Phytochemical Investigations into Roots and Rhizomes of *Matricaria recutita* L. and *Gelsemium sempervirens* (L.) J.St.-Hil. with Particular Focus on Secondary Metabolites, Fermentative Changes and Bioactivity Characteristics

Dissertation

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CONTENTS

ABBREVIATIONS

° C	Degrees Celsius
ACN	Acetonitrile
ATP	Adenosine triphosphate
B. subtilis	*Bacillus subtilis*
BPC	Base peak chromatogram
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
BuOH, *n*-BuOH	*n*-Butanol
d	Days
DAD	Diode array detector
DCM	Dichloromethane
DMF	Dimethylformamide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
E. coli	*Escherichia coli*
EI	Electron impact ionization
ESI	Electrospray ionization
EtOAc	Ethyl acetate
EtOH	Ethanol
G. sempervirens, GS	*Gelsemium sempervirens*
GC	Gas chromatography
GHP	German Homeopathic Pharmacopoeia
h	Hours
HPLC, LC	High performance liquid chromatography
J.ST.-HIL.	Jaume Saint-Hilaire, Jean Henri
L.	Linné, Carl von
LAB	Lactic acid bacteria
L. plantarum	*Lactiplantibacillus plantarum*
M. discoidea, MD	*Matricaria discoidea*
M. recutita, MR	*Matricaria recutita*
m/z	Mass-to-charge ratio
MeOH	Methanol
MIC	Minimum inhibitory concentration
min	Minutes

MS, MS/MS, MS^n	Mass spectrometry, tandem/multiple-stage MS
MTBE	Methyl *tert*-butyl ether
MW	Molecular weight
NCBI	National Center for Biotechnology Information
P. aeruginosa	*Pseudomonas aeruginosa*
Ph. Eur.	European Pharmacopoeia
P. pentosaceus	*Pediococcus pentosaceus*
ROS	Reactive oxygen species
rpm	Revolutions per minute
S. aureus	*Staphylococcus aureus*
SD	Standard deviation
TIC	Total ion current chromatogram
t_R	Retention time
TSA	Tryptic soy agar
UV	Ultraviolet
Vis	Visible
λ	Wavelength

LIST OF PUBLICATIONS

The work presented in this thesis consists of three full papers accepted and published in international peer reviewed journals, which are compiled below.

1) L. K. Mailänder, P. Lorenz, H. Bitterling, F. C. Stintzing, R. Daniels, D. R. Kammerer. Phytochemical Characterization of Chamomile (*Matricaria recutita* L.) Roots and Evaluation of Their Antioxidant and Antibacterial Potential. *Molecules* **2022**, *27*, 8508. https://doi.org/10.3390/molecules27238508

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AUTHOR CONTRIBUTION STATEMENT

Lilo K. Mailänder

Conceptualization and work plan, method development, harvest of plant material, preparation of extracts and fermentations, acquisition and interpretation of analytical data, writing of all manuscripts.

Peter Lorenz

Practical advice, discussions, proofreading of all manuscripts.

Hannes Bitterling

Support in GC-MS analyses, discussions and proofreading of manuscript 1.

Khadijeh Nosrati Gazafroudi

Data acquisition, discussions and proofreading of manuscripts 2 and 3.

Marit Greiß

Method development and data acquisition for the antioxidant assays of manuscript 2.

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Rolf Daniels

Discussions, proofreading of all manuscripts.

Dietmar R. Kammerer

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LIST OF POSTER PRESENTATIONS

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Novel insights into the roots of an ancient medicinal plant: *Matricaria recutita* L.

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Mercury) Extracts with Respect to Their Immunostimulating Activity. *Fermentation* **2023**, 9, 190. https://doi.org/10.3390/fermentation9020190

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Fermentierte Extrakte der Gewöhnlichen Rosskastanie (*Aesculus hippocastanum* L.) enthalten Indolessigsäure-N-glykoside und sind Quelle für natürliche Tenside

71st International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research 2023, Dublin, Ireland (July 2-5)

P. Lorenz, L. K. Mailänder, F. C. Stintzing, D. R. Kammerer

Phytochemical Characterization of Lactobacterial-Fermented Horse Chestnut (*Aesculus hippocastanum* L.) Extracts

1 INTRODUCTION

1.1 FUNDAMENTALS OF PHYTOCHEMISTRY

Phytochemistry, the chemistry of plants, refers to the science of natural chemicals in plants, their classification, function and biosynthesis [1]. Many of these substances possess biofunctional properties, which means they can influence physiological processes. So-called medicinal plants, which rely on the occurrence of bioactive components as active principles, have always been used by animals and humans for the treatment of a wide range of ailments [2,3]. The corresponding knowledge has been gained empirically and has been passed on from generation to generation. Consequently, phytomedical applications are still the primary form of healthcare today for up to 4 billion people worldwide [4,5]. And the demand for plant medicinal products as well as research interest in this particular field of therapy is rising, as can be deduced from the market expanding by about 6 % annually [5] and increasing numbers of publications each year [6], respectively. Moreover, phytochemistry also has a marked impact on modern medicine: numerous medicinal plants are processed to produce phytomedicinal preparations, i.e. aqueous, hydro-ethanolic or oily extracts for internal and external application, which are recovered either at low or elevated temperatures [2]. Furthermore, nearly 80 % of all synthetic drugs are derived from natural scaffolds [4], for example the anticancer drug vincristine from *Catharanthus roseus* (L.) G.DON or the anti-influenza drug oseltamivir from *Illicium verum* HOOK.F. [5]. Cinnamic acid is another example for the application of a plant secondary metabolite, which has been used as scaffold for producing eco-friendly and sustainable antiviral substances against the tobacco mosaic virus [7].

Thus, using modern analytical techniques, the ancient experiential knowledge of medicinal plants can nowadays be combined with comprehensive information on their secondary constituents to improve the understanding of active principles and expand the field of applications. Doing so, more than 200,000 secondary constituents have been isolated and structurally identified, among these about 8,000 phenolics, 29,000 terpenoids and 12,000 alkaloids [8,9].

1.1.1 Plant extracts

For standardizing pharmaceutical applications but also for phytochemical analysis and bioactivity testing, secondary metabolites are extracted, enriched or isolated from the plant matrix. Firstly, the fresh or dried plant material is comminuted to disrupt cell walls and facilitate the recovery of biofunctional and soluble constituents from the cellular matrix [2,10]. Solid-liquid extraction, i.e. the extraction of solid plant material with organic solvents, is most widely used for this purpose [10,11]. Conventional solid-liquid extraction techniques include infusion, decoction, percolation and maceration. However, to enhance extraction efficiency or automation, supercritical fluid, Soxhlet, microwave or ultrasound-assisted extractions may also be applied [2,10,12]. In contrast, alkaloids are often extracted by an acid/base approach, thus exploiting their particular physicochemical characteristics [2].

Extraction efficiencies are strongly dependent on the solvent type, temperature, pH value, duration, stirring rate and solvent/substrate ratio [12]. Thus, the solvent or solvent mixture has to be chosen carefully depending on the desired application or compound class, which often requires preliminary testing [2,11]. Acetone or ethanol mixtures with water are frequently used for this purpose [10]. Elevated temperatures may improve the extraction but may also lead to degradation of thermolabile substances [12]. Upon aqueous extraction, enzymatic breakdown may also occur along with oxidation of sensitive components in the presence of UV light and/or oxygen [2].

After the first extraction step, the solid plant matrix residue is removed by filtration [11]. To fractionate and enrich analytes from the obtained filtrate, the organic phase is usually removed by rotary evaporation and the residual aqueous phase subjected to liquid-liquid extraction. Ideally, three to four solvents of increasing polarity are selected and used successively for the selective recovery of different substance classes [10].

However, this approach is quite laborious and solvent-intensive. To improve the sustainability of extraction, solvents may be redistilled and reused. Furthermore, the use of green and sustainable methods is on the rise, i.e. the use of smaller volumes and less toxic solvents [13].

1.1.2 Analytical methods

Different analytical techniques may and should be applied for the comprehensive analysis of a complex sample, because they provide complementary information about different substance classes, i.e. of metabolites characterized by different polarity and volatility [14]. For unambiguous compound assignment, previous chromatographic separation of the complex mixture is inevitable and may be achieved e.g. by gas chromatography (GC) or high performance liquid chromatography (HPLC).

GC is a highly effective chromatographic separation method which is commonly used to analyze substances that can be volatilized without thermal degradation [15]. In addition, derivatization techniques such as silylation can be employed to convert poorly volatile compounds into volatile derivatives for analysis [15]. The sample is vaporized in the injector and forms the mobile phase with an inert carrier gas, typically helium. The mobile phase flows through the capillary column, which is coated with the stationary phase. This thin liquid layer often consists of polysiloxanes, the polarity of which can be adjusted by inserting methyl or phenyl groups [15,16]. Separation occurs due to differences in boiling points of the compounds and their adsorption onto the stationary phase [17]. Additionally, by using a temperature gradient in the column oven, the optimum boiling range for each individual fraction or component can be met, resulting in distinct, ideally baseline-separated peaks for each component of the mixture [15]. A non-specific flame ionization detector is commonly used for quantitative GC measurements, while coupling with a mass spectrometer is preferred for qualitative analyses and constituent profiling [15]. In GC-MS, electron impact ionization (EI) is commonly used. Due to the standardized ionization procedure, results are highly transferable between different institutions, instruments and samples, which facilitates automated substance assignment through the use of databases [18].

HPLC is an efficient, selective and sensitive technique and therefore highly relevant in the analysis of complex mixtures of non-volatile analytes [4,6]. The samples are injected and separated in the liquid phase. Reversed-phase (RP)-HPLC, which involves flowing a polar mobile phase through a non-polar stationary phase in the column, is the most commonly applied procedure [15]. Nowadays, core-shell columns often replace fully porous columns, for example to separate (poly)phenolic compounds from various food matrices [19]. Core-shell columns are filled with particles whose solid spherical cores are surrounded by a thin, porous shell. This reduces the longitudinal

diffusion in the column. As a result, peak sharpening and high efficiency at high operating pressures can be achieved [19,20].

In the case of RP chromatography, the mobile phase is composed of water and water-miscible solvents in various volume ratios, occasionally with the addition of organic acids or buffers [15,21]. The retention time of an analyte in HPLC is directly proportional to its polarity. For detection, diode array detectors are commonly used to obtain UV-VIS spectra between 200-800 nm, while coupling with mass spectrometers provides information about the molecular weight and structure of the analytes [15,22]. Mass spectrometers generate a beam of gaseous ions from the sample and separate them based on their mass-to-charge ratios (*m/z*). Depending on the measurement mode, either positively or negatively charged ions are detected [15,16].

For LC-MS coupling, analytes need to be transferred from the liquid to the gas phase in the mass spectrometer operating under high vacuum conditions. Electrospray ionization (ESI) is often used for this purpose. While the negative ionization mode is suitable for a wide range of natural substances such as (poly)phenolics, the detection of N-containing compounds is particularly sensitive in the positive ionization mode [22]. ESI is a soft ionization technique often producing quasimolecular ions $[M-H]^-$ or $[M+H]^+$. However, matrix or eluent components frequently form adducts with analytes, such as $[M+\text{formic acid-H}]^-$. Furthermore, large analytes may carry multiple charges [22,23].

Precursor ions can be isolated and fragmented in MS/MS or MS^n experiments using an ion trap, for example. From the product ions formed upon collision induced dissociation, conclusions can be drawn about the molecular structure of unknown analytes in addition to their molecular weight [23].

The resulting total ion current chromatograms (TIC) display the sum of all signal intensities as a function of retention time. Individual mass spectra are also recorded and stored in the TIC within a selected mass-to-charge ratio range, providing decisive information on the molecular structure of the respective compound in addition to its retention time [18]. In contrast, base peak chromatograms (BPC) are obtained by recording only the base peak intensity in each of a series of mass spectra and, therefore, are often less complex [24].

1.2 MEDICINAL PLANTS

Of the more than 370,000 plants described, an estimated 20-40,000 are used as medicinal plants [25,26]. These are used, for example, as tea, in phytopharmaceuticals or in cosmetics. In order to standardize the production of phytomedicinal preparations in Europe, the Committee on Herbal Medicinal Products (HMPC) was founded within the European Medicines Agency (EMA) in 2004 [27]. So far, the HMPC published about 170 herbal monographs [28]. In addition, 128 monographs of the German Homeopathic Pharmacopoeia (GHP) describe the manufacturing of homeopathic preparations [29,30]. For the present thesis, two medicinal plants were selected, i.e. *Matricaria recutita* and *Gelsemium sempervirens*, the underground plant parts of which form part of presently marketed medicinal products as aqueous extracts obtained via a fermentation process. Both representatives further have in common that the aforementioned plant parts have not yet been sufficiently investigated and described in the literature. The comparative study of these two plants from different origins and plant families should significantly expand our knowledge of secondary constituents and their fermentative metabolism.

1.2.1 *Matricaria recutita* L.

German chamomile *Matricaria recutita* L., also known as *Chamomilla recutita* or *Matricaria chamomilla*, is an annual plant within the Asteraceae (Compositae) family. It can be discriminated from related species by its narrow leaves and hollow yellow-white flower heads (Figure 1). Originating from Southern and Eastern Europe, chamomile is now widespread throughout Europe, Asia and America as well as in Australia and New Zealand [31,32].

Figure 1. Flowers of *M. recutita* L. and blue essential oil obtained from them by aqueous steam distillation.

Chamomile is one of the most important and best studied medicinal plants [33] with a global flower production of 7,000 – 8,000 tons per year [31,34]. Already in the first century AD, the Greek physician Dioscorides mentioned chamomile in his encyclopedia “De Materia Medica” and prescribed decoctions of chamomile flowers, herbs and roots as tonic, for the treatment of ulcers, spasms and inflammation of the urinary tract and against recurrent fever [35]. Today, infusions of chamomile are among the most frequently consumed single-ingredient herbal teas [36] and are applied for the treatment of gastrointestinal, skin and mucous membrane disorders, among many others [37,38].

Accordingly, extensive studies on the secondary metabolite profile of aerial parts, especially flowers, and their *in vitro* and *in vivo* antioxidant, antimicrobial and pharmacological activities have been and are still being conducted [38,39]. For example, the beneficial effects of chamomile tea are mainly attributed to polyphenols, especially flavones such as apigenin-7-glucoside, and phenolic acids [36,40,41]. Moreover, hydroethanolic and subcritical water extracts of chamomile flowers revealed pronounced antioxidant activities in the DPPH radical scavenging assay, probably also due to their phenolic contents [42,43]. The anti-inflammatory activity of fermented chamomile extracts relied on the modulation of primary human T cells, particularly by apigenin [44].

Chamomile flowers contain 0.2 – 1.9 % essential oil, which is mostly used in the cosmetic industry [31,32]. Its dark blue color (Figure 5) is due to chamazulene, which is formed from the sesquiterpene lactone matricin upon distillation. Other constituents of the essential oil comprise for example sesquiterpenoids such as farnesene, *α*-bisabolol and its oxides and polyacetylenic compounds. It has eczema reducing, spasmolytic, anti-inflammatory and antiseptic activities [32] partly due to the COX-2 inhibiting action of bisabolol [45]. Depending on the ratio of *α*-bisabolol and the bisabolol oxides A and B in the essential flower oil, chamomile cultivars are assigned to different chemotypes [46].

In contrast, studies on the phytochemical composition of chamomile roots are scarce. Early investigations of Reichling et al. showed that oil cells in the root cortex contain 0.04–0.09 % essential oil [47–49]. Contrary to the flower oil, the pale-yellow root oil is devoid of bisabolol and matricin. It contains up to 45 % *β*-farnesene, various other sesquiterpenes and polyacetylenes [49,50]. Phenolic acids such as protocatechuic, syringic, *p*-coumaric and ferulic acids have been found as main constituents in

aqueous chamomile root extracts [51]. Noteworthy, freshly harvested chamomile roots are traditionally applied in the manufacturing of aqueous-fermented extracts produced according to the GHP [29]. These are used for preparing calming and antispasmodic medicinal products and for treating hypersensitive sensory organs and nerves [52]. To complement current scientific knowledge, a comprehensive phytochemical characterization of chamomile root extracts and the fermentative metabolism thereof is presented in chapters 3.1 and 3.2.

1.2.2 *Gelsemium sempervirens* (L.) J.ST.-HIL.

The Gelsemiaceae family consists of only three highly toxic species, the Asian *G. elegans* (GARDNER & CHAMP.) BENTH. and two American species, *G. sempervirens* (L.) J.ST-HIL. and *G. rankinii* SMALL [53]. While *G. rankinii* has only been very scarcely investigated, *G. elegans* and *G. sempervirens* have been studied by different research groups with a particular focus on alkaloids and the corresponding pharmacological activities [53]. Among these species, the focus of the present work was laid on *G. sempervirens* (GS). GS, also known as yellow jessamine, is an evergreen vine with a cylindrical rhizome and wiry roots [54]. It is native to the southern regions of the United States [53], where with its fragrant yellow flowers it is often used as an ornamental plant (Figure 2) [54].

Figure 2. Flowers of *G. sempervirens*.
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Indigenous Americans called GS tincture bebo-sito, glass coffin. It was used in sacrificial rituals and paralyzed the victim while being fully conscious [55]. Despite its toxicity, GS is traditionally applied for the treatment of neuralgia and fever [53]. Low doses of GS were shown to have anxiolytic effects and increase exploratory behavior

in mice [56,57]. In complementary medicine, homeopathic preparations made of GS are prescribed as remedy against neuralgia, migraine and influenza [53,57].

The described pharmacological effects are generally attributed to the more than 120 different monoterpenoid indole alkaloids (chapter 1.3.3), which have been isolated from all plant organs of *G. elegans* and GS. As an example, the antinociceptive effects of the plant are often, but not exclusively, attributed to the alkaloids gelsemine and koumine and rely on the modulation of spinal glycine receptors [58–61]. Glycine and $GABA_A$ receptors belong to the same Cys-loop receptor family [62]. Interestingly, studies of Marileo et al. pointed to the role of $GABA_A$ receptors in gelsemine toxicity, while presynaptic activity might explain the analgesic and anxiolytic effects of the alkaloid [58,63].

Apart from alkaloids, only about ten iridoids and steroids and a few coumarins, phenolic acids and fatty acids have been assigned in GS so far [64]. A detailed phytochemical characterization of further non-alkaloid secondary metabolites was therefore performed and is outlined in chapter 3.3.

1.3 PLANT SECONDARY METABOLITES

For decades, it has been consent that primary constituents, i.e. lipids, proteins and carbohydrates, are of nutritional importance for the plants, while secondary metabolites account for biotic and abiotic plant-environment interactions [10,65]. The latter may also serve as taxonomic markers since they are often species-specific [2]. Thirdly, hormones exert regulatory functions in the metabolism [65]. However, recent research has demonstrated that secondary metabolites may also be integrated into metabolic and regulatory pathways, thus contributing to plant development, and may serve as precursors in the biosynthesis of primary compounds [65]. Despite the differentiation between these compound classes being increasingly blurred, the term ´secondary metabolites´ is used in its traditional meaning in this thesis.

With their biological and pharmacological activities, secondary metabolites are produced either as constitutive metabolites or as response to both biotic and abiotic influences such as infection, temperature, drought stress and many others, i.e. as induced secondary metabolites [12,66]. Accordingly, their biosynthesis is also subject to seasonal fluctuations as well as affected by the global climate change. For example, elevated temperatures and light intensities led to increased alkaloid contents in *Duboisia myoporoides* R.BR. and *Mahonia* ssp., while the phenolic content of *Mentha piperita* L. and *Chrysanthemum morifolium* (RAMAT.) KITAM. decreased upon drought stress and increased upon UV irradiation, respectively [8].

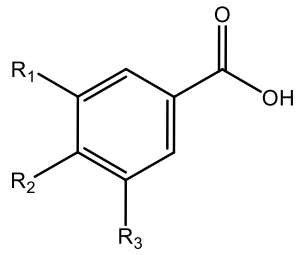
The following chapters provide an overview of the most important substance classes among the plant secondary metabolites discussed in this work.

1.3.1 Phenolic acids and depsides

Phenolic compounds consist of one or more aromatic rings with at least one hydroxyl group [12]. They are among the most diverse and abundant classes of secondary metabolites in all plant organs [11,12,26], where they are mostly formed via the phenylpropanoid/shikimate pathway [67,68]. (Poly)phenolics often occur bound to saccharides, organic acids, alcohols or cell wall polysaccharides [69,70] and fulfill diverse biological roles such as UV protection, coloration and defense against predators [12,71]. Phenolic compounds can be classified into phenolic acids, flavonoids, tannins, stilbenes and lignans [11,72], with the focus of this thesis being on phenolic acids.

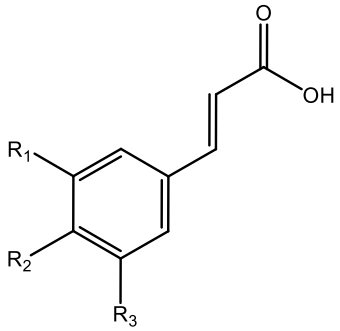
Phenolic acids are simple phenolic compounds but important allelochemicals, occurring ubiquitously in plants and soil [9,73]. Caffeic acid and its esters caffeoylquinic acid, i.e. chlorogenic acid, are among the most widespread representatives of this phenolic subclass [67]. Phenolic acids can be further classified into hydroxybenzoic and hydroxycinnamic acids [11,12]. Some typical compounds are displayed in Figure 3. For both subgroups a plethora of physiological functions has been described. The best-studied activity is their pronounced antioxidant effect due to their ability to scavenge free radicals [11,74]. In addition, antibacterial, antiviral, cytoprotective, blood-pressure lowering and antihypertensive effects were demonstrated for caffeic and chlorogenic acids in rats [75–79], while gallic acid was shown to improve insulin signaling and combat inflammation in obese humans [80].

Hydroxybenzoic acids



	R_1	R_2	R_3
Protocatechuic acid	OH	OH	H
Gallic acid	OH	OH	OH
Vanillic acid	H	OH	OCH_3
Veratric acid	H	OCH_3	OCH_3
Syringic acid	OCH_3	OH	OCH_3

Hydroxycinnamic acids



	R_1	R_2	R_3
p-Coumaric acid	H	OH	H
Caffeic acid	OH	OH	H
Ferulic acid	OCH_3	OH	H
Sinapic acid	OCH_3	OH	OCH_3

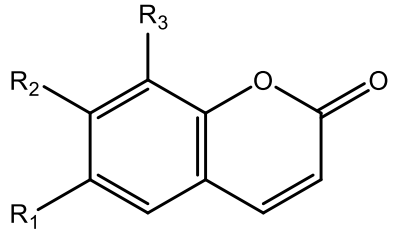
Figure 3. Structures of ubiquitous hydroxybenzoic and hydroxycinnamic acids [11].

Esters of two or more phenolic acids are referred to as depsides. This term was introduced early in the 20th century by Emil Fischer and Karl Freudenberg [81]. Depsides have been reported in more than 500 lichen [82,83] and about 30 fungal [84] species, where they mostly consist of derivatives of orsellinic acid, i.e. 2,4-dihydroxy-6-methylbenzoic acid [81,82]. In contrast, depsides of classical phenolic acids, i.e. without methyl substitution of the aromatic ring, have been found in various higher plants such as aronia [85], rosemary [86], sage [87] and pineapple [88]. For example, rosmarinic acid, the ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, was isolated from rosemary (*Rosmarinus officinalis* L.) in 1958 [89]. A plethora of interesting

bioactivities has been reported for all kinds of depsides. While analgesic, antimicrobial, antimalarial, neuroprotective and wound healing activities have been demonstrated for lichen depsides [83], rosmarinic acid was shown to alleviate inflammatory conditions such as colitis or arthritis due to its antioxidant and anti-inflammatory activity [90].

1.3.2 Coumarins

Coumarins are named after their simplest representative coumarin, *2H*-1-benzopyran-2-one, which was isolated from tonka beans (*Dipteryx odorata* (AUBL.) WILLD.) in 1820 (Figure 4) [91]. Since then, about 1300 different representatives of this phenolic subclass have been identified in plants, fungi and bacteria, among these furano-, pyrano-, phenyl- and biscoumarins [92]. Within the plant kingdom, coumarins are widely distributed, especially in the Apiaceae, Asteraceae and Rutaceae families [91,92]. They mainly serve as chemical defense against predators and mostly occur as glycosides, from which the aglycon is released upon injury or wilting due to enzymatic activities [71,93]. Moreover, natural furocoumarins were shown to protect essential agrumen oils from oxidation [94]. With their aromatic flavor, coumarins are used in the food and cosmetic industries [93], but pharmaceutical applications have also been reported [95]. The antioxidant and anti-inflammatory activity of coumarins is attributed to their ability to scavenge free radicals, inhibit lipoxygenase and cyclooxygenase activities and enhance cytokines such as TNF-α and IL-6 [92,96]. Antibacterial activity has also been shown, firstly by suppression of quorum sensing, which leads to the inhibition of biofilm formation, and secondly by inhibition of DNA gyrase and thus disturbance of transcription, replication and repair of bacterial DNA [26,97].



	R_1	R_2	R_3
Coumarin	H	H	H
Aesculetin	OH	OH	H
Aesculin	O-Glc	OH	H
Scopoletin	OCH_3	OH	H
Scopolin	OCH_3	O-Glc	H
Fraxetin	OCH_3	OH	OH
Fraxin	OCH_3	OH	O-Glc

Figure 4. Structures of representative simple coumarins and their glucosides (O-Glc) [98].

1.3.3 Alkaloids

Alkaloids are a heterogenous group of nitrogen-containing organic compounds [26,68]. Some examples with common core structures are displayed in Figure 5. They may serve as chemotaxonomic markers since they are often characteristic of individual plant families [99]. For example, the Solanaceae (nightshades) contain various steroid alkaloids [100], while the Gelsemiaceae are rich in indole alkaloids [101].

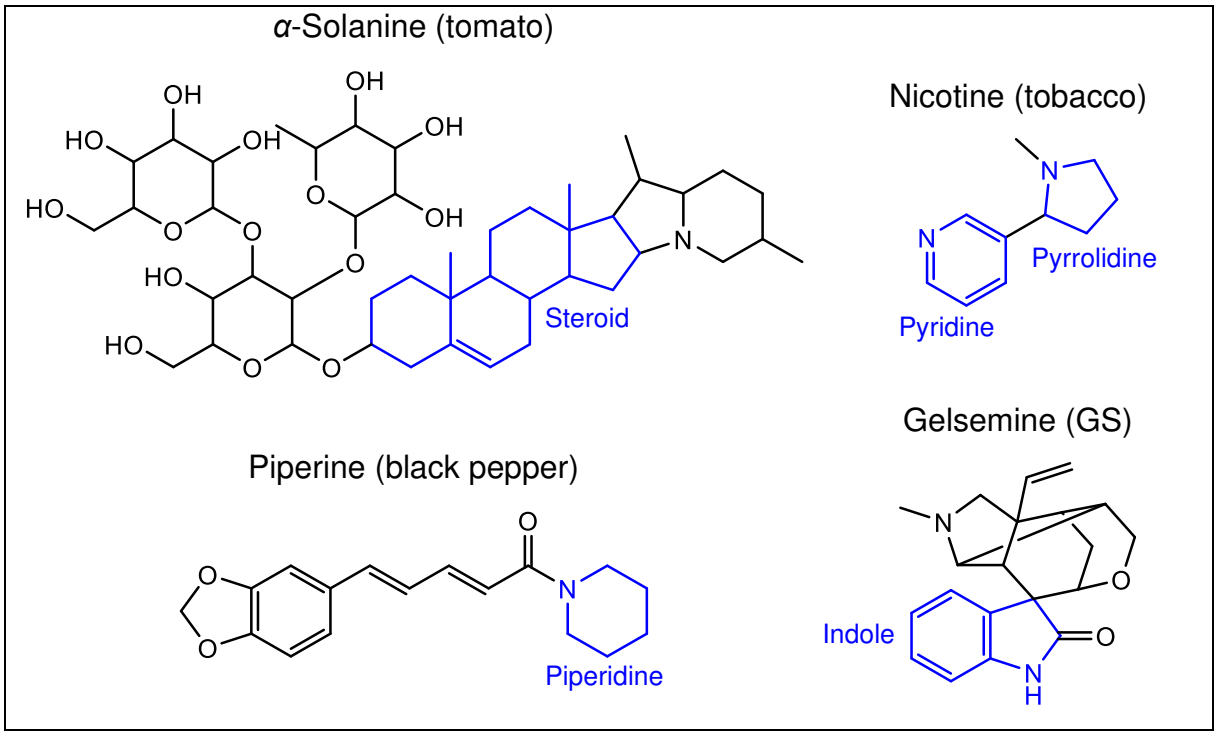


Figure 5. Examples of some natural alkaloids. Core structures are displayed in blue [100,102].

As displayed in Figure 6, alkaloids are defense-related metabolites, which can be accumulated in case of pathogen or herbivorous attacks [68,99]. In addition, their allelopathic activity may inhibit the growth of other plants [99].

Approx. 50 % of all plant-derived pharmaceutical substances are alkaloids, which may be attributed to their pronounced pharmacological effects [103]. Among others, alkaloids may inhibit enzymes such as acetylcholinesterase, or act as opioid, acetylcholine, glycine or adenosine receptor antagonists [58,99]. The manifold resulting pharmacological effects thus comprise strong analgesic, narcotic, spasmolytic and stimulant activities [26,59,99].

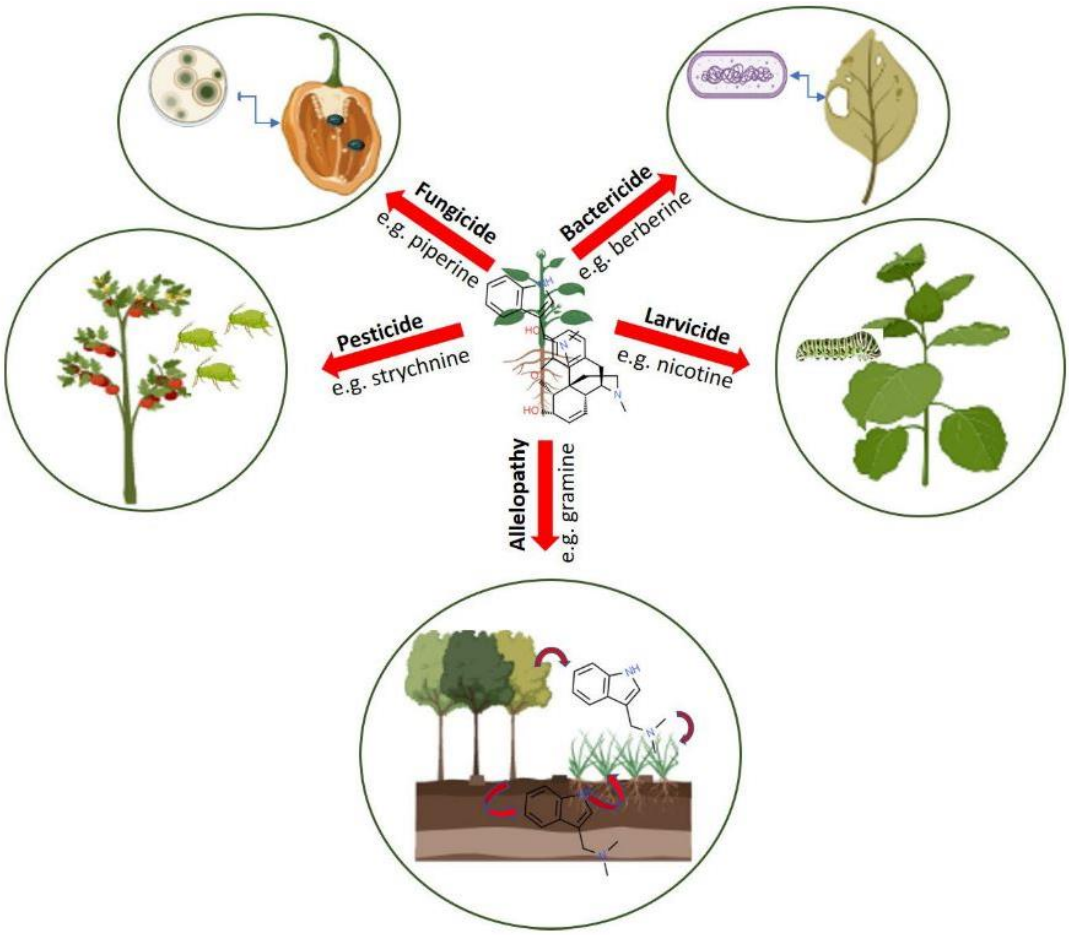


Figure 6. Representative alkaloids from different plant families exemplifying the defense strategy of plants. From Bhambhani et al. [99].

1.4 A LOOK FROM BELOW: ROOTS AND RHIZOMES

While the aerial parts of many medicinal plants, such as chamomile, marigold and kidney vetch, have been extensively studied, little attention is mostly paid to their underground plant parts, even though these may also contain interesting biofunctional secondary metabolites [104–106]. The main functions of roots are the attachment of the plant to the soil and the absorption of water and nutrients. In addition, secondary metabolites and plant hormones are often biosynthesized in the roots. In contrast, rhizomes are an underground part of the shoot axis and may be discriminated from roots, e.g. by their peripheral vascular bundles. Rhizomes facilitate hibernation and therefore often serve as storage organs [107]. However, the soil that surrounds roots and rhizomes is not only a mineral nutrient source, but a complex ecosystem of the so-called rhizosphere [106]. Figure 7 illustrates potential interactions between underground plant parts, soil and microorganisms. Plants shape and interact with their soil microbiome by root exudates, i.e. a variety of signaling molecules [108,109]. For example, protocatechuic and *p*-coumaric acids from *Vigna mungo* (L.) HEPPER roots were found to stimulate auxin production and regulate the morphogenesis of their symbiont *Rhizobium* [9]. The secretion of coumarins plays a role in iron uptake [91,110]. Furthermore, roots are often colonized with symbiotic mycorrhizal fungi which may increase plant growth and nitrogen availability [34]. Roots produce secondary metabolites for the defense against pathogenic microorganisms or herbivorous insects [106]. It is therefore not surprising, that a plethora of substances with potent pharmaceutical activities cannot only be extracted from flowers, but also from roots and rhizomes of various medicinal plants [104,111].

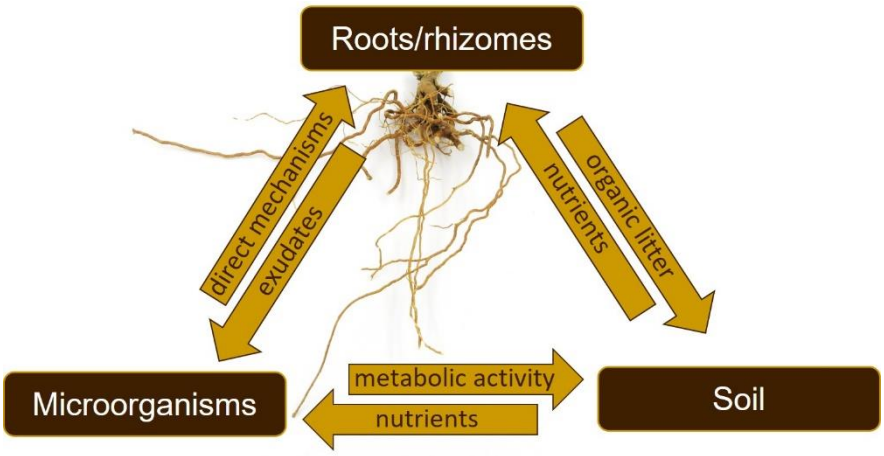


Figure 7. Interactions between roots/rhizomes, microorganisms and soil. Adapted from Jacoby et al. [108].

1.5 FERMENTATION

Fermentation is one of the oldest methods for preserving food and other natural products. Still today, fermentation forms an integral part of our daily diet, as can be deduced from the importance of foods such as vinegar, yogurt and cheese or sourdough bread, just to name a few, whose production mainly relies on fermentative processes [112,113]. However, the current focus of research into fermentation processes is not only on preservation, but mainly on improving the organoleptic and biofunctional properties of the respective substrate [114–116]. The impact of fermentation on the secondary metabolite and bioactivity profiles of many popular foods has therefore been well studied [113–115]. More recently, research interest in fermented edible herbs is rising [117]. Among the main products formed upon homo- or heterofermentative lactic acid fermentation are lactic, acetic and other organic acids as well as ethanol, which are produced from carbohydrate metabolism [114,118]. However, this only describes the conversion of major metabolites. Even more interestingly, the release of further plant constituents, such as phenolic compounds, upon cell wall polysaccharide hydrolysis in the course of fermentation may be enhanced, thus potentially improving their bioavailability. Further, enzymatic reduction, hydrolysis, decarboxylation, or (de)methylation of free polyphenols may then enhance their antioxidant and anti-inflammatory effects [69,119–121]. The bioavailability of minerals and vitamins may also be improved due to their release from bound forms or their microbial metabolism [69,114,122]. Toxic or anti-nutritional substances may be degraded upon fermentation, as has been demonstrated for oxalic acid in purslane juice, the content of which decreased by about 30 % [123]. In addition, fermented foods may be a source of probiotic bacteria, especially of Lactobacilli [119].

In contrast to the broad application of fermentation in the food industry, its use in the pharmaceutical industry has often been neglected and has so far been inadequately investigated [52].

1.5.1 Fermentation in the manufacturing of pharmaceutical preparations

In phytotherapy as well as homeopathy, herbal remedies are mainly produced as hydroethanolic extracts. However, according to the German Homeopathic Pharmacopoeia (GHP), pharmaceutical preparations from medicinal plants can also be produced based on fermentation processes [29]. The corresponding procedure was developed by Rudolf Hauschka and Ita Wegmann in the 1930ies, who aimed at preserving the healing power of medicinal plants in aqueous extracts, at the same time maximizing extract stability without the addition of preserving agents such as ethanol [52].

To produce so-called fermented mother tinctures, the fresh or dried plant material is comminuted and mixed with water and, depending on the production protocol of the GHP, with honey, lactose or whey [29]. The mixture is then subjected to a rhythmic process consisting of alternating warm and cold, calm and agitated and light and dark phases. The phyllosphere of the raw material consists of a plethora of different microorganisms initially contributing to the fermentation process. However, the controlled process conditions, especially the combination of warm and cold periods, then favor the growth of desired lactic acid bacteria (LAB) [124]. After a fermentation period of seven days, the suspension is filtered and the filtrate kept in the dark for maturation for a minimum of six months [125]. Finally, the mother tincture is sterile filtered prior to is use in the manufacturing of homeopathic and anthroposophic medicinal products such as ointments, gels or globules [29].

1.5.2 Metabolism of secondary constituents

The fermentation of both, foods and medicinal plants, may result in significant alterations to their primary and secondary metabolite profiles [126–129]. Initially, crushing of the plant material results in decompartmentalization, which permits the leakage of secondary metabolites, for example from the vacuole, into the aqueous medium. In addition, genuine plant enzymes and their substrate, which are separated in intact plant compartments, may get in contact. Microbial enzymes may further contribute to plant matrix degradation, which facilitates extraction and enhances the release of plant constituents into the solution [130,131].

Besides lactic and acetic acids, further organic aids, especially hydroxy-carboxylic acids, may also be formed: from short-chain acids such as 2-hydroxybutyric acid to

long-chain hydroxy fatty acids [132,133]. Looking at secondary metabolites, esters and glycosides may be hydrolyzed in the presence of unspecific microbial esterases [125]. As an example, gallotannins in witch hazel (*Hamamelis virginiana* L.) were gradually degraded to glucogallin, monosaccharides and gallic acid [126]. Mercurialis acid and phaseolic acid, two caffeic acid derivatives occurring in dog's mercury (*Mercurialis perennis* L.), were partly cleaved upon fermentation. It is noteworthy that the reaction rate was high at the beginning of fermentation and the reaction slowed down during storage, without entire degradation of the aforementioned substances [132]. The depside rosmarinic acid was also found to be easily hydrolyzed to yield the corresponding phenolic acids [86]. In sea onion (*Drimia maritima* L. STEARN), bufadienolides, exhibiting a steroidal core structure, were shown to be deglycosylated prior to dehydroxylation and deacetylation of the aglycones to less complex compounds [128]. Accordingly, the sugar moiety of flavonoid glycosides was released prior to further metabolization of the aglycones e.g. to phloroglucinol or hydroxybenzoic acids. This has been demonstrated upon fermentation of various medicinal plants such as deadly nightshade (*Atropa belladonna* L.) [130,134] or birch (*Betula pendula* ROTH) [135]. Microbial hydrolysis of glycosides and acetyl derivatives has also been observed in extracts of Christmas rose (*Helleborus niger* L.), although the steroidal aglycones remained unchanged thereafter [136]. Hermidine, the predominant alkaloid of *Mercurialis perennis*, is sensitive to oxidation and quickly reacts to cyanohermidine and hermidine quinones. Interestingly, upon fermentation of the corresponding extracts, the latter component was metabolized to ethylhermidine [132]. In contrast, the alkaloid atropine from *Atropa belladonna* was found to be stable against fermentative modifications [130].

To conclude, while glycosides and esters are easily hydrolyzed upon fermentation, the metabolism and reactivity of aglycons and further compounds strongly depends on their chemical structure. Fermentation may, thus, influence the antioxidant, antibacterial and other biological activities of plant extracts due to the formation or degradation of physiologically active metabolites such as hydroxybenzoic acids [123,126,129].

1.5.3 *Lactiplantibacillus plantarum*

Spontaneous or wild fermentations are initiated by the natural microbiome of the respective substrate. This may occasionally lead to faulty fermentations and the growth of undesirable, potentially pathogenic microorganisms [137,138]. To prevent this, the substrate is often inoculated with starter cultures in industrial fermentations, which then dominate the process and make it standardized, controllable and safe [138]. Nevertheless, also spontaneous fermentations may be regarded as safe if process conditions are strictly adhered to, which has led to the standards laid down in the GHP [29].

To eliminate the impact of potential variation of the natural plant microbiome, all fermentation batches investigated in the present work were incolulated with the lactic acid bacterium (LAB) *Lactiplantibacillus plantarum. L. plantarum* is one of the most important starter cultures used in the food industry. It occurs ubiquitously on plants, such as raw vegetables, as well as in the human gastrointestinal tract [70,124,139,140]. This species is regarded as safe by the US Food and Drug Administration (FDA) as well as by the European Food Safety Authority (EFSA). Its use is favored by its tolerance to low pH values and high salt concentrations [141]. Health benefits of foods fermented with *L. plantarum* include probiotic effects, suppression of human colon cancer cell growth and increased anti-inflammatory activities [140,142]. In addition, *L. plantarum* is able to produce bacteriocins against foodborne or spoilage bacteria, thus prolonging the shelf life of fermented goods [140,143] and to metabolize phenolic compounds such as hydroxybenzoic and hydroxycinnamic acids by various esterases and decarboxylases [69].

2 OBJECTIVES

Plants and preparations thereof are traditionally used in the treatment of various ailments and diseases, for example to promote wound healing or against gastrointestinal disorders or pain [144]. However, their use is often based on empirical studies. Phytochemical investigations are therefore crucial to link their traditional use with the pharmacological effects of their secondary constituents and for a toxicological risk assessment [144–146].

In many cases, this traditional use is limited to distinct plant organs. E.g., while the aerial parts or flowers of many medicinal plants have been well studied, little interest is often paid to their roots. Yet these are crucial for anchoring plants in the soil, as well as for absorbing nutrients and defense against microorganisms or predators.

Two traditional medicinal plants with considerable pharmaceutical potential, whose roots and rhizomes are used in the manufacturing of aqueous-fermented mother tinctures according to the German Homeopathic Pharmacopoeia, are *Matricaria recutita* L. from the Asteraceae family and *Gelsemium sempervirens* J.ST.-HIL. from the Gelsemiaceae family. The underground plant organs of both plants have been inadequately investigated to date.

In order to shed light on these subjects, a comprehensive phytochemical characterization of different solvent extracts should be performed using GC-MS and HPLC-DAD-MS^n. Furthermore, the metabolism of secondary constituents should be monitored in the course of lactic acid fermentation. In addition, these studies were to be extended by the assessment of biofunctional properties such as antioxidant, antibacterial and anti-inflammatory activities using various *in vitro* approaches.

Consequently, the aim of the present thesis was to significantly increase our knowledge of secondary constituents in *M. recutita* and *G. sempervirens* and demonstrate the pharmaceutical potential of their underground plant organs. In the case of chamomile in particular, this may also contribute to a more sustainable and economical cultivation.

3 PUBLICATIONS

3.1 PHYTOCHEMICAL CHARACTERIZATION OF CHAMOMILE (*MATRICARIA RECUTITA* L.) ROOTS AND EVALUATION OF THEIR ANTIOXIDANT AND ANTIBACTERIAL POTENTIAL

Mailänder, L. K.; Lorenz, P.; Bitterling, H.; Stintzing, F. C.; Daniels, R.; Kammerer, D. R. Phytochemical Characterization of Chamomile (*Matricaria recutita* L.) Roots and Evaluation of Their Antioxidant and Antibacterial Potential. *Molecules* **2022**, 27, 8508. https://doi.org/10.3390/molecules27238508

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Article

Phytochemical Characterization of Chamomile (*Matricaria recutita* L.) Roots and Evaluation of Their Antioxidant and Antibacterial Potential

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Abstract: *Matricaria recutita* L., German chamomile, is one of the most widely used medicinal plants, whose efficacy has been proven in numerous studies. However, its roots have attracted only little interest so far, since mainly above-ground plant parts are used for medicinal purposes. To broaden the knowledge of chamomile roots, a profound phytochemical characterization was performed along with a bioactivity screening of corresponding root extracts. While volatile constituents such as chamomillol and polyynes were detected using GC-MS, HPLC-MS^n analyses revealed the occurrence of four coumarin glycosides, more than ten phenolic acid esters and five glyceroglycolipids. Furthermore, the antioxidant activity of the extracts was evaluated. Polar extracts revealed IC_{50} values ranging from 13 to 57 µg/mL in the DPPH radical scavenging assay, which is in the same range as reported for chamomile flower extracts. In addition, superoxide radical scavenging potential and mild antibacterial effects against *S. aureus* und *B. subtilis* were demonstrated. Moreover, to assess interspecies variation in chamomile roots, extracts of *M. recutita* were compared to those of *M. discoidea* DC. Interestingly, the latter revealed stronger antioxidant activity. The presented results aim at the valorization of chamomile roots, previously discarded as by-product of chamomile flower production, as a sustainable source of bioactive phytochemicals.

Keywords: *Matricaria chamomilla* L.; *Matricaria discoidea* DC.; phytoextract; HPLC-MS; GC-MS; bioactive constituents; biological activity

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1. Introduction

Matricaria recutita L., also known as German chamomile, is an annual plant belonging to the *Asteraceae* (*Compositae*) family. It has yellow-white flowers, bi- to tripinnate leaves, and can be distinguished from related species by its hollow flower heads. Originating from Southern and Eastern Europe, chamomile is now widespread from Europe to India, throughout America as well as in Australia and New Zealand [1,2]. Chamomile is among the most important medicinal plants [3] with a production quantity of 7000–8000 tons per year [2]. For this reason, the secondary metabolite profile of aerial parts, especially flowers, together with their antioxidant, antimicrobial and pharmacological activities have been extensively studied in vitro and in vivo and remain a current research topic [4,5]. Infusions of chamomile are among the most consumed single-ingredient herbal teas [6] and, according to the European Medicines Agency monograph, are used for the treatment of gastrointestinal, mouth, throat, and skin disorders, minor wounds, or colds [7]. The beneficial effects are mainly attributed to the presence of phenolic compounds, such as apigenin-7-glucoside or hydroxycinnamic acid derivatives [8]. Moreover, alcoholic chamomile extracts

have been proven to show cardioprotective, neuroprotective, antispasmodic and antitumor effects [5]. The dark blue essential flower oil contains chamazulene, which is derived from the sesquiterpene lactone matricin during distillation. Furthermore, sesquiterpenoids such as farnesene, α-bisabolol and its oxides and acetylene derivatives such as polyynes have been detected in the essential oil. It has spasmolytic, anti-inflammatory and antiseptic activities and is often applied for cosmetic purposes [1]. Depending on the ratios of α-bisabolol and the bisabolol oxides A and B in the essential flower oil, chamomile cultivars are assigned to different chemotypes [9].

Besides *M. recutita*, other *Matricaria* species are occasionally used in folk medicine. For instance, the flowers of *M. discoidea* (pineapple weed) have a strong chamomile odor, but lack the white petals. The aerial parts of this species contain about 10% polyphenols, among others hydroxycinnamic acid derivatives, and the coumarins herniarin and umbelliferone [10]. β-Farnesene, geranyl-isovalerate and the (Z)-spiroether are the main components of *M. discoidea* essential oil [11]. Cantrell et al. demonstrated its strong insect-repellent activity [12]. *M. aurea* (golden chamomile) is another species used for medicinal purposes, the extracts of which exhibit antioxidant activity, inhibit the growth of *Bacillus subtilis* and *Staphylococcus aureus,* and even show antiproliferative activities on cancer cells [13]. Last but not least, *M. pubescens* (hairy chamomile), which is used in traditional Algerian medicine, contains similar flavonoids as *M. recutita*. It exhibits a protective effect against mild toxic doses of UV-A light on 3T3 fibroblasts [14].

In the 1st century AD, Dioscorides recommended not only decoctions of chamomile flowers, but also of the herb and roots as tonic and for treating urinary tract disorders, i.e., inflammation, spasms, ulcers. Topical applications included the treatment of wounds and burns. Furthermore, Dioscorides prescribed chamomile suppositories against recurrent fever [15,16]. Nowadays, aqueous fermented extracts prepared from chamomile roots are still used in complementary medicine. Indications are similar to those of flower preparations, i.e., the treatment of cramps, gastrointestinal and biliary problems, flatulence, menstrual cramps, teething problems, and sleep disorders of young children [17].

Due to their limited use, only few studies on chamomile roots have been reported. Early investigations showed that they contain 0.04–0.09% essential oil, which is localized in oil cells in the root cortex [16,18,19]. This pale yellow oil is mostly obtained by steam distillation in a Clevenger-type apparatus. It contains up to 45% β-farnesene and various other sesquiterpenes, but is devoid of bisabolol and chamazulene [19,20]. The content of chamomillol in essential root oil increases from early growth stages until the end of flowering, although chamomillaester and spiroether contents decrease [18]. In aqueous chamomile root extracts, cinnamic and benzoic acid derivatives such as chlorogenic, caffeic, ferulic, protocatechuic, vanillic and syringic acids were detected by HPLC-MS in concentrations of 1.5–20.4 $\mu g \cdot g^{-1}$ dry weight [21]. Further investigations into chamomile roots have focused on the impact of abiotic stress factors from an agricultural perspective. As an example, nitrogen deficiency enhances root growth and total phenolic accumulation as it suppresses soluble protein contents [22]. Chamomile is a known heavy metal accumulator. Although copper accumulation causes oxidative stress and leads to increased malondialdehyde concentrations in the roots [23,24], chamomile is tolerant to high cadmium concentrations [25]. Further investigations into chamomile roots, especially a comprehensive phytochemical characterization and an evaluation of their bioactivity profile, have not yet been conducted. Therefore, the present study focused on a broad GC-MS and HPLC-DAD-MS^n screening of secondary metabolites in mid-polar and polar *M. recutita* and *M. discoidea* root extracts. Furthermore, their antioxidant potential as well as antibacterial activity against the Gram-positive bacteria *B. subtilis* and *S. aureus* were assessed.

2. Results and Discussion

2.1. Secondary Metabolites in M. recutita Roots at Different Developmental Stages

For GC-MS analyses, essential root oils obtained by steam distillation were analyzed in *n*-hexane/ethyl acetate. DCM extraction of fresh roots yielded 0.20% (m/m) of a highly

viscous residue, which was dissolved in chloroform at concentrations of 5 mg/mL for direct analysis, or derivatized to obtain trimethylsilyl esters. The compound profiles of volatile secondary metabolites were identical in essential root oils and DCM extracts. Most volatile constituents were assigned by GC-MS analysis through their retention times and MS data, which were compared with the NIST database (National Institute of Standards and Technology, match factor > 800). A typical chromatogram together with the assigned compounds is displayed in Figure 1 with the corresponding mass spectral data being displayed in Table 1. Figure 2 illustrates structures of typical representatives of such extracts.

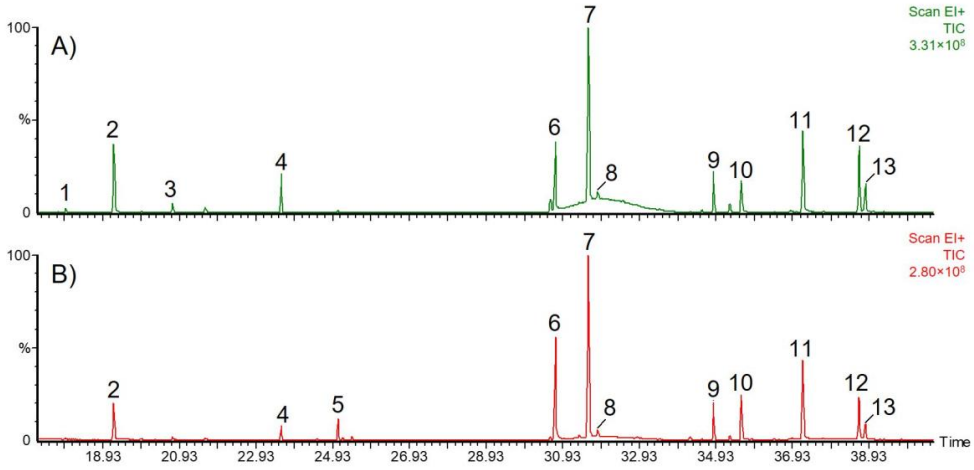

Figure 1. GC-MS total ion current chromatograms of *M. recutita* root dichloromethane extracts after silylation. Roots harvested (**A**) in March and (**B**) in June. Peak numbers refer to Table 1.

Table 1. Volatile compounds in *M. recutita* root DCM extracts assigned based on their GC-MS characteristics. Base peaks are displayed in bold.

No.	Compound	t_R (min)	MW (g/mol)	*m/z* (M^+ Int. %)
1	Berkheyaradulene	17.9	204	204 (15%), 189, **162**, 147, 134, 119
2	*β*-Farnesene	19.2	204	204 (10%), 161, 133, 120, 107, 93, 79, **69**, 55
3	*α*-Farnesene	20.8	204	204 (1%), 161, 119, 107, **93**, 79, 69, 55
4	Neryl-isovalerate	23.6	238	238 (1%), 136, 121, 107, 93, 85, **69**, 57
5	Chamomillol	25.1	222	222 (10%), 204, 179, 161, **119**, 105, 81
6	*Not identified*	30.7	220	**220** (100%), 190, 178, 136
7	*cis*-Spiroether	31.6	200	**200** (100%), 170, 157, 128, 115,76
8	*trans*-Spiroether	31.8	200	**200** (100%), 170, 157, 128, 115,76
9	Palmitic acid *	34.9	328	328 (20%), 313, 145, 161, 117, 73, 55
10	Chamomillaester I	35.6	228	228 (20%), 168, **153**, 141, 128, 115, 91, 77
11	Chamomillaester II	37.2	228	228 (25%), 168, **152**, 141, 128, 115, 91, 77
12	Linoleic acid *	38.7	352	352 (10%), 337, 262, 220, 129, 81, **73**, 67
13	Linolenic acid *	38.8	350	350 (10%), 335, 157, 129, 108, 95, **73**, 55

* Trimethylsilyl ester.

The sesquiterpenes berkheyaradulene (compound **1**), *β*-farnesene (**2**) and *α*-farnesene (**3**) were detected besides neryl-isovalerate (**4**) and traces of other terpenes. Terpenoids are prevalent in the plant kingdom, where they serve as plant hormones and signaling molecules. For example, they are often released upon damage of plant tissues in order to induce defence mechanisms. Terpenoid composition and concentration may vary substantially depending on the growth stage [26]. High amounts of farnesene are presumably due to premature harvesting [4]. Indeed, we found that farnesene concentration in DCM extracts decreased by about half from March to June. Chamomillol (**5**) was identified upon comparison of its fragmentation pattern with that published by Reichling et al. [18], who demonstrated an increase in the content of this sesquiterpene alcohol in chamomile

roots from early growth stages until the end of flowering. Accordingly, we detected this compound in roots harvested in May and June, just before and during flowering, but not in March and April. Compound **6** was tentatively assigned to a sesquiterpene oxide. Its fragmentation pattern, however, does not correspond to that of caryophyllene oxide, which has previously been identified in chamomile roots [18]. Further, two spiroether isomers could be distinguished by their retention times. Both compounds were assigned based on the fact that the *cis* isomer (**7**) is more abundant than the *trans* isomer (**8**) [18,27]. In addition, the trimethylsilyl esters of palmitic (**9**), linoleic (**12**) and linolenic acids (**13**) were identified after derivatization of the extract compounds with *N*,*O*-bis (trimethylsilyl)-trifluoroacetamide (BSTFA). Compounds **10** and **11** revealed an M^+ ion at m/z 228, which could not be further characterized. Based on their molecular mass and fragmentation patterns, these two substances were assigned to chamomillaester I and II, which have been previously described in *Matricaria* roots [18,28]. Although Das et al. reported the occurrence of bisabolol and its oxides in essential root oil [19], those compounds were detected neither in our investigations nor in those of Reichling et al. [18].

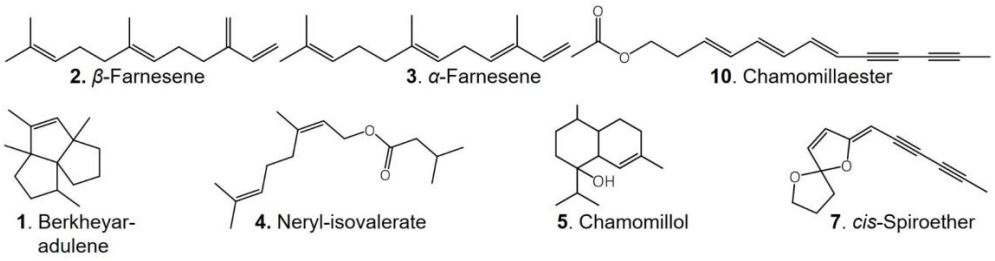

Figure 2. Structures of selected representatives characterized in *M. recutita* root dichloromethane extracts.

The yields of EtOAc and *n*-BuOH extractions were 0.05% and 0.12% (m/m), respectively. For HPLC-DAD-MS^n analyses, plant extracts were dissolved in purified water or methanol. Individual metabolites were characterized based on their retention times, UV spectra and fragmentation behavior in comparison with literature data or analytical standards. Base peak and UV chromatograms of representative EtOAc and BuOH extracts (March harvest) are illustrated in Figure 3 and peak assignment is displayed in Table 2.

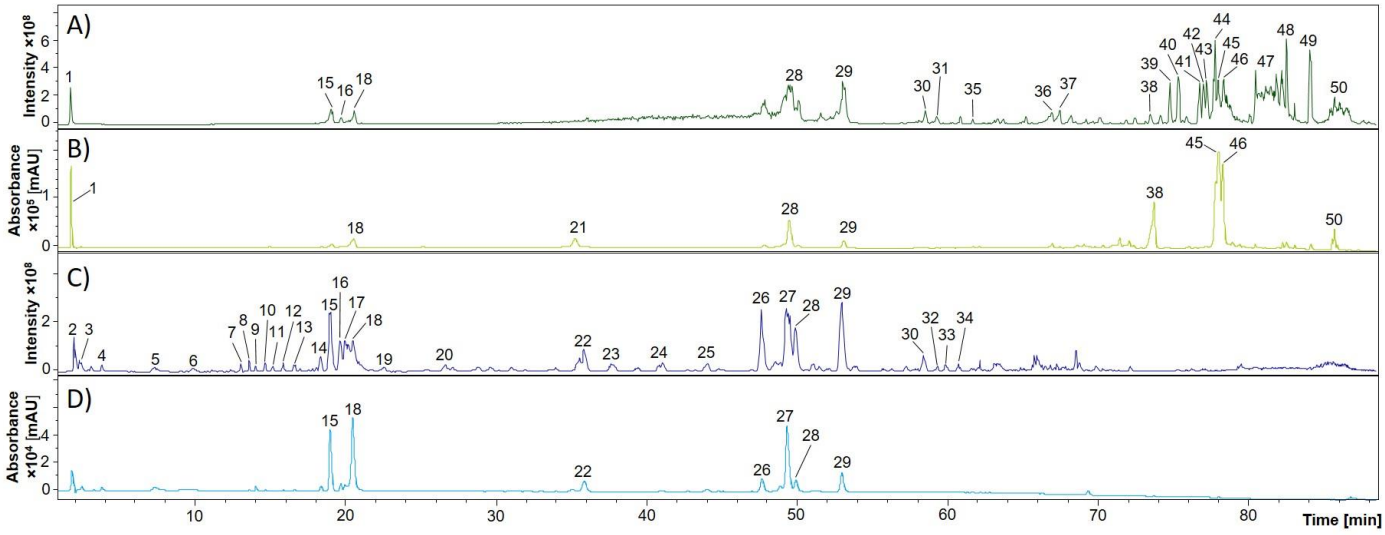

Figure 3. Secondary metabolites in root extracts of *M. recutita* analyzed via RP-HPLC-DAD-ESI-MS^n. The peak numbering corresponds to Table 2. (**A**) Base peak chromatogram (BPC) of an ethyl acetate extract; (**B**) Corresponding UV chromatogram (200–600 nm); (**C**) BPC of an *n*-butanol extract; (**D**) Corresponding UV chromatogram (200–600 nm). Peak numbers refer to Table 2.

Table 2. HPLC-DAD-MS^n data of compounds detected in ethyl acetate and *n*-butanol extracts of *M. recutita* roots in negative ionization mode.

EtOAc Extract (A) [a]	BuOH Extract (C) [a]	t_R (min)	Substance	UV Maxima (nm) [b]	Mass Spectrometric Data (*m/z*) [c]			Reference
					MS^1	MS^2	MS^3	
1		1.7	Chlorogenic acid hexoside	234, 324	**515**	353	191, 135	[29]
	2	1.9	Sucrose	-	683, **533**, 439, **404**	**341**, 179	143	[30]
	3	2.4	1-Kestose	-	**637**, 549, 503	**503**, **464**, 323		[30]
	4	3.7	Uridine	202, 262	**243**	**200**, 152	138, 110	[31]
	5	7.3	*trans*-Zeatin riboside	204, 258	533, **312**	**266**, 134	134	MassBank PR100614
	6	9.9	Ellagic acid	ND [d]	**347**	**301**	223, 161, 139	[32]
	7	12.8	Galloyl hexoside	ND [d]	**331**	**169**, 161	152, 139	[33]
	8	13.5	Galloyl-3-*O*-*β*-D-glucuronide	ND [d]	**391**	**345**, 207, 183	331, 183	[34]
	9	14.0	*L*-Tryptophan	220, 278	**203**	**159**, 158		[35,36], standard
	10	14.6	3-*O*-Caffeoylquinic acid	324	**353**	**191**, 179, 135	85	[37,38]
	11	15.1	Fraxin sulfate	206, 230, 288	**449**	**369**, 241	207, 192	[39]
	12	15.6	Aesculin	290 sh, 342	**339**	**177**	133	[40,41], standard
	13	16.4	Caffeoyl-Fraxetin	259, 305	**387**, 339	207, **179**	164, 161, 146	Tentative
	14	18.4	Scopolin	205, 226, 288 sh, 338	443, 419, **399**	353, 237, **191**, 176	176	[42]
15	15	18.9	5-*O*-Caffeoylquinic acid	218, 235 sh, 290 sh, 324	**707** *	**353**	191, 173, 135	[43], standard
16	16	19.7	Fraxin	208, 230, 300	**369**, 221	**207**	192	[42], standard
	17	19.9	Fraxetin sulfate	206, 230, 338	**287**	**207**	192	[39]
18	18	20.6	Isofraxidin-7-hexoside	208, 228 sh, 294, 334 sh	**429**, 383, 287, 221	**221**	206, 191	[42]
	19	22.6	4-*O*-Caffeoylquinic acid	324	**353**	191	173, 93	[44]
	20	26.7	Fraxetin derivative	ND [d]	**585**	**377**	**329**, 314	Tentative
21		35.4	3,5-Dicaffeoylquinic acid (3,5-diCQA)	218, 236, 322	533, **515**	**353**, 335	191, 179, 135	[44]
	22	35.9	Ferulic acid hexoside	223 sh, 236, 295 sh, 318	**711**	**355**	193, 149	[45,46]
	23	37.7	Acetylquinic acid	ND [d]	489, **233**	171, 143, 127		[40]
	24	41.1	Dimethyl lithospermate	226, 276	**565**, 467	**339**, 327	323, 309, 294	[47]
	25	43.9	Tricaffeoyl-quinic acid	322	**677**	515, **353**	191, 179, 135	[48]
	26	47.4	1,4-diCQA	218, 242, 300sh, 324	**515**	**353**, 335	191, 179, 173, 135	[44,46]
	27	49.1	1,3-diCQA	218, 236 sh, 300 sh, 326	**515**	**353**, 191	191, 179, 135	[44]
28	28	49.5	1,5-diCQA	218, 242, 300 sh, 326	**515**	**353**, 335, 191	191, 179, 135	[44]
29	29	52.8	4,5-diCQA	220, 242, 300 sh, 326	**515**	**353**, 203	191, 179, 173, 135	[44]
30	30	58.5	3,4-diCQA	280, 322	**515**, 439, 345	**353**, 191, 173	191, 179, 173, 135	[6,44]
31		59.2	Caffeoyl-feruloylquinic acid	328	**529**, 439	**367**, 349	334, 191, 179, 161	[40,49]
	32	59.3	unknown	ND [d]	**439**	393, **379**	349, 235, 217	
	33	59.9	Chicoric acid (acetyl derivative)	ND [d]	**515**, 455	473, 353, 311, **263**, 221, 179	203, 179, 161, 143	[36,50]
	34	60.7	Caffeic acid derivative	ND [d]	707, **519**	**477**	263, 221, 179, 161	Tentative
35		61.8	Sinapoyl-feruloyl-caffeoylquinic acid	242, 328	**735**	**559**	517, 337, 235, 193	[32,51]
36		66.9	Coumaroyl-feruloylquinic acid	238, 324	**707**, 427	**513**, 367	**367**, 173	[48]
37		69.9	Diferuloylquinic acid	242, 318	707, **645**	**543**	367	[48,49]
38		73.5	Linoleic acid diglycosyl monoglyceride	228, 238, 316	**723**	**677**, 397	415, 397, 235	[35]
39, 40		74.8 75.4	Linolenic acid monoglycosyl monoglyceride isomers	240, 313	**559**	513, **277**, 253	259, 233	[35,52]
41		76.8	Linoleic acid monoglycosyl monoglyceride	238, 250, 314	**561**	515, **279**	261, 205	[35,53]
42		77.0	Linoleic acid derivative	238, 314	**529**	511, **279**, 249	261, 205	Tentative
43		77.3	Linoleic acid monoglycosyl monoglyceride	240, 316	**561**	515, **279**	261, 205	[35,53]
44		77.8	Linoleic acid derivative	242, 254, 324	**529**	511, **279**, 249	261, 205	Tentative

Table 2. *Cont.*

EtOAc Extract (A) [a]	BuOH Extract (C) [a]	t_R (min)	Substance	UV Maxima (nm) [b]	Mass Spectrometric Data (*m/z*) [c]			Reference
					MS^1	MS^2	MS^3	
45, 46		78.0 78.4	Phosphoglyceride isomers	242, 250, 324	**431**	171, **153**	97, 79	[52]
47		82.1	Phosphoglyceride	314	**433**, 399	171, **153**	79	[52]
48		82.5	Linolenic acid	<200, 242	311, **277**	259, **233**, 205	191, 179	[35,54]
49		84.2	Linoleic acid	<200	**279**	**261**	243	[35,54]
50		85.7	Dihydroxy-linolenic acid	226	**325**, 281	**183**		[35], tentative

[a] For peak labeling see Figure 3; [b] UV and BPC intensities may differ due to differences in analyte ionizability, concentrations, molar extinction coefficients, etc.; [c] bold numbers: ion further fragmented in CID experiments; [d] not detected; * dimer is an artifact produced during ionization.

A number of compounds with similar fragmentation patterns and UV spectra were eluted in a retention time range of 15–21 min. Based on neutral losses of 162 Da resulting in $[M-H-hexosyl]^-$ ion species in the first fragmentation step and the mass-to-charge ratios of the corresponding aglycons, four coumarin glycosides, namely aesculin (compound **12**, t_R 15.6 min, *m/z* 339), scopolin (**14**, t_R 18.4 min, *m/z* 399), fraxin (**16**, t_R 19.7 min, *m/z* 369), and isofraxidin-7-glucoside (**18**, t_R 20.6 min, *m/z* 383) were assigned (Figure 4). The identity of aesculin and fraxin was verified using analytical reference standards. Compounds **11** and **17** revealed losses of 80 Da (sulfate or phosphate) upon collision-induced dissociation (CID). Since coumarin sulfates have been described earlier in *Pelargonium* species [55] and are formed in coumarin metabolism [56], the two substances were tentatively assigned to fraxin and fraxetin sulfate. Additionally, neutral losses of 208 Da for compounds **13** and **20** indicated the presence of further fraxetin derivatives. However, these could not be more closely identified. The coumarins herniarin, umbelliferone, esculetin, scopoletin and daphnetin, together with some of their glycosides, have previously been detected in chamomile flowers [57,58]. To the best of our knowledge, coumarins in general have not been detected in chamomile roots so far, and also fraxidin and fraxetin in *M. recutita* are described here for the first time. This is of particular interest, since in the plant kingdom coumarins play a role in iron uptake and bioactivities reported in in vitro studies are, among others, antimicrobial and anticoagulant [59].

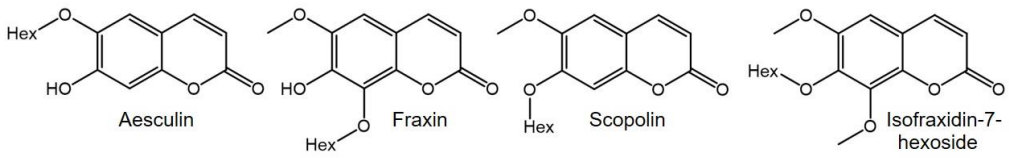

Figure 4. Structures of coumarin hexosides detected in *M. recutita* roots.

Furthermore, a number of caffeoylquinic acids (CQA) were characterized in EtOAc and BuOH extracts. These show interesting bioactivity characteristics, such as antiphlogistic and enzyme-inhibiting properties [60]. Molecular ions at *m/z* 353 with intense signals at *m/z* 191 in MS^2 experiments (compounds **10, 15, 19**) indicated the presence of 3-, 4- and 5-*O*-chlorogenic acids, respectively. For compounds **21** and **26**–**30**, fragmentation of the $[M-H]^-$ ions at *m/z* 515 yielded daughter ions at *m/z* 353 ($[M-H-162]^-$, loss of caffeoyl moiety). Together with UV maxima at 218 and 322 nm, the compounds were assigned to different isomers of dicaffeoylquinic acids (diCQA). The constitutional isomers were differentiated based on their MS^2 and MS^3 fragment ion intensities according to Clifford et al. [44]. The occurrence of mono- and diCQA in chamomile roots has been reported previously [60]. A decrease in diCQA contents was found to be the main difference between plants of various growth stages from March (before the shoot of the stem) to June (flowering stage). As deduced from signal intensities of UV chromatograms, 1,4-, 1,3- and 1,5-diCQA decreased by approximately 30%, the 4,5-isomer even by 80% (data not shown).

In a retention time range of 59 to 70 min, several esters of caffeic, ferulic, sinapic and *p*-coumaric acids were characterized based on their fragmentation patterns. These

hydroxycinnamates are known to serve as defence against herbivores and microorganisms, for protection from UV-B radiation as well as response to mechanical damage [26]. Flavonoids such as apigenin and its glucoside have been described as bioactive polyphenols in chamomile flower extracts and decoctions [8]. They were, however, not detected in chamomile roots.

In the last part of the chromatogram of EtOAc extracts, a number of glyceroglycolipids and phospholipids containing linoleic and linolenic acid moieties were eluted. Interestingly, when linoleic acid diglycosyl monoglyceride (**38**) was fragmented, the fatty acid moiety was released as a neutral loss and the polar head was further fragmented (Figure 5). In contrast, for linolenic and linoleic acid monoglycosyl monoglycerides (**39**–**41**, **43**), the fatty acid served as base peak in MS^2 experiments and was further fragmented, although the polar head was also detected in the MS^2 spectrum. Similar representatives of these compound classes have been described in other *Asteraceae* species. For example, glyceroglycolipids have been extracted from dandelion (*Taraxacum mongolicum* L.) [53] and glycerophospholipids from red lettuce (*Lactuca sativa* L. var. *crispa*) leaves and sunchoke (*Helianthus tuberosus* L.) roots [36].

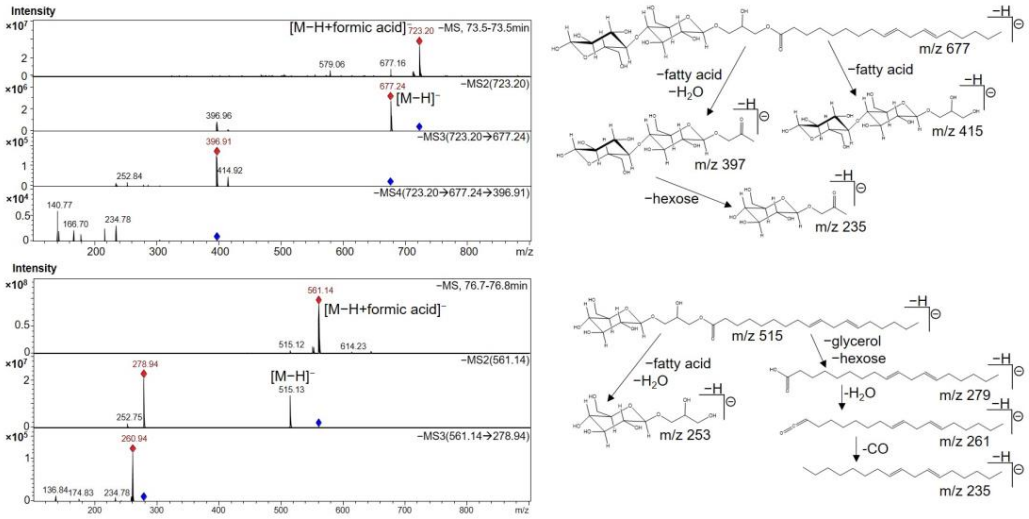

Figure 5. MS^n spectra of two glyceroglycolipids (compounds **38** and **41**) and postulated fragmentation pathways.

2.2. Phytochemical Comparison of Different Chamomile Varieties

Based on the chemical composition of the essential flower oil, chamomile varieties are classified into different chemotypes [9]. In this study, two different cultivars of *M. recutita* and one of *M. discoidea* were compared. In order to determine the chemotypes of the investigated samples, essential flower oil was obtained by aqueous steam distillation and analyzed by GC-MS. The dark blue essential flower oil of *M. recutita* grown in Bad Boll contained equal amounts of the bisabolol oxides A and B. The plant was therefore identified as chemotype D according to Schilcher et al. [9]. In contrast, α-bisabolol was the main compound in the essential flower oil of the chamomile cultivar from Sulzemoos, indicating chemotype C [9]. Interestingly, the essential flower oil of pineapple weed (*M. discoidea*) lacked the blue colour and thus chamazulene, but also bisabolol and its oxides. Instead, the terpenes *β*-pinene, *β*-cubebene and *tr*-nerolidol were detected. However, the relationship with other *Matricaria* species was evident from the presence of its main metabolites *β*-farnesene and *cis*-spiroether.

Contrary to the essential flower oils, the volatile secondary metabolites were identical in DCM root extracts of these three varieties. Chamomillaesters I and II (Figure 2) were identified in all samples along with *β*-farnesene, the unidentified sesquiterpene oxide (Table 1), *cis*- and *trans*-spiroether and the free fatty acids palmitic, linoleic and linolenic acid. Also, the HPLC-DAD-MS^n screening of root extracts of increasing polarity revealed similar

fingerprints of the three investigated varieties, except for coumarins and caffeoylquinic acids, where differences were particularly apparent. Figure 6 displays the corresponding section of the HPLC UV trace of roots harvested at flowering stage (May/June). Although at this harvest time mainly 1,3-dicaffeoylquinic acid was present in the BuOH extracts of both *M. recutita* cultivars, *M. discoidea* extracts additionally contained the 1,4- and 4,5-isomers in almost equal amounts as deduced from the signal intensities recorded at 320 nm. In contrast, the isofraxidin-7-hexoside content was lowest in *M. discoidea*.

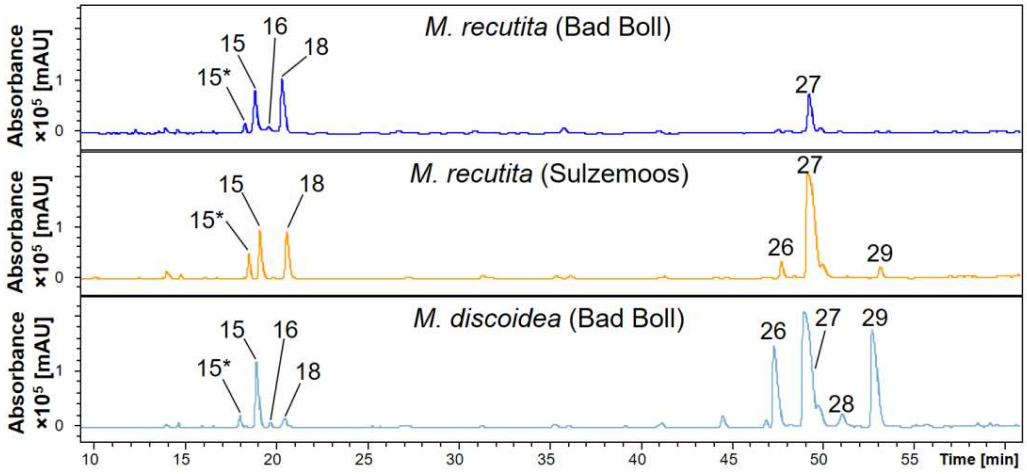

Figure 6. HPLC-DAD UV chromatograms (200–600 nm) showing coumarins and caffeoylquinic acids in *n*-butanol extracts of different chamomile varieties. Peak numbers refer to Table 2. 15: 5-*O*-caffeoylquinic acid (* formic acid adduct); 16: fraxin; 18: isofraxidin-7-hexoside; 26–29: 1,4-/1,3-/1,5-/4,5-dicaffeoylquinic acids.

2.3. Antioxidant Potential of Chamomile Root Extracts

2.3.1. DPPH Assay

The DPPH radical scavenging assay is very common for the determination of antioxidant activities of plant extracts in vitro, although results published in literature may vary due to a lack of standardization and access to different extraction techniques, solvents and chemicals [61]. This assay has been performed in a methanolic solution or on TLC plates assessing various chamomile extracts [36,62,63]. IC_{50} values amounted to 6.8 ± 0.01 µg/mL and 8.5 ± 0.7 µg/mL for the two reference substances trolox and chlorogenic acid, respectively (Figure 7). DCM extracts of the studied chamomile varieties revealed the highest IC_{50} values of 279–290 µg/mL and, thus, the weakest antioxidant activity. *M. discoidea* EtOAc and BuOH extracts had the strongest DPPH scavenging activity with IC_{50} values of 12.7 ± 3.8 and 13.8 ± 0.4 µg/mL, respectively.

Different solvent extracts from aerial plant parts of chamomile have been evaluated with regard to their DPPH scavenging potential in a large number of studies. The IC_{50} values of essential oil and methanol extract of *M. recutita* leaves was reported to be 4.18 µg/mL and 1.83 µg/mL, respectively [64]. Al-Dabbagh and co-workers determined an IC_{50} value of 26.7 µg/mL for a hydroethanolic chamomile flower extract [65]. Subcritical water extracts of chamomile flowers revealed IC_{50} values of 10–45 µg/mL, depending on the extraction temperature [66]. Thus, the IC_{50} values determined in our study for EtOAc and BuOH extracts are in the same range as those of the flowers. Generally, infusions and decoctions, i.e., aqueous solutions, possess higher antioxidant activities than methanol extracts [45] and IC_{50} values decrease with the increasing polarity of the solvent used [62]. Accordingly, in this study, extracts of increasing polarity showed lower IC_{50} values, indicating stronger antioxidant properties.

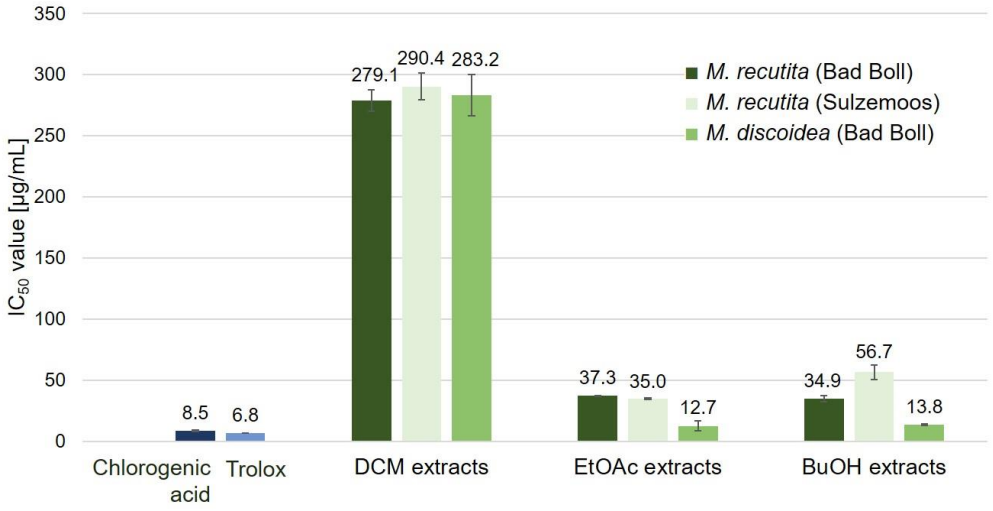

Figure 7. IC_{50} values of different chamomile root extracts, trolox and chlorogenic acid determined applying the DPPH radical scavenging assay (n = 3).

Phenolic acids and flavonoids have been identified as main contributors to the antioxidant activity of various chamomile extracts [6]. The radical scavenging effect of ethyl acetate and butanol extracts is probably due to coumarins and the abundant mono- and diCQA derivatives identified by HPLC-DAD-MS^n. The stronger effect of *M. discoidea* extracts may be due to the fact that they contain higher amounts of 1,4- and 4,5-dicaffeoylquinic acids than the *M. recutita* cultivars (Figure 6), and the 4,5-isomer has been shown to have strongest DPPH scavenging activity among the diCQA isomers [67]. However, a direct comparison of reference substances with plant extracts, which are complex mixtures of numerous metabolites, remains challenging, since synergistic, additive or antagonistic effects may also affect the final read-out values.

2.3.2. Superoxide Assay

The superoxide radical $O_2^{\bullet -}$ belongs to the reactive oxygen species (ROS) and is generated in cells by mitochondrial electron transfer systems, NADPH oxidase and xanthine oxidase. Consequently, antioxidants and radical scavenging enzymes, which protect cells from oxidative stress, are crucial for preventing adverse effects such as increased ageing and Alzheimer's disease [68]. In contrast to the DPPH assay, the superoxide assay is performed under physiological conditions. This allows a better understanding of the antioxidant effects of chamomile root extracts in vivo. Although strong antioxidant activities have been determined for caffeoylquinic acids in general [69], chlorogenic acid as pure compound did not show any effect in this assay. Since trolox also had no effect, gallic acid was used as reference substance. Additionally, aesculin, one of the coumarins detected in the extracts, was tested as second reference substance. Due to the insufficient solubility of DCM and EtOAc extracts in the buffer solution, which led to turbidity, only BuOH extracts were investigated. The relative inhibition of formazan formation by the different samples is displayed in Figure 8. The least amount of formazan was formed in samples containing 5–30 µg/mL gallic acid, thus absorbances remained low and relative inhibition was highest. This indicates that among all samples gallic acid had the strongest superoxide scavenging activity. This is in accordance with the findings of Furuno et al., that the pyrogallol moiety strongly contributes to superoxide radical scavenging activity [70]. In comparison, BuOH extracts showed moderate superoxide scavenging activity. Similar to the DPPH assay, *M. discoidea* exerted the most pronounced antioxidant effects among the chamomile samples studied. The different *M. recutita* samples showed similar results, regardless of origin or harvest date. Aesculin as reference standard showed lowest inhibition and, thus, a very weak superoxide scavenging effect. The extracts studied are complex mixtures whose

antioxidant effects are probably caused by the sum of their individual components such as gallic acid and other phenolic acids, coumarins and further metabolites.

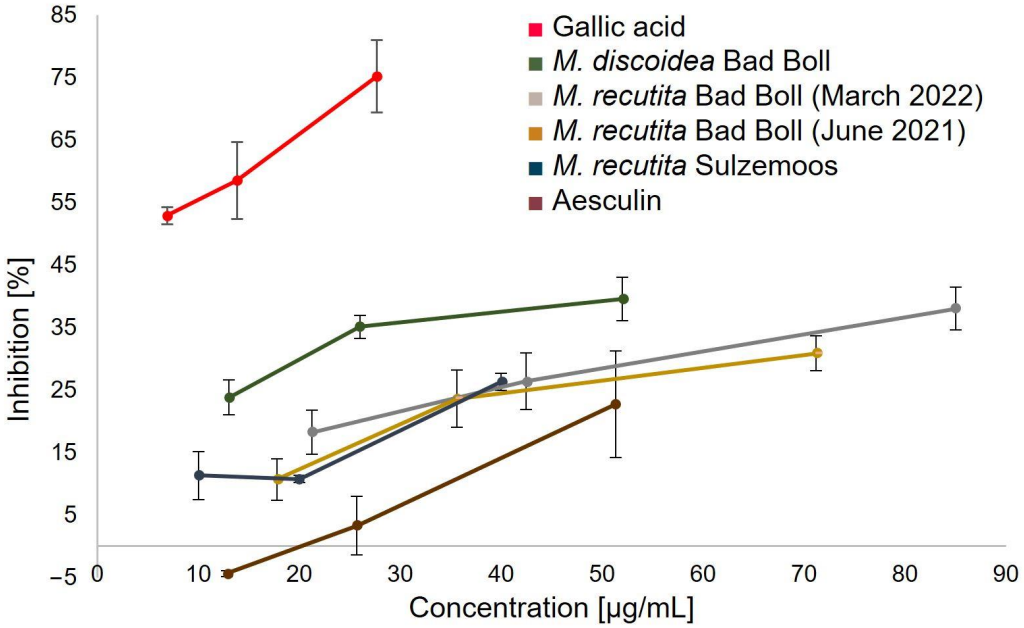

Figure 8. Superoxide anion radical scavenging activity of various chamomile root butanol extracts, aesculin and gallic acid. Results represent mean ± SD (n = 3). Negative inhibition values result from mathematical calculation of the relative inhibition.

The superoxide scavenging activity of chamomile has not been widely assessed. Merely Cvetanovic et al. determined IC_{50} values between 30 and 100 µg/mL in electron spin resonance (ESR) studies [71]. Physiological antioxidant effects of chamomile flower essential oil and extracts have been investigated in different studies. As an example, Sebai et al. tested the impact of chamomile flower decoction against oxidative stress in rats. The authors showed, that chamomile decoction protected the animals from castor oil-induced diarrhea and intestinal fluid accumulation but also prevented the reduction of the activity of antioxidant enzymes such as catalase and superoxide dismutase [72]. Accordingly, administration of chamomile flower decoction protected these enzymes from ethanol-induced injury and prevented lipoperoxidation in the liver [73]. The effects were attributed to phenolic compounds, which also occur in chamomile roots.

Antioxidant properties are desired not only in medicinal applications, but also in the cosmetics and food sector. Many slightly or more highly processed products require the addition of stabilizing, coloring or preserving ingredients [74]. However, there is a growing consciousness for natural formulations without synthetic additives. Therefore, plant extracts, e.g., rosemary essential oil, are increasingly incorporated as natural antioxidant compounds in different food and cosmetic matrices [75,76]. In the case of German chamomile, research is again focused on extracts or essential oils from flowers or aboveground plant parts, e.g., to enhance the stability of dairy products without changing their nutritional value [77]. In the light of the present study, root extracts with their comparably potent antioxidant activity should also be considered in the future.

2.4. Antibacterial Potential of Chamomile Roots

Due to increasing resistance to conventional antibiotics, the use of natural products for their supplement or substitution is a promising research topic [26,62]. For a first evaluation of the potential antibacterial activity of different *M. recutita* root extracts, disk diffusion experiments were performed. All samples inhibited the growth of Gram-positive bacterial strains of *B. subtilis* and *S. aureus* in amounts $\geq$ 0.8 mg per disk, as shown in Table 3. Antibacterial effects were comparable for both susceptible strains. Except for *M. recutita* grown

in Bad Boll, DCM and EtOAc extracts showed stronger inhibition than BuOH extracts. This is not surprising since the antibacterial effects of many essential oils have already been described [78] and the main constituents of these, e.g., terpenoids, are also present in nonpolar extracts. Interestingly, DCM and EtOAc root extracts of *M. discoidea* showed the strongest effects, but the corresponding BuOH extract was completely inactive. Inhibiting effects could neither be detected against Gram-negative bacteria strains *P. aeruginosa* and *E. coli* nor against *C. albicans* (data not shown).

Table 3. Mean inhibition zones in mm against Gram-positive bacterial strains of *B. subtilis* and *S. aureus* ($n = 3$).

Extract	*S. aureus* 0.8 mg/Disk	*S. aureus* 1.6 mg/Disk	*S. aureus* 3.2 mg/Disk	*B. subtilis* 0.8 mg/Disk	*B. subtilis* 1.6 mg/Disk	*B. subtilis* 3.2 mg/Disk
			M. recutita Bad Boll			
DCM	8 ± 0	9 ± 0	9 ± 2	6 ± 0	7 ± 0	8 ± 0
EtOAc	8 ± 0	7 ± 1	9 ± 1	-	8 ± 1	10 ± 1
BuOH	7 ± 0	8 ± 1	10 ± 1	7 ± 1	8 ± 0	9 ± 1
			M. recutita Sulzemoos			
DCM	7 ± 1	9 ± 1	10 ± 1	8 ± 0	9 ± 0	9 ± 1
BuOH	-	8 ± 1	9 ± 1	-	7 ± 0	8 ± 1
			M. discoidea			
DCM	9 ± 1	10 ± 1	11 ± 1	9 ± 2	9 ± 2	9 ± 1
EtOAc	7 ± 0	9 ± 1	10 ± 0	8 ± 0	9 ± 0	10 ± 0
BuOH	-	-	-	-	-	-

The antibacterial effects of various compound classes are based on different mechanisms. Essential oil constituents such as terpenes can pass or interact with bacterial cell membranes, which may go along with disruption or leakage. Inside the cells, oxidative stress and disturbance of protein metabolism and mitochondria may occur, among others [79]. Cinnamic and chlorogenic acids are also known to disrupt bacterial cell membranes, thus increasing their fluidity and permeability [80]. Furthermore, some coumarins have been reported to inhibit DNA gyrase, which normally causes negative supercoiling of the DNA [81].

Although the antimicrobial potential of chamomile flowers has been extensively studied, information about the roots is scarce. An antibacterial potential of chamomile roots has been described against *Pseudomonas syringae* pv. *maculicola*. The effects could be attributed to the presence of spiroethers and coumarins, but have not been studied further [82]. In contrast, roots of other members of the *Asteraceae* family have been assessed in more detail. For example, dandelion (*Taraxacum officinale* L.) roots inhibited *S. aureus* and *B. cereus* growth, presumably due to the presence of hydroxylinoleic and hydroxylinolenic acids, vanillin and coniferylaldehyde [83]. The inhibitory effect of tansy (*Tanacetum vulgare* L.) root extracts against *B. subtilis* and two plant pathogens could be attributed to different polyacetylenic compounds [63].

A lipophilic chamomile flower extract obtained by supercritical CO_2 extraction inhibited the growth of different crop-borne fungi by 80–100% [84]. Roby et al. compared the antibacterial potential of different chamomile flower extracts. Consistent with all other studies, the extracts were more effective against Gram-positive than against Gram-negative bacteria. Very low amounts of 7.5–20 µg per disk inhibited the growth of various bacterial strains and *C. albicans* [62]. Higher concentrations were used by Abdoul-Latif et al.: 300 µg leaf methanol extract or 10 µL essential oil per disk inhibited the growth of different Gram-positive and Gram-negative bacterial strains, with the essential oil showing stronger effects [64]. Interestingly, bisabolol oxides negatively influenced antibacterial activity [4], indicating that the activity strongly depended on the compound profile of the respective sample. Thus, for an appropriate use, the chemotype of the essential flower oil as well

as season of harvest and the extraction procedure have to be chosen carefully [85]. The presented results show that, in addition to chamomile flowers and leaves, the roots also have promising potential with regard to their antibacterial properties. Thus, the use of chamomile roots for the preparation of phytomedicinal products contributes to a sustainable cultivation and use of this important medicinal plant, although further studies are needed, e.g., to determine minimal inhibitory concentrations of the respective extracts, allowing a profound assessment of the antibacterial potential.

3. Materials and Methods

3.1. Chemicals and Reagents

Acetone, acetonitrile, *n*-butanol (BuOH), dichloromethane (DCM), dimethylsulfoxide (DMSO), chloroform, ethanol, ethyl acetate (EtOAc), methanol (MeOH) and toluene were purchased from Chemsolute (Th. Geyer GmbH & Co., KG, Renningen, Germany). Nitrotetrazolium blue chloride (NBT), gallic acid monohydrate and TRIS hydrochloride were obtained from Carl Roth GmbH & Co., KG (Karlsruhe, Germany). *N*,*O*-Bis (trimethylsilyl)-trifluoroacetamide (BSTFA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hypoxanthine, tryptophane and xanthine oxidase (XOD, grade III from bovine milk) were from Sigma-Aldrich (St. Louis, MO, USA), and formic acid from Fluka (Sigma Aldrich, St. Louis, MO, USA). Trolox was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), and chlorogenic acid hemihydrate from Alfa Aesar (Karlsruhe, Germany). Fraxin and aesculin analytical standards were obtained from PhytoLab GmbH & Co., KG (Vestenbergsgreuth, Germany). *N*,*N*-Dimethylformamide (DMF), sodium sulfate, Tryptic Soy Agar (TSA) and Sabouraud Dextrose Agar (SDA) broth and agar plates were from Merck KGaA (Darmstadt, Germany). Ampicillin sodium salt and gentamicin were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

3.2. Plant Material and Extraction

Roots of *M. recutita* were harvested monthly between March and June 2021 and in March 2022 in the medicinal plant garden of WALA Heilmittel GmbH (Bad Boll/Eckwälden, Germany). Further, roots of *M. discoidea* were harvested in the same place in June 2021. Additionally, roots of a bisabolol-rich *M. recutita* variety were harvested at Kistler & Co., GmbH in Sulzemoos, Germany, in June 2021. The plant material was rinsed with tap water, drained, packed in freezer bags and stored at −80 °C until investigation. Voucher specimens were deposited at the herbarium of the Institute of Botany, Hohenheim University (Stuttgart, Germany). The identity of the plant material was confirmed by Dr. R. Duque-Thüs (*M. recutita* Bad Boll, voucher number: HOH-022871; *M. recutita* Sulzemoos, voucher number: HOH-022870; *M. discoidea* Bad Boll, voucher number: HOH-022872).

100 g of fresh plant material was mixed with acetone/water (500 mL, 60/40, *v*/*v*). The material was minced for three min using an Ultra-Turrax (17,000 rpm; IKA Werke GmbH and Co., KG, Staufen, Germany). Prior to and after comminution, the mixture was bubbled with nitrogen for 15 min to avoid oxidative degradation of the plant constituents. The slurry was stored at 4 °C overnight and then filtered over Celite® (Carl Roth GmbH + Co., KG, Karlsruhe, Germany). Solid residues were extracted a second time in the same manner. Both brown-coloured filtrates were combined and acetone was removed by rotary evaporation.

Subsequently, the obtained aqueous extract was successively extracted with 3 × 100 mL each of dichloromethane, ethyl acetate and *n*-butanol, using a separating funnel. Dichloromethane and ethyl acetate extracts were dried over anhydrous sodium sulfate and filtered over a glass frit (Por. 3, ROBU® Glasfilter-Geräte GmbH, Hattert, Germany). The solvents were then removed in vacuo to obtain dry extracts for further investigations. Extraction was performed in duplicate for all three chamomile species.

Additionally, 50 g of either chamomile roots or flowers, stems and leaves in 200 mL water were distilled in a Clevenger-type apparatus for four hours. Essential oils were trapped in *n*-hexane/ethyl acetate 3/1 (*v*/*v*) and dried over anhydrous sodium sulfate.

3.3. GC-MS Analysis of Volatile Constituents

Crude extracts obtained by solvent extraction were dissolved in chloroform at concentrations of 5 mg/mL for direct analysis. Essential oils in *n*-hexane/ethyl acetate, recovered upon distillation as described above, were directly injected into the GC. To obtain trimethylsilyl derivatives of individual compounds, crude extracts (3–5 mg) were dissolved in DMF (500 µL) and 200 µL BSTFA were added. The solution was heated to 105 °C for 15 min and subsequently analyzed via GC/MS.

GC/MS analyses were conducted with a PerkinElmer *Clarus 500* gas chromatograph (PerkinElmer, Inc., Shelton, CT, USA) with split injection (split ratio 30:1, injection volume 1.0 µL) coupled to a single quadrupole mass spectrometer operating in electron ionization (EI) mode at 70 eV. A *Zebron ZB-5MS* capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness, 5% phenylpolysiloxane and 95% dimethylpolysiloxane coating; Phenomenex, Torrance, USA) was used as a stationary phase, helium served as carrier gas at a flow rate of 1 mL/min. The injector temperature was 250 °C, the temperature program of the column oven was 100–320 °C, applying a linear gradient of 4 °C/min and a final holding time of 30 min. Data were acquired and processed using the software *TurboMass* (v.5.4.2, PerkinElmer, Inc., Waltham, MA, USA).

3.4. RP-HPLC-DAD-ESI-MS^n Analysis

High performance liquid chromatographic analyses were carried out on an Agilent 1200 HPLC system (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with binary pump, micro vacuum degasser, autosampler, thermostatic column compartment and UV/VIS diode array detector (DAD). A *Kinetex*® C18 reversed-phase column (2.6 µm particle size, 150 mm × 2.1 mm i.d., Phenomenex Ltd., Aschaffenburg, Germany) and a pre-column of the same material were used for chromatographic separation at 25 °C and a flow rate of 0.21 mL/min. The mobile phase consisted of 0.1% formic acid in water (eluent A) and acetonitrile (eluent B). The injection volume of each sample was 10 µL. The gradient was as follows: 0–8 min, 0–10% B; 8–20 min, 10% B; 20–51 min, 10–23% B; 51–70 min, 23–60% B; 70–80 min, 60–100% B; 80–85 min, 100% B; 85–90 min, 100–0% B; 90–100 min, 0% B.

The LC system was coupled to an *HCTultra* ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with an ESI source. All extracts were analyzed in negative ionization mode using a capillary voltage of 4000 V, a dry gas (N_2) flow of 9.00 L/min with a capillary temperature of 365 °C and nebulizer pressure of 35 psi. Full scan mass spectra (mass range m/z 50–1000) of HPLC eluates were recorded during chromatographic separation yielding $[M–H]^-$ ions. MS^n data were acquired in the auto MS/MS mode by collision-induced dissociation (CID). The instruments were controlled by *ChemStation for LC 3D systems* (Rev. B01.03 SR1 (204)) and *EsquireControl* software (V7.1).

Samples were dissolved in water (BuOH extracts) or methanol (all other extracts) to reach a concentration of 5 mg/mL.

3.5. 2,2- Diphenyl-1-picrylhydrazyl (DPPH) Assay

The DPPH free radical scavenging assay is based on the ability of antioxidant components to reduce the artificial stable DPPH radical, going along with a change of colour from deep purple to yellow and, thus, a strong decrease in absorbance at 516 nm. The half maximal inhibitory concentration (IC_{50}) is the amount of sample needed to reduce the initial DPPH content by 50% and an indication of the antioxidant potential of individual compounds or complex plant extracts. For the assay, DPPH was dissolved in methanol at a concentration of 100 mM. The plant extracts were dissolved at concentrations of 1–4 mg/mL in methanol and diluted to five appropriate concentrations. Then, 200 µL of the test or reference solution or methanol as blank sample were added to 1800 µL DPPH solution. The sample was incubated at 38 °C for 30 min and then analyzed at 516 nm using a spectrophotometer (Lambda 2, Perkin Elmer Ltd., Waltham, MA, USA) as reported previously [86]. Trolox was used as reference compound preparing solutions at five different concentrations

ranging from 3–100 mM. Absorbance values for each sample were plotted against the concentrations, and IC_{50} values were calculated from the formula of the linear trend line at 50% of the maximum absorbance value. Analyses were performed in triplicate.

3.6. Superoxide Assay

The ability of BuOH extracts to scavenge the superoxide radical $O_2^{\bullet-}$ was investigated using a modified version of the procedure described by Lorenz et al. [87]. Superoxide was generated enzymatically using a hypoxanthine/xanthine oxidase (XOD) system and analyzed by the reduction of NBT to form a blue formazan product. The latter was detected using a spectrophotometer (Lambda 2, Perkin Elmer Ltd., Waltham, MA, USA). 50 mM TRIS buffer at pH 7.4 containing 539 µM hypoxanthine and 111 µM NBT was used as solvent. Solid BuOH root extracts were dissolved in DMSO and diluted to three different concentrations in the range of 1–9 mg/mL. Subsequently, 1960 µL buffer solution was mixed with 20 µL sample solution and 20 µL enzyme solution (3.4 U/mL). Samples were incubated at 37 °C for exactly 7 min after enzyme addition and immediately analyzed spectrophotometrically at 560 nm against a blank control not containing the enzyme. Gallic acid and aesculin were used as reference compounds. Analyses were performed in triplicate. The percentage inhibition of formazan formation was calculated using the following equation:

$$\text{Inhibition (\%)} = (A_{control} - A_{sample})/A_{control} \times 100$$

where $A_{control}$ and A_{sample} were the absorbance values of the control solution with pure DMSO and the sample solution, respectively.

3.7. Antimicrobial Assay

Disk diffusion tests were performed to evaluate the antimicrobial activity of different chamomile root extracts against four common bacteria strains. Among these, Gram-negative strains, i.e., *Pseudomonas aeruginosa* ATCC 9027 and *Escherichia coli* ATCC 8739, and Gram-positive strains, i.e., *Staphylococcus aureus* ATCC 6538 and *Bacillus safensis* ATCC 6633 were tested (Leibniz Institute, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). Additionally, one fungal strain (*Candida albicans* strain ATCC 10231) was tested. Cell material was taken from pure cultures and incubated in 4 mL TSA broth (bacteria) or SDA broth (*C. albicans*) at 37 °C for 24 h. Colony-forming units were determined by serial dilution to 10^6–10^8 (*B. safensis*, *E. coli*, *C. albicans*) and 10^9 (*S. aureus, P. aeruginosa*). Thus, the latter were diluted with TSA broth (1:10, v:v) prior to usage. Plant extracts were suspended in MeOH at a concentration of 80 mg/mL. Sterile antimicrobial test disks (Oxoid™ blank, Thermo Fisher Diagnostics GmbH, Waltham, MA, USA) were loaded with 10–40 µL suspension (0.8–3.2 mg dry extract) and dried. Pure MeOH (10 µL) served as negative control, and the antibiotics gentamicin (0.5 mM, 1.0 mM, 1.5 mM; 10 µL) and ampicillin (0.1 mM, 10 µL) were used as positive controls for *S. aureus*, *E. coli*, *P. aeruginosa* and *B. safensis*, respectively. Subsequently, 100 µL of the bacterial suspension was spread on a TSA agar plate and allowed to dry briefly. SDA agar plates were used for *C. albicans*. Disks with negative and positive controls as well as three extract concentrations were placed on each plate. Inhibition zones (diameter in millimeter including the test disk) were measured after incubation at 37 °C for 20 h. The assay was conducted in triplicate for all samples.

4. Conclusions

In the present study, the roots of two *Matricaria recutita* and one *M. discoidea* accessions were investigated for their secondary metabolite composition and bioactivity characteristics. Interestingly, although the volatile constituents in essential flower oils varied considerably between the three varieties, all roots contained similar principal constituents. Among others, β-farnesene, chamomillol, spiroether and chamomillaester were detected by GC-MS. Additionally, HPLC-DAD-MS^n analyses revealed the presence of the coumarin glycosides

aesculin, scopolin, fraxin and isofraxidin-7-hexoside along with other coumarin derivatives, caffeoylquinic acids, phospho- and glyceroglycolipids in the roots.

EtOAc and BuOH root extracts showed a DPPH radical scavenging activity comparable to that of chamomile flowers. Thus, middle polar extracts may be incorporated into emulsions or oil-based cosmetic products to improve their stability and antioxidant properties. The BuOH extracts also had scavenging effects on the superoxide ($O_2^{\bullet -}$) radical when evaluated under physiological conditions in buffered solution at pH 7.4. This may point to an antioxidant potential of the extracts in vivo. Moreover, moderate antibacterial activity of chamomile root extracts against the Gram-positive bacterial strains *S. aureus* and *B. subtilis* was observed. Chamomile roots are a by-product of chamomile tea and essential oil production. Their use in phytomedicinal or cosmetic preparations thus contributes to a more sustainable agricultural production. However, the efficacy of such preparations should be evaluated in further studies.

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3.2 IMPACT OF FERMENTATION ON THE PHYTOCHEMICAL PROFILE AND BIOACTIVITY CHARACTERISTICS OF AQUEOUS *MATRICARIA RECUTITA* L. ROOT EXTRACTS

Mailänder, L. K.; Nosrati Gazafroudi, K.; Greiß, M.; Lorenz, P.; Nicolay, S.; Gründemann, C.; Stintzing, F. C.; Daniels, R.; Kammerer, D. R. Impact of Fermentation on the Phytochemical Profile and Bioactivity Characteristics of Aqueous *Matricaria recutita* L. Root Extracts. *Chemistry & Biodiversity* **2024**, e202400159. https://doi.org/10.1002/cbdv.202400159

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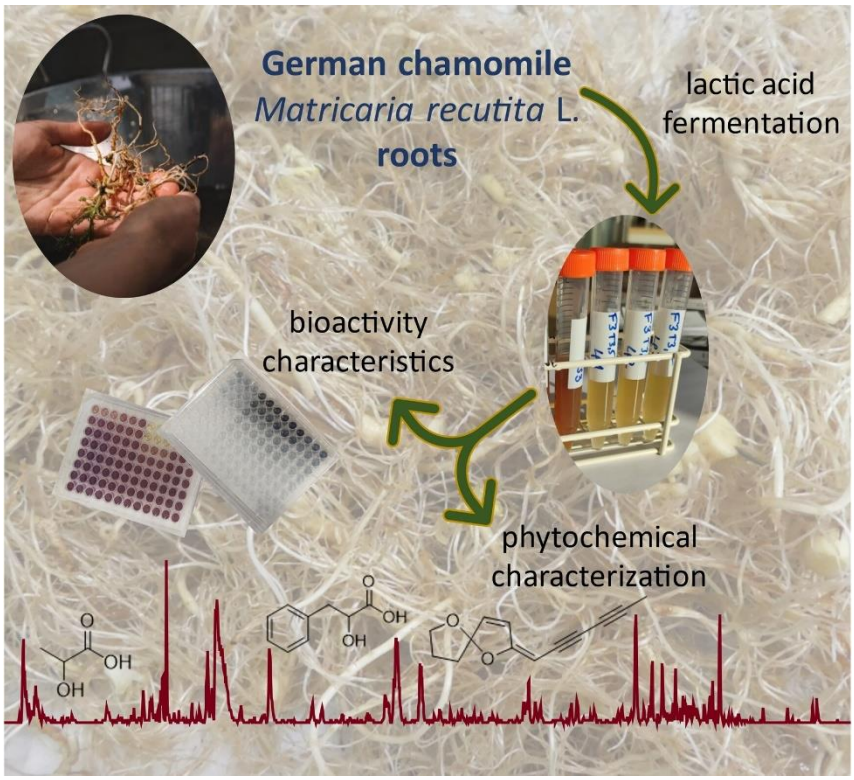

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Impact of Fermentation on the Phytochemical Profile and Bioactivity Characteristics of Aqueous *Matricaria recutita* L. Root Extracts

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While the flowers of *Matricaria recutita* L., German chamomile, are widely used for medicinal and cosmetic purposes, little is known about its roots, which are used in complementary medicine for the preparation of aqueous fermented extracts for the treatment of cramps and anxiety. To broaden the understanding of the active principles involved, a model fermentation approach was developed and fermentates were compared to commercially manufactured tinctures. Coumarins and hydroxycinnamates were among the major secondary metabolites characterized using HPLC-MS^n. After six months of fermentation and storage, low-molecular organic acids were detected by GC-MS. Fermentation contributed to the stabilization of antioxidant and radical scavenging activities, which were in a range of about 8–10 mg gallic acid equivalents/g dry weight and 20–24 mg trolox equivalents/g dry weight, determined by Folin-Ciocalteu and DPPH assays, respectively. In addition, antibacterial activities of the extracts against Gram-positive and -negative bacteria increased during the first week of fermentation. Fermentates were neither cytotoxic nor pro- or anti-inflammatory. Thus, fermentation of chamomile roots is a suitable method for the safe production of biofunctional aqueous chamomile root extracts that remain stable without the addition of synthetic preservatives.

Introduction

Lactic acid fermentation has been used since ancient times to preserve food and other natural products. Nowadays, with the predominance of further preservation techniques, research on fermentation is mainly focused on its potential to improve the bio- and technofunctional properties of the respective substrate.[1–3] Typical changes occurring upon fermentation include carbohydrate metabolism going along with the formation of lactic and other organic acids, ethanol, peptides and vitamins. In human nutrition, the bioavailability of nutrients such as minerals or vitamins may be improved.[1,4] The effects of fermentation on bioactivity have been studied for various substrates, with a strong focus on popular fermented foods, i.e. yoghurt and other dairy products, cereals and vegetables.[1,2] For instance, fermentative hydrolysis, decarboxylation, or (de)methylation of polyphenols may improve mitochondrial function and enhance antioxidant and anti-inflammatory effects.[5,6]

Fermentation may occur either after inoculation or spontaneously. Inoculation involves the addition of starter cultures into the medium, which then dominate the fermentation process, making the fermentation safe, controllable and reproducible.[7] Starter organisms strongly influence the pH and chemical composition of the fermentate. E.g., different *Lactobacillus* species and even different strains may vary in the amounts of free amino and fatty acids, esters and aldehydes produced, as demonstrated for purslane juice[8] and may also vary in their enzymatic activities.[9] *Lactiplantibacillus plantarum*[10] (formerly *Lactobacillus plantarum*[11]) is an ubiquitous LAB species often used as a starter culture in the food industry. This species is regarded as safe by the US Food and Drug Administration as well as by the European Food Safety Authority. *L. plantarum* is commercially used as probiotic.[12] This use is favored by its tolerance to low pH values and high salt concentrations.[13] Further, health benefits upon fermentation with *L. plantarum* have been described, including suppression of human colon

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cancer cell growth and anti-inflammatory activity.[14] In addition, *L. plantarum* exhibits antioxidant activity and is able to produce bacteriocins against foodborne or spoilage bacteria, thus prolonging the shelf life of fermented goods.[12]

In contrast, spontaneous or wild fermentation is driven by a consortium of microorganisms naturally occurring on the plant surface. This microbial community is often more robust, even against bacteriophages, than is an isolated starter culture.[1] However, the sensory outcome may vary and has often not been studied in further detail.[1] Spontaneous fermentation is frequently applied in the production of many traditional foods and beverages,[1] traditional Chinese medicine[15] and European complementary medicine. In the latter case, so-called mother tinctures are produced, the manufacturing of which is described in the European Pharmacopoeia and in national pharmacopoeias, such as the German Homeopathic Pharmacopoeia (GHP).[16] Aqueous fermented chamomile (*Matricaria recutita* L.) root extracts are one example of such mother tinctures obtained by spontaneous fermentation. According to GHP method 33c, freshly harvested roots are minced, then honey, lactose and water are added. The specified process then favors the growth of lactic acid bacteria (LAB) from the rhizosphere. The pH drops to values below 4.0 due to the formation of lactic acid or other acids. LAB support fermentative extraction by degrading the plant matrix and cell walls. This enhances extraction yields, thus, increasing the content of secondary metabolites in the corresponding extracts.[4] After one week the fermentative extraction is stopped by filtration and the filtrate is stored for at least six months.[16] During this time, further transformation or degradation of secondary metabolites may occur.[17] Although fermented chamomile root extracts are applied in complementary medicine for their antispasmodic and soothing properties,[18] to the best of our knowledge nothing is known about fermentative changes occurring upon manufacturing.

In contrast, the fermentation of chamomile flowers has been investigated in a number of studies, although revealing inconclusive results. For example, fermentation of chamomile flower extracts was found to either enhance or suppress their antioxidant, antimicrobial and cytotoxic activities.[19,20] Spontaneous fermentation based on the genuine microbiome in a 2.5% sodium chloride solution resulted in the release of apigenin from its glucoside. This was expected to enhance antioxidant effects, however, due to an overall decrease in total phenolic content, unfermented samples showed stronger activity.[4] In contrast, flavonoid content and DPPH scavenging activity increased upon fermentation of chamomile flowers with *L. plantarum.*[19]

To fill the aforementioned knowledge gap with regard to chamomile roots, we recently investigated the phytochemical composition and biofunctional properties of fresh chamomile roots.[21] In continuation of these analyses, the present study focused on a detailed investigation of changes in the phytochemical profile by GC-MS and LC–MS^n and in antibacterial, antioxidant and antiinflammatory properties of aqueous chamomile root extracts obtained upon fermentation with *L. plantarum.* These fermentation experiments were performed in small-scale model systems and compared to commercial mother tinctures produced according to the GHP. Such studies are expected to significantly increase the knowledge on phytomedicinal preparations and contribute to the production of extracts with well-defined and known chemical composition and bioactivity.

Results and Discussion

To improve our understanding of the process of mother tincture production, a simplified modification of the procedure described in the GHP was developed first. For this purpose, aqueous chamomile root extracts were inoculated with *L. plantarum*, and a fermentation temperature of 33 °C for the first three days with subsequent storage at room temperature turned out to be suitable.

The initial pH value of fresh root extracts was slightly above 6. During the first 24 h, the pH decreased to 3.6–3.7. From day three, all *L. plantarum* fermented (LF) extracts had pH values of 3.4 ± 0.1 and a lactic acid content of 4.43 ± 0.01 mg/mL. Both parameters remained unchanged during the whole storage time of 6–24 months. Mother tinctures (MT) prepared at WALA Heilmittel GmbH according to the official GHP method 33c had pH values of 3.5 ± 0.25, indicating a similar course of fermentation in the simplified model process.

The dry matter content of the obtained LF extracts was found to be 15 mg/mL on the first day and 17–19.5 mg/mL during the whole fermentation and storage period. This increase is presumably due to the enhanced release of solubles as a result of the fermentative breakdown of cell wall and middle lamella polymers by LAB. In contrast, Park et al reported decreasing solid contents during three days of fermentation, which remained constant afterwards.[19] Possibly, compounds were precipitated from the solution in this latter study. All MT used for comparison in the present study had a dry weight of 12.9 ± 0.5 mg/mL. This difference is attributed to the comminution using an Ultra-Turrax which enables a more complete extraction than crushing in a mortar as specified in the GHP.

Changes in the Polar Secondary Metabolite Profile

Changes in the polar secondary metabolite profile upon fermentation were analyzed using RP-HPLC-DAD-ESI-MS^n in negative ionization mode. Figure 1 displays representative base peak chromatograms of aqueous *M. recutita* root extracts in the course of a six-month fermentation period and of two MT produced according to GHP. Peak numbers refer to assignments specified in Table 1. As can be seen in the chromatograms, major changes occurred within the first week of fermentation. More precisely, most metabolic reactions occurred between day one and three (data not shown), which goes along with a maximum in LAB vitality and maximal pH decrease. Upon further storage for six months, these changes were less pronounced than in the first week, although a significant decrease in chicoric acids contents could be observed. Couma-

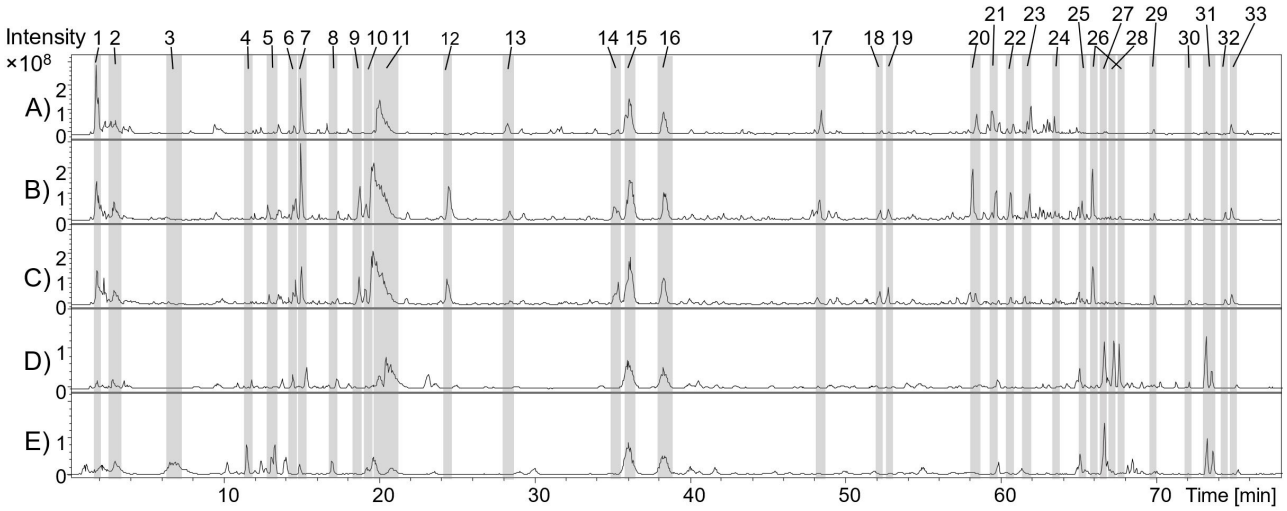

Figure 1. RP-HPLC-ESI-MS^n base peak chromatograms of aqueous *M. recutita* root fermentates on A) day 0, B) day 7 and C) day 180 of lactic acid fermentation in comparison to two mother tinctures produced according to GHP in D) 2019 and E) 2018.

rins and acetylquinic acids were among the main constituents in both, LF extracts and MT, while differences between both fingerprints were obvious in retention time (t_R) ranges between 10–18 min and 65–75 min, respectively.

At early retention times, a saccharide derivative (compound **1**) and citric acid (**2**) were assigned based on their fragmentation behavior and on literature data. As deduced from the intensities of these two signals, the saccharide was obviously metabolized, while citric acid contents slightly increased upon fermentation. Compound **3** was assigned to an organic acid ester due to neutral losses of 60 Da (acetic acid) and 90 Da (lactic acid). However, its structure could not unequivocally be assigned. Neutral losses of 60 and 90 Da often indicate the presence of C-glycosides.[34] However, the formation of C-glycosides by lactic acid bacteria in MT appears unlikely. Accordingly, compound **4** was tentatively assigned to a lactic acid ester due to a neutral loss of 90 Da. The retention time range of 18–35 minutes comprised several derivatives of coumarins and caffeic acid as deduced from the mass spectra of the respective compounds. Compounds **6**, **9** and **10** were assigned to caffeoylquinic acids (CQA), and compounds **12** and **14** to derivatives thereof. Interestingly, dicaffeoylquinic acids were not detected in this study, although these have previously been found in chamomile roots,[21,35] stems, leaves[35] and flowers.[36] This is probably the result of poor solubility of these compounds in aqueous systems. During the first days of fermentation, an increase in CQA was detected. Two more caffeoylquinic acid derivatives were obviously formed during this early fermentation stage, possibly products originating from LAB metabolism.

The MS^2 and MS^3 spectra of compounds **7** and **11** indicated the presence of the coumarin glucoside fraxin and its aglycon fraxetin, which were also characterized in our previous study in root EtOAc and BuOH extracts.[21] Due to neutral losses of 80 Da in the first fragmentation step, the respective coumarins were characterized as sulfuric acid esters.[21] Another fraxetin derivative (**13**) was assigned based on its MS^2 and MS^3 spectra, however, could not be unambiguously characterized. During fermentation, fraxin was partly converted into its aglycon. The occurrence of coumarins is of particular interest for applications in medical and cosmetic preparations, since these have been reported to possess anticoagulant, anti-platelet, anti-inflammatory and antimicrobial activities.[37] Further derivatives of this compound class, e.g. hydroxylated and methoxylated coumarins such as umbelliferone and herniarin, have also been detected in chamomile flowers.[38]

A variety of phenolic acid derivatives were eluted in a retention time range of 35–73 minutes. Compounds **15** and **16** showed identical fragmentation patterns. Compound **15** had distinct UV maxima at 238 and 218 nm, although with very low intensity, while no UV signal was detected for compound **16**. This is presumably due to the lower concentration of **16**, as indicated by the smaller peak in the UV trace. Based on the low UV absorbance and a comparison of the mass spectra with literature data, both compounds were tentatively assigned to acetylquinic acids, which were previously described in chamomile roots[21] and which remained stable throughout the fermentation. Compounds **18**, **20** and **21** exhibited similar fragmentation patterns in the MS^2-MS^4 spectra (Figure S1, supplementary material). Compounds **20** and **21** showed precursor ions at *m/z* 515, corresponding to dicaffeoylquinic acids. However, the most intense product ions were found at *m/z* 473, 311 and 179. Thus, both substances were tentatively assigned to acylated chicoric, i.e. dicaffeoyltartaric, acid isomers based on their mass spectral data, which were compared to literature findings. Chicoric acid has also been identified in various chamomile flower extracts.[39] In comparison, substance **22** revealed differences in its *m/z* ratios in the MS and MS^2 spectra of four Da as compared to compounds **20** and **21**, whereas subsequent fragments were identical to the aforementioned compounds. Thus, it was assigned to the tetrahydro derivative of **20**/**21**. Finally, compound **23** exhibited the same

Table 1. HPLC-DAD-ESI-MS^n data obtained in negative ionization mode of compounds detected in freshly prepared and fermented extracts of *M. recutita* roots.

#[a]	t_R [min]	Compound	λ_{max} [nm]	Mass spectrometric data [*m/z*]			Reference	Fresh roots[b]	Content[c]
				MS^1	MS^2	MS^3			
1	1.8	Saccharide	ND[d]	404[e,f]	179,[f] 161, 143		[22]	✓	↓
2	2.8	Citric acid	ND[d]	191[e,f]	173, 111[f]		[23]	×	↑
3	7.3	Acetic and lactic acid ester	ND[d]	545, 495[e,f]	405, 345[f]	220,[f] 119	tentative	×	MT only[g]
4	11.9	Lactic acid ester	ND[d]	537[e,f]	447, 345, 220[f]	119	tentative	×	MT only[g]
5	13.7	undefined	260, 346	439[e,f]	241[f]	223, 139, 97		×	↑
6	14.6	3-*O*-Caffeoylquinic acid	324	353[e,f]	191,[f] 179	171, 85	[24,25]	✓	↑
7	15.1	Fraxin sulfate	206, 228, 284	449[e,f]	369,[f] 241	207, 192	[21]	✓	↓
8	17.2	Peptide	ND[d]	451[e,f]	433, 335[f]	292, 173, 130	[26]	×	↑
9	18.9	5-*O*-Caffeoylquinic acid	218, 235sh, 290sh, 324	353[e,f]	191,[f] 173		[24]	✓	↑
10	19.1	4-*O*-Caffeoylquinic acid	238, 326	707, 353[e,f]	191, 173[f]	137	[24]	✓	↑
11	19.9	Fraxetin sulfate	206, 230, 338	287[e,f]	207[f]	192	[21]	✓	→
12	24.5	5-*O*-(4′-*O*-Hydroxy-dihydro-caffeoyl-glucosyl)quinic acid	283, 322	533[e,f]	371[f]	353, 209,[f] 191	[27]	×	↑
13	28.4	Fraxetin derivative	310	585, 517, 411[e,f]	433, 335, 207[f]	192	[28]	×	↑
14	35.4	Caffeoyl hexoside/dimer	208, 228, 342	341[e,f]	179, 161[f]	147, 119[f]	[29,30]	×	↓
15	36.2	Acetylquinic acid	236, 318	233[e,f]	171, 143[f]	59	[27,31]	✓	↑
16	38.3	Acetylquinic acid	ND[d]	233[e,f]	171, 143[f]	113, 73	[27,31]	✓	→
17	48.4	Dicoumaroyl-glycerol	ND[d]	427, 383[e,f]	368, 221, 161[f]	143,[f] 113	[32]	×	↓
18	52.2	Chicoric acid derivative	268	563, 519, 473[e,f]	263,[f] 221, 179	179,[f] 149, 113	[30]	×	↑
19	52.8	Caffeoyl-2,7-anhydro-2-octulo-pyranosonic acid	ND[d]	487, 443[e,f]	397[f]	353, 161,[f] 113	[33]	×	↑
20	58.5	Chicoric acid (acetyl derivative)	268	515[e,f]	473,[f] 305	311, 263, 221, 179	[30]	✓	↑↓
21	60.0	Chicoric acid (acetyl derivative)	268	515[e,f]	473,[f] 311, 263, 179	311	[30]	✓	↑↓
22	61.0	Tetrahydrochicoric acid	ND[d]	519[e,f]	477[f]	263, 221,[f] 179, 161	tentative	✓	↑↓
23	62.0	Chicoric acid derivative	268	645[e,f]	557,[f] 515, 353, 305	515,[f] 473, 305, 263, 161	tentative	×	↓
24	63.5	Caffeoylcinnamic acid derivative	316	535[e,f]	447, 405, 243[f]	183	tentative	×	↓
25	65.3	Undefined	222, 234, 278	373[e,f]	343, 314[f]	285,[f] 269, 255		×	↑↓
26	65.9 67.9	Coumaroyl-caffeoyl-hydroxy-phenol	268	417[e,f]	373, 355[f]	310, 279	tentative	×	↑
27	66.5	Trihydroxy-octadecenoic acid isomer	308	329[e,f]	311, 293, 229, 211,[f] 171, 155	183, 167, 107	[32]	×	MT only[g]
28	67.2	Dihydroferulic and dihydroxy-phenyllactic acid depside	268	345[e,f]	195[f]	177, 151,[f] 135	tentative	×	MT only[g]
29	69.9	Hydroxy-octadecatrienoic acid isomer	ND[d]	293[e,f]	236,[f] 221	218, 192[f]	[32]	×	↑

Table 1. continued

#[a]	t_R [min]	Compound	λ_{max} [nm]	Mass spectrometric data [*m/z*]			Reference	Fresh roots[b]	Content[c]
				MS^1	MS^2	MS^3			
30	72.5	9- or 12-Hydroperoxy-octadecadienoic acid	334	311[e,f]	293, 201[f]	155, 137	[32]	×	↑↓
31	73.5	Dihydroxy-octadecanoic acid	ND[d]	315[e,f]	297,[f] 185	279, 197, 185	tentative	×	MT only[g]
32	73.8	9,10-Dihydroxy-octadecenoic acid	ND[d]	313[e,f]	295, 201, 155[f]	127	[32]	×	↓
33	74.9	Dihydroxy-octadecenoic acid isomer	ND[d]	313[e,f]	297[f]	281, 265	[32]	×	→

[a] Peak labeling refers to Figure 1; [b] indicates if the substance has (✓) or has not (×) been described in EtOAc or *n*-butanol extracts of fresh chamomile roots[21]; [c] change in content of the respective compound during the six-month observation period as deduced from signal intensities; [d] not detected; [e] molecular ion $[M-H]^-$; [f] base peak which was further fragmented by collision induced dissociation; [g] only detected in fermented mother tinctures.

mass transition from *m/z* 515 to 473 as compounds **20** and **21** in the MS^3 experiments. It was therefore also tentatively assigned to a chicoric acid derivative. The MS^2 base peak at *m/z* 557 ($[M-H-88]^-$) was formed by the loss of two carboxyl groups, the MS^3 base peak at *m/z* 515 by the loss of an acetyl group. However, further structural details could not be deduced, which precludes exact structure assignment of this constituent. Interestingly, the contents of these compounds, which among others exhibit hypoglycemic and immunostimulating effects,[40] increased during the first week of fermentation. This is presumably due to successive extraction of the plant material and partial cell wall degradation as a result of microbial activity.[4] Over the next few months, chicoric acids were slowly degraded, possibly by enzymatic decarboxylation or side chain hydration by *L. plantarum* enzymes,[41] or by oxidation reactions.

Finally, compounds **27** and **29**–**33** were characterized as oxidized C18 fatty acid constituents. These compounds, which have a pronounced antifungal activity, can be produced by various lactobacilli and have also been found in sourdough[42] or fermented rice rinse water.[43] Here, the differences between LF extracts and MT were particularly pronounced. Three derivatives (compounds **27**, **28** and **31**) were only detected in MT. This may be due to oxidation reactions occurring during storage, or because the diverse community of microorganisms in the case of mother tincture fermentation has a more complex fatty acid metabolism.

Low Molecular Weight Compounds in Fermented Samples

For the analysis of volatile constituents, these were extracted with EtOAc from 12 months old LF extracts and MT and further analyzed by gas chromatography after derivatization with BSTFA. In unfermented aqueous extracts, hardly any substance could be detected following this procedure. In contrast, compounds from fermented extracts were eluted in two retention time ranges, 6.5–16 and 20–40 min, respectively. The corresponding chromatograms are displayed in Figure 2. Identification of the substances was facilitated by automated comparison with the NIST database (National Institute of Standards and Technology, Table 2). Interestingly, the en-yn-dicycloether was the only characteristic constituent assigned previously in chamomile flowers[44] and roots[21] while the other compounds were mostly formed during fermentation. Lactic acid (t_R 8.1 min) was by far the most abundant constituent in all extracts. Using an enzyme assay its concentration was found to be approx. 4.4 mg/mL, which is comparable to LAB fermented *Mercurialis perennis* extracts, where slightly lower concentrations were determined.[45] The content of succinic acid (t_R 14.5 min) was more than three times higher in MT, indicating that various heterofermentative microorganisms play a significant role in the spontaneous fermentation of *Matricaria* roots, whereas, unsurprisingly, such metabolites were less pronounced in the model experiments performed with an isolated microbial strain. Accordingly, succinic acid was also found as a pronounced constituent in spontaneously fermented *Hamamelis virginiana* extracts.[17] In general, alcohols and carboxylic acids were the most abundant compound classes in both, LF extracts and MT. These may originate from LAB metabolism or be formed upon fermentation from the phenolic compounds detected by HPLC-MS. Analogous compounds have also been detected in fermented foods such as tamarillo juice[46] or rice beer.[47] Phenyllactic and hydroxyphenyllactic acids are lactobacterial deamination products of the amino acids phenylalanine and tyrosine[48] with the former occurring in higher concentrations in MT. Pronounced antifungal activity of organic acids such as lactic, citric and phenyllactic acids have been demonstrated in numerous studies,[43] which presumably contributes to the microbial stability of the aqueous extracts examined in the present study. A slightly higher number of constituents have been detected in MT than in LF extracts (Table 2). This also points to the fact, that the microbiota involved was more heterogenous than in extracts fermented upon inoculation with *L. plantarum*. Nevertheless, the similarity between LF extracts and MT in their chromatographic profile is particularly interesting, because the starter culture may strongly influence the metabolism and content of secondary constituents, as has been demonstrated e.g. for kimchi.[49] Thus, it can be assumed that LAB closely related to *L. plantarum* are among the most important microorganisms in MT fermentation.

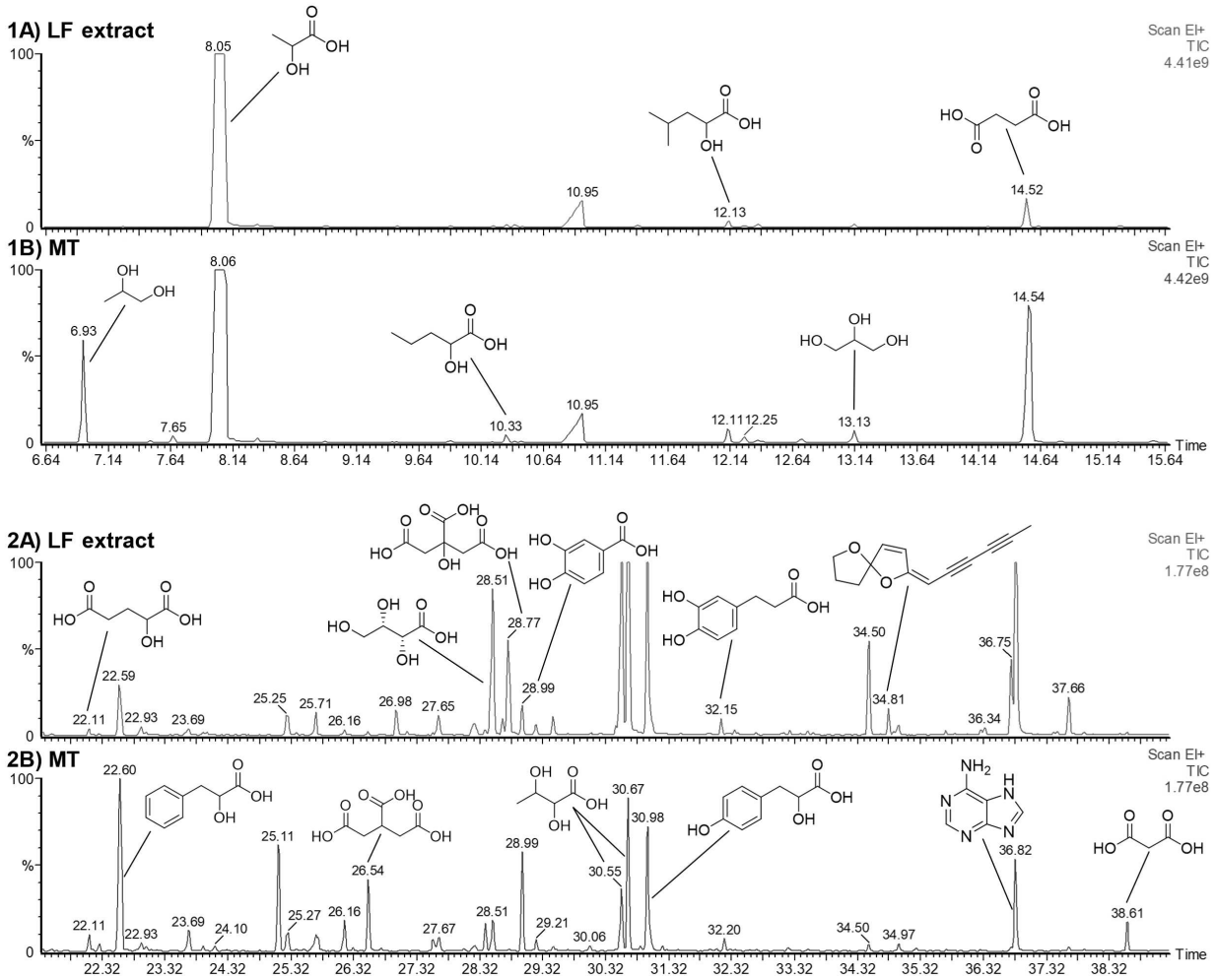

Figure 2. Relevant sections (**1** t_R 6.5–16 min, **2** t_R 20–40 min) of the chromatograms of **A)** lactobacteria fermented (LF) extracts and **B)** a mother tincture (MT) analyzed via GC-MS after EtOAc extraction and derivatization. For a better overview, structures are displayed in unsilylated form.

To conclude, despite some minor variations, model fermentation with *L. plantarum* was shown to be a suitable simplified model for studying the fermentation pathways of mother tinctures produced according to the GHP.

Reducing Properties and Antioxidant Activity of *M. recutita* Root Extracts

The Folin-Ciocalteu assay is based on the measurement of the reductive power of a sample. Since polyphenols in general are characterized by such properties, this assay is often used for the semi-quantitation of phenolic compounds. However, in the presence of further compounds displaying strong reducing activity, the assay is of limited significance.[50]

The phenolic content resp. antioxidant activity of chamomile root extracts was determined throughout two weeks of fermentation and upon storage for up to three months. Gallic acid was used as reference substance and results were expressed as gallic acid equivalents (GAE) per g dry weight (see Figure 3). Method details and calibration data can be found in the supporting information (Table S1–S4). Antioxidant activity was found to range from 8 to 10 mg GAE/g dry weight. Fluctuations of this parameter were observed during the first week of fermentation. Between days 14 and 30, the antioxidant

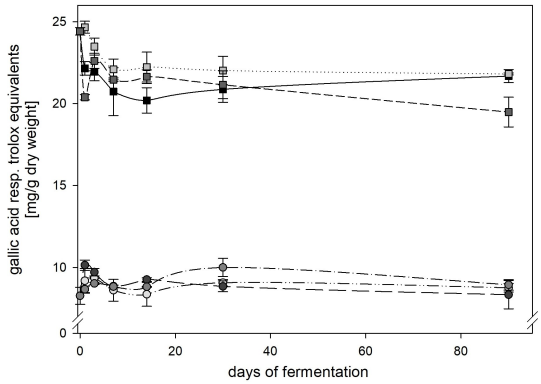

Figure 3. Phenolic contents (●) and DPPH radical scavenging activities (■) of aqueous *M. recutita* root extracts during three months of lactic acid fermentation. Results are expressed as mean ± SD ($n = 3$). Data are displayed for three representative fermentation batches.

Table 2. Compound assignment of volatile constituents in ethyl acetate extracts of lactobacteria fermented *M. recutita* root extracts (LF) and mother tinctures (MT) via GC-MS analysis after derivatization.

Constituent	t_R [min]	MW [Da]	Fragment *m/z* (Intensity %)	LF	MT
1,2-Propanediol	6.9	220.5	205 (5),[a] 147 (57),[b] 117 (100), 73 (78)[b]		✓
Lactic acid	8.1	234.4	219 (10),[a] 191 (28), 147 (100),[b] 117 (90), 73 (67)	✓	✓
2-Hydroxy-3-methylbutyric acid	10.3	262.5	247 (6),[a] 219 (12), 147 (71),[b] 145 (100), 73 (87)[b]		✓
N,N-Dimethylamino-methylidene-trifluoro-acetamide[c]	11.0	168.1	168 (2), 99 (100), 69 (24)	✓	✓
2-Hydroxyisocaproic acid	12.1	276.5	159 (81), 147 (55),[b] 103 (100), 73 (96)[b]	✓	✓
Ethylsuccinic acid	12.7	218.3	203 (34),[a] 175 (41), 157 (19), 129 (21), 75 (100), 73 (92),[b] 55 (37)		✓
Glycerol	13.1	308.6	218 (25), 205 (59), 147 (84),[b] 133 (20), 117 (33), 103 (33), 73 (100)[b]		✓
Butanedioic acid	14.5	262.4	247 (13),[a] 147 (100),[b] 73 (49)[b]	✓	✓
2-Hydroxyglutaric acid	22.1	364.2	349 (4),[a] 247 (66), 147 (70),[b] 129 (81), 73 (100)[b]	✓	✓
4-Hydroxy-phenylethanol	22.3	282.4	282 (17), 267 (12),[a] 193 (14), 179 (100), 103 (12), 73 (53)[b]		✓
β-Phenyllactic acid	22.6	310.5	267 (6), 220 (14), 193 (69), 147 (59),[b] 73 (100)[b]	✓	✓
Methylmaleic acid	22.9	274.5	247 (7), 184 (29), 147 (100),[b] 73 (98)[b]	✓	✓
p-Hydroxybenzoic acid	23.7	282.5	282 (28), 267 (53),[a] 268 (10), 193 (24), 73 (100)[b]	✓	✓
Cyclooctene diol	25.1	286.2	286 (35), 260 (22), 217 (23), 169 (45), 147 (38),[b] 116 (33), 73 (100)[b]		✓
Methylsuccinic acid	25.3	360.6	303 (51), 147 (46),[b] 73 (100)[b]	✓	✓
D-Erythro-pentonic acid	25.7	527.0	348 (34), 147 (33),[b] 129 (26), 103 (30), 73 (100)[b]	✓	✓
Suberic acid	26.2	318.6	303 (50),[a] 287 (20), 213 (29), 147 (61),[b] 103 (22), 75 (83), 73 (100),[b] 69 (50), 55 (51)	✓	✓
Propane-1,2,3-tricarboxylic acid	26.5	392.7	377 (35),[a] 217 (21), 185 (28), 147 (100),[b] 73 (80)[b]		✓
cis-Aconitic acid	27.0	390.7	375 (9),[a] 229 (31), 147 (69),[b] 73 (100)[b]	✓	
2-Hydroxysebacic acid	27.7	434.2	419 (3),[a] 317 (60), 147 (44),[b] 73 (100)[b]	✓	✓
Azelaic acid	28.4	332.6	317 (31,[a] 201 (27), 147 (30),[b] 129 (23), 117 (24), 75 (81), 73 (100),[b] 55 (35)		✓
L-Threonic acid	28.5	424.8	375 (4), 292 (34), 289 (34), 147 (55),[b] 73 (100)[b]	✓	✓
Citric acid	28.8	480.2	465 (6),[a] 273 (69), 147 (62),[b] 73 (100)[b]	✓	
Protocatechuic acid	29.0	370.1	370 (37), 355 (24),[a] 311 (21), 193 (100), 73 (85)[b]	✓	✓
Phthalic acid[d]	29.2	310.5	309 (25), 295 (30),[a] 207 (9), 147 (31,[b] 133 (11), 73 (100)[b]	✓	✓
Glyceric acid	29.5	322.6	292 (59), 220 (14), 147 (58),[b] 73 (100)[b]	✓	
Tartaric acid isomer	30.6	438.8	303 (24), 292 (55), 185 (19), 147 (53),[b] 95 (23), 73 (100)[b]	✓	✓
Tartaric acid isomer	30.7	438.8	303 (34), 292 (50), 221 (9), 147 (62),[b] 95 (17), 73 (100)[b]	✓	✓
4-Hydroxyphenyllactic acid	31.0	398.7	383 (2),[a] 355 (4), 308 (37), 179 (100), 147 (20),[b] 73 (49)[b]	✓	✓
Hydrocaffeic acid	32.2	398.7	398 (41), 280 (21), 267 (40), 179 (100), 73 (71)[b]	✓	✓
Undefined sesquiterpene lactone	34.5	220.4	220 (100), 192 (94), 178 (46), 164 (55), 150 (49), 135 (43), 122 (65), 108 (43), 97 (29)	✓	✓
En-yn-dicycloether	34.8	200.2	200 (97), 172 (96), 157 (28), 144 (33), 128 (56), 115 (100), 102 (32), 88 (46), 73 (68),[b] 63 (34)	✓	
Scopoletin	35.0	264.3	264 (39), 234 (100), 206 (47), 191 (8), 176 (11), 73 (43)[b]	✓	✓
Caffeic acid	36.7	396.7	396 (57), 381 (16),[a] 219 (100), 191 (16), 73 (95)[b]	✓	
Adenine	36.8	279.5	294 (36), 279 (26), 264 (100),[a] 249 (8), 236 (15), 221 (10), 193 (9), 73 (32)[b]	✓	✓
Linoleic acid	37.7	352.6	352 (33), 337 (100),[a] 73 (66)	✓	
Malonic acid	38.6	248.4	233 (64),[a] 147 (44),[b] 73 (100)[b]		✓

[a] $[M-CH_3]^+$; [b] *m/z* 73 $[C_3H_9Si^+]$, *m/z* 147 $[C_6H_{15}SiO_2^+]$; [c] silylation artifact, [d] plasticizer.

activity data stabilized and remained almost constant upon further storage. The same tendency was observed for the radical scavenging activity of the extracts determined by the DPPH assay. Here, the results were expressed as trolox equivalents (TE) in mg per g dry weight (Figure 3). Only one batch showed a decreasing activity during the first 24 h of fermentation, followed by an increase until day three and a further but very slight decrease during the next months. In all other cases, the radical scavenging activity decreased during the first week of fermentation and remained comparatively constant thereafter. The radical scavenging activity of all LF samples ranged from 19.5 to 24.5 mg TE/g dry weight. MT produced in 2011 and 2019 had activities of 20.1 and 14.2 mg TE/g dry weight and 10.3 and 11.8 mg GAE/g dry weight, respectively. This indicates that the initial radical scavenging activity may vary between different batches in absolute values, but remains fairly constant after the first fermentation period.

Among other components, phenolic metabolites, which were characterized by HPLC-MS^n, are covered when applying the Folin-Ciocalteu assay. The changes in the quantitative data observed within the first week of fermentation may therefore be a result of the metabolic conversion of individual phenolic compounds exhibiting different antioxidant activities, in combination with enhanced extraction of secondary metabolites from the plant matrix as a consequence of progressive enzymatic cell wall degradation. After one week of fermentation, the suspensions were filtered, thus, excluding further extraction of the solid plant material. Furthermore, the metabolic activity of LAB is expected to decrease upon prolonged fermentation and storage. Although aerobic storage would allow further oxidation reactions, the measured values reached a constant level after about two weeks, which indicates a mutual stabilization of the different components in the fermentate. In general, despite some initial variations, antioxidant activities determined via the Folin-Ciocalteu assay were found to be comparable in fresh and fermented chamomile root extracts. In contrast, total phenolic contents of chamomile ligulate flower extracts were previously found to decrease from 21.75 to 18.76 mg GAE/g upon fermentation with *L. plantarum* for 72 h.[19] This is in accordance with previous studies which revealed methanolic extracts of chamomile flowers harvested at different locations in Italy to roughly range from 5 to 25 mg GAE/g dry weight.[51] Thus, the phenolic contents and the associated antioxidant potential appear to be affected by the growth conditions and, of course, the respective plant parts under investigation, with flowers usually showing stronger effects than roots.

The antioxidant properties of coumarins as well as caffeic and quinic acid derivatives, which were among the main compounds in the extracts, have been extensively investigated in the past.[52] As these were only partly metabolized throughout storage, it was likely that the antioxidant properties of the extracts also changed only slightly. Indeed, the DPPH radical scavenging capacity slightly decreased within the first seven days of fermentation and remained constant thereafter. In contrast to our results, an increase in DPPH scavenging capacity has previously been reported for chamomile flower extracts upon fermentation.[4,19,53] This may, firstly, be attributed to the fact that a number of flavonoid glycosides such as apigenin-7-glucoside were found in the latter.[36] When the saccharide moiety is released as a result of microbial enzyme activities, the corresponding flavonoid aglycone is formed, which normally is characterized by stronger antioxidant activity.[53] Accordingly, the antioxidant activity of fermented chamomile flower extracts increased by approximately 11 % during 72 h of fermentation.[19] However, flavonoids could not be detected in chamomile root extracts via HPLC-MS^n. Secondly, the formation of soluble lignin oligomers with marked antioxidant properties was detected during a four-week LAB fermentation of chamomile flower extracts.[4] Some of the unknown components addressed in Figure 1 and Table 1 may result from lignin degradation, but these could not be unambigously characterized. Thus, the fluctuations observed in this study are probably due to changes in the phenolic profile occurring upon fermentation.

To conclude, fermentation of fresh chamomile roots is a convenient way of preserving their antioxidant activity and stabilizing phenolic constituents for an extended period of time. Since the latter are known as potentially health-promoting components,[54] fermentation appears to be a suitable method for the production of stable extracts with biofunctional properties, which may be used e.g. for the manufacturing of phytopharmaceutical preparations.

Antibacterial Activity

The antibacterial activity of aqueous chamomile root extracts was assessed during fermentation applying a microdilution assay. The results are displayed in Table 3. While inhibitory effects were not observed for fresh root extracts, the growth of *P. aeruginosa* was inhibited beginning with day one (minimal inhibitory concentration, MIC, 6.3 mg/mL) and of *E. coli*, *S. aureus* und *B. subtilis* beginning with day 3. According to current literature, an antibacterial effect of plant extracts is only considered relevant at MIC values below 1 mg/mL.[55] The values determined in this study were significantly higher. Thus, fermented chamomile root extracts are not suitable as potential alternatives to conventional antibiotics. Nevertheless, the results demonstrate that fermentation contributes to the microbiological stability of the obtained extracts.

Table 3. MIC values [mg/mL] of *M. recutita* root extracts evaluated during fermentation (three batches each in triplicate) in comparison to a mother tincture produced in 2018 and lactic acid as a reference.

	P. aeruginosa	*E. coli*	*S. aureus*	*B. subtilis*
Day 0	>6.3	>6.3	>6.3	>6.3
Day 1	6.3	>6.3	>6.3	>6.3
Day 3	4.2	4.2	6.3	6.3
Day 7	4.2	4.2	6.3	6.3
Day 30	4.2	4.2	6.3	6.3
Day 365	8.0	4.0	4.7	4.0
Mother tincture	3.9	3.9	3.9	3.9
Lactic acid	>4.5	1.6	1.6	1.6

Interestingly, during the first 30 days, MIC values for Gram-negative bacteria (*P. aeruginosa*, *E. coli*) were lower than those for Gram-positive strains (*S. aureus*, *B. subtilis*). In contrast, our previous study revealed only Gram-positive bacteria to be affected by different chamomile root extracts in an agar disk diffusion assay.[21] This may be due to diffusion effects of the active constituents, which is irrelevant in the microdilution assay. The MT had a similar MIC of 3.9 mg/mL on all strains tested. Lactic acid when tested as pure substance had a MIC value of 1.6 mg/mL for all strains except *P. aeruginosa*, whose growth was not affected by any of the concentrations tested.

In the model fermentation experiments, lactic acid accounted for approx. 0.9–1.3 mg/mL of the total extract dry weight. Thus, the content was below the MIC determined for the isolated compound. This suggests lactic acid to be partly, but not exclusively, responsible for the aforementioned effects of aqueous-fermented chamomile root extracts. In accordance with these results, fermentation of apple juice was found to enhance the inhibition of *E. coli* and *S. aureus*.[41] According to the authors of this latter study, this increased activity was due to progressive accumulation of lactic acid going along with decreasing pH values and the formation of phenolic metabolites exhibiting higher antimicrobial activity compared to their precursor compounds. Cvetanovic et al. compared the MIC values of fresh and spontaneously fermented chamomile flower extracts. In general, *E. coli* was most susceptible, while *S. aureus* and *B. subtilis* proved to be most robust against the extracts studied. Furthermore, aqueous extracts of the fermented samples revealed highest activity against *E. coli*, *C. albicans* and *A. niger*, whereas corresponding ethanol/water (70/30, w/w) extracts were most effective against *E. coli* and *B. subtilis*. All MIC values determined in this study ranged from 19 to 313 μg/mL.[20] In another investigation, the MIC of ethanolic *M. recutita* leaf extract in 5% DMSO was 12.5 mg/mL,[56] thus higher than the values determined in the present study. In general, extracts from chamomile flowers appear to have a stronger antimicrobial effect than extracts from leaves or roots, probably due to their flavonoid content. However, the solvent used for extraction also has a significant impact on the phytochemical composition and thus the respective activity.[57]

Cytotoxic and Inflammation-Modulating Effects of LF Extracts

For the cell culture studies, THP-1 cells were differentiated into macrophages with phorbol myristate acetate (PMA). Cytotoxicity of two different LF extracts after seven and 180 days of fermentation on these cells was assessed by WST assay and by flow cytometry. Fraxin, a coumarin glucoside, which has been detected in *M. recutita* roots, was used as reference. The WST assay is used to estimate the live cell count via their mitochondrial respiratory activity. Cellular mitochondrial dehydrogenases reduce a tetrazolium salt to the corresponding formazan, which can be quantitated colorimetrically. The larger the number of viable cells and thus the higher the activity of the mitochondrial dehydrogenases, the more formazan is formed.[58] Both assays consistently showed that none of the extracts was cytotoxic in the applied concentration range of 1–300 μg/mL (Figure 4). This is of particular interest since cytotoxic effects on various cell lines have been determined for aqueous chamomile flower extracts in concentrations < 100 μg/mL.[20,59]

Subsequently, potential immunomodulating effects of LF extracts were evaluated. The transcription factor NF-κB plays a key role in regulating immune responses upon stimulation. Stimuli that can trigger activation of NF-κB include growth factors, cytokines (e.g. TNF-α and IL-1), but also lipopolysaccharides (LPS) and UV radiation.[6] To study the effects on the NF-κB-eGFP reporter signal, cells were stimulated with LPS and immediately treated with LF extracts or fraxin. Dexamethasone (Dex) served as positive control. The results, relative to the stimulated and untreated control, may indicate a minor pro-inflammatory effect of the fermented chamomile extracts (Figure 5). However, this effect did not differ from that of pure DMSO and was not concentration-dependent. Thus, neither the tested extracts nor fraxin as a reference substance showed a significant influence on the *in vitro* inflammatory response.

In accordance with these results, a slight inhibition of IL-6 and TNF-α expression compared to the stimulated control may be assumed (supporting information; Figure S2). However, the

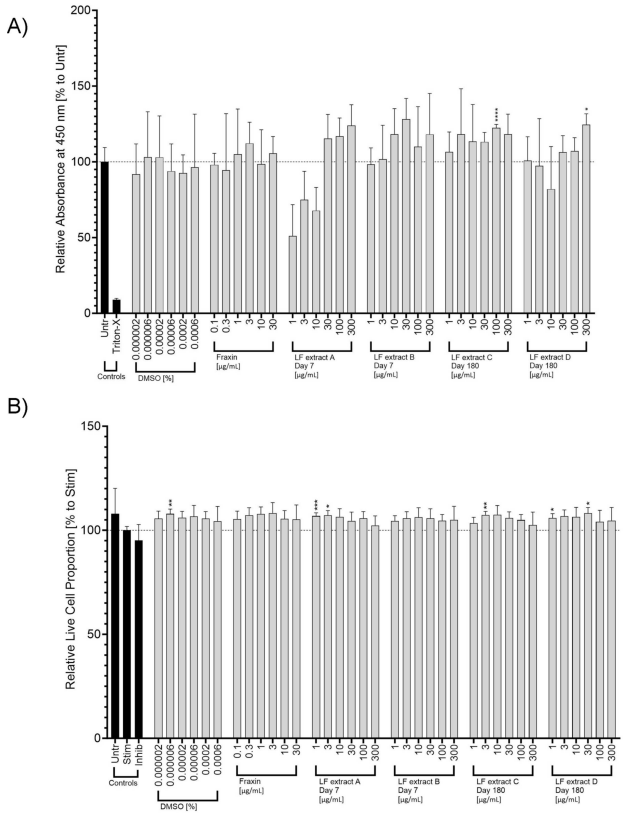

Figure 4. Effects of test substances on cell viability of THP1-NF-κB cells. The results are presented as mean ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. A) WST assay: Cells were incubated for 24 hours with medium (Untr.), triton-X, or samples. The absorbance of the medium at 450 nm was measured and set in relation to the absorbance of the untreated control; $n = 4$. B) Flow cytometry: Percentage of metabolically active cells relative to the unstimulated control. Cells were incubated for 24 hours with medium (Untr.), LPS (100 ng/mL, Stim.), LPS + dexamethasone (10 μM; Inhib.) and LPS + samples; $n = 5$.

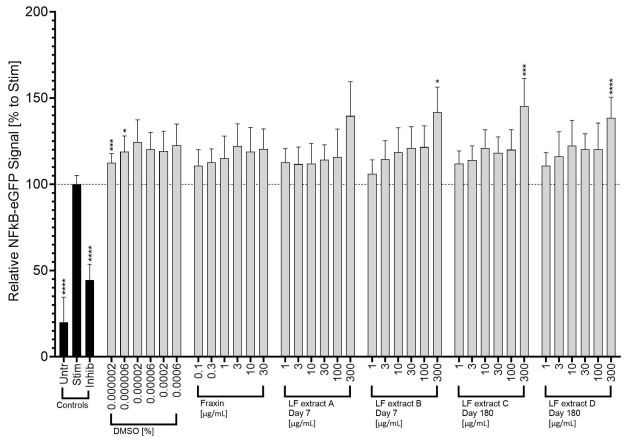

Figure 5. Influence of lactobacteria fermented (LF) chamomile root extracts, DMSO and fraxin on the NF-κB-eGFP reporter signal of THP1-NF-κB reporter cells. Cells were stimulated with LPS (100 ng/mL) and subsequently incubated for 24 h with samples or dexamethasone (10 μM, Inhib.). GFP expression was quantified by flow cytometry. The eGFP median fluorescence intensity of the whole cell population is expressed relative to the stimulated control as mean ± standard deviation. $n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

secretion was much less pronounced than the effect of Dex and was not consistently concentration dependent. At the highest LF extract concentration tested (300 μg/mL), increased secretion and thus proinflammatory activity was observed, most likely due to the endotoxin content which has been assessed by LAL assay (supporting information; Table S5). However, this does not explain the analogous behavior of the reference substance fraxin. In contrast, antioxidant and anti-inflammatory action has been reported in the literature for various coumarins due to their ROS scavenging activity.[60] In a mouse model, fraxin decreased not only the serum levels of TNF-α and IL-6, but also the nitric oxide production and LPS-induced endotoxic shocks.[61] In summary, only low pharmacological action and no cytotoxicity of LF extracts were found in the present study. To intensify the observed effects, the active secondary constituents may be further characterized and enriched from the extracts.

Conclusions

Fermentation is an ancient method for the preservation of food and feed. The present study demonstrated that lactic acid fermentation is also a convenient way of preserving the secondary metabolite profile as well as antioxidant and radical scavenging activities of aqueous medicinal plant extracts, as exemplified for chamomile. The fermentation process enhanced the microbial stability against Gram-positive and -negative bacteria. The aqueous extracts obtained were neither cytotoxic nor did they influence inflammatory processes. Comparison with chamomile root mother tinctures produced according to the GHP demonstrated, that the results obtained in model fermentation experiments with *L. plantarum* were similar to those obtained by spontaneous fermentation of this particular plant part. In summary, the present study highlights the toxicological safety and beneficial biofunctional effects of stable plant extracts that may be exploited in food, cosmetic or pharmaceutical applications. Synthetic preservatives may thus be replaced by plant-derived extracts and, in the case of chamomile roots, by-products originating from agricultural production may be further exploited. Thus, further studies should be conducted to exploit the full potential of fermented plant extracts in a one-health circular concept considering animal, human and environmental health.

Experimental

Chemicals and Reagents

Acetonitrile, ethyl acetate (EtOAc), methanol (MeOH), sodium carbonate and anhydrous sodium sulfate were purchased from Chemsolute (Th. Geyer GmbH & Co. KG, Renningen, Germany). Gallic acid monohydrate and lactose were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), dimethylformamide (DMF), *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), phorbol-12-myristate-13-acetate (PMA), penicillin, streptomycin, triton-X, L-glutamine, lipopolysaccharide (LPS; from O11:B4 *E. coli*) and dexamethasone (Dex) were from Sigma-Aldrich (St. Louis, Missouri, USA), lactic and formic acids from Fluka (Sigma Aldrich, St. Louis, Missouri, USA). Trolox was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), chlorogenic acid hemihydrate from Alfa Aesar (Karlsruhe, Germany), fraxin from PhytoLab (Vestenbergsgreuth, Germany). Folin-Ciocalteu's reagent and Mueller-Hinton-Bouillon (MHB) were from Merck KGaA (Darmstadt, Germany). RPMI medium was from Gibco (Waltham, MA, USA), foetal calf serum (FCS) was from BioConcept (Allschwil, Switzerland), WST-1 assay solution from Roche (Basel, Switzerland). Honey was obtained from the medicinal herb garden of WALA Heilmittel GmbH (Bad Boll/Eckwälden, Germany). Ultrapure water (ELGA Purelab Classic, High Wycombe, UK) was used for all experiments.

Plant Material and Fermentation

Roots of *M. recutita* were harvested in the medicinal plant garden of WALA Heilmittel GmbH (Bad Boll/Eckwälden, Germany) in March 2021. The plant material was rinsed with tap water, drained, packed in polyethylene freezer bags and stored at −70 °C until analysis. A voucher specimen was deposited at the herbarium of the Institute of Botany, Hohenheim University (Stuttgart, Germany). The identity of the plant material was confirmed by Dr. R. Duque-Thüs (voucher number: HOH-022871).

Three commercially produced mother tinctures (MT) were used for comparison with the model fermentations. These were obtained according to the GHP from plant material harvested in 2011, 2018 and 2019 (*Chamomilla recutita e radice ferm 33c*, WALA Heilmittel GmbH, Bad Boll/Eckwälden, Germany).

Fermentation of the plant material was performed according to the German Homeopathic Pharmacopoeia, with certain modifications. Briefly, 100 g of frozen chamomile roots were mixed with 0.75 g each of honey and lactose, subsequently 250 mL water were added. The material was minced for two minutes using an UltraTurrax (17,000 rpm; IKA Werke GmbH and Co. KG, Staufen, Germany). The slurries were then inoculated with 1 mL of a *Lactiplantibacillus plantarum* (previously *Lactobacillus plantarum*, GenBank accession number: MK841313.1; sequence length: 1083 base pairs; closest relative in National Center for Biotechnology Information: *Lactoba-*

cillus plantarum strain 2.7.17, MK611349.1; similarity 100%) suspension[7] in MRS broth (5×10^8 CFU/mL) and kept at 33 °C. On the third day of fermentation, the slurry was filtered through a cotton cloth yielding a turbid solution. From this point on, the solutions were stored in capped glass bottles (250 mL, DWK Life Sciences, Wertheim, Germany) in the dark at room temperature. After seven days, the turbid solution was filtered through filter paper (pore size 2, Whatman™, Buckinghamshire, UK) yielding a clear solution which was used for storage experiments. Three temporally independent fermentation trials were conducted, each of which was performed in duplicate or triplicate. Samples of the lactobacteria-fermented (LF) extracts were withdrawn on days 0, 1, 3, 7, 14 and 30 and every three months during six months of storage, centrifuged (7745×*g*, 10 min; Eppendorf 5430 R, Hamburg, Germany) and the supernatant was used for further investigations.

Determination of Dry Matter and Lactic Acid Contents

25 mL porcelain crucibles were heated at 105 °C for 3 h, cooled in a desiccator and weighed. Plant extracts were centrifuged as described above, 5 mL aliquots of the clear supernatants were pipetted into the dishes in duplicate, dried at 80 °C for one hour and at 105 °C for another two hours. After cooling in a desiccator, the dry matter was quantitated gravimetrically.

Lactic acid was determined using a commercial assay kit (Enzytec™ Liquid D-/L–Lactic acid, R-Biopharm AG, Darmstadt, Germany). Hereby, lactic acid is oxidized to pyruvate catalyzed by the enzyme lactate dehydrogenase in the presence of NAD^+. The reaction product NADH is then determined spectrophotometrically at 340 nm.

RP-HPLC-DAD-ESI-MS^n Analysis of Phenolic Compounds

High performance liquid chromatography with diode array detection was carried out as described previously.[21] For mass spectrometric detection, an HCTultra ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with an ESI source was used. All extracts were analyzed in negative ionization mode using a capillary voltage of 4000 V, a dry gas (N_2) flow of 9.00 L/min with a capillary temperature of 365 °C and nebulizer pressure of 35 psi. MS^n data were acquired in the auto MS/MS mode by collision induced dissociation (CID). The instruments were controlled by Agilent LC 3D systems (Rev. B01.03SR1 (204)) and Bruker Daltonics EsquireControl software (V7.1).

Samples were injected after filtration through a 0.45 μm syringe filter (regenerated cellulose, WICOM Germany GmbH, Heppenheim, Germany) without further pretreatment.

GC-MS Analysis of Volatile Compounds

For GC-MS analyses, 50 mL of fermented aqueous extracts were extracted with 3×20 mL EtOAc. The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated using a rotary evaporator. 10 mg of the highly viscous residue were dissolved in 500 μL DMF and derivatized with 200 μL BSTFA (105 °C, 15 min) to obtain trimethylsilyl derivatives. Analyses were conducted according to.[21]

Folin-Ciocalteu Assay for the Determination of Antioxidant Activity

The plant extracts were diluted fivefold with deionized water. Gallic acid was used as reference compound in concentrations ranging from 1.5 to 47 μg/mL. 20 μL of sample or reference solution was pipetted into a 96-well plate and 40 μL of Folin-Ciocalteu's reagent was added. The plate was shaken in the reader for one minute, and subsequently 160 μL of sodium carbonate solution (700 mM) were added. Absorbance at 765 nm was measured using a multiplate reader (Epoch2, Agilent Technologies Inc., Santa Clara, CA, USA) after incubation at 37 °C for 30 minutes. Absorbance values were plotted against gallic acid concentrations, and antioxidant activity was calculated as gallic acid equivalents [mg gallic acid/g dry weight]. Analyses were performed in triplicate. Absorbance values and calibration data are provided in the supplementary information.

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay for Assessing Radical Scavenging Capacity

DPPH was dissolved in MeOH at a concentration of 100 μM. The same sample dilutions applied in the Folin-Ciocalteu assay were also used in this test system. Trolox was used as reference compound preparing solutions at five different concentrations ranging from 12 to 100 μg/mL. 180 μL of DPPH solution was then added to 20 μL of the test or reference solution or water as a blank sample in a 96-well plate. These solutions were incubated at 37 °C for 45 minutes and then analyzed at a wavelength of 516 nm using a multiplate reader (Epoch2, Agilent Technologies Inc., Santa Clara, CA, USA). Absorbance values were plotted against trolox concentrations, and radical scavenging capacities of the samples were calculated as trolox equivalents [mg trolox/g dry weight]. Analyses were performed in triplicate. Absorbances values and calibration data are given in the supplementary information.

Determination of Antimicrobial Activity

Minimal inhibitory concentrations (MIC) of the plant extracts were determined according to a protocol of Wiegand et al.[62] Fermentation samples, mother tinctures and lactic acid solution (9 mg/mL) were filtered through 0.2 μm cellulose acetate filters and diluted with sterile deionized water (fermented samples 2.6–20 mg/mL, lactic acid 0.9–9 mg/mL). Bacterial strains *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538 and *Bacillus safensis* ATCC 6633 (Leibniz Institute, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) were inoculated in MHB for 4–6 h, adjusted to 0.5 McFarland by Densichek® (Biomérieux SA, Marcy-l'Étoile, France) and diluted 100-fold. 50 μL of sample solutions in triplicate or sterile water as growth control were pipetted on a 96-well plate and 50 μL of bacterial suspension was added to each well. 50 μL water and 50 μL MHB served as sterility control without the addition of bacteria. The plate was sealed with transparent foil (viewSEAL, Greiner Bio-One GmbH, Frickenhausen, Germany) and incubated at 37 °C for 20 h. Subsequently, turbidity was evaluated optically and MIC values were determined as least concentration with no visible bacterial growth.

Cell Culture

THP1-NF-κB reporter cells and THP1 monocytes were purchased from Merck KGaA (Darmstadt, Germany). The cells were cultured in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin in an incubator at 37 °C, in an atmosphere consisting of 5% CO_2 and 95% air.

WST Assay

THP1-NF-κB reporter cells were seeded at 50,000 cells/well in a 96-well plate. Different concentrations (300, 100, 30, 10 and 1 μg/mL) of LF extracts and different concentrations (30, 10, 1, 0.3 and 0.1 μg/mL) of fraxin were added immediately. 0.1% Triton was used as a control. Cells were incubated at 37 °C and 5% CO_2. After 24 h, cells were washed twice with PBS and incubated in phenol free culture medium supplemented with 10% WST-1 assay solution for 2 h at 37 °C and 5% CO_2. Finally, the absorption was measured at 450 nm using a Tecan Infinite M200 plate reader (Tecan, Männedorf, Switzerland).

THP1-NF-κB Reporter Assay

THP1-NF-κB reporter cells were seeded at 50,000 cells/well in a 96-well plate. The medium was supplemented with 1 μg/mL LPS, except for the unstimulated control. Different concentrations (300, 100, 30, 10 and 1 μg/mL) of LF extracts and different concentrations (30, 10, 1, 0.3 and 0.1 μg/mL) of fraxin were added immediately after stimulation. 10 μM dexamethasone was used as inhibitor control. Cells were incubated at 37 °C and 5% CO_2. After 24 h, cells were supplemented with 30% FACS buffer and transferred to a U-bottom 96-well plate. The eGFP-NF-κB reporter signal was measured using a CytoflexS Flow Cytometer (Beckman Coulter, Brea, CA, USA).

IL-6 and TNF-α Expression Assay

THP1 monocytes were seeded at 30 000 cells/well in a 96-well plate and differentiated to M0 macrophages with 100 nM PMA. Cells were incubated for 72 h at 37 °C and 5% CO_2. On the fourth day, cells were washed twice with PBS and stimulated with 1 μg/mL LPS (except for the unstimulated control) in serum free medium. Different concentrations (300, 100, 30, 10 and 1 μg/mL) of LF extracts and different concentrations (30, 10, 1, 0.3 and 0.1 μg/mL) of fraxin were added immediately after stimulation. 10 μM dexamethasone was used as inhibitor control. Cells were incubated at 37 °C and 5% CO_2. After 24 h, the supernatant was harvested and frozen at −80 °C. IL-6 and TNF-α concentrations were determined using Legendplex™ (BioLegend, San Diego, CA, USA).

Supporting Information

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.202400159.

Author Contributions

L. K. M. and D. R. K. designed the study. L. K. M. and M. G. prepared the extracts and performed and evaluated the analyses. S. N. and C. G. conducted the cell culture studies. L. K. M. wrote the draft. L. K. M., K. N. G., P. L., F. C. S., R. D. and D. R. K. evaluated the results and proofread the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interests

L. K. M., K. N. G., P. L., D. R. K. and F. C. S. are employed at WALA Heilmittel GmbH. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: biological activity · chamomile · *L. plantarum* · natural products

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Chemistry & Biodiversity

Supporting Information

Impact of Fermentation on the Phytochemical Profile and Bioactivity Characteristics of Aqueous *Matricaria recutita* L. Root Extracts

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Supporting information

Abbreviations:

c	concentration
DPPH	2,2-diphenyl-1-picrylhydrazyl
dw	dry weight
FC	Folin-Ciocalteu
GA	gallic acid
GAE	gallic acid equivalents
SD	Standard deviation
T	trolox
TE	trolox equivalents

HPLC-MS investigation of aqueous fermented *M. recutita* root extracts

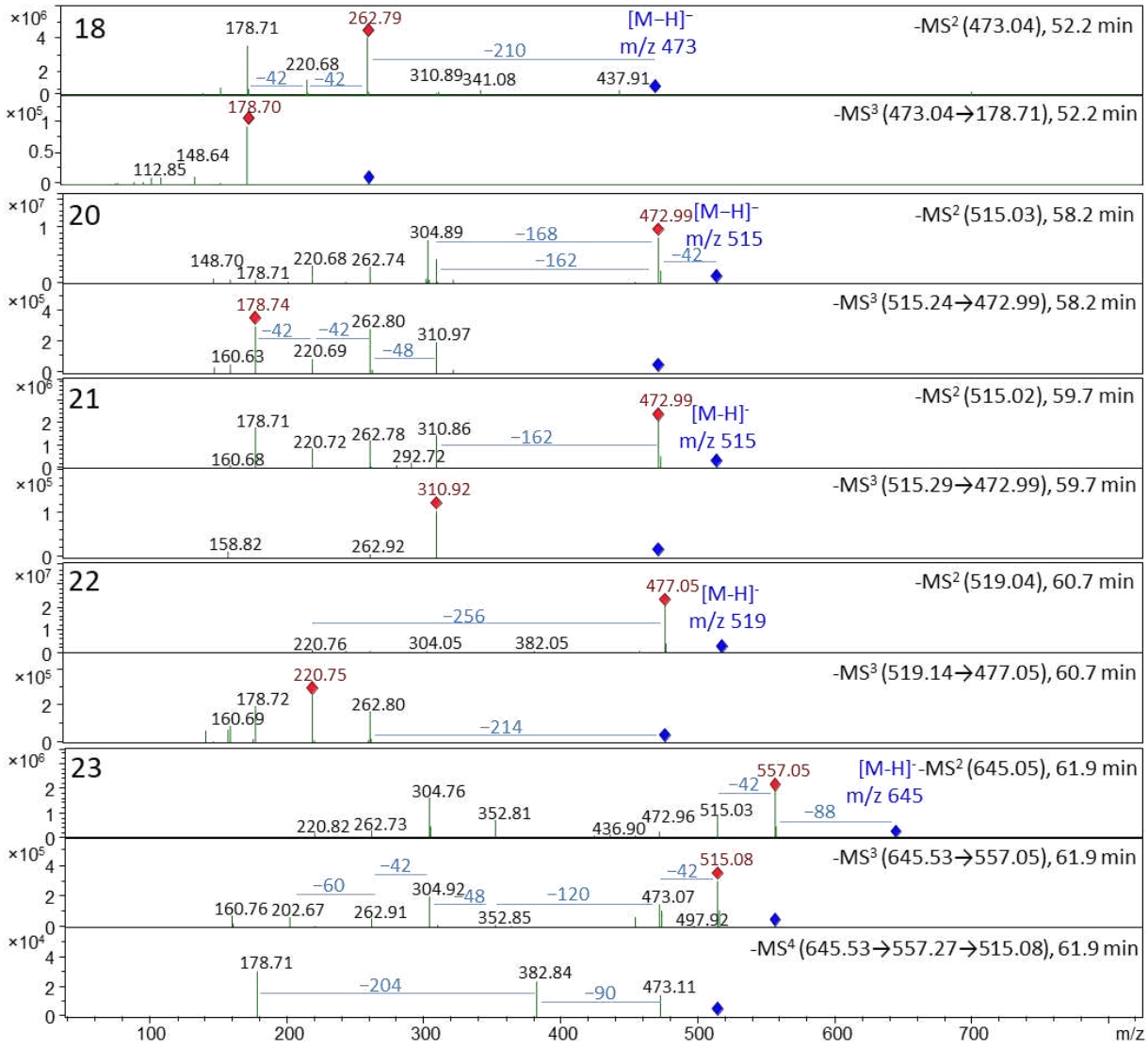

Figure S1: Fragmentation of caffeoyltartaric acids tentatively characterized in aqueous *M. recutita* root extracts during fermentation.

Total phenolic contents determined by Folin-Ciocalteu (FC) assay

Table S1: Calibration data for the Folin-Ciocalteu assay obtained with gallic acid as reference compound (GA, 3 – 47 µg/mL).

Curve Name	Formula	a	b	R^2
A	y=a*x+b	0.0063	-0.0029	0.992
B	y=a*x+b	0.0067	0.0005	0.995

Table S2A: Total phenolic contents of freshly prepared aqueous chamomile root extracts.

Day	Replicate	Absorbance (blanked)	Used calibration	c [µg GA/ mL sample]	GAE [mg GA/g dw)	Mean	SD
0	1	0.142	A	115.00	7.67	8.27	0.52
	2	0.160	A	129.29	8.62		
	3	0.158	A	127.70	8.51		

Table S2B: Total phenolic contents of batch 1 during six months of fermentation.

Day	Replicate	Absorbance (blanked)	Used calibration	c [µg GA/ mL sample]	GAE [mg GA/g dw)	Mean	SD
1	1	0.213	B	159.53	8.31	9.18	0.76
	2	0.247	B	185.06	9.64		
	3	0.246	B	184.31	9.60		
3	1	0.239	B	179.05	9.33	9.35	0.05
	2	0.239	B	179.05	9.33		
	3	0.241	B	180.56	9.40		
7	1	0.237	B	177.55	9.25	8.61	0.67
	2	0.222	B	166.29	8.66		
	3	0.203	B	152.03	7.92		
14	1	0.231	B	173.05	9.01	8.36	0.73
	2	0.194	B	145.27	7.57		
	3	0.218	B	163.29	8.50		
30	1	0.233	B	174.55	9.09	9.07	0.20
	2	0.227	B	170.05	8.86		
	3	0.237	B	177.55	9.25		
90	1	0.226	B	169.29	8.82	8.75	0.26
	2	0.217	B	162.54	8.47		
	3	0.230	B	172.30	8.97		

Table S2C: Total phenolic contents of batch 2 during six months of fermentation.

Day	Replicate	Absorbance (blanked)	Used calibration	c [µg GA/ mL sample]	GAE [mg GA/ g dw)	Mean	SD
1	1	0.237	A	190.40	9.92	10.14	0.31
	2	0.239	A	191.98	10.00		
	3	0.251	A	201.51	10.50		
3	1	0.238	A	191.19	9.96	9.71	0.23
	2	0.231	A	185.63	9.67		
	3	0.227	A	182.46	9.50		
7	1	0.210	A	168.97	8.80	8.86	0.06
	2	0.211	A	169.76	8.84		
	3	0.213	A	171.35	8.92		
14	1	0.218	A	175.32	9.13	9.26	0.11
	2	0.223	A	179.29	9.34		
	3	0.222	A	178.49	9.30		

30	1	0.202	A	162.62	8.47	8.84	0.33
	2	0.214	A	172.14	8.97		
	3	0.217	A	174.52	9.09		
90	1	0.217	A	174.52	9.09	8.33	0.86
	2	0.203	A	163.41	8.51		
	3	0.176	A	141.98	7.40		

Table S2D: Total phenolic contents of batch 3 during six months of fermentation.

Day	Replicate	Absorbance (blanked)	Used calibration	c [µg GA/ mL sample]	GAE [mg GA/g dw)	Mean	SD
1	1	0.205	A	165.00	8.59	8.66	0.20
	2	0.203	A	163.41	8.51		
	3	0.212	A	170.56	8.88		
3	1	0.217	A	174.52	9.09	9.02	0.06
	2	0.215	A	172.94	9.01		
	3	0.214	A	172.14	8.97		
7	1	0.210	A	168.97	8.80	8.83	0.09
	2	0.213	A	171.35	8.92		
	3	0.209	A	168.17	8.76		
14	1	0.210	A	168.97	8.80	8.81	0.02
	2	0.211	A	169.76	8.84		
	3	0.210	A	168.97	8.80		
30	1	0.238	A	191.19	9.96	10.00	0.56
	2	0.226	A	181.67	9.46		
	3	0.253	A	203.10	10.58		
90	1	0.236	B	176.80	9.21	8.93	0.32
	2	0.220	B	164.79	8.58		
	3	0.231	B	173.05	9.01		

Table S2E: Total phenolic contents of two mother tinctures.

Year	Replicate	Absorbance (blanked)	Used calibration	c [µg GA/ mL sample]	GAE [mg GA/g dw)	Mean	SD
2019 (prior to sterile filtration)	1	0.204	B	152.78	12.32	11.76	0.52
	2	0.187	B	140.02	11.29		
	3	0.193	B	144.52	11.65		
2011 (after sterile filtration)	1	0.181	B	135.51	10.93	10.28	0.64
	2	0.160	B	119.74	9.66		
	3	0.170	B	127.25	10.26		

DPPH radical scavenging assay

Table S3: Calibration data for the DPPH radical scavenging assay obtained with trolox (T, 3–200 µg/mL).

Curve Name	Formula	a	b	R^2
A	y=a*x+b	-0.00250	0.6111	0.999
B	y=a*x+b	-0.00257	0.9460	0.998
C	y=a*x+b	-0.00325	0.7360	0.997

Table S4A: DPPH radical scavenging capacity of freshly prepared aqueous chamomile root extracts.

Day	Replicate	Absorbance (blanked)	Used calibration	c [µg T/ mL sample]	TE [mg T/g dw)	Mean	SD
0	1	0.429	A	364.20	24.28	24.41	0.23
	2	0.429	A	364.20	24.28		
	3	0.426	A	370.20	24.68		

Table S4B: DPPH radical scavenging capacity of batch 1 during six months of fermentation.

Day	Replicate	Absorbance (blanked)	Used calibration	c [µg T/ mL sample]	TE [mg T/g dw)	Mean	SD
1	1	0.728	B	424.12	22.09	22.16	0.41
	2	0.731	B	418.29	21.79		
	3	0.723	B	433.85	22.60		
3	1	0.729	B	422.18	21.99	21.95	0.56
	2	0.735	B	410.51	21.38		
	3	0.724	B	431.91	22.50		
7	1	0.733	B	414.40	21.58	20.74	1.46
	2	0.733	B	414.40	21.58		
	3	0.758	B	365.76	19.05		
14	1	0.745	B	391.05	20.37	20.20	0.77
	2	0.74	B	400.78	20.87		
	3	0.755	B	371.60	19.35		
30	1	0.745	B	391.05	20.37	20.87	0.79
	2	0.744	B	393.00	20.47		
	3	0.731	B	418.29	21.79		
90	1	0.732	B	416.34	21.68	21.68	0.41
	2	0.728	B	424.12	22.09		
	3	0.736	B	408.56	21.28		
180	1	0.738	B	404.67	21.08	20.54	0.69
	2	0.741	B	398.83	20.77		
	3	0.751	B	379.38	19.76		

Table S4C: DPPH radical scavenging capacity of batch 2 during six months of fermentation.

Day	Replicate	Absorbance (blanked)	Used calibration	c [µg T/ mL sample]	TE [mg T/g dw)	Mean	SD
1	1	0.371	A	480.20	25.01	24.66	0.37
	2	0.378	A	466.20	24.28		
	3	0.374	A	474.20	24.70		
3	1	0.389	A	444.20	23.14	23.48	0.51
	2	0.388	A	446.20	23.24		
	3	0.380	A	462.20	24.07		
7	1	0.392	A	438.20	22.82	22.09	0.63
	2	0.403	A	416.20	21.68		
	3	0.402	A	418.20	21.78		
14	1	0.400	A	422.20	21.99	22.23	0.91
	2	0.388	A	446.20	23.24		
	3	0.405	A	412.20	21.47		
30	1	0.405	A	412.20	21.47	22.02	0.87
	2	0.404	A	414.20	21.57		
	3	0.390	A	442.20	23.03		
90	1	0.402	A	418.20	21.78	21.82	0.06
	2	0.401	A	420.20	21.89		
	3	0.402	A	418.20	21.78		

Table S4D: DPPH radical scavenging capacity of batch 3 during six months of fermentation.

Day	Replicate	Absorbance (blanked)	Used calibration	c [µg T/ mL sample]	TE [mg T/g dw)	Mean	SD
1	1	0.415	A	392.20	20.43	20.39	0.16
	2	0.417	A	388.20	20.22		
	3	0.414	A	394.20	20.53		
3	1	0.389	A	444.20	23.14	22.61	0.45
	2	0.397	A	428.20	22.30		
	3	0.396	A	430.20	22.41		
7	1	0.406	A	410.20	21.36	21.47	0.10
	2	0.404	A	414.20	21.57		
	3	0.405	A	412.20	21.47		
14	1	0.408	A	406.20	21.16	21.64	0.42
	2	0.401	A	420.20	21.89		
	3	0.401	A	420.20	21.89		
30	1	0.410	A	402.20	20.95	21.16	0.85
	2	0.415	A	392.20	20.43		
	3	0.399	A	424.20	22.09		
90	1	0.749	B	383.27	19.96	19.49	0.91
	2	0.764	B	354.09	18.44		
	3	0.748	B	385.21	20.06		

Table S4E: DPPH radical scavenging capacity of two mother tinctures.

Year	Replicate	Absorbance (blanked)	Used calibration	c [µg T/ mL sample]	TE [mg T/g dw)	Mean	SD
2019 (prior to sterile filtration)	1	0.622	C	175.38	13.60	14.16	0.43
	2	0.614	C	187.69	14.55		
	3	0.618	C	181.54	14.07		
	4	0.615	C	186.15	14.43		
2011 (after sterile filtration)	1	0.569	C	256.92	19.92	20.10	0.87
	2	0.564	C	264.62	20.51		
	3	0.560	C	270.77	20.99		
	4	0.577	C	244.62	18.96		

Endotoxin (LAL Limulus) assay

The endotoxin content was analyzed according to the manufacturer's protocol (Charles River Endosafe Charleston, SC, USA). 100 µL amoebocyte lysate were added to the samples (diluted 1:100 v/v), endotoxin standard, negative control (water) and a standard spike (sample + 0.5 EU/mL endotoxin) on a 96-well plate. Turbidity was measured at 340 nm upon incubation at 37 °C until coagulation was complete. Concentrations were calculated from the Michaelis-Menten kinetic by linear regression for a calibration range of 0.005 – 5 EU/mL with an r value of -0.9963.

Table S5: Endotoxin content of the lactobacteria fermented (LF) samples evaluated in cell culture studies.

	Endotoxin content [EU/mL]	Recovery [%]
LF extract A, day 7	< 387.9	-
LF extract B, day 7	54.1	88
LF extract C, day 180	144.1	73
LF extract D, day 180	12.1	104

Effect of lactobacteria fermented (LF) extracts on cytokine expression

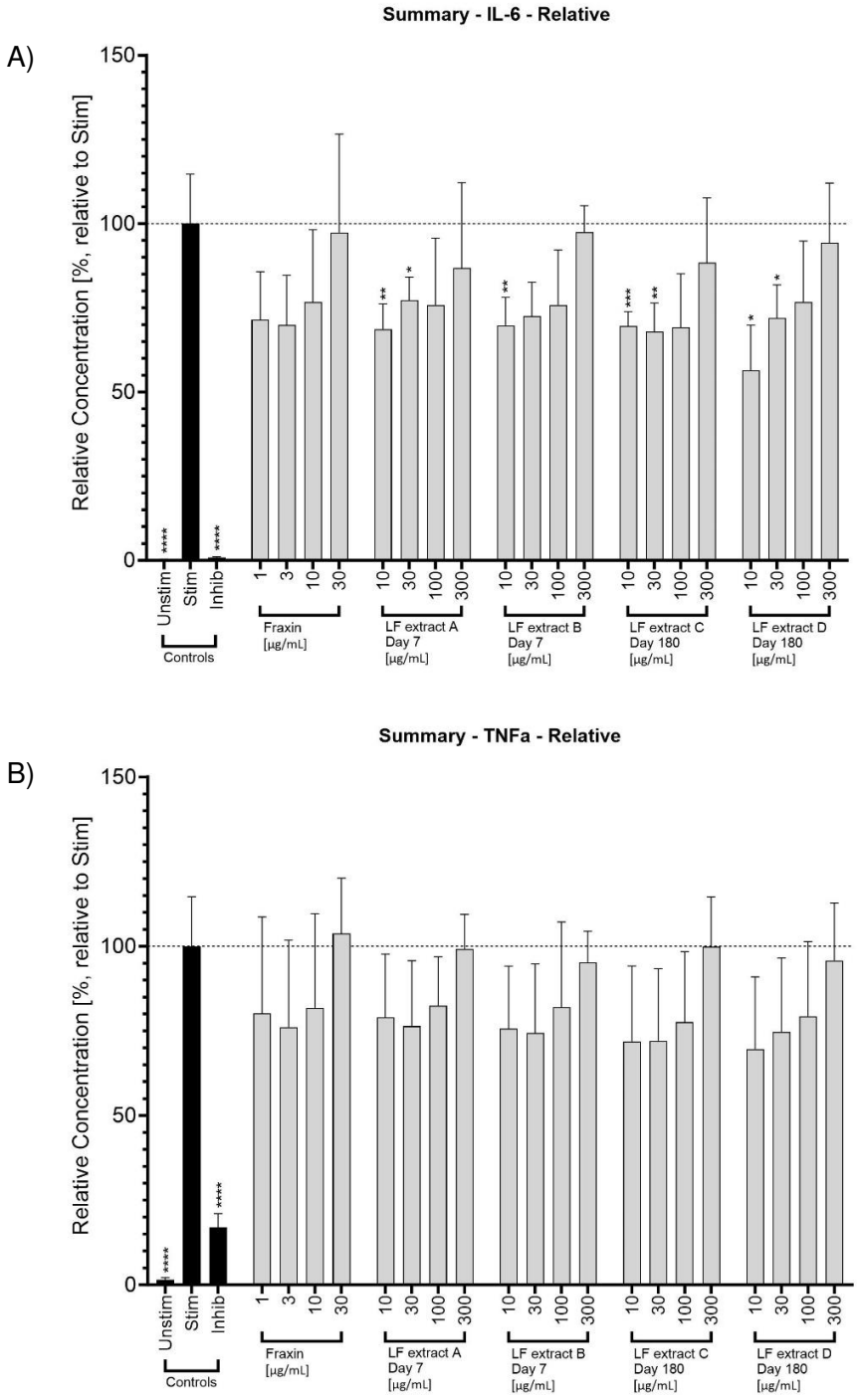

Figure S2: Effects of LF extracts on cytokine expression of THP-1 monocyte derived M0 macrophages. Except for the untreated control (Unstim), the macrophages were stimulated with LPS (1 µg/mL) and incubated for 24 h with serum-free medium (Stim), dexamethasone (Inhib; 10 µM) or samples. The levels of IL-6 (A), and TNF-α (B) in the supernatants were determined by flow cytometry (Legendplex™). Results are presented as mean ± SD in comparison to the stimulated control (n = 3).

3.3 IT IS NOT ALL ABOUT ALKALOIDS – OVERLOOKED SECONDARY CONSTITUENTS IN ROOTS AND RHIZOMES OF *GELSEMIUM SEMPERVIRENS* (L.) J.ST.-HIL.

Mailänder, L. K.; Nosrati Gazafroudi, K.; Lorenz, P.; Daniels, R.; Stintzing, F. C.; Kammerer, D. R. It Is Not All about Alkaloids − Overlooked Secondary Constituents in Roots and Rhizomes of *Gelsemium sempervirens* (L.) J.St.-Hil. *Plants* **2024**, 13, 2208. https://doi.org/10.3390/plants13162208

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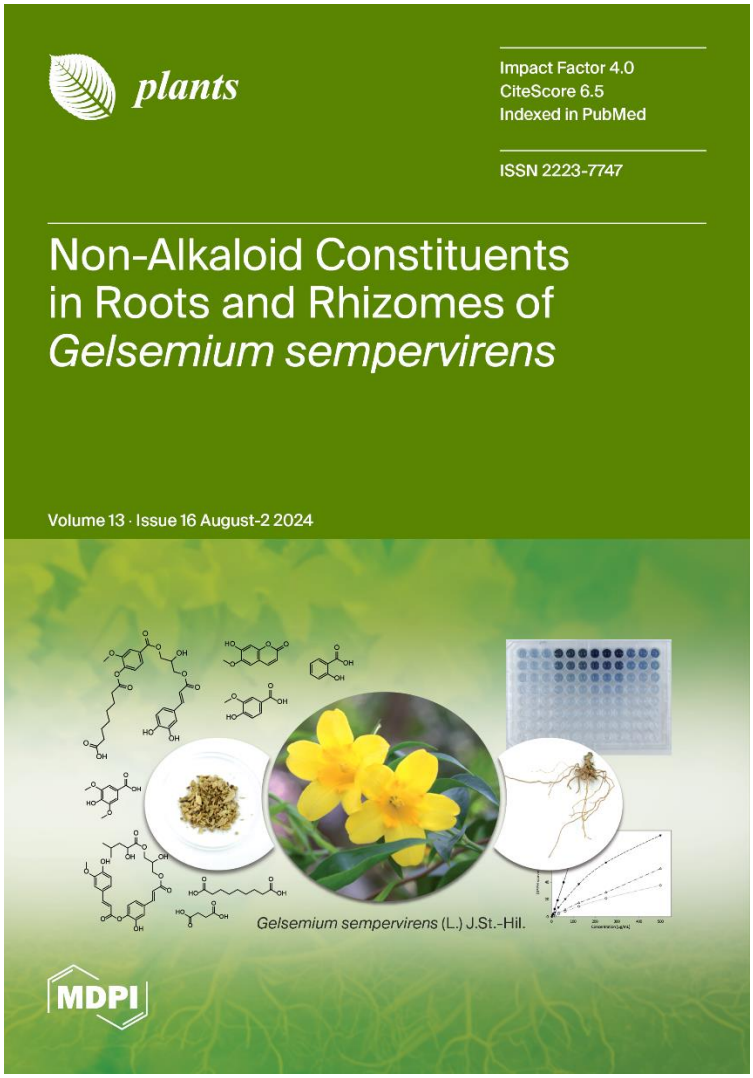

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It Is Not All about Alkaloids—Overlooked Secondary Constituents in Roots and Rhizomes of *Gelsemium sempervirens* (L.) J.St.-Hil

Lilo K. Mailänder [1,2,*], Khadijeh Nosrati Gazafroudi [1,2], Peter Lorenz [1], Rolf Daniels [2,†], Florian C. Stintzing [1,†] and Dietmar R. Kammerer [1,*,†]

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† The authors are members of the MOCS ('more than one constituent substances') initiative (www.stintmed.de/initiative-vielstoffgemische).

Abstract: *Gelsemium sempervirens* (L.) J.St.-Hil. is an evergreen shrub occurring naturally in North and Middle America. So far, more than 120 alkaloids have been identified in this plant in addition to steroids, coumarins and iridoids, and its use in traditional medicine has been traced back to these compound classes. However, a comprehensive phytochemical investigation of the plant with a special focus on further compound classes has not yet been performed. Therefore, the present study aimed at an extensive HPLC-MS^n characterization of secondary metabolites and, for the first time, reports the occurrence of various depsides and phenolic glycerides in *G. sempervirens* roots and rhizomes, consisting of benzoic and cinnamic acid derivatives as well as dicarboxylic acids. Furthermore, mono- and disaccharides were assigned by GC-MS. Applying the Folin–Ciocalteu assay, the phenolic content of extracts obtained with different solvents was estimated to range from 30 to 50% calculated as chlorogenic acid equivalents per g dry weight and was related to the DPPH radical scavenging activity of the respective extracts. Upon lactic acid fermentation of aqueous *G. sempervirens* extracts, degradation of phenolic esters was observed going along with the formation of low-molecular volatile metabolites.

Keywords: depsides; phenolic glycerides; mass spectrometry; natural products

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1. Introduction

G. sempervirens (L.) J.St.-Hil. (GS), also known as yellow jessamine, is an evergreen vine with a cylindrical rhizome and wiry roots [1]. With its fragrant yellow flowers, it is often used as an ornamental plant [1]. GS belongs to the genus *Gelsemium*, the only genus within the Gelsemiaceae plant family, which comprises only three highly toxic species [2]. While *G. elegans* (Gardner and Champ.) Benth. is distributed in Southern China and Southeast Asia, GS (Figure 1) and *G. rankinii* Small originate from North and Middle America [1,2]. Among these species, *G. elegans* has been phytochemically best studied, mainly by Asian research groups [3,4]. In contrast, fewer data are available on GS, while *G. rankinii* has been very scarcely investigated [2]. Previous publications have mainly focused on indole and oxindole alkaloids, of which more than 120 different constituents have been isolated from GS and *G. elegans* [2]. Based on their complex core structures, these have been classified into six different types: gelsemine, koumine, gelsedine, humantenine, sarpagine, and yohimbane. In addition, approximately ten steroids, twenty-five iridoids, and five coumarins have also been characterized in both species [5–7]. Further constituents

such as phenolic acids, lignans, and saccharides have only been assigned in *G. elegans* using HPLC-MS [8,9].

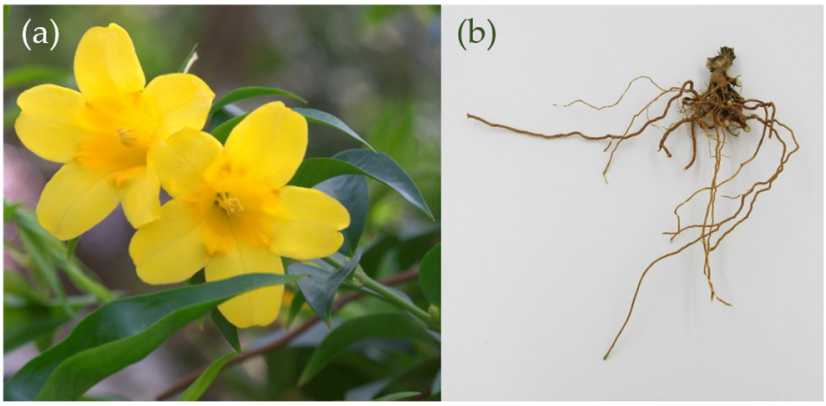

Figure 1. (**a**) Flowers of *G. sempervirens*. © Horst Arne Schneider. (**b**) Roots and rhizome of *G. sempervirens*. Photo: L. Mailänder.

Various pharmacological effects have been described for the three *Gelsemium* species. Different solvent extracts as well as isolated alkaloids have been found to exhibit anti-inflammatory, cytotoxic, and immunostimulatory activities and modulate noradrenaline and serotonin uptake, among many others [1,2]. The antinociceptive effects of the plant are often, but not exclusively, attributed to the alkaloids gelsemine and koumine and rely on the activation of spinal glycine receptors [10–12], which may also explain its neurotoxicity. Despite its toxicity, GS is traditionally applied as a medicinal plant for the treatment of neuralgia and fever [2] and was shown to have anxiolytic effects in mice [13].

Many of the phenolic compounds assigned in this study belong to the depsides, a substance class not previously described in the Gelsemiaceae. Depsides are defined as condensation products of two or more aromatic hydroxycarboxylic acids connected by an ester bond [14]. Depsides occur in many lichen species, where they are mainly composed of methyl- or alkyl-substituted dihydroxybenzoic acids but have also been found in fungi and higher plants [14]. Various interesting bioactivities, such as analgesic, antimalarial, neuroprotective, and wound healing activities, have been demonstrated for lichen depsides [15]. Rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, is one of the best-studied non-lichen depsides, which was first isolated from rosemary (*Rosmarinus officinalis*) in 1958 [16]. Its antioxidant and anti-inflammatory activities have been exploited for the treatment of inflammatory diseases such as colitis or arthritis [17]. It is, however, sensitive to oxidation and easily degraded by fermentation or digestion processes [18].

To obtain information on the enzyme-catalyzed conversion of GS depsides and other constituents, aqueous GS extracts were subjected to a model fermentation using the ubiquitous lactic acid bacterium (LAB) *Lactiplantibacillus plantarum*. Contrary to most other LABs, *L. plantarum* is able to metabolize phenolic compounds such as ferulic, coumaric, caffeic, and gallic acids [19,20]. This metabolism normally comprises an esterase activity followed by decarboxylation reactions [21] resulting in the production of, e.g., ethyl or vinyl phenols or pyrogallol [19]. These volatile metabolites are also known from red wine [22], for example.

To the best of our knowledge, no comprehensive phytochemical analysis of GS roots and rhizomes with a focus on non-alkaloid metabolites has been reported so far. Consequently, this study aimed to expand the phytochemical knowledge of the Gelsemiaceae plant family and provide new perspectives on composition, antioxidant activity, and the fermentative metabolism of GS root and rhizome constituents. Therefore, an exhaustive extraction of the phytoconstituents with solvents of different polarity, i.e., dichloromethane, ethyl acetate, and *n*-butanol, was performed followed by thorough HPLC-DAD-MS^n and

GC-MS analyses. In this way, both primary and secondary metabolites were assigned, with the focus of this study being clearly on the latter. The presented results may form a basis for further pharmacological studies supporting and potentially expanding the aforementioned medicinal applications, although the toxicity of the plant limits its use.

2. Results

2.1. Estimation of Total Phenolic Contents by Folin–Ciocalteu Assay

The Folin–Ciocalteu colorimetric assay was applied to estimate the phenolic contents of the GS extracts. Absorbance values and calibration data are displayed in the Supporting Information (Part I). Gallic and chlorogenic acids were used for calibration purposes, and total phenolic contents were calculated as gallic acid equivalents (GAE) or chlorogenic acid equivalents (CAE). As displayed in Figure 2, GAE and CAE differed by almost 50%. Ethyl acetate (EtOAc) extracts showed the highest phenolic contents of 536.6 µg CAE/mg dry weight and 274.0 µg GAE/mg dry weight, respectively. The lowest phenolic contents were determined in *n*-butanol (*n*-BuOH) extracts, while dichloromethane (DCM) extracts ranged in between.

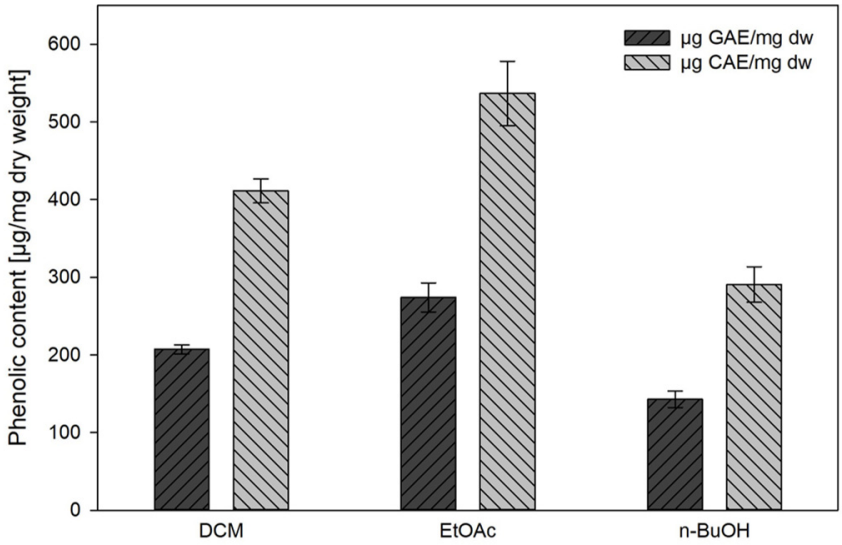

Figure 2. Total phenolic contents of dichloromethane (DCM), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) extracts of *G. sempervirens* roots and rhizomes. Results are expressed as µg gallic acid equivalents (GAE)/mg dry weight (dw) and µg chlorogenic acid equivalents (CAE)/mg dw, respectively; mean ± SD; $n = 3$.

2.2. DPPH Radical Scavenging Activity of GS Extracts

In addition to their phenolic contents, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activities of the aforementioned extracts and trolox as reference compounds were determined spectrophotometrically at 516 nm (Supporting Information Part II). The percentage of scavenged DPPH was calculated relative to the maximum amount scavenged and is displayed in Figure 3. The correlation between extract concentration and DPPH scavenging activity was linear for trolox ($r^2 = 1$) and the BuOH extract ($r^2 = 0.99$) in the entire concentration range examined. For the EtOAc and DCM extracts, linearity was only found at concentrations < 100 µg/mL. Generally, EtOAc extracts exhibited the highest radical scavenging activities. While the BuOH extract had the least total phenolic content, its antioxidant activity was stronger than that of the DCM extract. This is presumably due to differences in the phenolic composition of both extracts as our analyses have shown (Sections 2.3 and 2.4).

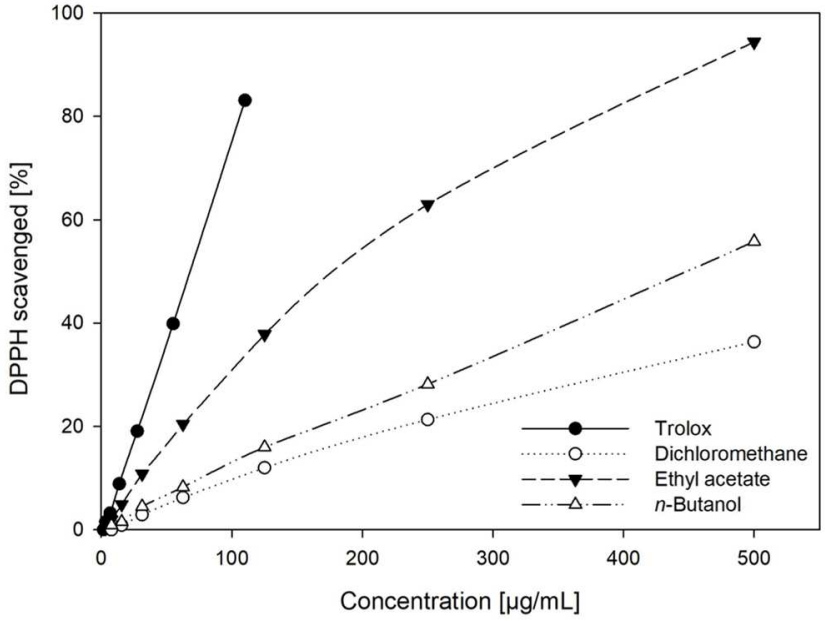

Figure 3. Percentage of DPPH scavenged by different solvent extracts from *G. sempervirens* roots and rhizomes in comparison to trolox as reference compound.

2.3. Analysis of Low Molecular Constituents by GC-MS

DCM, EtOAc, and BuOH extracts were analyzed by GC-MS after the silylation of individual constituents with BSTFA (Figure 4). For characterizing individual compounds, the obtained mass spectra (Table 1) were compared with the NIST database (National Institute of Standards and Technology, match factor > 800) and with MassBank Europe (massbank.eu, version 2.2.3). In DCM extracts, scopoletin (t_R 32.6 min) was by far the most abundant component, followed by citral (t_R 27.1 min). A variety of low molecular phenolic substances were eluted in a retention time range of 13–30 min. Among these, the three most abundant signals were attributed to coumarin (t_R 16.6 min), salicylic acid (t_R 18.2 min), and veratric acid (t_R 20.8 min). Between 34 and 37 min, adenine (t_R 34.3 min) and various fatty acids were eluted. Among the latter, linoleic, oleic, and stearic acids were the predominant compounds assigned based on their specific mass spectra. At higher retention times, mass spectrometric investigations indicated the presence of sterols. However, with match factors < 600, their exact identity could not be clarified. Various dicarboxylic and phenolic acids as well as scopoletin were characterized as main constituents in the chromatograms of EtOAc extracts. Finally, the polar BuOH extracts mainly contained a number of saccharides. Pentoses such as xylose and arabinose and hexoses such as fructose and glucose were eluted between 23 and 31 min. Disaccharides such as sucrose or trehalose were detected between 44 and 52 min. Furthermore, for aglycone analysis, the components of EtOAc and BuOH extracts were hydrolyzed with hydrochloric acid. The corresponding results can be found in the Supporting Information (Part III).

Table 1. Compound assignment of substances detected using GC-MS in (a) dichloromethane, (b) ethyl acetate, and (c) *n*-butanol extracts after silylation. Corresponding chromatograms are illustrated in Figure 4.

t_R [min]	Constituent (TMS Derivative)	MW [Da]	Fragment *m*/*z* (Intensity %)
		(a) Dichloromethane	
10.0	2-Octenoic acid	214.1	214 (38), 199 (82), 124 (100), 109 (62), 73 (88), 55 (28)
11.0	Benzoic acid	194.3	194 (7), 179 (100), 135 (66), 105 (65), 77 (49)

Table 1. *Cont.*

t_R [min]	Constituent (TMS Derivative)	MW [Da]	Fragment *m*/*z* (Intensity %)
16.8	Coumarin	146.1	146 (96), 118 (100), 89 (45), 75 (12), 77 (49)
18.2	Salicylic acid	282.5	267 (100), 209 (10), 73 (82)
19.2	Vanillin	224.3	224 (26), 209 (46), 194 (100), 73 (21)
20.8	Veratric acid	254.4	254 (44), 239 (100), 136 (93), 73 (90)
27.1	Citral	152.2	152 (53), 107 (26), 84 (73), 69 (100)
28.6	Pyrogallol	342.7	329 (36), 239 (34), 209 (21), 147 (49), 93 (32), 73 (100)
28.8	Syringic acid	342.5	342 (67), 327 (100), 312 (79), 297 (63), 253 (39), 73 (71)
32.6	Scopoletin	264.3	264 (48), 234 (100), 206 (37), 191 (8), 176 (10), 73 (36)
34.3	Adenine	279.5	294 (37), 279 (25), 264 (100), 73 (30)
36.2	Linoleic acid	352.3	337 (31), 129 (37), 117 (32), 95 (44), 81 (58), 73 (100), 55 (54)
36.3	Oleic acid	354.3	354 (2), 339 (51), 129 (68), 117 (91), 73 (100), 55 (63)
36.8	Stearic acid	356.3	356 (6), 341 (78), 132 (63), 117 (100), 73 (80), 55
40.3	Undefined sterol		440 (4), 369 (8), 225 (75), 130 (23), 93 (17), 73 (100)
50.2	Undefined sterol		386 (7), 371 (37), 281 (36), 269 (46), 207 (42), 73 (100)
			(b) Ethyl acetate
12.5	Succinic acid	262.4	247 (11), 147 (100), 73 (35)
13.5	2-Hydroxy-isocaproic acid	276.5	247 (66), 159 (82), 147 (47), 115 (19), 73 (100)
21.6	Salicylic acid	282.5	282 (22), 267 (38), 193 (18), 73 (100)
23.9	Suberic acid	318.6	303 (58), 213 (30), 147 (71), 73 (100), 69 (44), 55 (66)
25.0	2,5-Dimethoxy-phenylacetic acid	268.4	268 (16), 253 (32), 209 (38), 134 (25), 105 (36), 91 (29), 73 (100)
25.3	Vanillic acid	312.1	312 (57), 297 (100), 282 (34), 267 (67), 253 (51), 223 (51), 126 (31), 73 (49)
25.5	Gentisic acid	370.6	370 (4), 355 (100), 73 (60)
26.2	Azelaic acid	332.6	317 (51), 201 (52), 147 (26), 129 (31), 117 (29), 73 (100), 55 (48)
26.7	Protocatechuic acid	370.6	370 (49), 355 (26), 311 (21), 193 (100), 73 (53)
27.1	Citral	152.2	152 (42), 107 (23), 93 (23), 84 (67), 81 (29), 75 (60), 69 (100)
28.7	Pyrogallol	342.7	329 (27), 239 (29), 209 (18), 147 (46), 143 (34), 119 (28), 103 (28), 73 (100)
28.8	Syringic acid	342.5	342 (66), 327 (100), 312 (76), 297 (66), 253 (43), 149 (32), 141 (33), 73 (77)
32.4	Scopoletin	264.3	264 (50), 234 (100), 206 (40), 191 (8), 176 (11), 73 (31)
32.5	Vanillylmandelic acid	414.7	428 (64), 297 (100), 73 (83)
34.5	Caffeic acid	396.7	396 (71), 381 (19), 219 (100), 191 (16), 73 (79)
43.5	Methoxysalicylic acid	312.5	297 (100), 73 (48)
51.2	Saccharide derivative		331 (30), 253 (85), 217 (100), 204 (21), 147 (31), 103 (25), 93 (27), 73 (89)
			(c) *n*-Butanol
11.3	Glycerol	308.6	218 (19), 205 (58), 147 (90), 133 (20), 117 (33), 103 (31), 73 (100)
23.7	Xylose	438.8	217 (39), 204 (100), 191 (41), 147 (33), 73 (66)
25.1	Arabinose	438.8	217 (45), 204 (100), 191 (45), 147 (34), 73 (83)
26.3	Fructofuranose	541.1	437 (13), 217 (78), 147 (28), 73 (100)
26.4	Fructopyranose	541.1	437 (24), 217 (27), 204 (78), 147 (37), 73 (100)
28.4	Glucose	541.1	217 (18), 204 (100), 191 (50), 147 (24), 73 (62)
28.6	Galactose	541.1	329 (22), 239 (23), 217 (18), 204 (63), 191 (35), 147 (56), 143 (27), 73 (100),
30.6	Glucopyranose	541.1	217 (19), 204 (100), 191 (54), 147 (24), 73 (62)
31.0	Myo-Inositol	613.2	318 (23), 305 (32), 217 (70), 191 (27), 147 (48), 133 (33), 73 (100)
44.4	Sucrose	919.7	437 (18), 361 (100), 217 (43), 147 (25), 103 (19), 73 (81)
46.6	Unknown	394.5	394 (22), 351 (21), 323 (16), 134 (16), 108 (100), 73 (43)
49.4	Disaccharide	919.7	361 (94), 340 (38), 251 (38), 217 (33), 204 (20), 191 (28), 147 (31), 73 (100)
50.1	Disaccharide	919.7	373 (19), 217 (18), 204 (100), 147 (16), 73 (47)
51.2	Saccharide derivative		331 (29), 253 (85), 217 (100), 204 (22), 147 (26), 103 (23), 93 (24), 73 (83)

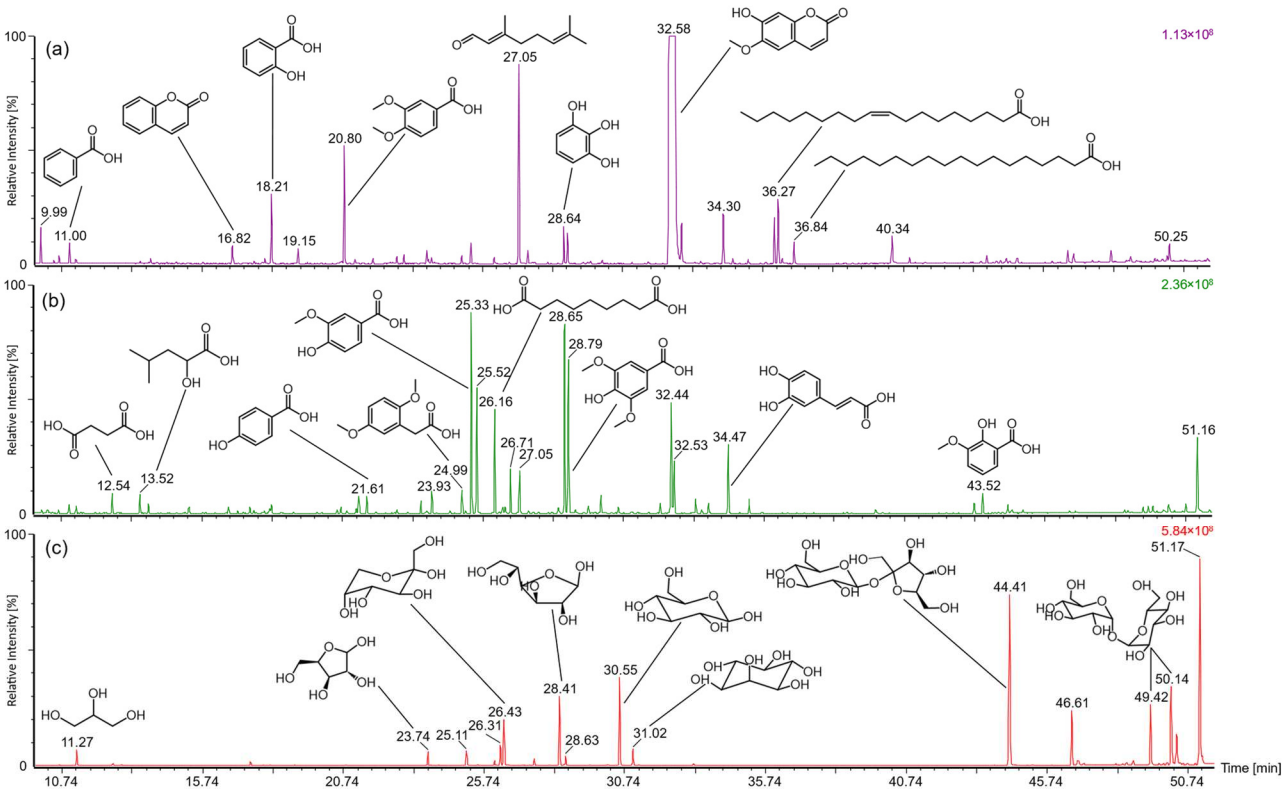

Figure 4. GC-MS profiles of secondary constituents in (**a**) dichloromethane, (**b**) ethyl acetate, and (**c**) *n*-butanol extracts after silylation. Compound assignment is presented in Table 1.

2.4. Analysis of Polar Secondary Constituents by HPLC-DAD-MS^n

Applying HPLC-DAD-ESI-MS^n in negative ionization mode, a variety of mostly phenolic substances were characterized based on the mass-to-charge ratios (*m*/*z*) of their precursor and fragment ions in comparison to the constituents and aglyca assigned in EtOAc and BuOH extracts by GC-MS. Moreover, for tentative structure elucidation, the fragmentation behavior of individual components in combination with UV spectral characteristics was compared to data published in the literature. A plethora of substances was assigned to depsidic structures, i.e., esters of two or three phenolcarboxylic acids, as displayed in Figure 5. Comprehensive HPLC-DAD-MS^n characteristics are displayed in Table 2.

Table 2. HPLC-DAD-MS^n characteristics of individual secondary metabolites in ethyl acetate (EtOAc) and *n*-butanol (BuOH) extracts from *G. sempervirens* roots and rhizomes obtained in negative ionization mode. Corresponding chromatograms are illustrated in Figure 5.

Peak Number		t_R [min]	λ_{max} [nm]	Negative Ionization *m*/*z*			Compound Assignment
EtOAc [a]	BuOH [b]			MS^1	MS^2	MS^3	
	1	11.1	-	421 [c], 411	225, 179 [c]	161, 143 [c], 119	Saccharide
	2	12.4	204	393 [d]	347 [c]	185, 161 [c], 143	Saccharide
	3	12.5	ND [e]	375 [c]	213 [c]	169 [c]	Gallic acid hexoside
	4	14.2	206, 230, 308	421 [c]	179 [c]	143 [c]	Methylgallic acid hexoside glycerol ester
	5	14.9	204, 324	353 [c]	191 [c], 179	173 [c]	4-*O*-Caffeoylquinic acid
6	6	15.5	238	375 [c]	213, 169 [c]	151, 125 [c], 109	Gallic acid hexoside
	7	16.1	252	375 [c]	213 [c], 169	125, 107 [c]	Gallic acid hexoside
	8	16.6	208, 254	421 [c]	179 [c]	143 [c], 119, 89	Methylgallic acid hexoside glycerol ester
	9	17.1	209, 254	407 [c], 397	343 [c]	179 [c], 161, 143, 119	Veratric acid hexoside derivative
	10	17.7	204, 288	407 [c]	343, 179 [c]	143, 119, 89, 83	Veratric acid hexoside derivative

Table 2. *Cont.*

Peak Number		t_R [min]	λ_{max} [nm]	Negative Ionization *m*/*z*			Compound Assignment
EtOAc [a]	BuOH [b]			MS^1	MS^2	MS^3	
	11	18.0	204, 225, 290sh, 328	377 [d]	331 [c]	161 [c]	Hydroxycoumarin and azelaic acid ester
12	12	18.5	218, 236, 312, 328	707, 353 [c]	191	173, 127, 111, 93	5-*O*-Caffeoylquinic acid
13	13	19.4	220, 240sh, 290sh, 326	353 [c]	191, 173 [c]	127, 93	4-*O*-Caffeoylquinic acid
	14	19.6	224, 324	403 [c], 353	237, 195 [c]	165, 151, 97	Dihydroferuloyl-dihydrosinapinic acid
15		21.1	216, 274	557 [c]	197 [c]	181, 153, 137 [c]	Syringyl-galloyl-dihydrosinapinic acid
16		21.4	222, 296	505 [c], 405	145 [c]		Succinyl-galloyl-dihydrosinapinic acid derivative
17	17	21.7	226, 302	405 [c]	225, 179 [c]	89 [c]	Gallic and caffeic acid glyceride
	18	23.0	230, 326	403 [d]	357, 195 [c], 179	151, 125 [c]	Caffeoyl-dihydroferulic acid
19		23.1	230, 316	353 [c]	191 [c]	171, 127	3-*O*-Caffeoylquinic acid
	20	24.1	308	537 [c]	311 [c]	293, 233, 149 [c], 101	Caffeoyl-galloyl-tartaric acid glyceride
21		25.1	218, 316	391 [f], 195 [c]	151 [c]		Dihydroferulic or dimethylphenyl-acetic acid
	22	26.8	238, 324	517 [c]	193 [c]	176, 149, 134 [c]	Dicaffeoyl-ferulic acid
23		27.6	226, 264	363 [c]	315 [c], 272	300, 272, 256 [c]	Methylellagic acid derivative
	24	29.3	238, 324	547 [c]	367, 325, 295, 265, 223 [c]	205, 163 [c]	Dicaffeoyl-sinapinic acid
25	25	30.3	212, 324	367 [c]	191 [c], 173	173 [c], 93	Feruloylquinic acid
26		32.4	207, 228, 296, 342	191 [c]	176 [c]		Scopoletin
	27	35.4	308	359 [c]	197 [c]	153 [c], 135, 109	Caffeoylsyringic acid
28		35.9	204, 234, 332	543, 367 [c]	179 [c], 161	135 [c]	Feruloyl-caffeoyl-hydroxyisocaproic acid glyceride
	29	37.6	224, 282	467 [d]	421 [c], 293	293 [c], 191, 149	Tartaric and methylcinnamic acid ester
30	30	43.5	224, 282	495 [c]	463, 327 [c]	311, 183	Digalloylcinnamic acid derivative
31		44.0	222, 260sh, 286	509 [c]	327 [c]	183 [c]	Digalloylcinnamic acid derivative
32		44.6	222, 264, 300	465 [c]	433 [c]	289, 271 [c], 179	Caffeoylsyringic acid glyceride
	33	45.5	228	549 [d]	503 [c]	371 [c], 161	Dihydroferuloyl-caffeoyltartaric acid
	34	48.6	232	481 [d]	435 [c], 293	293 [c], 149	Tartaric and methylcinnamic acid ester
35		49.0	222, 235, 300sh, 326	515 [c]	353 [c]	191 [c], 179, 135	3,5-Di-*O*-caffeoylquinic acid
36		51.2	222, 282	539 [c], 515	523 [c], 341	197 [c]	Di-dihydrocaffeoyl-syringic acid methyl ester
	37	51.5	224, 288	541 [c]	523 [c]	197 [c]	Di-dihydrocaffeoyl-syringic acid methyl ester derivative
38		51.8	232, 292	533 [c]	371 [c]	197, 173 [c]	Syringyl- caffeoyl-quinic acid
39	39	52.5	220, 240, 300sh, 326	515 [c]	353 [c]	173 [c]	4,5-Di-*O*-caffeoylquinic acid
40		55.1	232, 324	491 [c]	315 [c]	153 [c]	Feruloyl-caffeoyl-protocatechuic acid
41		57.8	234, 324	559 [c]	397 [c]	223, 173 [c]	Caffeoyl-vanillyl-suberic acid glyceride
42		58.4	232, 290, 340	515 [c]	353 [c]	191 [c], 179, 173	3,4-Di-*O*-caffeoylquinic acid
43		62.7	232, 326	501 [c]	483 [c], 465, 439	419 [c], 403	Gelse-norursane A
44		65.0	-	329 [c]	211, 293, 229 [c], 171, 158		Trihydroxy-octadecenoic acid isomer
45		67.7	-	483 [c]	419, 391 [c], 379, 203	321 [c]	Gelse-norursane derivative
46		68.2	-	515 [c]	471 [c]	453 [c], 427	Gelse-norursane C derivative
47		69.1	-	485 [c]	467 [c]	437 [c], 355	Gelse-norursane B
48		69.4	-	485 [c]	467, 405 [c]	363 [c]	Gelse-norursane B
49		70.9	-	469 [c]	405 [c]	375 [c]	Gelse-norursane E

[a] Signals in ethyl acetate extract, see Figure 2; [b] signals in *n*-butanol extract; [c] ion isolated for subsequent fragmentation; [d] formic acid adduct $[M-H+HCOOH]^-$; [e] not detected; [f] dimeric ion $[2M-H]^-$.

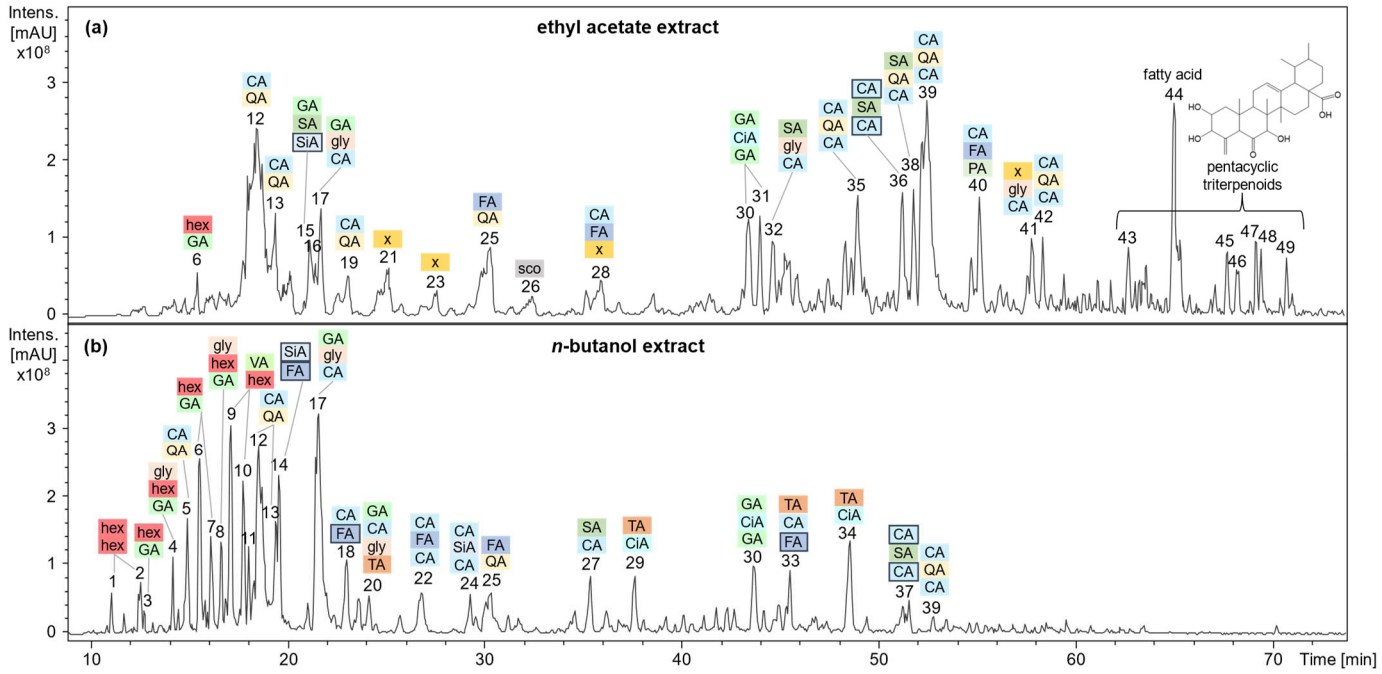

Figure 5. HPLC-MS^n base peak chromatograms of an (**a**) ethyl acetate and (**b**) *n*-butanol extract of dried *G. sempervirens* roots and rhizomes showing the occurrence of di- and tridepsides. The most abundant monomeric constituents are designated as follows: CA caffeic acid; CiA cinnamic acid; FA ferulic acid; GA gallic acid; gly glycerol; hex hexose; PA protocatechuic acid; QA quinic acid; SA syringic acid; sco scopoletin; SiA sinapinic acid; TA tartaric acid; VA veratric acid; x minor constituent (see Table 2); dihydro derivatives are displayed in bordered boxes.

Hydroxybenzoic acids such as gallic acid and its derivatives are characterized by two marked UV maxima at around 215 and 270 nm. Hydroxycinnamic acids such as ferulic and caffeic acids typically exhibit absorption maxima at approx. 220 and 320 nm [23]. The coumarin scopoletin has a characteristic UV spectrum with maxima at 207, 228, 296, and 342 nm [24]. However, at low analyte concentrations, UV spectra become less conclusive due to poor signal intensities.

The characterization of phenolic esters and depsides was based on their molecular masses and neutral losses upon fragmentation and is exemplified in Figure 6 for compounds **28** and **41**. The most frequent molecular ions $[M-H]^-$ were detected at *m/z* 149 (tartaric acid), 169 (gallic acid), 179 (caffeic acid or hexose, distinguishable by the UV spectrum), 191 (quinic acid), 193 and 195 (ferulic and dihydroferulic acid) and 197 (syringic acid), the corresponding neutral losses were 17 Da less [25–30]. Interestingly, various neutral losses (compounds **4**, **8**, **16**, **17**, **20**, **28**, **32**, **41**) pointed to constituents being composed of a phenolic acid moiety and glycerol [26,31], which was also detected by GC-MS. In accordance with the literature, we assumed that the phenolic acids are esterified with the primary hydroxy groups of glycerol as a consequence of their higher reactivity [31,32].

In the following section, compound assignment is exemplified for some representative substances. Compound **4** (t_R 14.2 min) exhibited a neutral loss of 242 Da in the MS^2 experiment, which may be due to glycerol (92−17−17) and methyl gallic acid (184) moiety, producing a hexoside fragment ion (*m/z* 179). An isomer of this compound was eluted after 16.6 min (**8**). The compounds eluting next, **9** and **10** (t_R 17.1 and 17.7 min), revealed similar fragmentation patterns. A loss of 64 Da in the first fragmentation step pointed to sulfite or furan derivatives, the subsequent loss of 164 Da may be due to veratric, i.e., dimethoxybenzoic, acid, which has also been detected by GC-MS. As above, the MS^3 base peak at *m/z* 179 indicated hexosides. Then, compound **16** (t_R 21.4 min), exhibiting an *m/z* at 505, showed a base peak at *m/z* 145 in the MS^2 experiment which corresponds to $[M-H-360]^-$, possibly [M−H−gallic acid−dihydrosinapinic acid]$^-$. This is equivalent

to the molar weight of ethylsuccinic or methylglutaric acid, both being aliphatic dicarboxylic acids. As a neutral loss of succinic acid (100 Da) may be assumed from the MS^1 spectrum and succinic acid was also found by GC-MS, the substance was characterized as ethylsuccinyl-galloyl-dihydrosinapic acid. A neutral loss of 226 Da (gallic acid + glycerol) indicated compound **17** (t_R 21.7 min) to be a gallic and caffeic acid glyceride and compound **20** (t_R 24.1 min) to be a tartaric acid ester thereof. In contrast, compound **27** (t_R 35.4 min) had an $[M-H]^-$ ion at *m/z* 359 and showed a loss of caffeic acid in the first fragmentation step. This may point to either caffeoyl-dihydroxyphenyllactic, i.e., rosmarinic, or caffeoyl-syringic acid. The latter assignment appears more plausible due to accordance with the GC-MS analyses. Furthermore, compounds **29** and **34** (t_R 37.6 and 48.6 min) were detected as formic acid adducts $[M-H+46]^-$ of the respective molecular ions. Based on the results of GC-MS analyses and the findings for compound **16**, the neutral loss of 128 Da (compound **29**) was assigned to ethylsuccinic acid, and 142 Da (compound **34**) to propylsuccinic acid. The neutral loss of 144 Da in the MS^3 experiments may be due to a hydroxycoumarin or a methylcinnamic acid moiety (compounds **29**–**31**, **34**). However, due to the lack of distinct UV maxima >300 nm, which would be expected for coumarins, the latter was assumed. Compounds **30** and **31** (t_R 43.5 and 44.0 min) could not be unambiguously characterized. Losses of 168 and 182 Da, possibly methyl- and dimethylgallic (syringic) acid, yielded MS^2 base peaks at *m/z* 327. This ion was further fragmented yielding an MS^3 base peak at *m/z* 183 (neutral loss of 144 Da, see above), which may either be ascribed to trihydroxyphenylacetic or methylgallic acid. Finally, the molecular ions of compounds **43** (t_R 62.7 min) and **45**–**49** (t_R 67.7–70.9 min) indicated the presence of pentacyclic triterpenoids such as gelse-norursanes, which have been described earlier [9,33].

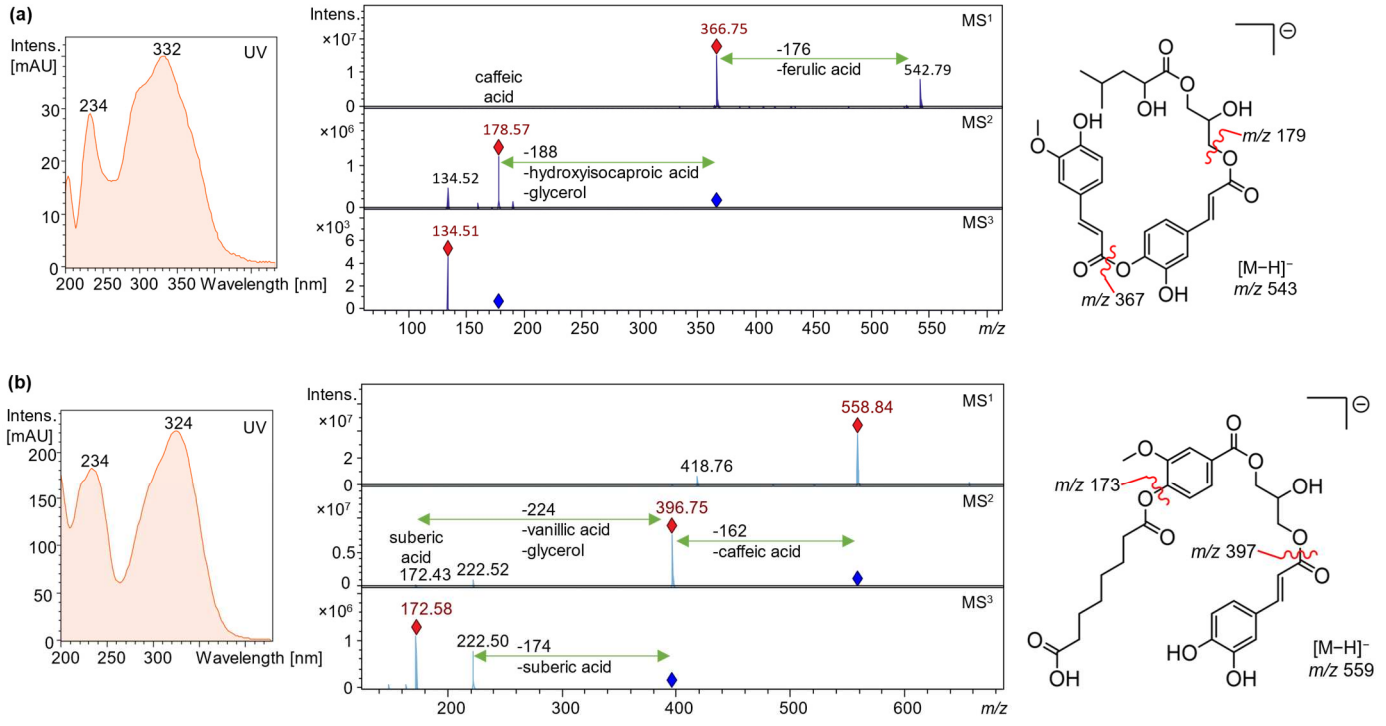

Figure 6. UV spectral and mass spectrometric structure assignment exemplified for compounds **28** (**a**) and **41** (**b**).

Alkaloids form the most intensely investigated group of secondary metabolites in *Gelsemium* species. Expectedly, these were only detected by LC-MS in positive ionization mode [4,8,9]. However, since these have been well investigated and described in the literature, they were outside the focus of the present study. For chromatograms and a peak list please refer to the Supporting Information (Part IV). As deduced from the intensities of the base peak chromatograms, the alkaloid concentrations were higher in

BuOH than in EtOAc extracts (Supporting Information Figure S4.1). In contrast, the latter were characterized by a more complex profile of individual compounds, particularly in a later retention time range. Gelsemine, N-methylgelsedilam, gelsemicine, and sempervirine were the main alkaloids detected in the BuOH extracts.

2.5. Metabolism of Phenolic Constituents upon Lactic Acid Fermentation

During the experiments, the progressive formation of a pleasant and spicy flavor of aqueous GS extracts was noticed. This observation is presumably due to the metabolic conversion of phenolic compounds caused by the microbial flora or endogenous plant enzymes after cell decompartmentation. Interestingly, the volatile compound formation was accelerated by inoculating the suspended plant material with *L. plantarum*, going along with a pH drop from 4.9 to 3.4 within three days as a result of lactic acid formation.

It is well known, that depsides and other esters are unstable upon enzymatic digestion and are rapidly hydrolyzed [18,34]. Accordingly, the HPLC-MS^n base peak chromatogram (Figure 7) showed a marked degradation of phenolic compounds, especially depsides and glycerides, within 30 days of lactofermentation. As can be deduced from the UV traces (Supporting Information Figure S4.2), the chlorogenic acid content decreased by almost 50% within seven days but then slowed down with declining microbial viability. While the caffeic acid content increased correspondingly, the coumarin scopoletin was obviously not metabolized. Except for a slight decrease in gelsemine and sempervirine contents, only minor changes were monitored in the alkaloid spectrum.

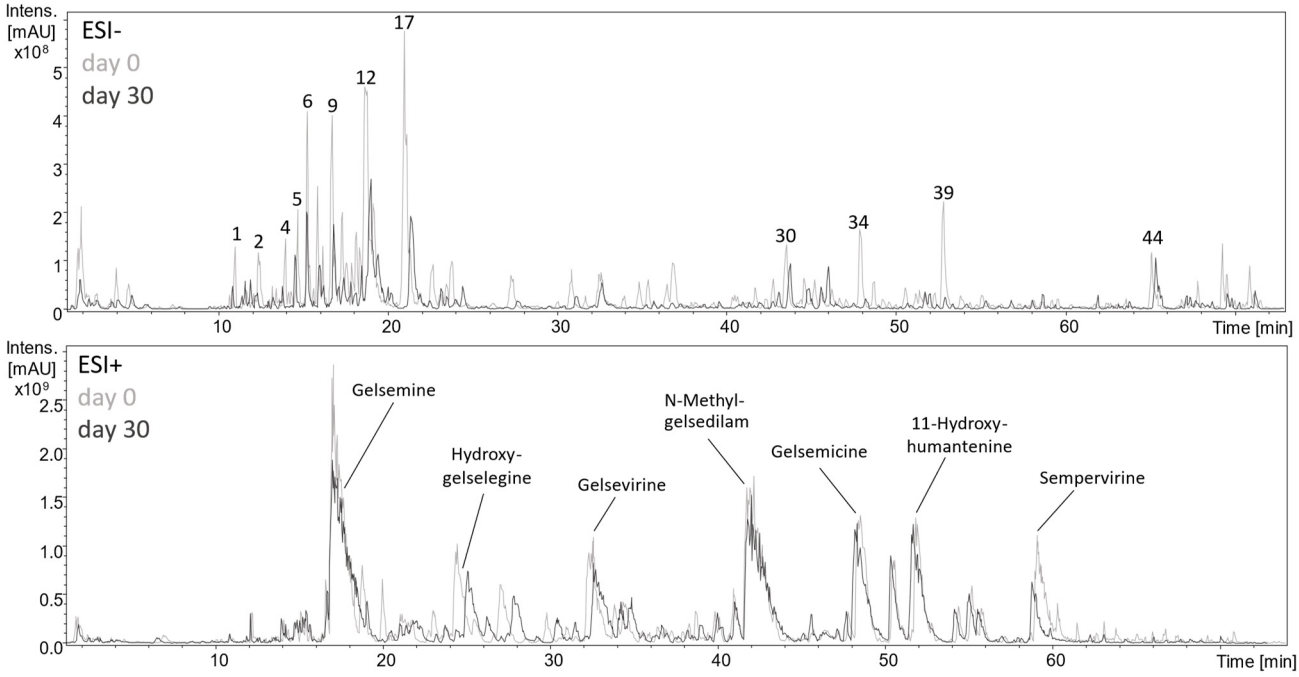

Figure 7. HPLC-MS^n base peak chromatograms of fermentation samples on day 0 (light grey) and 30 (dark grey) were recorded in negative (ESI−; top) and positive (ESI+; bottom) ionization mode. Peak numbers correspond to Table 2, LC-MS data of the alkaloids can be found in the Supporting Information (Table S4.1).

For analyzing the volatile constituents contributing to the intense smell of fermented aqueous GS extracts, these were extracted with diethyl ether and injected into the GC-MS system without prior derivatization. As described for DCM and EtOAc extracts, benzoic, salicylic, isovanillic, azelaic, protocatechuic, syringic, ferulic, and caffeic acids were assigned (Figure 8, for mass spectral data, see Supporting Information Table S3.2). In addition, aliphatic compounds such as propylcyclohexene, methylethylidene-cyclohexane, and dimethyloctene were assigned based on a comparison of mass spectral data with the NIST database. Among oxygen-

containing metabolites, ethylcatechol, hydroxy-methylbenzaldehyde, and the sesquiterpenoid oxo-α-ionol were detected. Two isomeric compounds at retention times of 36.2 and 36.3 min, exhibiting a molecular mass of 222 Da, could not be further characterized. However, the molecular weight could indicate a hydroxy-dimethoxy-coumarin [35].

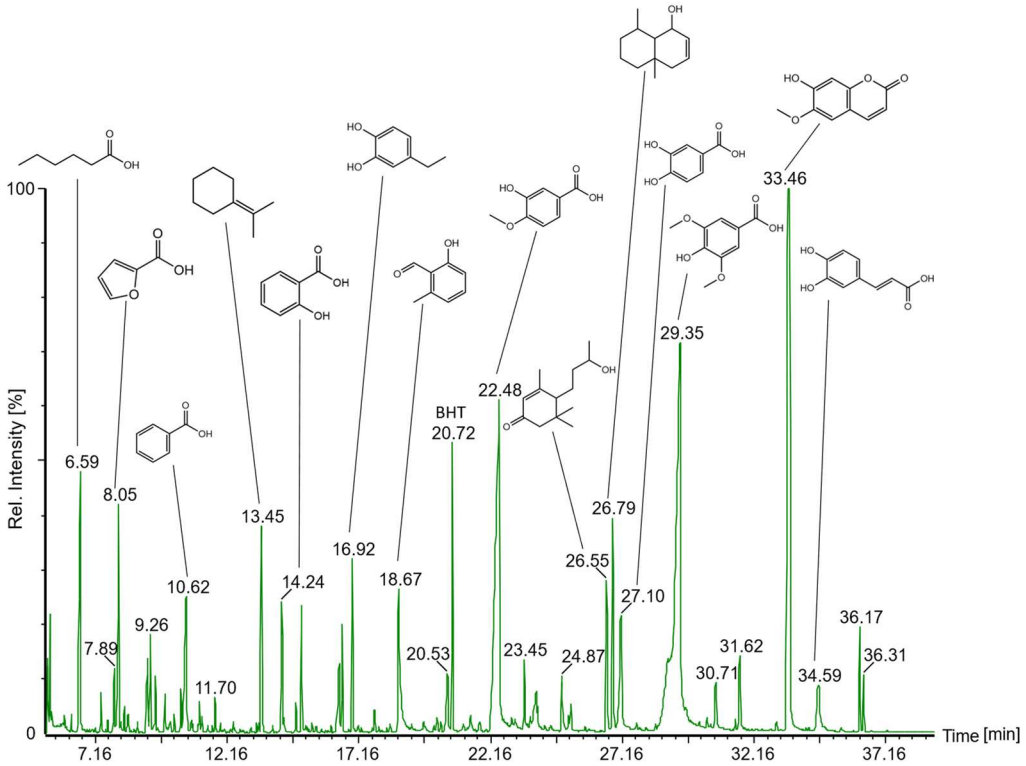

Figure 8. GC-MS total ion current chromatogram of volatile compounds extracted with diethyl ether from six months old aqueous fermented *G. sempervirens* extracts.

3. Discussion

3.1. Phenolic Content and Antioxidant Activity

The Folin–Ciocalteu (FC) and the DPPH assay are both used for assessing the antioxidant activity of plant extracts [36]. The FC assay corroborated the high content of phenolic constituents in GS extracts. More precisely, when calculated as chlorogenic acid equivalents, the dry matter of EtOAc extracts was composed of about 50% phenolic substances, the corresponding values of DCM and BuOH extracts amounted to 40% and 30%, respectively. However, it should be kept in mind that due to the unspecific redox reaction of the Folin assay, it may only be regarded as a semiquantitative method and rather as an indication of the reductive potential of the sample [37,38]. Although phenolic substances generally have strong antioxidant effects, the extent markedly depends on the molecular structure, e.g., the number of phenolic hydroxyl groups [37]. Consequently, the results calculated as gallic and chlorogenic acid equivalents differed by almost 50%. This phenomenon is well known and is due to the higher reducing capacity of a galloyl group compared to catechol or hydroxycinnamic acid groups and has been documented in the literature [37,39]. These effects were also reflected by the fact that despite higher phenolic contents of DCM extracts as deduced from the FC assay, their DPPH radical scavenging activity was weaker than that of BuOH extracts. Accordingly, differences in the phenolic profiles of the extracts could be shown in our analyses. Furthermore, while trolox and BuOH extracts showed a linear correlation between the percentage of scavenged DPPH and concentration in the entire concentration range tested, the curves of EtOAc and DCM flattened at concentrations > 100 µg/mL. This indicates that not only concentration but also the exact chemical

composition affects the characteristics of such extracts and that concentration-dependent interactions between individual components may occur.

3.2. Phytochemical and Bioactivity Profiling of G. sempervirens Roots and Rhizomes

Saccharides were detected in the GC-MS chromatograms of crude BuOH extracts as well as in the aqueous residues after solvent partitioning. Rhizomes often contain high amounts of starch since they commonly serve as storage organs [40]. However, the extraction procedure and derivatization prior to analysis discriminate oligomeric and polymeric saccharides. This is the reason why only low molecular saccharides were covered in the present analysis. Among these, various ubiquitous pentoses and hexoses as well as disaccharides could be assigned. The occurrence of significant amounts of sugars in rhizomes is also known from other plants such as *Polygonatum* species [29]. Interestingly, while only saccharides were characterized in crude BuOH extracts, various phenolic constituents were detected after hydrolysis with hydrochloric acid and extraction with ethyl acetate. This indicates that part of the phenolics naturally occur in bound forms but can be released by enzymatic or acidic cleavage. In contrast, chromatograms of EtOAc extracts were comparable prior to and after hydrolysis in terms of peak profile and compound assignment.

Among phenolics, a variety of benzoic and cinnamic acid derivatives were assigned via GC-MS analysis, with vanillic, gentisic, syringic, and caffeic acids being the main phenolic acids in both, EtOAc and BuOH extracts. Furthermore, coumarins, fatty acids, dicarboxylic acids, saccharides, and glycerol were assigned based on comparison with the NIST database. A number of these substances such as scopoletin, vanillic acid, and chlorogenic acid have previously been assigned in *G. elegans* [8,9,41], which in combination with the alkaloids emphasizes the phytochemical similarity of the two *Gelsemium* species despite their origin from different continents [1]. The monoterpene citral, a mixture of the isomers geranial and neral, presumably contributes to the smell of GS roots but has not been detected in its flowers so far [42].

The LC-MS investigations performed in the present study expanded the knowledge of the complex composition of the phenolic profile, as not only monomers but also higher molecular weight compounds can be detected using this technique. The assignment of most substances was based on their mass spectrometric behavior and UV spectra, which was aligned with the findings for the monomers obtained by GC-MS in crude and hydrolyzed EtOAc and BuOH extracts. Many of the compounds characterized according to this procedure belong to the depsides, i.e., esters of two or more phenolic acids [43]. Depsides have particularly been found in many lichen [15,44] and fungal [14] species, where they mostly consist of orsellinic, i.e., 2,4-dihydroxy-6-methylbenzoic, acid [14]. However, depsides of phenolic acids without methyl substitution of the aromatic ring have also been found in various higher plants such as aronia [45], rosemary [18], sage [46], or pineapple [47], thus across a wide range of plant families. The manifold bioactivities described for this compound class include cytotoxic, antimicrobial, analgesic, hepato-, nephro-, and neuroprotective as well as anti-inflammatory effects [15,17,46], which renders GS extracts potentially interesting from a pharmacological viewpoint.

Phenolic glycerides are another substance class characterized for the first time in the Gelsemiaceae in the present study. They have, however, also been detected in other plant families such as the Liliaceae [48], Bromeliaceae [49], and Asparagaceae [26], and are characteristic of propolis [31,50]. Like other phenolic substances, they exhibit marked antioxidant and anti-inflammatory activities [51,52]. Thus, while the toxicity of GS is attributed to alkaloids, the described inflammation, and eczema-reducing activity [2] may also be due to the phenolic constituents herein or due to an interplay of the different compound classes in the complex mixture.

Nor-ursane type triterpenoids such as the gelse-norursanes assigned in this study have been isolated from representatives of approx. 15 different plant families, mostly but not exclusively occurring in tropical and subtropical regions [33]. The natural habitat of

GS from Florida to Virginia also fits in this climate zone [2]. Interesting bioactivities have been described for similar pentacyclic triterpenoids, for example, antidiabetic effects due to inhibition of the insulin-resistance-promoting enzyme tyrosine proteinase [53]. In addition, hepatoprotective effects [54] as well as cytotoxicity against leukemia, liver, breast, and colon cancer cells [55,56] were demonstrated.

3.3. Fermentation of Aqueous GS Root Extracts

The rapid formation of an aromatic odor within one week of fermentation in combination with a marked pH decrease indicated that the growth and viability of *L. plantarum* were not affected by the high alkaloid contents of GS. GC-MS analyses of the volatile compounds in fermented GS extracts revealed the presence of various low-molecular phenolic acids and metabolites thereof. As an example, 4-ethylcatechol may be produced from hydroxycinnamic acids by *L. plantarum* and is a well-known off-flavor component in various fermented foods [57]. γ-Amylbutyrolactone (γ-nonalactone) is an odor-active compound also found in whiskey [58], the sesquiterpenoid oxo-α-ionol is a metabolite produced by yeasts during winemaking [59]. However, it was not investigated if only a few character impact compounds account for the spicy smell of the fermented extracts, or if it is caused by a more complex variety of compounds detected in larger concentrations.

Using HPLC-MS^n analysis in negative ionization mode, a degradation of saccharides, glycerides as well as depsides was observed as can be deduced from the base peak chromatograms. This is not surprising, since several esterases have been described in *L. plantarum* [21,60], which, among others, leads to the release of further compounds that may serve as substrates for bacterial metabolization. In contrast, only minor changes were detected in the alkaloid spectrum analyzed in positive ionization mode. The pH decline from 4.9 to 3.4 upon fermentation may have a stabilizing effect on the alkaloids, as they are protonated at lower pH values, increasing their solubility in water. Still, the decrease in gelsemine and sempervirine is most likely due to their poor solubility in aqueous systems, which leads to precipitation. Accordingly, both components were also found in the sediment formed during storage, after extraction of the latter with methanol. To conclude, fermentation appears to be a suitable method for the extraction of secondary metabolites from the plant matrix of GS as well as for preserving the obtained aqueous extracts, despite the metabolic degradation, particularly of phenolic constituents. The obtained extracts may, among others, be used for pharmaceutical applications, although their toxicity due to the alkaloid fraction must be considered.

4. Materials and Methods

4.1. Chemicals and Reagents

Acetone, acetonitrile, *n*-butanol (BuOH), chloroform, dichloromethane (DCM), ethyl acetate (EtOAc), hydrochloric acid, methanol (MeOH), methyl-*tert*-butyl ether (MTBE), sodium carbonate and sodium sulfate were purchased from Chemsolute (Th. Geyer GmbH & Co. KG, Renningen, Germany). Diethyl ether, gallic acid monohydrate (GA), and lactose were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). *N,O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA), dimethylformamide (DMF), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were from Sigma-Aldrich (St. Louis, Missouri, USA), formic acid from Fluka (Sigma Aldrich, St. Louis, MO, USA). Trolox was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), and chlorogenic acid hemihydrate (CA) from Alfa Aesar (Karlsruhe, Germany). Folin–Ciocalteu's reagent was from Merck KGaA (Darmstadt, Germany). Ultrapure water was produced with an ELGA Purelab Classic system (High Wycombe, UK) and used for all experiments.

4.2. Plant Material and Extraction

Dried and cut *G. sempervirens* roots and rhizomes (Figure 9) were obtained from a commercial supplier (Albert Stephan export–import, Zweibrücken, Germany). A sample

was deposited at the herbarium of the Institute of Botany, University of Hohenheim, Stuttgart (voucher number: HOH-022975).

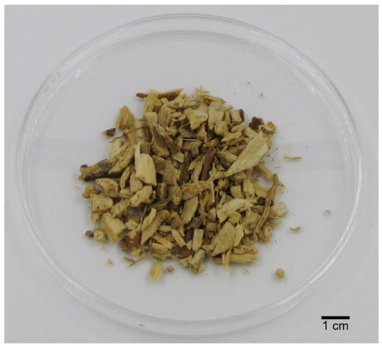

Figure 9. Dried *G. sempervirens* plant material used in this study.

For the extraction, 35 g plant material was mixed with 500 mL 70% acetone (*v*/*v*), bubbled with nitrogen for 5 min, and minced by Ultra-Turrax treatment (IKA Werke GmbH and Co. KG, Staufen, Germany; 3 min, 17,000 rpm). After another 10 min bubbling with nitrogen, the slurry was stored at 4 °C overnight. The mixture was then filtered over Celite® (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and the filter cake was re-extracted analogously. Then, the two filtrates were combined, the acetone was removed by rotary evaporation, and the residual aqueous phase was extracted successively with 3 × 100 mL of DCM, EtOAc, and *n*-BuOH. DCM and EtOAc extracts were dried over anhydrous sodium sulfate and filtered through filter paper (Whatman™ qualitative filter paper 2, GE Healthcare, Buckinghamshire, UK). Solvents were removed in vacuo and the dry residues were used for further experiments. Extraction was performed in duplicate.

For aglycone analyses, 50 mg of EtOAc or BuOH extracts were dissolved in 1 N hydrochloric acid (20 mL) and kept at 105 °C for 1 h. Aglycones were then extracted with 2 × 30 mL EtOAc, the organic phase was dried over sodium sulfate, filtered, and the solvent removed by rotary evaporation.

4.3. Fermentation Experiments

For the fermentation experiments, 20 g of dried roots were mixed with 500 mL of water containing 0.75% (*w*/*v*) lactose as substrate for microbial fermentation. The material was minced using an UltraTurrax (2 min, 17,000 rpm). This suspension was then inoculated with 1 mL of *Lactiplantibacillus plantarum* (previously *Lactobacillus plantarum*, GenBank accession number: MK841313.1; sequence length: 1083 base pairs; closest relative in National Center for Biotechnology Information: *Lactobacillus plantarum* strain 2.7.17, MK611349.1; similarity 100%) in MRS broth (5×10^8 CFU/mL). After three days at 33 °C, the slurry was filtered through a cotton cloth yielding a turbid solution and the filtrate was kept in glass bottles at room temperature in the dark. After one week the turbid solution was filtered through filter paper yielding a clear solution, which was stored for a minimum of six months. Three temporally independent fermentations were conducted, each in duplicate or triplicate.

4.4. Estimation of the Total Phenolic Content by Folin–Ciocalteu Assay

The dried DCM, EtOAc, and BuOH extracts were dissolved in MeOH (250 µg/mL). Gallic acid (GA) and chlorogenic acid (CA) were used as reference substances in concentrations ranging from 3.5 to 55 µg/mL (GA) and 10–160 µg/mL (CA), respectively. A 20 µL amount of sample or reference solution and 40 µL of Folin–Ciocalteu's reagent were mixed on a 96-well plate. The plate was shaken in the reader for one minute, and subsequently, 160 µL of sodium carbonate solution (700 mM) was added. After incubation (37 °C, 30 min), the absorbance at 765 nm was measured using a multiplate reader (Epoch2, Agilent Technologies Inc., Santa Clara, CA, USA). Calibration equations of GA and CA were calculated by plotting the absorbance values against the concentrations. The total

phenolic content of the samples was then calculated as GA or CA equivalents [mg/g dry weight] by inserting the sample absorbance values into the regression equations. Analyses were performed in triplicate. Absorbance values and calibration data are provided in the Supporting Information (Part III).

4.5. Determination of the DPPH Radical Scavenging Activity

The DCM, EtOAc, and BuOH extracts were dissolved in MeOH and diluted to seven concentrations between 7.8 and 500 µg/mL. Trolox (1.7 to 110 µg/mL) was used as reference compound. 180 µL of DPPH solution (100 µM in MeOH) was then added to 20 µL of the test or reference solution or methanol as a blank sample in a 96-well plate. The plate was incubated at 37 °C for 45 min and then analyzed colorimetrically at 516 nm using a multiplate reader Epoch2. The percentage of scavenged DPPH was calculated from the maximum and sample absorbances (A) using the formula

$$\text{DPPH scavenged [\%]} = (A_{max} - A_{sample})/A_{max} \times 100\%.$$

Analyses were performed in triplicate. Absorbance values are provided in the Supporting Information (Part IV).

4.6. GC-MS Analyses

For GC-MS analyses, crude DCM, EtOAc, and BuOH extracts and their aglycones (5–10 mg) were dissolved in DMF (500 µL) and mixed with BSTFA (200 µL). The solution was heated to 105 °C for 15 min in order to obtain trimethylsilyl derivatives of individual compounds.

For the extraction of volatile aroma composites from the fermented solutions, 40 mL of each sample was extracted with 2 × 10 mL diethyl ether. The solvent was removed under reduced pressure, and the residue was dissolved in 1 mL methyl-*tert*-butyl ether (MTBE) and directly used for analysis.

GC-MS analyses were conducted with a PerkinElmer Clarus 500 gas chromatograph (PerkinElmer, Inc., Shelton, CT, USA) coupled to a single quadrupole mass spectrometer operating in electron ionization (EI) mode at 70 eV according to [61]. Split injection (split ratio 30:1, injection volume 1.0 µL) was applied and a Zebron ZB-5MS capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness, 5% phenylpolysiloxane and 95% dimethylpolysiloxane coating; Phenomenex, Torrance, CA, USA) was used as a stationary phase. Helium served as carrier gas at a flow rate of 1 mL/min. The injector temperature was 250 °C, and the temperature of the column oven was 100–320 °C with a linear gradient of 4 °C/min. Data were acquired and processed using the software TurboMass (v.5.4.2, PerkinElmer, Inc., Waltham, MA, USA).

4.7. HPLC-DAD-ESI-MS^n Analyses

For HPLC analyses, EtOAc and BuOH extracts were dissolved in MeOH or water (5 mg/mL), respectively, and aqueous samples were directly injected after filtration through a syringe filter (perfect-flow RC, 0.45 µm, WICOM, Heppenheim, Germany).

Reversed phase high performance liquid chromatography was carried out as described previously [61]. In brief, an Agilent 1200 HPLC system (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with binary pump, micro vacuum degasser, autosampler, thermostatic column compartment and UV/VIS diode array detector (DAD); a Kinetex® C18 reversed-phase column (2.6 µm particle size, 150 mm × 2.1 mm i.d., Phenomenex Ltd., Aschaffenburg, Germany); and a pre-column of the same material were used for chromatographic separation at 25 °C and a flow rate of 0.21 mL/min. 0.1% formic acid in water and acetonitrile were used as mobile phase.

For mass spectrometric detection, an HCTultra ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with an ESI source was used. All extracts were analyzed in positive and negative ionization mode using a capillary voltage of + or −4000 V, respectively. The dry gas (N_2) flow was 9.00 L/min, capillary temperature 365 °C, and

nebulizer pressure 35 psi. MS^n data were generated by performing collision-induced dissociation (CID) experiments. The instruments were controlled by Agilent LC 3D systems (Rev. B01.03SR1 (204)) and Bruker Daltonics EsquireControl software (V7.1).

5. Conclusions

In the investigation presented here, secondary metabolites in GS roots and rhizomes were comprehensively characterized with a special focus on phenolic constituents. Applying the Folin–Ciocalteu assay, total phenolic contents of 411, 537, and 291 µg chlorogenic acid equivalents per mg dry weight were determined in DCM, EtOAc, and BuOH extracts, respectively. Accordingly, pronounced antioxidant activity was determined using the DPPH antioxidant assay. Interestingly, the correlation between concentration and DPPH scavenging activity was not strictly linear for EtOAc and DCM extracts, indicating concentration-dependent interactions between individual components.

The identity of the phenolic compounds was studied by GC-MS and HPLC-DAD-MS^n analyses and could mainly be assigned to depsides and phenolic glycerides consisting of various hydroxybenzoic, hydroxycinnamic, and dicarboxylic acids. A plethora of bioactivities have been reported for the aforementioned constituents, such as anti-inflammatory, analgesic, and neuroprotective action. They may therefore also contribute to the pharmacological effects described for GS, which have previously been attributed mainly to alkaloids.

Upon lactic acid fermentation with *L. plantarum*, depsides, glycerides, and other esters were rapidly degraded. Subsequently, the formation of low-molecular phenolic metabolites could be shown in GC-MS analyses. The obtained extracts remained microbially stable during the six-month period of investigation. The presented results expand the knowledge on the traditional medicinal plant GS and may open new perspectives of use, despite its toxicity limiting pharmaceutical applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants13162208/s1, Table S1.1. Calibration data for the Folin–Ciocalteu assay obtained with gallic acid. Table S1.2. Calibration data for the Folin–Ciocalteu assay obtained with chlorogenic acid. Table S1.3. Total phenolic content of *G. sempervirens* DCM, EtOAc, and BuOH extracts (250 µg/mL) calculated as µg gallic or chlorogenic acid equivalents per mg dry weight. Table S2.1. Calibration data for the DPPH radical scavenging assay obtained with trolox. Table S2.2. DPPH radical scavenging capacity of the DCM extract at different concentrations. Table S2.3. DPPH radical scavenging capacity of the ethyl acetate extract at different concentrations. Table S2.4. DPPH radical scavenging capacity of the *n*-butanol extract at different concentrations. Figure S3.1. GC-MS total ion current chromatograms of hydrolyzed (a) *n*-butanol and (b) ethyl acetate extracts after silylation. Table S3.1. Aglycones detected in ethyl acetate and *n*-butanol extracts after hydrolysis and silylation using GC-MS. Compound assignment was achieved by comparison with the NIST database (match factor >800). Table S3.2. GC-MS data of volatile compounds extracted with diethyl ether from aqueous fermented samples. Compound assignment was achieved by comparison with the NIST database (match factor >800). Figure S4.1. HPLC-DAD-ESI-MS^n base peak chromatograms of (A) ethyl acetate and (B) n-butanol extracts of *G. sempervirens* roots and rhizomes recorded in positive ionization mode. Table S4.1. HPLC-DAD-MS^n characteristics of individual secondary metabolites in ethyl acetate and n-butanol extracts from *G. sempervirens* roots and rhizomes obtained in positive ionization mode. Figure S4.2. Stacked display of UV chromatograms (328–332 nm) of aqueous fermented *G. sempervirens* extracts within 30 days of fermentation. References [62,63] are cited in the supplementary materials.

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Conflicts of Interest: L.K.M., K.N.G., P.L., F.C.S. and D.R.K. are employed at WALA Heilmittel GmbH, they declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. R.D. declares no conflict of interest.

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Supporting information

Abbreviations:

A	absorbance
BuOH	*n*-butanol
c	concentration
CA	chlorogenic acid
CAE	chlorogenic acid equivalents
DCM	dichloromethane
DPPH	2,2-diphenyl-1-picrylhydrazyl
dw	dry weight
EtOAc	ethyl acetate
GA	gallic acid
GAE	gallic acid equivalents
MW	molecular weight
TMS	trimethylsilyl
t_R	retention time
SD	standard deviation

Part I: Folin-Ciocalteu assay

Table S1.1. Calibration data for the Folin-Ciocalteu assay obtained with gallic acid.

c [µg/mL]	A_{765nm}	A_{Blank}	Mean	SD
55	0.416	0.334	0.315	0.019
	0.377	0.295		
	0.398	0.316		
36.7	0.329	0.247	0.227	0.028
	0.289	0.207		
	0.406	0.323*		
27.5	0.242	0.16	0.151	0.009
	0.224	0.142		
	0.232	0.15		
13.8	0.189	0.107	0.084	0.023
	0.166	0.084		
	0.143	0.061		
6.9	0.123	0.041	0.031	0.009
	0.109	0.027		
	0.106	0.024		
3.4	0.091	0.009	0.006	0.004
	0.084	0.002		
	0.089	0.007		

*Value was not considered in calculation.

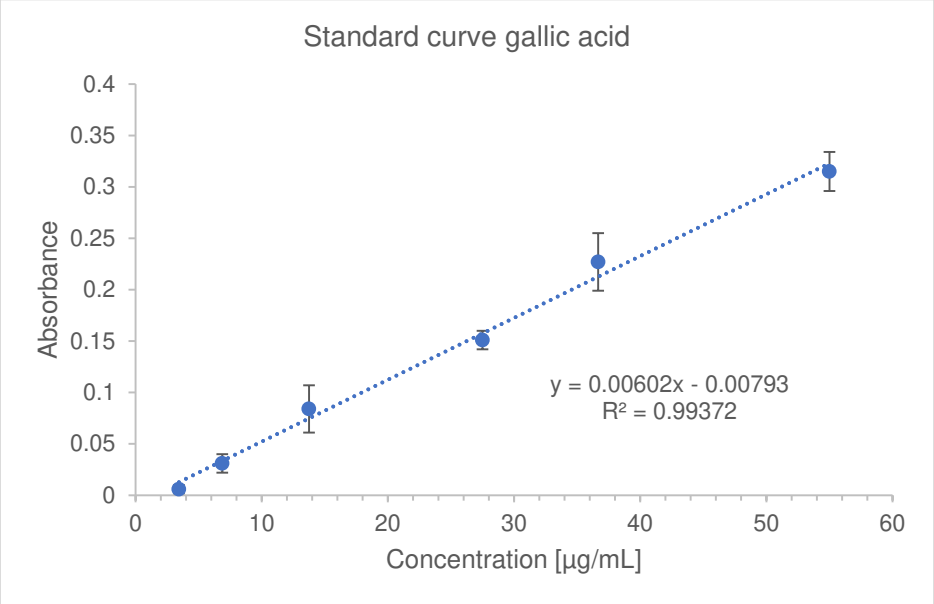

Table S1.2. Calibration data for the Folin-Ciocalteu assay obtained with chlorogenic acid.

c [µg/mL]	A_{765nm}	A_{Blank}	Mean	SD
160	0.639	0.509	0.493	0.035
	0.583	0.453		
	0.647	0.517		
80	0.344	0.214	0.238	0.04
	0.345	0.215		
	0.414	0.284		
53.3	0.277	0.147	0.153	0.029
	0.257	0.128		
	0.314	0.185		
40	0.236	0.106	0.100	0.006
	0.225	0.095		
	0.229	0.099		
20	0.232	0.102	0.061	0.037
	0.164	0.034		
	0.175	0.046		
10	0.116	-0.014	0.001	0.025
	0.117	-0.012		
	0.16	0.03		

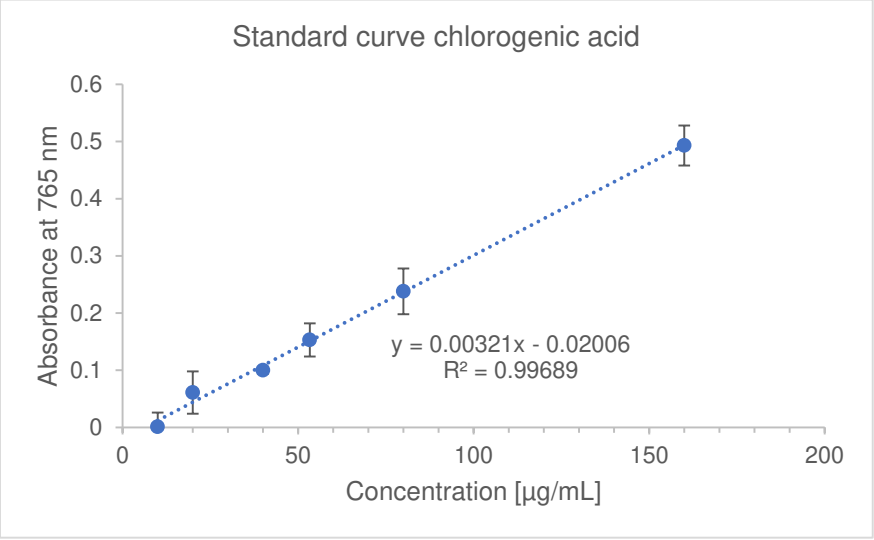

Table S1.3. Total phenolic content of *G. sempervirens* DCM, EtOAc and BuOH extracts (250 µg/mL) calculated as µg gallic or chlorogenic acid equivalents per mg dry weight.

Extract	A_{Blank}	µg GA/ mL	µg GA/ mg dw	Mean	SD	µg CA/ mL	µg CA/ mg dw	Mean	SD
DCM	0.31	52.8	211.2	207.1	5.8	102.8	411.3	411.1	15.6
	0.303	51.6	206.6			100.6	402.6		
	0.288	49.2	196.6			96.0	383.9		
EtOAc	0.394	66.8	267.1	274.0	18.8	129.0	516.0	536.6	41.5
	0.366	62.1	248.5			120.3	481.1		
	0.385	65.3	261.1			126.2	504.7		
BuOH	0.2	34.5	138.2	142.9	10.6	68.6	274.2	290.7	22.7
	0.196	33.9	135.5			67.3	269.2		
	0.215	37.0	148.1			73.2	292.9		

Part II: DPPH assay

Table S2.1. Calibration data for the DPPH radical scavenging assay obtained with trolox.

c [µg/mL]	A_{516nm}	A_{Blank}	Mean	SD	DPPH scavenged [%]
110	0.244	0.164	0.158	0.006	83.1
	0.232	0.152			
	0.238	0.158			
55	0.636	0.556	0.562	0.007	39.8
	0.649	0.570			
	0.639	0.559			
27.5	0.828	0.748	0.756	0.01	19.1
	0.832	0.752			
	0.846	0.767			
13.8	0.928	0.848	0.851	0.004	8.9
	0.930	0.850			
	0.935	0.856			
6.9	0.982	0.902	0.904	0.003	3.2
	0.983	0.903			
	0.987	0.907			
3.4	0.995	0.915	0.92	0.004	1.5
	0.999	0.920			
	1.004	0.924			
1.7	1.007	0.928	0.934	0.007	0.0
	1.013	0.934			
	1.021	0.941			

Table S2.2. DPPH radical scavenging capacity of the DCM extract at different concentrations.

c [µg/mL]	A_{516nm}	A_{Blank}	Mean	SD	DPPH scavenged [%]
500	0.673	0.593	0.595	0.002	36.3
	0.677	0.597			
	0.674	0.594			
250	0.821	0.741	0.735	0.009	21.3
	0.818	0.738			
	0.804	0.725			
125	0.897	0.817	0.822	0.004	12.0
	0.902	0.822			
	0.905	0.825			
62.5	0.954	0.874	0.876	0.004	6.2
	0.953	0.874			
	0.961	0.881			
31.3	0.991	0.911	0.907	0.004	2.9
	0.986	0.907			
	0.983	0.903			

c [µg/mL]	A_{516nm}	A_{Blank}	Mean	SD	DPPH scavenged [%]
15.6	1.004	0.924	0.926	0.003	0.9
	1.003	0.924			
	1.009	0.929			
7.8	1.015	0.935	0.934	0.004	0.0
	1.016	0.936			
	1.009	0.929			

Table S2.3. DPPH radical scavenging capacity of the ethyl acetate extract at different concentrations.

c [µg/mL]	A_{516nm}	A_{Blank}	Mean	SD	DPPH scavenged [%]
500	0.132	0.052	0.053	0.001	94.4
	0.132	0.053			
	0.566	0.487			
250	0.425	0.346	0.346	0.002	63.0
	0.424	0.344			
	0.428	0.348			
125	0.658	0.578	0.581	0.003	37.8
	0.661	0.581			
	0.663	0.584			
62.5	0.823	0.743	0.743	0.004	20.4
	0.818	0.738			
	0.826	0.747			
31.3	0.909	0.829	0.833	0.003	10.8
	0.912	0.832			
	0.916	0.836			
15.6	0.967	0.887	0.888	0.003	4.9
	0.966	0.886			
	0.971	0.891			
7.81	0.996	0.916	0.916	0.004	1.9
	0.992	0.912			
	0.999	0.919			

Table S2.4. DPPH radical scavenging capacity of the *n*-butanol extract at different concentrations.

c [µg/mL]	A_{516nm}	A_{Blank}	Mean	SD	DPPH scavenged [%]
500	0.497	0.418	0.413	0.007	55.8
	0.495	0.416			
	0.485	0.405			
250	0.76	0.681	0.671	0.01	28.2
	0.753	0.673			
	0.74	0.661			
125	0.871	0.792	0.785	0.007	16.0
	0.865	0.786			
	0.857	0.777			
62.5	0.939	0.859	0.857	0.002	8.2
	0.936	0.856			
	0.935	0.855			
31.3	0.975	0.895	0.892	0.004	4.5
	0.974	0.894			
	0.967	0.888			
15.6	1.009	0.929	0.920	0.008	1.5
	0.998	0.918			
	0.993	0.914			
7.8	1.011	0.931	0.926	0.005	0.9
	1.001	0.921			
	1.006	0.927			

Part III: GC-MS analyses

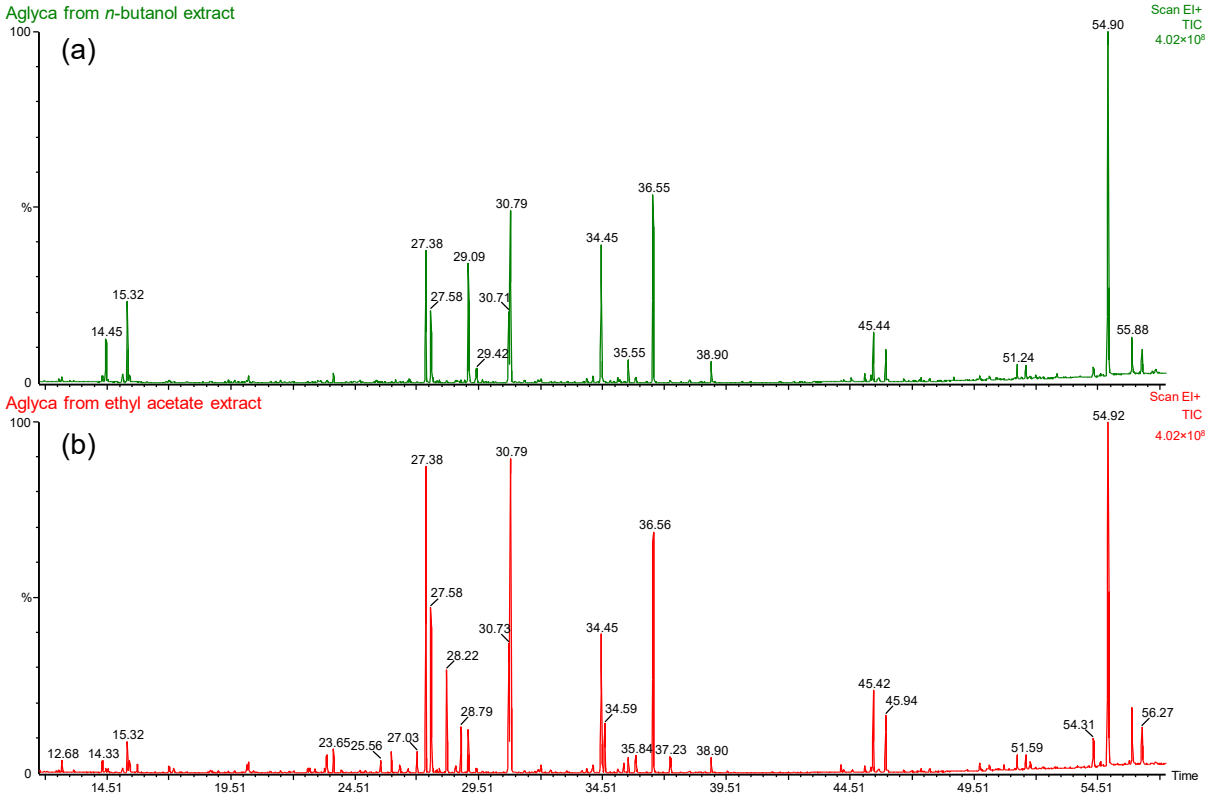

Figure S3.1. GC-MS total ion current chromatograms of hydrolyzed (a) *n*-butanol and (b) ethyl acetate extracts after silylation.

Table S3.1. Aglycones detected in ethyl acetate and *n*-butanol extracts after hydrolysis and silylation using GC-MS. Compound assignment was achieved by comparison with the NIST database (match factor >800).

t_R [min]	Constituent (TMS derivative)	MW [Da]	Fragment *m/z* (Intensity %)
12.7	Benzoic acid	194.3	194 (3), 179 (100), 135 (57), 105 (82), 77 (60)
14.3	Succinic acid	262.4	247 (11), 147 (100), 73 (35)
14.5	3-Methylfurancarboxylic acid	198.3	183 (100), 169 (16), 139 (12), 109 (47), 73 (47)
15.3	Linalool	226.4	226 (9), 143 (100), 94 (13), 81 (31), 73 (74)
23.4	*m*-Salicylic acid	282.5	282 (14), 267 (22), 193 (16), 73 (100)
23.7	*p*-Salicylic acid	282.5	282 (22), 267 (100), 223 (81), 193 (62), 73 (80)
25.6	Suberic acid	318.6	303 (58), 287 (17), 213 (30), 147 (71), 117 (22), 73 (100), 69 (44), 55 (66)
26.0	Suberic acid	318.6	303 (32), 213 (20), 147 (50), 73 (100)
27.0	2,5-Dimethoxy-phenylacetic acid	268.4	268 (1), 253 (17), 209 (20), 134 (18), 105 (33), 73 (100)
27.4	Vanillic acid	312.1	312 (57), 297 (100), 282 (34), 267 (67), 253 (51), 223 (51), 126 (31), 73 (49)
27.6	Gentisic acid	370.6	370 (4), 355 (100), 73 (60)
28.2	Azelaic acid	332.6	317 (51), 201 (52), 147 (26), 129 (31), 117 (29), 73 (100), 55 (48)
28.8	Protocatechuic acid	370.6	370 (49), 355 (26), 311 (21), 193 (100), 73 (53)
29.1	Citral	152.2	255 (18), 152 (32), 107 (22), 84 (51), 75 (52), 73 (41), 69 (100)

t_R [min]	Constituent (TMS derivative)	MW [Da]	Fragment *m/z* (Intensity %)
29.4	Farnesol	294.5	255 (43), 152 (27), 107 (25), 93 (23), 84 (47), 75 (68), 73 (55), 69 (100)
30.7	Pyrogallol	342.7	329 (27), 239 (29), 209 (18), 147 (46), 143 (34), 119 (28), 103 (28), 73 (100)
30.8	Syringic acid	342.5	342 (62), 327 (100), 312 (76), 297 (66), 253 (44), 149 (32), 141 (29), 73 (86)
34.5	Scopoletin	264.3	264 (48), 234 (100), 206 (37), 191 (8), 176 (10), 73 (36)
34.6	Hydroxy-dihydroferulic acid	428.3	428 (46), 297 (86), 73 (100)
35.6	Ferulic acid	338.5	338 (76), 323 (52), 308 (51), 293 (36), 249 (47), 147 (28), 73 (100)
35.8	Esculetin	322.5	322 (35), 307 (30), 73 (100)
36.6	Caffeic acid	396.7	396 (71), 381 (19), 219 (100), 191 (16), 73 (79)
37.2	Linoleic acid	352.3	352 (32), 337 (100), 73 (84)
38.9	Sinapinic acid	368.6	368 (84), 353 (46), 338 (100), 323 (29), 279 (24), 162 (25), 73 (100)
45.4	Unknown		560 (41), 545 (8), 471 (17), 306 (24), 219 (12), 73 (100)
45.9	Unknown		560 (38), 545 (8), 471 (19), 307 (7), 219 (6), 73 (100)
51.2	Vanillylmandelic acid	414.7	401 (1), 297 (100), 73 (49)
51.6	Vanillylmandelic acid	414.7	401 (1), 297 (100), 73 (54)
54.3	Trihydroxyphenylacetic acid	400.7	562 (8), 532 (7), 472 (44), 442 (11), 73 (100),
54.9	Chlorogenic acid	787.4	419 (2), 397 (6), 345 (61), 307 (33), 255 (45), 219 (16), 191 (11), 147 (26), 73 (100)
55.9	Unknown		489 (5), 324 (16), 307 (85), 255 (51), 219 (14), 147 (16), 73 (100)
56.3	Unknown		473 (3), 447 (8), 345 (47), 307 (64), 255 (24), 147 (21), 73 (100)

Deviations from retention times of Table 1 (main manuscript) are due to application of a new GC column.

Table S3.2. GC-MS data of volatile compounds extracted with diethyl ether from aqueous fermented samples. Compound assignment was achieved by comparison with the NIST database (match factor >800).

t_R [min]	Constituent (not silylated)	MW [Da]	Fragment *m/z* (Intensity %)
5.4	Valeric acid	102.1	102 (6), 73 (44), 60 (100), 55 (16)
6.6	Caproic acid	116.2	87 (12), 73 (56), 60 (100), 55 (19)
7.9	Ethyl-3-methyl-butenoate	128.1	128 (51), 113 (58), 95 (44), 83 (100), 67 (65), 55 (83)
8.1	2-Furancarboxylic acid	112.1	112 (100), 95 (61), 55 (10)
9.3	Menthol isomer	156.3	142 (18), 113 (100), 95 (45), 67 (36), 55 (64)
10.6	Benzoic acid	122.1	122 (84), 106 (11), 105 (100), 77 (91)
11.1	2-Acetylphenol	136.1	136 (43), 121 (100), 93 (19), 65 (20), 56 (11)
11.7	3-Propylcyclohex-1-ene	124.2	124 (20), 81 (100), 71 (30), 53 (17)
13.5	1-Methylethylidene cyclohexane	124.2	124 (63), 109 (12), 93 (32), 81 (100), 67 (23), 55 (38)
14.2	*o*-Salicylic acid	138.1	138 (61), 120 (90), 92 (100), 64 (32), 63 (22)
15.0	3,7-Dimethyloct-1-ene	140.3	100 (44), 84 (17), 69 (29), 55 (100)
16.4	2,4-Octadienoic acid	140.1	140 (22), 97 (76), 81 (100), 79 (66), 69 (53), 55 (54)
16.5	γ-Amylbutyrolactone	156.2	85 (100), 55 (11)
16.9	4-Ethylcatechol	138.2	138 (30), 123 (100), 77 (15)

t_R [min]	Constituent (not silylated)	MW [Da]	Fragment *m/z* (Intensity %)
18.7	2-Hydroxy-6-methylbenzaldehyde	136.1	136 (100), 89 (34), 79 (16), 63 (13)
20.5	*p*-Salicylic acid	138.1	138 (51), 121 (100), 93 (26), 65 (18)
20.7	Butylhydroxytoluol*	220.4	220 (24), 205 (100), 177 (15), 145 (14), 57 (17)
22.5	Isovanillic acid	168.1	168 (100), 153 (100), 125 (33), 97 (34), 79 (11), 52 (12)
23.5	3,4,5-Trimethoxyphenol	184.2	184 (73), 169 (100), 141 (52), 126 (27), 111 (27), 69 (43)
23.9	Azelaic acid	188.2	168 (39), 152 (40), 124 (22), 111 (32), 83 (53), 73 (36), 69 (56), 60 (55), 55 (100)
24.9	Oxo-α-ionol	208.3	152 (11), 134 (12), 109 (20), 108 (100), 91 (19)
26.6	7,8-Dihydro-3-oxo-α-ionol	210.3	210 (23), 177 (27), 150 (54), 135 (100), 123 (84), 108 (80), 93 (92), 79 (52)
26.8	Dehydrogeosinin or 4-ethylcamphor	180.2	180 (96), 151 (18), 122 (19), 109 (100), 81 (50), 69 (34), 53 (55)
27.1	Protocatechuic acid	154.1	154 (82), 137 (100), 109 (29), 81 (23), 79 (6), 53 (17)
29.4	Syringic acid	198.1	198 (100), 183 (43), 127 (28), 109 (21), 81 (16)
30.7	Ferulic acid	194.2	194 (100), 179 (34), 133 (23), 77 (30)
31.6	Dimethyloctahydroiso-benzofuran-4-ol	170.2	170 (15), 140 (18), 107 (32), 95 (32), 81 (100), 69 (30), 55 (44)
33.5	Scopoletin	192.2	192 (100), 177 (59), 164 (28), 149 (58), 121 (29), 69 (37)
34.6	Caffeic acid	180.2	180 (100), 163 (27), 134 (58), 89 (32), 77 (25)
36.2	Unknown		222 (100), 207 (52), 179 (20), 123 (36), 95 (23), 79 (19)
36.3	Unknown		222 (100), 207 (23), 179 (23), 123 (15), 95 (12), 79 (12)

*Stabilizing agent in diethylether.

Part IV: RP-HPLC-DAD-ESI-MS^n analyses

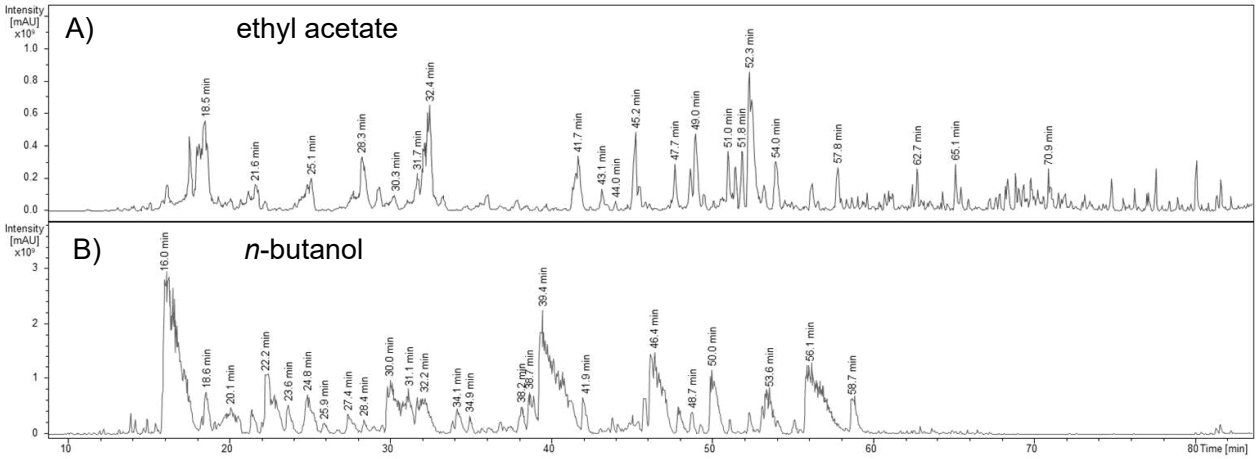

Figure S4.1. HPLC-DAD-ESI-MS^n base peak chromatograms of A) ethyl acetate and B) *n*-butanol extracts of *G. sempervirens* roots and rhizomes recorded in positive ionization mode.

Table S4.1. HPLC-DAD-MS^n characteristics of individual secondary metabolites in ethyl acetate and *n*-butanol extracts from *G. sempervirens* roots and rhizomes obtained in positive ionization mode.

Substance	t_R [min]	extract		UV [nm]	Mass spectrometric data *m/z*			Ref.
		EtOAc	BuOH		MS^1	MS^2	MS^3	
Gelsemine	16.0		X	208, 252, 250, 325	**323**	274, **236,** 196, 190		[1,2]
5-O-Caffeoylquinic acid	18.5	X	X	218, 236, 312, 328	**355**, 163	163	145	[3]
Koumidine	20.1		X	222, 288	387, **295**	297, 265		[2]
Caffeic acid	21.6	X	X	226, 302	**181**	163, 137, 93		
Hydroxygelselegine	22.2		X	224, 290	**375**	**344**	315, 270, 217, 176, 148	[2]
Unknown	23.6		X	230, 322	**391**	**360**, 331	341, 331, 313, 244	
15-Hydroxy-humantenine	24.8		X	-	**371**	**340**	326, 295, **277**, 246	[2]
Dihydroferulic or dimethylphenylacetic acid	25.1	X		218, 316	**197**	**179**		[4]
Koumidine	25.9		X	-	**295**	277, **254,** 225, 171, 144, 132		[2]
19-(Z)-Akuammidine	27.4 28.3	X	X		**353**	333, **323**, 166, 157		[2]
19-(Z)-Anhydrovobasinediole	28.4		X	-	**309**	291	175	[1]
Gelsevirine	30.0		X	216, 324	367, **353**	**322**	294, 289	[2]
Feruloylquinic acid	30.3	X		238, 326	369, **177**	**145**	89	[5]
Gelsempervine A	31.1 32.2		X	226, 264, 340	**383,** 193	**180**	**165,** 148	[1,2]
Gelsevirine	31.7	X		204, 228, 255sh, 295sh, 342	**353,** 193	**322**	307, 304, 391, 189, 281, 264, 261, 233, 223, 251, 132	[1,2]

Substance	t_R [min]	extract		UV [nm]	Mass spectrometric data *m/z*			Ref.
		EtOAc	BuOH		MS^1	MS^2	MS^3	
Scopoletin	32.4	X		207, 228, 296, 342	**193**	**173**		[6]
1-Methoxy-21-oxo-gelsemine	34.1		X	-	**367**	**334**, 180	304	
Gelsedine	38.2		X	226, 288	**329**	**296**	256 224 162	[1]
Sarpagan-16-carboxylic acid, 17-oxo-, methyl ester	38.7		X	252, 304	**351**	**333**	319	
N-Methylgelsedilam	39.4		X	208, 258	351, **329**	**298,** 269	278, 268,	[1]
	41.7	X					242	
Gelegamine C	41.9		X	224	**389**	**339**	226, **175**	[2]
	43.1	X		232, 286	285, **245**	227, 225, **171**	125	
Hydroxygelsenicine	44.0	X		222, 260sh, 286	**343**	325, 181		[1]
Unknown	45.2	X		224, 288	**535**	343, **181**	153	
Gelsemicine	46.4		X	234	**359**	328	298, 245, 166, 152	[1,2]
Gelsemicine	47.7	X		234	**359**	**328**	298, 284, 236, 202	[1,2]
14-Acetoxygelsenicine or 11-Methoxy-humantenine	48.7		X	230, 286, 342	**385,** 371, 315	**354**	321	[1,2]
Dicaffeoylquinic acid isomer	49.0	X		222, 235, 300sh, 326	517, **499**	**319**	299, 163	[3]
11-Hydroxyhumantenine	50.0		X	224, 290,	**371**	**340**	175, 105	[1,2]
or Humantenirine	51.0	X		326				
Dicaffeoylquinic acid isomer	51.8	X		232, 292	535, **517**	**337**	181	[3]
Dicaffeoylquinic acid isomer	52.3	X		220, 240, 300sh, 326	**517**, 499	319, **163**	145, 117	[3]
Tabersonine	53.6		X	234, 322	**337**	305, **180**		[7]
Gelsempervine	54.0	X		234, 322	**425**	**383**, 305, 206	208, 173, 146	[1]
Sempervirin	56.1		X	240, 292, 336, 382	**273**	**257**	185, 159	[1,2]
Unknown	57.8	X		234, 324	561, **543**	363, **207**	175	
Ourouparine	58.7		X	204, 236, 284, 340	**329,** 273	314, 295, 283		[8]
Gelse-norursane A	62.7	X		232, 326	**503**	**439**	421	[1]
Trihydroxy-octadecenoic acid isomer	65.1	X		-	**294**	**277**	131, 107	[4]
Gelse-norursaneE	70.9	X		-	**471**	**453**	407, 217	[1]

Ions displayed in **bold** were isolated for further fragmentation.

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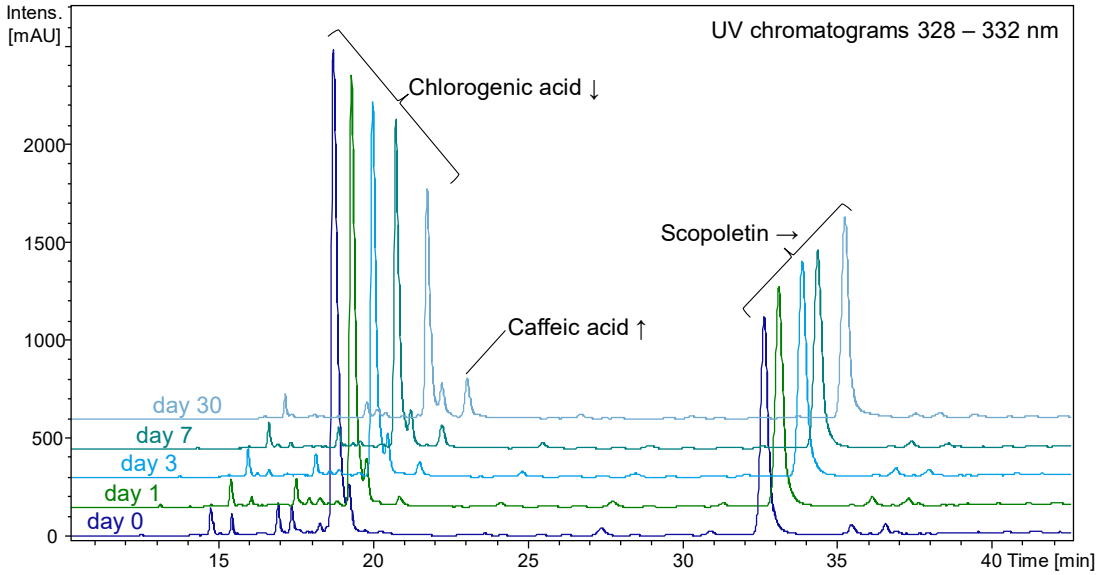

Figure S4.2. Stacked display of UV chromatograms (328 – 332 nm) of aqueous fermented *G. sempervirens* extracts within 30 days of fermentation.

4 GENERAL DISCUSSION

The present thesis describes detailed investigations into underground plant parts of *Matricaria recutita* L. (MR) and *Gelsemium sempervirens* J.ST.-HIL. (GS). Although both of them are important medicinal plants [32,53], the roots of MR are only considered a by-product of chamomile flower production so far and have therefore not aroused large research interest. In contrast, secondary metabolites and physiological effects of GS roots and rhizomes have been widely studied. The focus, however, was always on alkaloids, which is why other compound classes remained mostly overlooked [53,147]. These knowledge gaps have been covered in three publications (chapters 3.1 – 3.3) and are further discussed in the following chapters.

4.1 COMPARISON OF SECONDARY METABOLITES FROM BOTH SPECIES

Although the species investigated are not related, some similarities in their secondary metabolite profiles could be detected using GC-MS and HPLC-DAD-MS^n. These involved mainly ubiquitous substances belonging to the phenolics and the coumarins. (Poly)phenolic compounds are among the principal groups of secondary metabolites and occur throughout most plant families and organs [11,12,26]. Due to their toxic effect on many microorganisms, these substances are predestined root constituents to support their resistance in the soil [11,12]. Accordingly, phenolic acids were among the main secondary metabolites in roots and rhizomes of both plant species studied in the present work. More precisely, acetyl-, caffeoyl- and dicaffeoylquinic acids as well as chicoric acid derivatives were found as main metabolites in EtOAc and BuOH extracts from MR (chapter 3.1). Aqueous extraction discriminated dicaffeoylquinic acids, whereas caffeoylquinic, chicoric and acetylquinic acid derivatives were also assigned in fermentation samples (chapter 3.2). Depsides consisting of hydroxybenzoic, hydroxycinnamic and dicarboxylic acids made up almost the entire base peak chromatograms (negative ionization mode) of EtOAc and BuOH extracts from GS (chapter 3.3). Interestingly, tridepsides consisting of sinapic, ferulic, caffeic and coumaric acids have also been assigned in MR (chapter 3.1). A literature study revealed that similar compounds have also been found in other plant families and plant parts. As an example, twelve depsides with neuroprotective activity were assigned in the roots of *Salvia miltiorrhiza* BUNGE (Lamiaceae) [87]. Depsides consisting of various chlorogenic acid isomers and rosmarinic acid were found in the fruits of *Aronia* ssp.

MEDIK. (Rosaceae) [85], while peels and crowns of *Ananas comosus* (L.) MERR. (Bromeliaceae) fruits were rich in *p*-coumaric, ferulic and caffeic acid depsides [88].
In addition to phenolic acids, coumarins were also present in both plant species investigated in the studies presented here. Coumarin is particularly known from tonka beans (*Dipteryx odorata* (AUBL.) WILLD.) and cassia cinnamon (*Cinnamomum cassia* (L.) D.DON), where contents range up to 3 %, and derivatives thereof have been found in more than 150 plants of about 30 families [148]. In MR, different coumarin glycosides, i.e. aesculin, fraxin, scopolin and isofraxidin-7-glycoside, were characterized based on their UV spectra, fragmentation behavior and a comparison with fraxin as reference compound (chapter 3.1). In comparison, the aglycon scopoletin was the major compound in GC-MS spectra of DCM extracts from GS (chapter 3.3). Glycosides thereof have been identified previously in aerial parts of GS [149]. The coumarins mentioned above have also been found in other plants such as *Arabidopsis thaliana* (L.) HEYNH. (Brassicaceae) or *Fraxinus* ssp. (Oleaceae) [98,150,151]. They therefore appear to be quite widespread in the plant kingdom. Coumarins are important secondary root metabolites since they may enhance iron uptake from the soil and have protective effects against predators and microorganisms [91]. Accordingly, more than ten coumarins and furocoumarins were identified in the roots of *Levisticum officinale* W.D.J.KOCH, a popular food and spice plant [152], while prenylated coumarins were found in the roots of *Nicotiana tabacum* L. [153]. The presence of coumarins in a large number of plants, including the two investigated in this thesis, illustrates that their occurrence is not species-specific, but rather that different plant families take advantage of their biofunctional properties.

4.2 VARIATIONS IN SECONDARY METABOLITES

4.2.1 Differences between plant organs

Secondary metabolites fulfill a plethora of functions in plants [71]. For example, while colorful compounds in the flowers attract pollinators [154], root metabolites are important for nutrient uptake and defense against pathogenic soil bacteria [106,108]. Therefore, the compound profiles in different organs of one plant may vary greatly. This has been demonstrated, e.g., for *Juniperus communis* L., where the essential oil content was highest in the needles and mainly consisted of monoterpenes, while the lowest yield was obtained from bark and wood with the corresponding oil being rich in sesquiterpenoids [155]. In *Rumex* ssp., the phenolic content was highest in flowers and fruits, followed by young and old leaves, while roots and stems contained less phenolic substances [156].

During the investigations for chapters 3.1 – 3.3, similarities and differences between underground and aerial parts were also found for MR and GS. Figure 8 displays GC-MS chromatograms of DCM extracts of MR roots harvested in a medicinal plant garden (Bad Boll) and at a commercial field in Sulzemoos (Bavaria). *Matricaria discoidea* DC. (MD) roots harvested in Bad Boll were used as comparison. As can be seen, all roots lacked bisabolol and its oxides, matricin/chamazulene and flavonoids such as apigenin, all of which are abundant in aerial plant parts and considered important active constituents in MR flowers [32,157]. In contrast, polyacetylenic compounds such as en-yn-dicycloether and chamomillaester, which are characteristic of chamomile flowers, could also be assigned in the roots (chapter 3.1). Alkaloids and phenolics were the main constituents in GS roots and rhizomes (chapter 3.3), but have also been found in aerial parts such as stems and leaves [64]. On the contrary, benzenoids, terpenoids and some fatty acid derivatives were exclusively detected in GS flowers after solid phase microextraction [158].

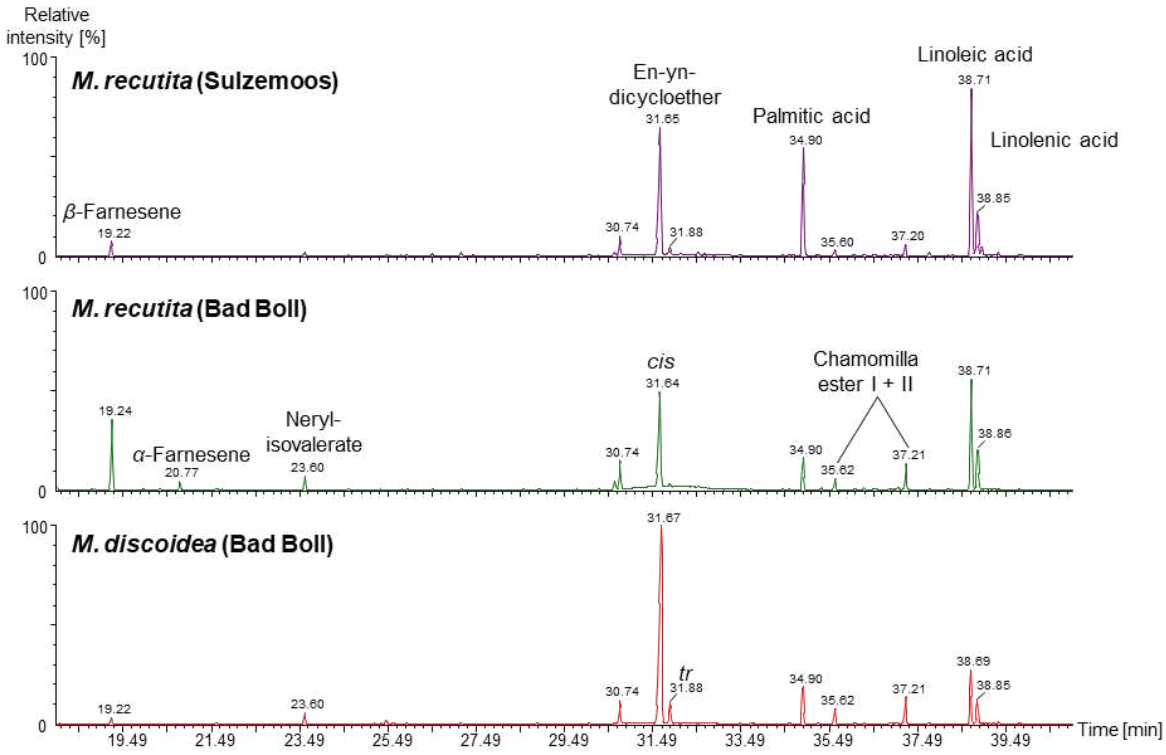

Figure 8. Relevant sections of GC-MS total ion current chromatograms of chamomile root DCM extracts after silylation with BSTFA (*cis*, *tr*: *cis*/*trans* isomer).

4.2.2 Differences between chemotypes

Within one plant species, different chemotypes may be assigned based on qualitative differences in their secondary metabolite profile [71]. For example, in the case of rosemary [159], thyme [160] or chamomile [46], the composition of the essential oil is decisive. More precisely, the essential flower oil of different chamomile chemotypes may differ significantly in its contents of chamazulene, *α*-bisabolol and bisabolol oxides A and B [46]. Accordingly, pronounced differences could be detected in essential oils of the three chamomile varieties studied in chapter 3.1. As displayed in Figure 9, the essential flower oil of the variety harvested in Sulzemoos (Bavaria) was particularly rich in *α*-bisabolol and chamazulene, indicating chemotype C according to Schilcher [46]. The essential oil from flowers harvested in Bad Boll contained equal amounts of the bisabolol oxides A and B and therefore belonged to chemotype D. The essential oil of MD flowers was completely devoid of these constituents but resembled the other oils in its *β*-farnesene and *cis*-spiroether contents. In contrast to these observations, the volatile constituents in chamomile roots were rather similar for the three varieties despite their origin from different locations and soil types (Figure 8). The chemotypes can therefore not be distinguished on the basis of the root constituents. This also

shows that less attention needs to be paid to the chemotype when using the roots than when using the flowers. However, HPLC-DAD-MS^n analyses showed that both MR species contained more coumarins than MD, while the latter was richer in dicaffeoylquinic acids (chapter 3.1). In contrast, no dependence of the volatile metabolite profile could be detected in GS flowers of different population types (wild or cultivated). However, variations were particularly present between long and short flower morphologies [158].

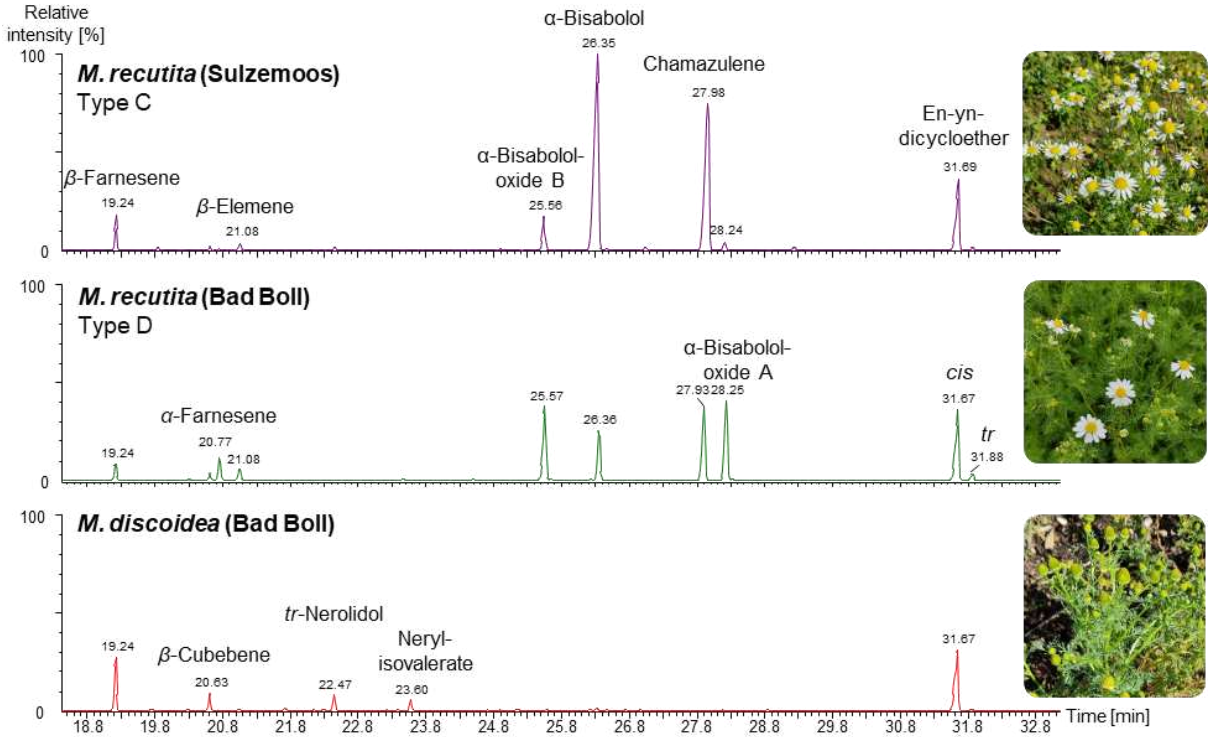

Figure 9. Relevant sections of GC-MS total ion current chromatograms of essential chamomile flower oils obtained by aqueous steam distillation (*cis*, *tr*: *cis*/*trans* isomer).

4.2.3 Impact of growing conditions and harvest time on the secondary metabolite profile

Even within a single chemotype, the content of secondary constituents may vary in terms of composition and quantity [71]. Fluctuations in concentration are particularly common. These may occur over several months, depending on the growth stage and environmental factors such as light, humidity, temperature and salinity. Short-term and diurnal fluctuations may be caused, for example, by herbivore attacks or serve to attract pollinators [71,161,162]. Such fluctuations may affect all parts of the plant. *Scrophularia ningpoensis* HEMSL. is a traditional medicinal plant in China. Containing iridoid glycosides, phenylpropanoids, and flavonoids, it was shown to have various biological activities. Interestingly, the content of secondary metabolites in leaves,

stems and roots increased between April and June. Thereafter, the contents in the roots remained constant, while those in the leaves increased moderately and those in the stems rose sharply. After reaching a maximum in August, the levels dropped to below those of the roots [163]. As another example, in dandelion (*Taraxacum officinale* L.) root latex, the secondary metabolite content was directly influenced by temperature, suggesting that the metabolism of the plant corresponds to seasonal growth requirements [164]. In *Echinacea* and *Astragalus* roots, phenolic contents were higher during fruiting than in the vegetative stages. In roots of perennial herbs, the secondary metabolite content often increases during growth, as has been demonstrated for *Panax ginseng* C.A.MEY. [162].

Fluctuations in the secondary metabolite profile were also observed in the present studies in MR roots harvested between March and June. More precisely, the *β*-farnesene concentration decreased during the growth season from March to June, while chamomillol could only be detected in May and June, thus just before and during flowering (chapter 3.1). In contrast to chamomile, there are hardly any studies on GS in this respect. It has merely been shown previously that the geographic location did not influence the constituent composition of GS flowers [158]. Whether the roots are affected by seasonal or environmental fluctuations may be subject of further investigations.

4.3 BIOFUNCTIONAL PROPERTIES OF ROOT EXTRACTS

Roots and rhizomes of a plant are confronted with completely different conditions than its aerial parts, e.g., darkness, humidity and pathogenic microorganisms of the rhizosphere. One way of dealing with such adversities is the biosynthesis of secondary metabolites with protective and biofunctional properties [165]. The food, cosmetic and pharmaceutical industries may take advantage of this in various ways. This is discussed in the following sections taking antioxidant and antibacterial activities as an example.

4.3.1 Antioxidant activity

Difficult growth conditions such as nutrient deficiencies, heavy metals, drought, salinity or elevated temperatures lead to oxidative stress in plants, which is due to the formation of reactive oxygen species [165]. These free radicals may damage or modify biomolecules with possible effects ranging from cell dysfunction to cell death [166]. To cope with this, plants have developed enzymatic and non-enzymatic strategies [165]. In the latter, phenolic compounds play a major role by their ability to donate electrons or hydrogen atoms and because they form relatively stable radical intermediates [167,168].

However, natural antioxidants and radical scavengers, respectively, are not only important to protect the plant, but they also have nutritional value and may partly prevent aging and ROS related diseases [166]. In addition, clean-label products are progressively desired for food and cosmetics, which means that the ingredient list should be devoid of synthetic additives, transparent and understandable for consumers. Therefore, conventional antibiotics or preservatives are increasingly replaced with antimicrobial natural plant extracts such as essential oils or fermentates, the latter for their content of organic acids and bacteriocins [169–171]. For example, rosemary extracts are exploited as natural preservatives in the food and cosmetics industries [172,173], a chamomile decoction enhanced the shelf life of cottage cheese without altering the nutritional profile [174]. This aroused interest in investigating the antioxidant effect of MR and GS roots in more detail.

To assess the *in vitro* antioxidant capacity of plant-derived extracts, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is a frequently used single electron transfer method. Other possibilities are, e.g., the ferric reducing antioxidant power (FRAP) or azinobis-

(3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay. However, the obtained results may not be directly compared between the different approaches due to lacking standardization, different reaction mechanisms and conditions and because the results reported in the literature are not consistently related to mg dry matter or a defined volume of extract, the concentration of which also often remains unclear [165].

For the DPPH assay, sample solution and methanolic DPPH solution are simply mixed. Antioxidant constituents may then reduce the artificial, stable DPPH radical, which is going along with a color change from dark purple to yellow and thus with a decrease in absorbance at 516 nm [165,166]. The measurement is performed in cuvettes [175,176], on 96-well plates [177,178] or on TLC plates [179,180]. The investigations described in chapter 3.1 were performed using cuvettes and a classical spectrophotometer, while a microplate reader was used for the antioxidant assays described in chapters 3.2 and 3.3. The small volumes on the plate require some practice in pipetting. However, the advantage is that large sample numbers can be analyzed quickly and in parallel with little effort and low solvent consumption.

Different strategies have been published in the literature for the evaluation of the obtained results. Either the absorbance is determined for three to five different sample concentrations and the IC_{50} value is calculated from the linear regression equation at half maximal absorbance [176,178]. However, applying this method, results vary greatly between different research groups and should therefore always be related to standard substances such as trolox [166]. Accordingly, IC_{50} values between 2 – 45 µg/mL have been published for various chamomile flower extracts [42,43,181] with the values determined for chamomile root EtOAc and BuOH extracts in chapter 3.1 lying in the same range. To contextualize these findings, Trinh et al. investigated the DPPH scavenging activity of crude ethanolic extracts of nine different Asteraceae species. The lowest IC_{50} value was 17.3 µg/mL (*Tagetes erecta* L.), all others were >100 µg/mL [182]. A comparison with these results reveals that chamomile flower and root extracts exert significant antioxidant effects.

Alternatively, a calibration curve can be created by determining the absorbance of a reference compound in solutions of different concentrations. Typical reference substances include trolox, ascorbic acid, gallic acid or chlorogenic acid. The sample is then diluted until its absorbance is within the calibration range and the concentration is calculated from the regression equation and expressed as equivalents of the corresponding reference substance per g dry sample weight [175,183]. This approach

was chosen for the experiments reported in chapter 3.2. Here, the aim was not to calculate any specific IC_{50} value, but rather to illustrate changes in antioxidant activity during the course of fermentation. In this way, fluctuations in the DPPH radical scavenging activity were detected during a two-week lactic acid fermentation of aqueous MR extracts. An increase in activity may be explained by enhanced extraction of secondary metabolites from the plant cells due to progressive cell wall degradation by microbial enzymes. The metabolism of phenolic constituents may lead to the formation of various products with stronger or weaker activity than their precursor compounds. Upon storage, the values reached a constant level indicating mutual stabilization of the secondary components (chapter 3.2).

Lastly, the inhibition of the different sample concentrations can be calculated relative to the maximum inhibition, which allows a very clear representation of the curves and a comparison between samples and reference compounds [177,184]. In this way, not only the different antioxidant activities of GS DCM, EtOAc and BuOH extracts could be demonstrated in chapter 3.3, but also the curve form can be evaluated. Interestingly, the curves of DCM and EtOAc extracts were not strictly linear but flattened at higher concentrations. This may indicate concentration-dependent interactions of the secondary constituents (chapter 3.3).

The strong antioxidant activity of phenolic compounds is well-known. Thus, the antioxidant activity of plant extracts is often directly correlated to their total phenolic contents [185]. However, the activities of individual phenolic compounds vary and increase with the degree of hydroxylation and upon methoxylation of hydroxy groups. Hydroxycinnamic acids have stronger effects than hydroxybenzoic acids [11,74,186]. Unsurprisingly, pronounced antioxidant activities have been determined for a plethora of medicinal plants, although systematic studies using identical methodologies are scarce [187]. Investigations into different plant parts of quinoa (*Chenopodium quinoa* WILLD.) have shown, that the roots exhibited stronger antioxidant activities in the DPPH assay than all other plant organs [188].

It is not surprising, that the phenol-rich MR, MD and GS root extracts showed pronounced antioxidant activities in the DPPH assay (chapters 3.1 – 3.3). Generally, aqueous extracts such as decoctions and infusions possess higher antioxidant activities than methanol extracts [177] and IC_{50} values decrease with an increasing polarity of the solvent used [170]. Accordingly, DCM extracts of GS and MR revealed weakest antioxidant activities, while extracts of increasing polarity (EtOAc, BuOH)

exhibited stronger effects (chapters 3.1 and 3.3). MD had slightly higher activities than MR, possibly due to the higher content of dicaffeoylquinic acids (chapter 3.1). To conclude, the presented results extend our knowledge of antioxidant plant extracts and indicate, that the antioxidant effects of MR roots are not only comparable to those of the flowers, but also to other medicinal plants. They may therefore be exploited as natural preservatives in the food or cosmetic industries.

4.3.2 Antibacterial activity

Extensive use and misuse of antibiotics have resulted in the emergence of antibiotic resistance, which is why new antibacterial substance classes or targets are urgently needed [26,189,190]. Promising approaches might be, e.g., the inhibition of LpxC, a key enzyme in the biosynthesis of the outer membrane of Gram-negative bacteria, or the development of pathoblockers. These substances do not kill bacteria, but merely eliminate the toxins they produce [191,192]. The use of plant extracts alone or in combination with common antibiotics is another promising approach due to possible synergistic actions and because they may simultaneously target different bacterial resistance mechanisms [193–195]. Antibacterial plant extracts and essential oil constituents such as terpenes often impair the integrity and structure of the cytoplasmic membrane and thus disrupt its functionality. Inside the cell these may disturb protein and mitochondrial functions [196,197]. Phenolic compounds such as cinnamic acid derivatives also affect the permeability and fluidity of bacterial cell membranes [198]. Other mechanisms include inhibition of protein and DNA synthesis, efflux pumps or quorum sensing [199,200]. Thus, *in vivo* studies reported significantly lower MIC values when combining antibiotics and plant extracts [26,197]. For example, carsonic acid from *Rosmarinus officinalis* increased the effect of the macrolide antibiotic erythromycin by a factor of 16 to 32 [201], and synergistic effects between essential oils of, among others, oregano, thyme or cloves and antibiotics have been monitored *in vitro* [197]. However, the use of plant extracts for antimicrobial applications remains challenging due to natural variations in secondary metabolites (harvesting season, cultivation conditions) and the complexity of the mixture. Furthermore, procedures should be standardized and the access to the plant material has to be regulated and sustainable [202,203].

In order to investigate the antibacterial effects of MR and GS roots and rhizomes, disk diffusion assays were carried out on agar plates and broth microdilution assays on 96-

well plates. DCM, EtOAc and BuOH extracts of fresh MR roots slightly inhibited the growth of the Gram-positive bacteria *S. aureus* and *B. subtilis* in disk diffusion experiments (Figure 10). Gram-negative bacteria, which possess an additional protective membrane, were not affected in this experiment (chapter 3.1).

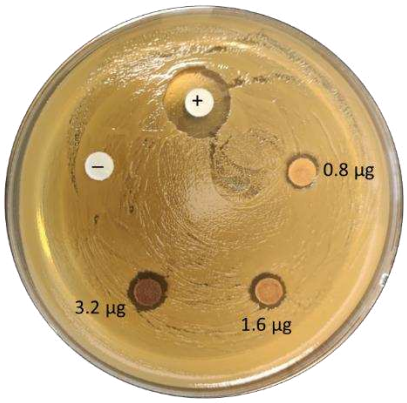

Figure 10. Disk diffusion experiment with *S. aureus* on tryptic soy agar. The disks were loaded with solvent as negative control (−), the antibiotic gentamicin as positive control (+) and different concentrations of a chamomile DCM extract and dried prior to placing them on the bacterial layer.

Interestingly, applying a minimal inhibitory concentration (MIC) assay in 96-well plates, growth inhibition could be detected for *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa*, thus Gram-positive and -negative bacteria (chapter 3.2). This discrepancy may probably be due to the limited diffusion of the active constituents through the agar plate.

The antibacterial potential of aqueous chamomile extracts increased upon fermentation. A comparison with lactic acid as reference compound showed that this effect could be partially, but not completely, attributed to it and the low pH value. It therefore appeared that other secondary ingredients such as spiroethers, coumarin glycosides or glyceroglycolipids were also involved in the effect (chapter 3.2). In the current literature, only MIC values below 1 mg/mL are considered clinically relevant [204]. MIC values below this threshold have been found for some chamomile extracts [157,171]. The values determined for MR in the present study were between 4 and 6 mg/mL and no synergism with the antibiotics ampicillin and gentamicin could be observed in a checkerboard assay. The extracts are therefore not suitable as a substitute for conventional antibiotics. Nevertheless, the metabolism of primary and secondary metabolites, especially phenolic compounds, by *L. plantarum* may contribute to the formation of antibacterial metabolites and thus to the stability of the obtained extracts (chapter 3.2), as has also been shown for canola meal [205].

GS extracts were also tested for their antibacterial effect on the above-mentioned Gram-positive and-negative bacteria; however, no effect was visible in the same concentrations as tested with chamomile. This is surprising since antimicrobial effects of many alkaloids have been proven, e.g. by efflux pump inhibition [102].

To conclude, antimicrobial substances are synthesized by many higher plants as effective defense strategy. In general, these belong to different substance classes, i.e. phenolics, alkaloids, saponins or terpenoids [206]. Chassagne et al. summarized that MIC values below 500 µg/mL have been reported for various plant families, especially the Asteraceae, Fabaceae and Lamiaceae, and that cinnamon, thyme and rosemary were the most intensively studied species [207]. In contrast, despite their high content of phenolic and alkaloid constituents, the extracts investigated in the present work did not exhibit relevant antibacterial activities. This indicates that not only the quantity, but the exact chemical composition of secondary metabolites is decisive for their effects.

4.4 FERMENTATION OF MEDICINAL PLANT EXTRACTS

Lactic acid fermentation of plants and plant-derived substrates has been applied since ancient times in the food sector and fermentative changes as well as health benefits arising thereof have been widely investigated [123,142,208,209]. However, fermentation is also applied in the manufacturing of phytomedicines, e.g., in Traditional Chinese Medicine or anthroposophic medicine [29,136,210].

Fermentation experiments have been performed for both plants, MR and GS, investigated in the studies presented here. According to GHP manufacturing method 33c, fresh MR roots are mixed with honey, lactose and water and are subjected to a seven-day rhythmic procedure with one filtration step after 3.5 days. In contrast, according to method 35b, GS mother tinctures are produced in a slightly different way. Here, dried plant material, water and honey are split evenly into seven subsets. The first subset is mixed and subjected to a rhythmic treatment. For one week, the solution is filtered daily with subsequent addition of another quantity of raw material [29].

For the laboratory fermentation experiments described in chapters 3.2 and 3.3, various pretests have been performed. Spontaneous fermentation as well as batches fermented after inoculation with isolated strains of *L. plantarum* or *P. pentosaceus* were evaluated. Spontaneous fermentation caused by the autochthon microflora of the plants did not always lead to reproducible pH curves as displayed in Figure 11. Fermentation with the two isolated bacterial strains was similar in terms of pH decrease and secondary metabolite profiles (GC-MS, LC-MS). Therefore, only *L. plantarum* was chosen for further fermentation experiments to monitor the impact of process conditions on fermentation. Among others, different incubation temperatures between room temperature and 37 °C have been assessed. Incubation at 33 °C with subsequent storage at room temperature led to a quicker pH decrease than incubation at room temperature, while spoilage occurred more frequently at 37 °C. Therefore, a temperature of 33 °C was chosen for all further experiments.

With around 6, the initial pH value of chamomile root extracts was slightly higher than that of GS extracts with 4.8 (Figure 11). Addition of further GS plant material on the third day of fermentation led to a pH decrease in pH (Figure 11 C), which was not monitored without addition (Figure 11 B). However, after 14 days, mold was only visible on the surface of this batch (Figure 11 C). Despite the lower pH, which normally enhances stability, the extract solution has presumably been spoilt with fungal spores upon repeated addition of plant material. After inoculation with *L. plantarum*, the pH of

the extracts decreased to 3.4 – 3.5 within three days independent of the plant (Figure 11 D, chapters 3.2 and 3.3).

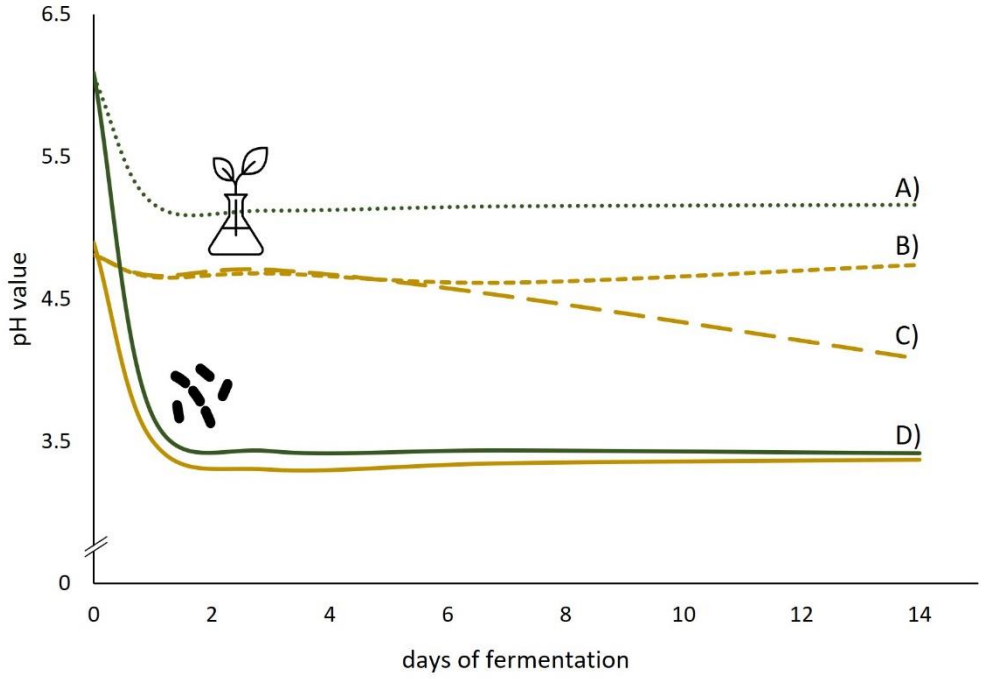

Figure 11. Mean pH values of aqueous MR (green) and GS (yellow) root extracts during lactic acid fermentation. A) Spontaneous MR fermentation; B) Spontaneously fermented GS batch filtered through cotton on the third day,without further addition of material; C) Further addition of dried GS, water and honey after filtration on day three; D) MR and GS after inoculation with *L. plantarum*.

A look at fermentative changes firstly revealed the formation of lactic acid and other low-molecular organic acids from saccharides, which was reflected in the pH decrease. In accordance with previously published data [111,126] and as discussed in chapter 1.4.2, depsides and other esters as well as glycosides were found to be quickly degraded to aglycones and monomeric substances. Major changes occurred within one week after inoculation, i.e. with high LAB activity and increasing extraction of the plant material, with the obtained extracts remaining stable for at least 12 months (chapters 3.2 and 3.3).

In fermented MR extracts mainly aliphatic hydroxycarboxylic and dicarboxylic acids were characterized using GC-MS (chapter 3.2). Fermented GS extracts, however, were rich in volatile phenolic acids and their degradation products such as 4-ethylcatechol (chapter 3.3). These compounds may be formed by enzymes from *L. plantarum* [69,142,211] and are well-known from fermented plants and foods such as rice beer [212], wine [213], tamarillo juice [214], or witch hazel extracts [126]. In contrast, only a slight decrease in indole alkaloid contents of aqueous *Gelsemium sempervirens* extracts was observed (chapter 3.3). This is most probably not due to

alkaloid metabolism but can be explained by progressive precipitation due to polymerization reactions or poor solubility. Presumably, the slightly acidic pH value of the fermentate is not sufficient to fully protonate the alkaloids and thus increase their solubility in water. Accordingly, six alkaloids were detected in the sediment, which was formed upon storage, after rinsing and extraction of the latter with 65 % MeOH (Figure 12).

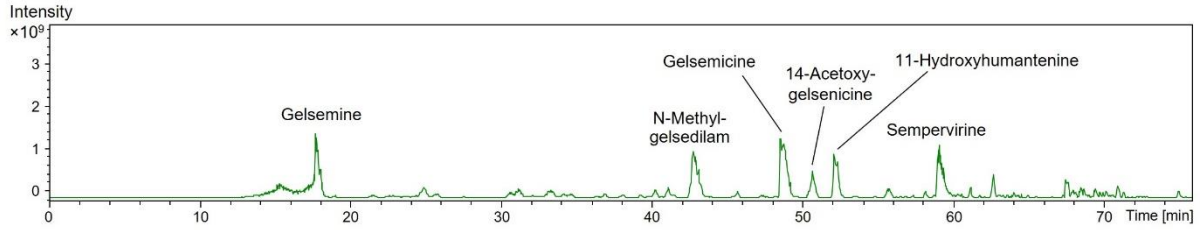

Figure 12. HPLC-MS^n base peak chromatogram (positive ionization mode) of a 65 % MeOH extract of sediment from a GS fermentation sample.

The metabolism of secondary constituents may affect bioactivities of the fermented extracts. Numerous studies reported enhanced antioxidant activity, for example in fermented black soy milk due to the formation of isoflavone aglycones [209] and in jujube-wolfberry juice due to increasing total phenolic contents upon fermentation with *L. plantarum* [215]. The same trend was observed in fermented apple [216] and red beetroot [217] juices as well as in kimchi [208]. In contrast, phenolic contents and antioxidant activity decreased upon fermentation of cocoa beans [218]. Furthermore, fermentation of chamomile lingulate flower extracts did not significantly improve their antioxidant activity [157] and, accordingly, antioxidant activities of MR extracts remained relatively stable upon fermentation (chapter 3.2).

At the same time, the antibacterial potential of MR extracts against *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. aureus* increased upon fermentation (chapter 3.2). In accordance with these findings, fermented chamomile extracts were previously shown to be more potent than native extracts against *B. subtilis* and *E. coli* [157]. This has also been shown for other substrates. E.g., *L. plantarum* fermented canola meal increased its activity against *Salmonella enterica* and *Campylobacter jejuni* [205], fermented rosemary extract moderately inhibited methicillin-resistant *Staphylococcus aureus* (MRSA) [86].

To conclude, for both plants studied, lactic acid fermentation turned out to be a rather simple method for the production of stable aqueous extracts in a cost-efficient manner and in absence of organic solvents and synthetic preservatives.

4.5 TOWARDS A MORE SUSTAINABLE INDUSTRY

500 million tons of residual biomass are generated annually as by-product from agricultural production, which has a huge impact on economy, environment and society [219–221]. Chamomile, as an example, is mainly cultivated for its flower heads and essential flower oil. The latter is commonly obtained by hydrodistillation of the upper plant parts. The remaining plant parts, such as lower stems and leaves or roots are discarded or composted, as is the distillation residue [222]. However, these by-products may contain valuable phytochemicals which could be used in the pharmaceutical or cosmetic industries as active ingredients [219,222]. As an example, the distillation residues of Roman chamomile (*Chamaemelum nobile* L.) were shown to exhibit strong antioxidant effects due to their high contents of caffeoylquinic acids [223]. Other investigations demonstrated that chamomile distillation residues may also be used as a source of pectic substances [222] and other polysaccharides [224]. In addition, chamomile roots were shown to be a rich source of antioxidant and antibacterial phenolic compounds (chapter 3.1). Enzymatic extraction or microbial bioconversion of these plant organs may further enhance the yield and bioactivity of the corresponding extracts by releasing phenolics from their bound forms and transforming them into more potent bioactive compounds [120,225]. The findings reported in chapter 3.1 may therefore not only contribute to sustainable and circular chamomile cultivation, but also add economic benefits to producers through the use of currently underutilized plant parts [219,226].

Among the side streams, wastewater originating from the food, pharmaceutical and cosmetics industries must also be considered in terms of sustainability. Most wastewater treatment plants today are not designed for the removal of anthropogenic trace contaminants such as contraceptives, analgesics or antibiotics [227]. For such pharmaceutical residues, a fourth purification stage is often demanded, which allows their removal through advanced oxidation processes such as ozonation and photoelectrocatalytic reactions or adsorption onto activated charcoal [228]. In contrast to synthetic cosmetic or pharmaceutical ingredients, plant-derived extracts as those discussed in chapters 3.1 to 3.3 can be considered naturally biodegradable and are degraded in the second purification stage. Thus, not only the production of plant extracts for industrial applications, but also their disposal may be regarded as sustainable [228].

5 SUMMARY

In the present study, root and rhizome extracts of the traditional medicinal plants chamomile (*Matricaria recutita* L.) and yellow jessamine (*Gelsemium sempervirens* (L.) J.ST.-HIL.) were subjected to a comprehensive phytochemical characterization by GC-MS and HPLC-DAD-MS^n and their antioxidant activity was investigated. In chamomile roots, polyacetylenic compounds, which are characteristic of chamomile, were characterized besides various coumarin glycosides, caffeic acid derivatives and glyceroglycolipids. In *Gelsemium*, mainly indole alkaloids have been described to date. Hence, in this work, special attention was paid to other constituents not reported so far. The occurrence of various di- and tridepsides was of special interest. These phenolic acid esters are particularly known from lichen species, but also from few higher plants. As the large number of characterized phenolic compounds already suggested, the ethyl acetate and butanol extracts of both plants showed strong antioxidant effects.

In a second step, aqueous root extracts of the two plants were subjected to lactic acid fermentation, based on the manufacturing monographs 33 and 35 of the German Homeopathic Pharmacopoeia. To maximize reproducibility, the preparations were inoculated with the lactic acid bacterium *L. plantarum*. This led to a rapid decrease in pH as a result of lactic acid formation. Within one week, hydrolysis of glycosides and esters, including depsides, could be monitored using HPLC-DAD-MS^n. While hydroxy- and dicarboxylic acids could be assigned in fermented chamomile extracts using GC-MS, a pronounced metabolic conversion of phenolic substances into volatile, aroma-active compounds was observed in fermented extracts of yellow jessamine. Although total phenolic contents and antioxidant activity of the chamomile extracts exhibited fluctuations during fermentation, they remained relatively constant in general. The antibacterial activity increased upon fermentation, which was only partly due to the lactic acid formed and is probably also due to the metabolic conversion of other compounds.

In summary, the presented results expand our knowledge of the underground plant parts of these two medicinal plants, demonstrate their pharmaceutical potential, and may also contribute to sustainable and economical plant cultivation through the use of previously underutilized plant parts.

6 ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurden Extrakte aus Wurzeln und Rhizomen der traditionellen Heilpflanzen Echte Kamille (*Matricaria recutita* L.) und Gelber Jasmin (*Gelsemium sempervirens* (L.) J.ST.-HIL.) mittels GC-MS und HPLC-DAD-MS^n umfassend phytochemisch charakterisiert sowie auf ihre antioxidative Aktivität untersucht. In den Wurzeln der Echten Kamille konnten dabei neben den kamillentypischen Polyacetylenverbindungen verschiedene Coumarin-Glycoside, Kaffeesäurederivate und Glyceroglycolipide charakterisiert werden. In *Gelsemium* wurden bisher überwiegend Indolalkaloide beschrieben. In dieser Arbeit wurde daher ein besonderer Fokus auf weitere Inhaltsstoffe gelegt. Besonders hervorhebenswert war das Vorkommen verschiedener Di- und Tridepside. Diese Phenolsäure-Ester sind insbesondere aus Flechten, aber auch aus wenigen höheren Pflanzen bekannt. Wie die große Anzahl an charakterisierten phenolischen Verbindungen bereits vermuten ließ, zeigten die Ethylacetat- und Butanol-Extrakte beider Pflanzen eine starke antioxidative Wirkung.

Im zweiten Schritt wurden wässrige Wurzel- bzw. Rhizom-Extrakte der beiden Pflanzen einer milchsauren Fermentation unterzogen, angelehnt an die Herstellmonographien 33 und 35 des Homöopathischen Arzneibuchs. Um die Reproduzierbarkeit zu erhöhen, wurden die Ansätze mit dem Milchsäurebakterium *L. plantarum* inokuliert. Dies führte zu einer raschen pH-Absenkung durch Milchsäurebildung. Innerhalb einer Woche konnte mittels HPLC-DAD-MS^n eine Hydrolyse von Glycosiden und Estern wie den Depsiden nachgewiesen werden. Während mittels GC-MS in fermentierten Kamillen-Extrakten überwiegend Hydroxy- und Dicarbonsäuren charakterisiert wurden, war beim Gelben Jasmin eine metabolische Umwandlung phenolischer Substanzen zu flüchtigen, aromaaktiven Verbindungen zu beobachten. Gesamtphenolgehalt und antioxidative Aktivität der Kamillenextrakte schwankten zwar während der Fermentation, blieben insgesamt aber relativ konstant. Die antibakterielle Wirkung hingegen nahm im Verlauf der Fermentation zu, was nur teilweise auf die gebildete Milchsäure zurückzuführen war und wahrscheinlich auch durch weitere Stoffwechselmetaboliten bedingt wird.

Zusammenfassend erweitern die hier vorgestellten Ergebnisse unser Wissen über die unterirdischen Pflanzenteile der beiden untersuchten Arzneipflanzen, zeigen deren pharmazeutisches Potential und könnten durch die Verwendung bisher wenig oder nicht genutzter Pflanzenteile auch zu einem nachhaltigen und wirtschaftlicheren Pflanzenanbau beitragen.

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8 LIST OF FIGURES