the PuriFlash® 4250 fraction collector and its integrated detectors e.g., ELSD to monitor the chromatographic process.

The HPCCC run was performed in normal phase mode, with the stationary phase being the polar LL. At the beginning, the semi-preparative column was filled with stationary phase (LL) with a flow rate of 10 ml/min and rotational speed of 200 rpm for 30 min, which was increased to 1450 rpm as the mobile phase was pumped into the system at a flow rate of 6 ml/min. After 30-60 min the hydrodynamic equilibrium occurred, which means that no more stationary phase eluted. Next, the sample was injected into the system. 240 mL of the four UL were consecutively added to the system in descending order (UL 9, 7, 5, 4) using. For gradient elution, the following procedure was performed. After the consumption of 180 mL of the respective UL, 60 mL of the next UL was added. When 60 mL of this mixture was consumed, the remaining volume of the next UL was added. At the very end of the fractioning process, elution extrusion was performed for 30 minutes.

A mixture of MeOH/ddH2O (1:1) was pumped through the system, while the rotation level decreased to 200 rpm and the flow rate increased to 10 mL/min. Fractions were collected by the fraction collector (1 tube per minute) of the Interchim device, totalling 174 fractions. All fractions were evaporated to dryness (40°C) using the GeneVac® evaporation device.

Table 14: HPCCC setting for Galsyl_EaBu_3 fractionation.

Instrument	Dynamic Extractions Spectrum HPCCC coupled with Interchim PuriFlash® 4250
Mode	normal phase / semi-preparative
Total column volume [ml]	136
Flow rate [ml/min]	6
Injection volume [ml]	10
Applied amount before centrifugation [mg]	~ 400
Rotation speed [rpm]	1600
Max pressure [bar]	13
Collected volume per tube [ml]	6

6.4.4.4 Pooling of fractions

All obtained HPCCC fractions were reconstituted in MeOH and analysed by TLC. Collected fractions with similar fingerprint were pooled. The collected fractions were combined as shown in Table 15 for a total of 13 HPCCC fractions.

Table 15: Yields and pooling of HPCCC fractions.

Fraction name	Fractions	Yield [mg]
GalsylHM_EaBu_#1	1-3	16,3
GalsylHM_EaBu_#2	4 & 5	11,55
GalsylHM_EaBu_#3	6-9	6,86
GalsylHM_EaBu_#4	10 & 11	4,5
GalsylHM_EaBu_#5	12-15	16,85
GalsylHM_EaBu_#6	16-26	51,64
GalsylHM_EaBu_#7	27-40	13,47
GalsylHM_EaBu_#8	41-59	15,82
GalsylHM_EaBu_#9	60-68	17,07
GalsylHM_EaBu_#10	69-89	11,96
GalsylHM_EaBu_#11	90-120	66,09
GalsylHM_EaBu_#12	121-154	20,74
GalsylHM_EaBu_#13	155-174 + elution extrusion	5,73

6.5 Biological activity experiments

6.5.1 Cell Culture and Biological Assays

All biological experiments were conducted by Mag. Dr. Benjamin Kirchweger and Giulia Verrilli in the laboratory of Prof. R. Fürst at the Department of Pharmacy, Ludwig-Maximilians-Universität München. Human peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte reduction system cones derived from buffy coats (University Hospital of LMU Munich). The study was approved by the institutional ethics committee (24-0133, 28/03/2024). PBMCs were obtained by density gradient centrifugation using lymphocyte separation medium. Leukocytes were seeded in Petri dishes for 2 h (37 °C, 5% CO_2) to allow monocyte adhesion. Non-adherent lymphocytes were removed by washing and centrifugation. Both lymphocytes and monocytes were cultured separately in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO_2 until further use. Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell, Germany. They were maintained on collagen G–coated lab ware in endothelial growth medium (ECGM) supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (2.5 µg/mL).

6.5.2 Proliferation

Human umbilical vein endothelial cells (HUVECs) were seeded in collagen G–coated 96-well plates (1,500 cells/well) and cultured for 24 h in supplemented ECGM. Cells were treated with test samples for 72 h; vehicle controls received 0.1% DMSO. At 0 h and 72 h, cells were fixed, stained with 0.5% crystal violet, and dried. Later the dye was solubilized in sodium citrate and absorbance was measured at 595 nm. Proliferation was calculated by subtracting baseline (0 h) values and normalizing to vehicle control.

6.5.3 Migration

HUVECs (2.5×10^4 cells/well) were grown to confluence in collagen G-coated 96-well plates. A standardized scratch was introduced, and cells were treated with samples in supplemented ECGM. Negative controls received ECGM without FCS (0.1% DMSO), and positive controls received fully supplemented ECGM (0.1% DMSO). Images were recorded at 0 h and 16 h. Scratch closure was quantified using ImageJ and expressed relative to vehicle control.

6.5.4 Leukocyte–Endothelial Adhesion

HUVECs were seeded in collagen G–coated 48-well plates and cultured until confluence. Both HUVECs and primary leukocytes (monocytes or lymphocytes, as indicated) were treated with the respective samples for 24 h. HUVECs were additionally stimulated with TNF (10 ng/mL), while primary monocytes were stimulated with LPS (100 ng/mL).

Primary leukocytes were labelled with 2.5 µM 5-chloromethylfluorescein diacetate and 1×10^5 labelled cells were added per well onto the endothelial monolayer. After 15 min incubation at 37 °C, non-adherent cells were carefully removed by washing with PBS+. Adherent fluorescent leukocytes were quantified by fluorescence measurement (Ex 485 nm/Em 535 nm). For selected experiments, images of the well center were acquired using the green channel of a Tecan Spark Cyto system and analysed with Fiji software. Images were binarized using Otsu thresholding, cell clusters were separated by the watershed algorithm, and cells were quantified using the “Analyse Particles” function. All results were normalized to the positive control (0.1% DMSO with TNF stimulation). Data represent the mean of at least two independents

6.6 Instruments, Solvents, Reagents

6.6.1 Instruments and lab ware

Device	Description
Analytical Balance	Secura A225D-15, Sartorius
Balance	BCE42021-15, Sartorius
Centrifugal evaporator	EZ-2 plus, GeneVac®
Centrifuge	miniSpin, Eppendorf
Centrifuge (big)	Laborfuge 400 Function Line, Heraeus
Eppendorf tubes	1.5 ml Micro tube, SARSTEDT AG & Co. KG
Flash Chromatography	PuriFlash 4250 by Interchim
Grinder	Caso design, electric grinder
Heat gun	Steinel HG 2000 E
HPCCC	Dynamic Extractions Spectrum
HPCCC – Chiller	Accel 500 LC, Thermo Scientific
Microcapillary for TLC	Micropipettes 1/2/3/4/5* µL, BLAUBRAND®intraMARK, BRAND®
Orbital Shaker	GFL®-3005
Pipette	Eppendorf Research (1000 µL, 100 µL, 10 µL)
Pipette Tips	Eppendorf; Sarstedt; StarLab
Rotary Evaporator	R-210 and Heating Bath B-491, Büchi Laboratorium Technologie AG
TLC Spray Cabinet	CAMAG® TLC Spray Cabinet II
Ultrasonic Bath	Transsonic T 460, Elma
UPLC	Waters Aquity UPLC H-Class, modules: SM, QSM, PDA, ELSD, ISM, QDa
UPLC	Acquity UPLC H-Class System by Waters
UV-Lamp	CAMAG®
Vacuum Pump	V-710, Büchi
Vortex	Heidolph REAX 2000

6.6.2 Solvents and Reagents

Solvent/Reagent	Description
Acetic Acid	≥ 98%, p.a., ACS, Carl Roth GmbH®
Butanol	AnalaR Normapur, VWR, 99,9%
DCM	AnalaR, VWR® Reag. Ph. Eur., ACS
ddH_2O	double-destilled water, purified by ion exchanger
Ethyl acetate	AnalaR, VWR® Reag. Ph. Eur., ACS
Formic Acid 99%	≥ 98%, p.a., ACS, Carl Roth GmbH®
MeOH	HiPerSolv Chromanorm ≥ 99.9%, for HPLC
n-hexane	HiPerSolv. Chromanorm, VWR®
Nature substance reagent A (solid)	≥98%, p. a., Roth®
Vanillin	99%, ReagentPlus®, Sigma-Aldrich®

6.6.3 TLC spraying solvents

Vanillin/H2SO4 spraying reagent

Solution 1: vanillin 1% in methanol

Solution 2: sulfuric acid 5% in methanol

NSR/PEG400 spraying reagent

Solution 1: natural substance reagent A 1% in methanol

Solution 2: polyethylene glycol 400 in 96% ethanol

6.6.4 TLC plates

Merck TLC Silica Gel 60 F254 20 x 20 cm TLC Plates

6.6.5 UPLC column

Waters ACQUITY UPLC® BEH C18 1.7 µm; 2,1 x 100mm Column, S/No: 02523502115739

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8 Acknowledgments

First and foremost, I would like to thank **Univ.-Prof. Dr. Judith M. Rollinger**, for sparking my interest in phytochemistry, phytotherapy and pharmacognosy and later giving me the opportunity to write my master´s thesis in her research group at the Division of Pharmacognosy. With her leading, support and excellent expertise every day was an opportunity to learn something new. I am very thankful that I was given the opportunity to be part of this amazing group.

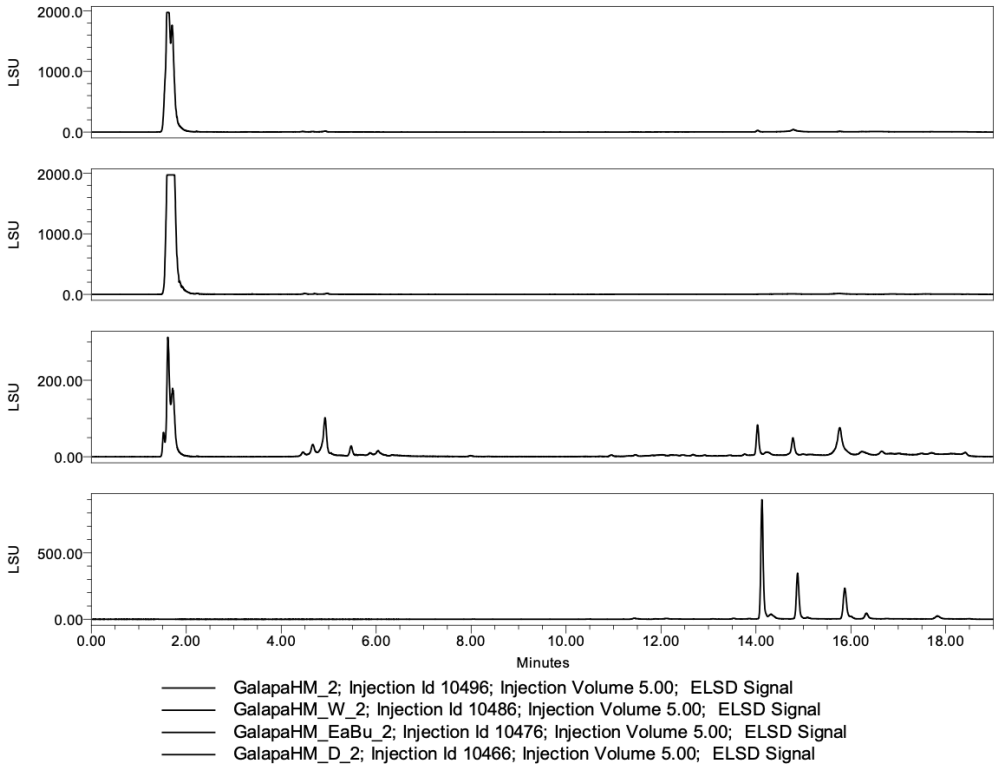
Mag. Dr. Andreas Wasilewicz deserves my deepest gratitude for the most excellent supervision, his helping hand and invaluable support throughout the completion of my thesis. He left no questions unanswered, making sure I fully understand each topic. His love and passion for this field was contagious and made work very enjoyable. I highly appreciate all his trust and patience.

Many thanks to all members of the **Division of Pharmacognosy**, especially the team of the Phytochemistry and Biodiscovery research group for making me always feel welcome and part of the team. Furthermore, I appreciate the great time with my **fellow students**, especially **Philip Unzeitig**, helping and motivating me other. Without him every day at university would have been half the fun.

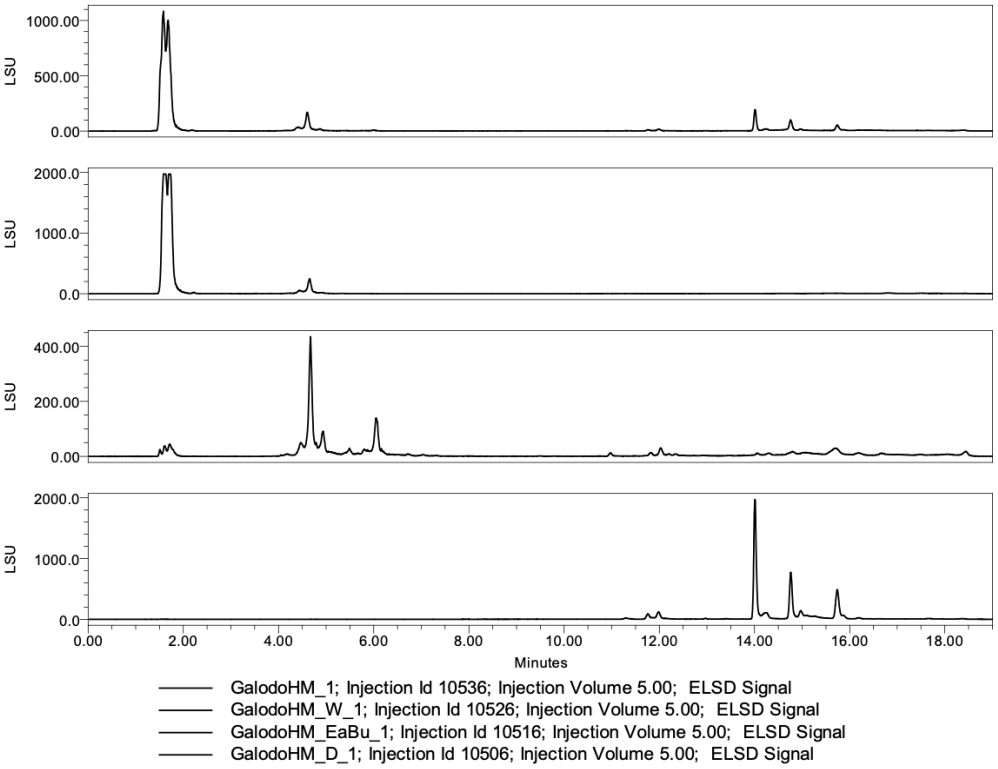
Last but not least, I am eternally grateful my **family** and my **wife Mara**. Thank you for the endless support and encouragement from the very start of my studies.

9 Appendix

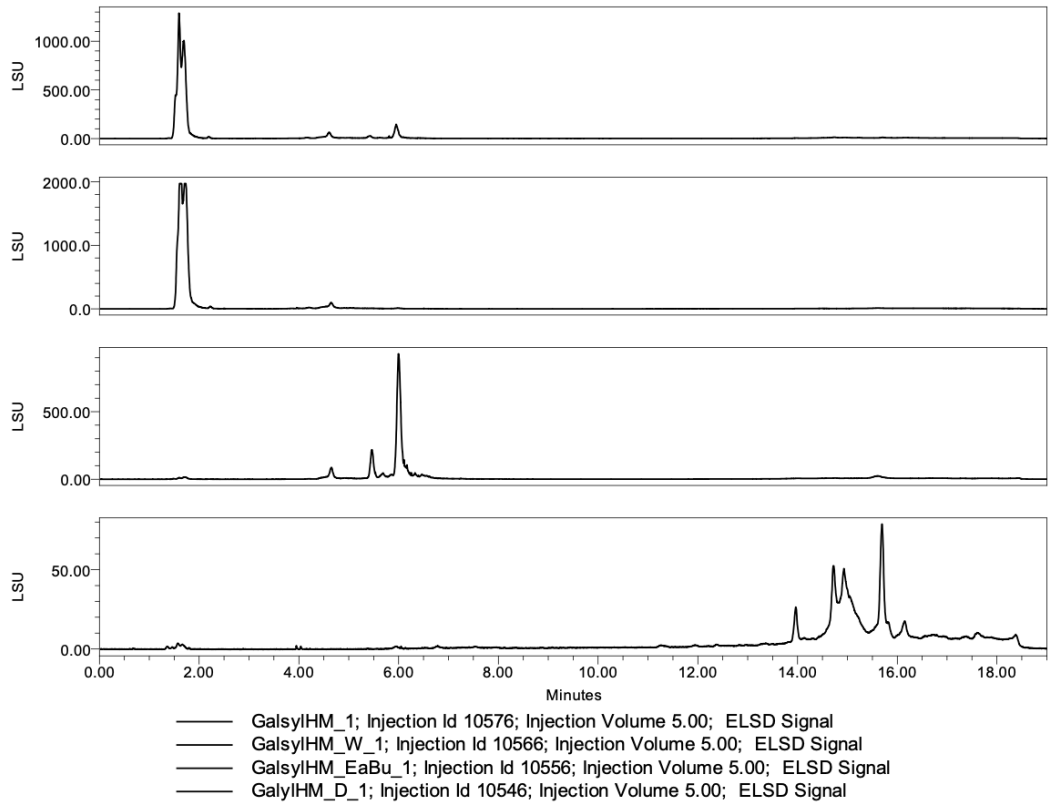
UHPLC-ELSD chromatograms of GalapaHM_2



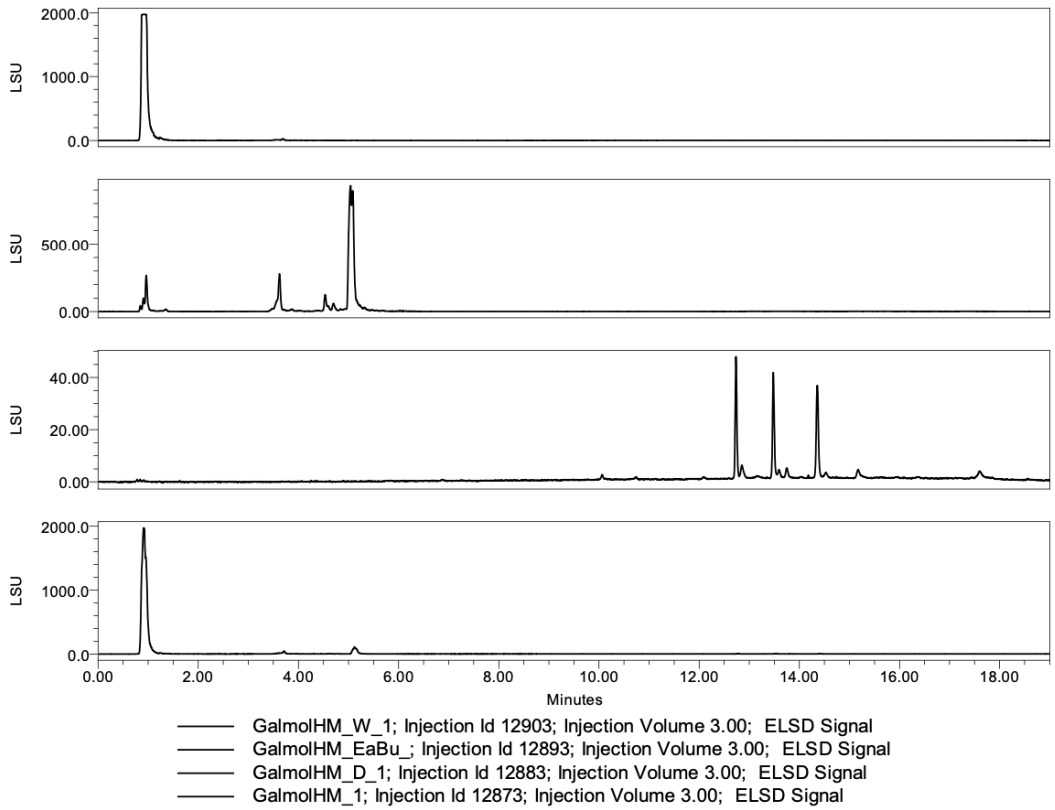
UHPLC-ELSD chromatograms of GalodoHM_1



UHPLC-ELSD chromatograms of GalsylHM_1

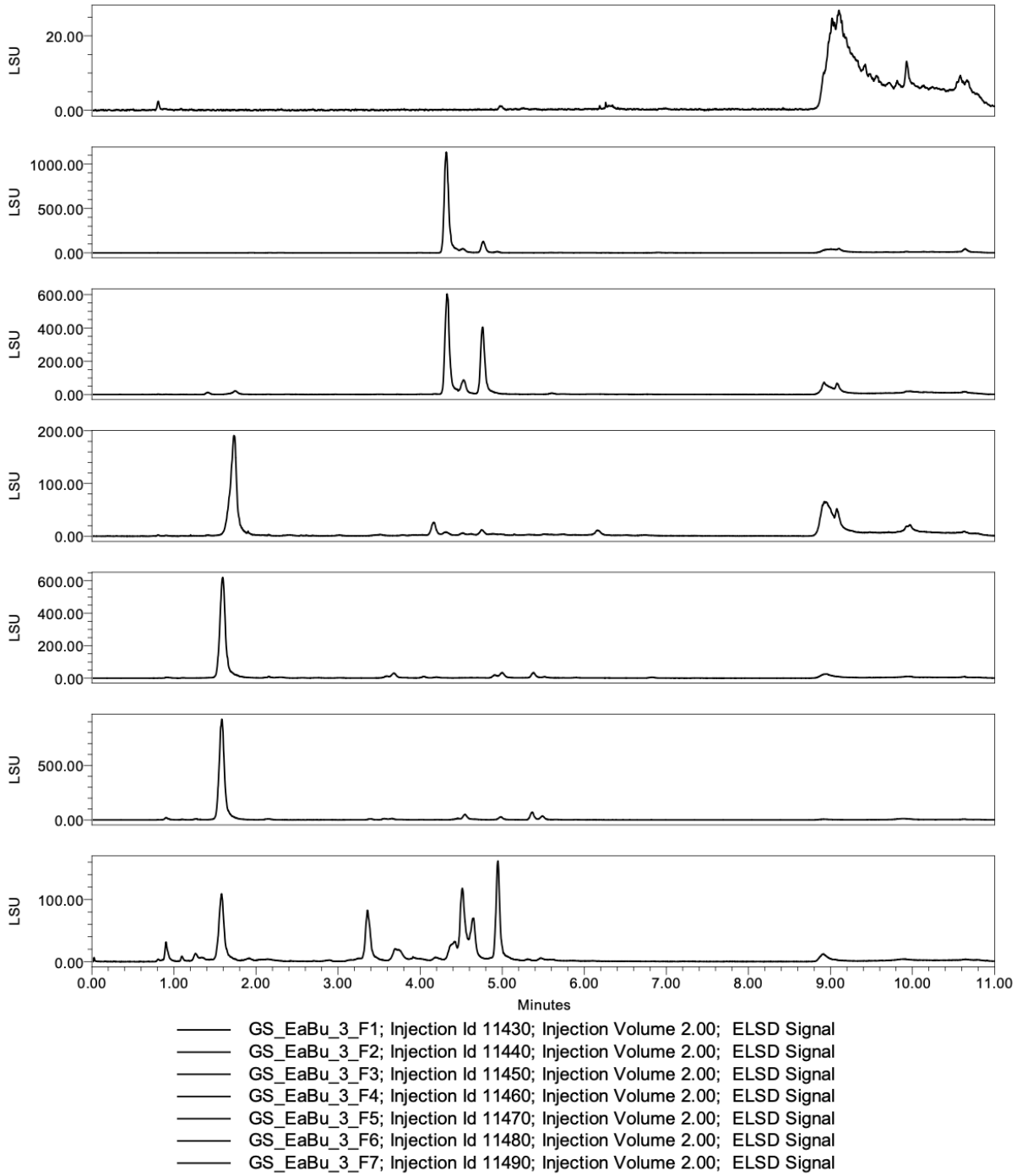


UHPLC-ELSD chromatograms of GalmolHM_1

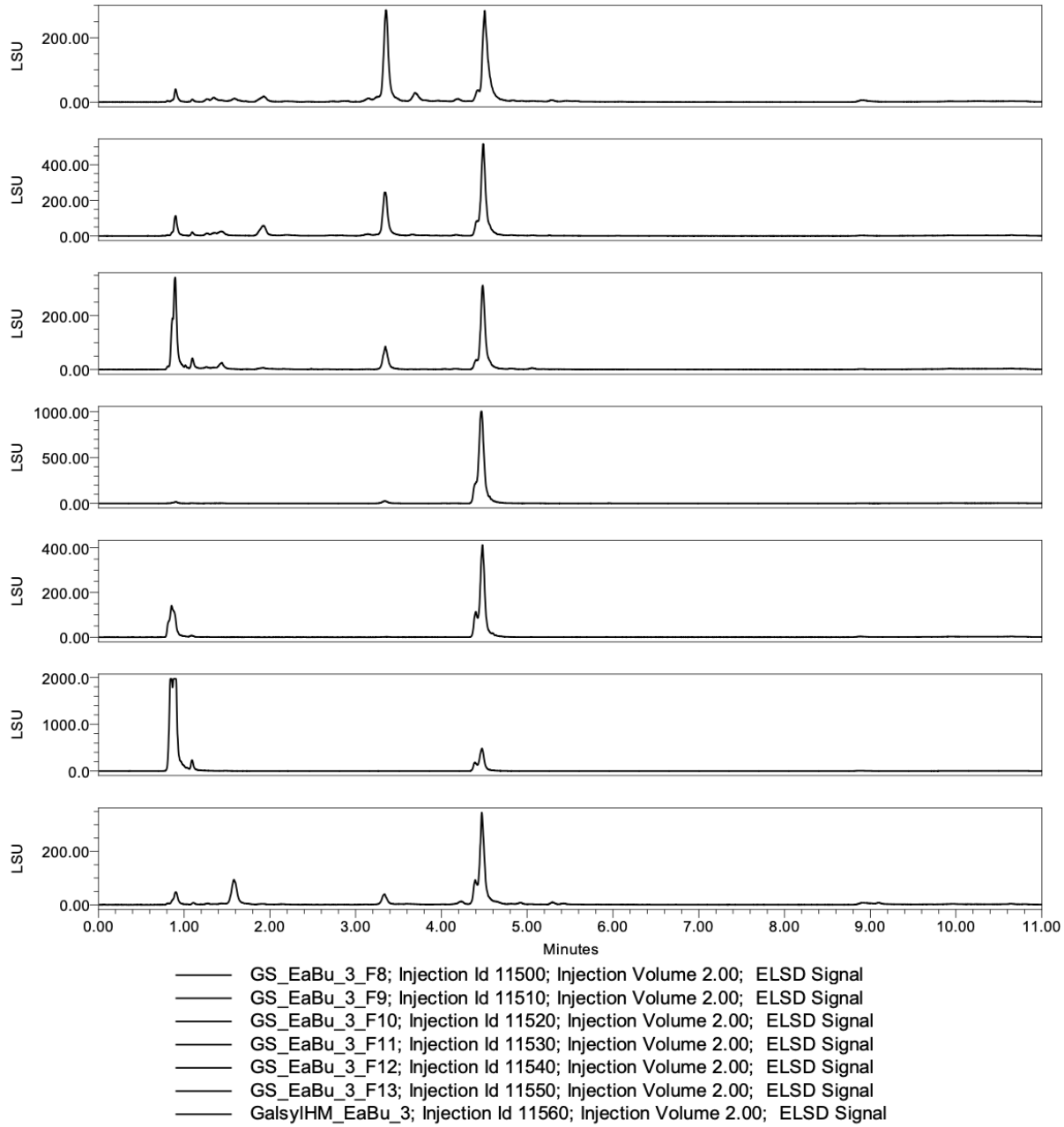


UHPLC-ELSD chromatogram of HPCCC pooled fractions

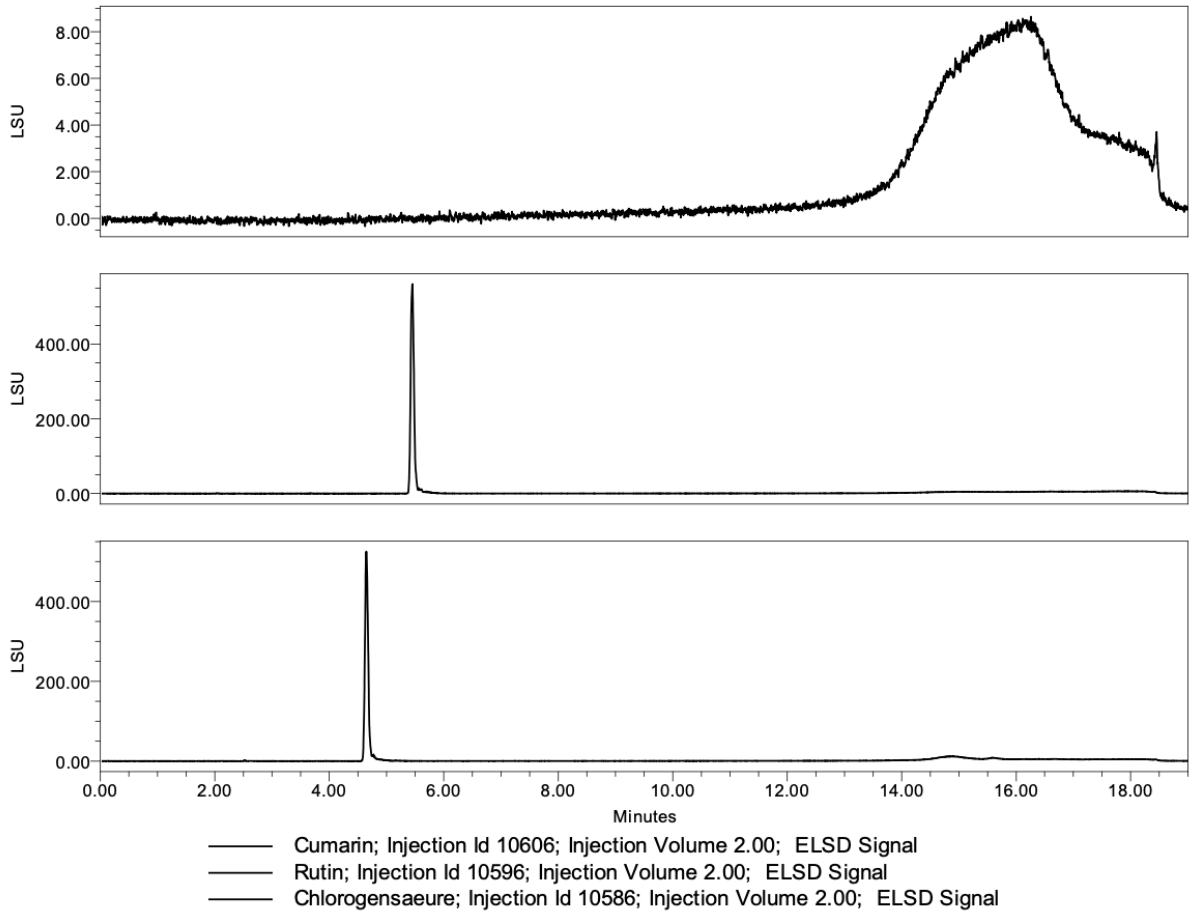
Fractions 1 to 7



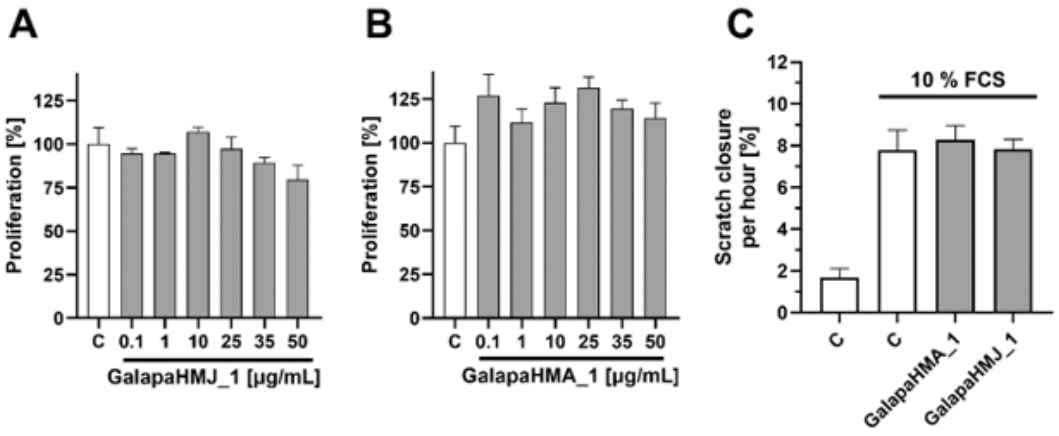
Fractions 8-13 & R



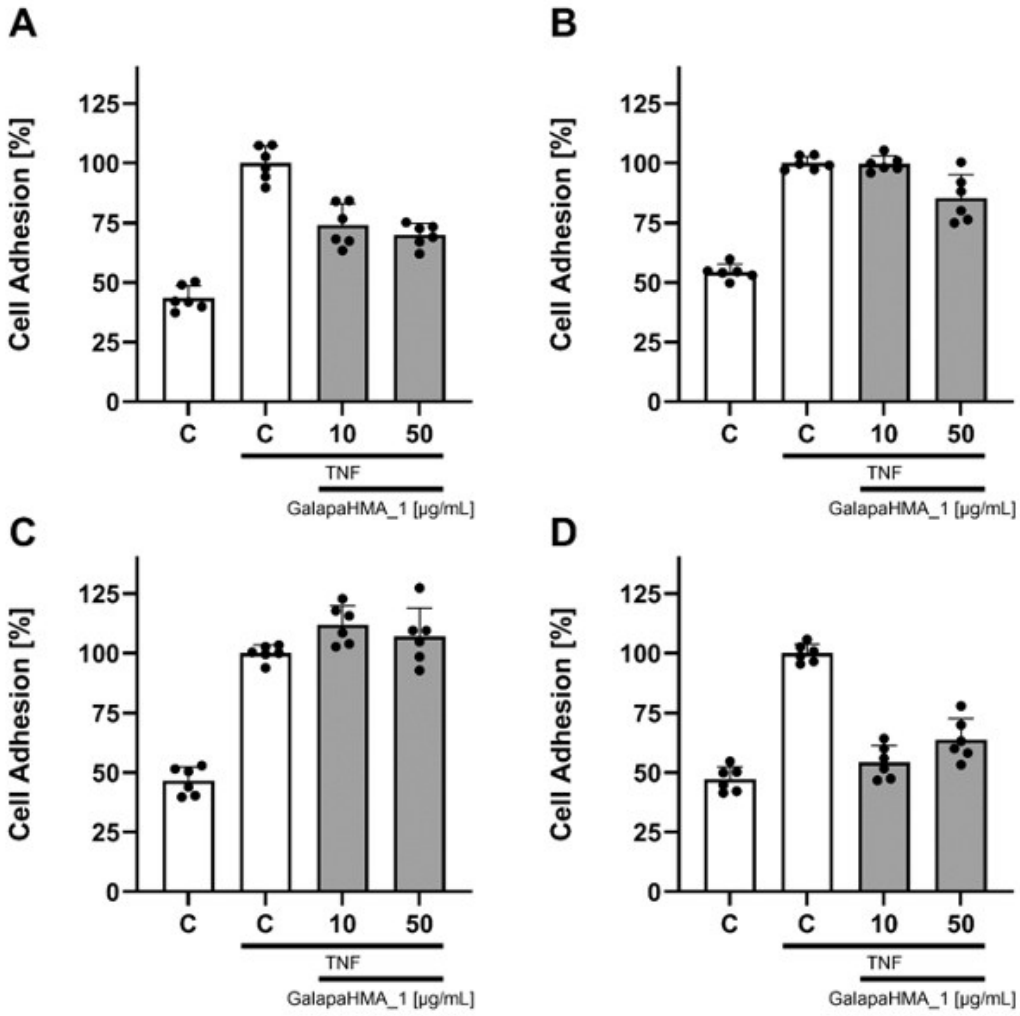
UHPLC-ELSD reference substances



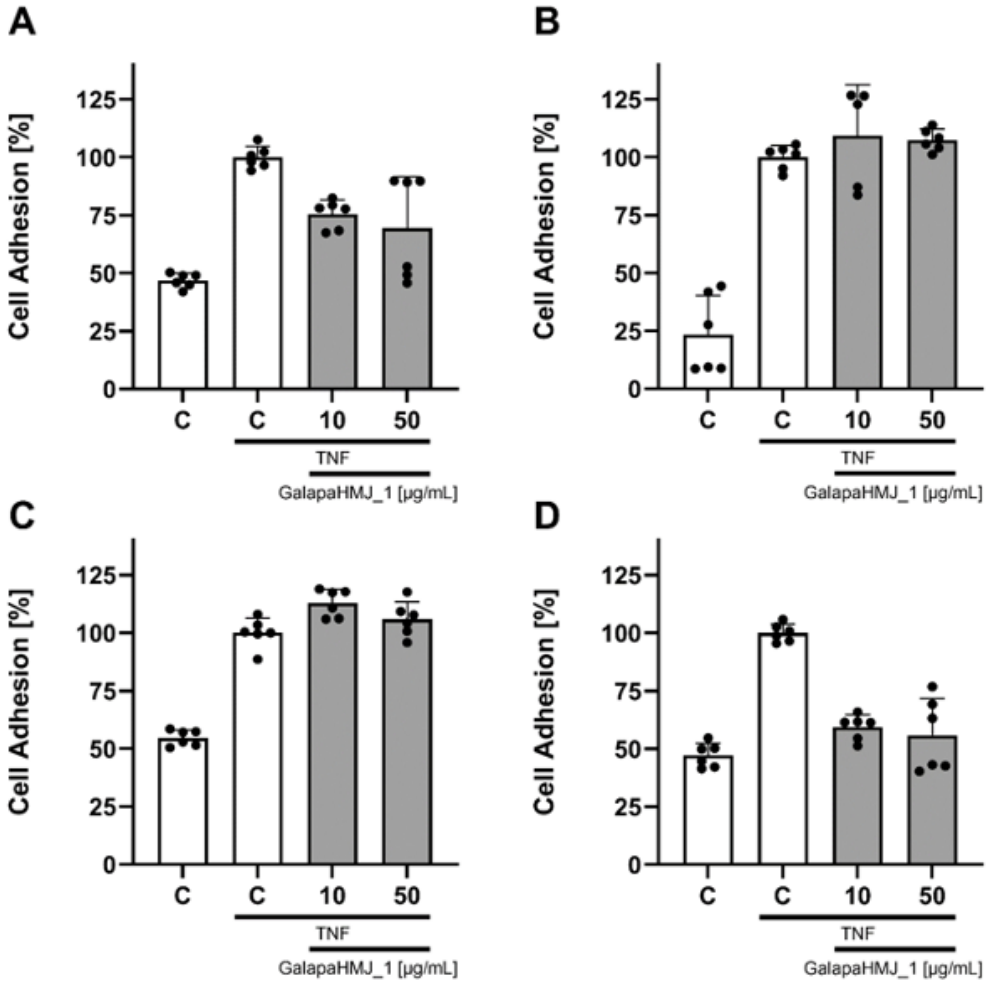
Preliminary bioactivity testing Galapa extracts on endthelial cell function



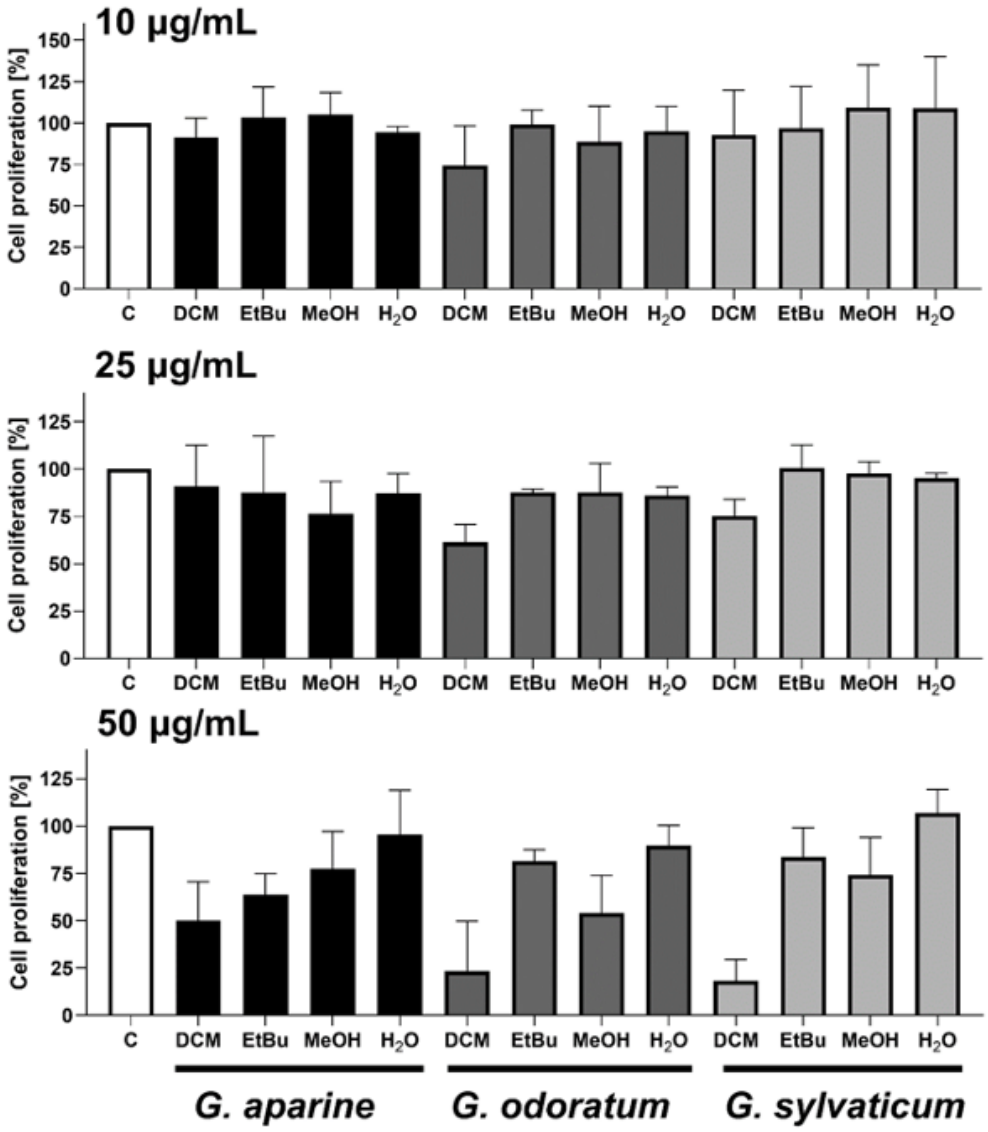
Preliminary bioactivity testing GalapaHMA_1 on HUVEC/PBMC leukocyte cell adhesion



Preliminary bioactivity testing GalapaHMJ_1 on HUVEC/PBMC leukocyte cell adhesion



Preliminary bioactivity testing *Galium* extracts on HUVEC proliferation



Preliminary bioactivity testing *Galium* extracts on HUVEC/PBMC lymphocyte cell adhesion