DISSERTATION | DOCTORAL THESIS

Titel | Title

Traditional Austrian Herbal Medicines for Wound Healing – Analytics and Bioactivity

verfasst von | submitted by

Mag.pharm. Elisabeth Eichenauer

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"Die Fragen sind es, aus denen das, was bleibt, entsteht."
Erich Kästner

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Abstract

Herbal medicines are valued as alternative therapies for wound healing. This doctoral thesis examines traditional wound-healing plants in Austrian folk medicine. Hence, the VOLKSMED database was screened for wound-healing indications. Seven lesser-known plant genera were surveyed regarding their status in the published literature on wound healing (**Publication I**). Three of these almost-forgotten genera were further investigated in this thesis.

Norway spruce (*Picea abies* (L.) H.Karst.) balm, traditionally prepared as a lard-based ointment, was analyzed for its phytochemical composition and potential to enhance re-epithelialization *in vitro*. Lignans and diterpene resin acids were found to be the main bioactive components (**Publication II**). These diterpene resin acids represent the major substance class found in Norway spruce balm and seem appropriate as marker compounds for quality control. Therefore, we developed a rapid supercritical fluid chromatography protocol to separate and quantitate these substances in different balm samples and commercial products (**Publication III**). Additionally, this thesis compared the phytochemical profiles and pro-re-epithelialization properties of Norway spruce balm and resin, larch balm, and black pine resin. Hence, appropriate chromatographic methods were developed. Significant *in vitro* results were found for larch balm, revealing still unknown components (**Publication IV**). Lastly, different semi-solid vehicles were analyzed as potential alternatives to the traditional lard. The most stable one, wool wax/castor oil, was chosen for permeation studies using Franz-diffusion cells and compared to a lard-based commercial product. More polar balm constituents showed higher total permeated amounts from lard than wool wax/castor oil, whereas the more apolar substances revealed a comparable permeation potential from both formulations (**Publication V**).

The aerial and underground parts of sanicle (*Sanicula europaea* L.) are traditionally used to treat wounds. However, the term "Radix Saniculae" is synonymously used for drooping bittercress (*Cardamine enneaphyllos* (L.) Crantz) in the Austrian vernacular language. Therefore, chromatographic methods were established to compare their main constituents. Besides sugars, hydroxycinnamic acid derivatives and triterpene saponins were detected in sanicle versus glucosinolates in drooping bittercress (**Manuscript I**).

Fresh leaves of white cabbage (*Brassica oleracea* var. *capitata* L. f. *alba*) are traditionally applied as compresses on wounds. The active principle underlying this tradition was investigated via bioactivity-guided fractionation using different *in vitro* assays assessing

bioactivities relevant to wound healing (i.e., keratinocyte migration/proliferation, activation of Nrf2- and inhibition of NF-κB signaling). The most active extracts and fractions were characterized using various chromatographic techniques. Moreover, the major volatile compounds were analyzed (**Manuscript II**).

The results of this thesis substantiate the use of these wound-healing traditions, and the established chromatographic methods provide a basis for quality control during the reintroduction of these applications in modern times.

Zusammenfassung

Heilpflanzen sind geschätzte alternative Therapieoptionen zur Wundheilung. Diese Doktorarbeit untersucht traditionelle wundheilungsfördernde Pflanzen der österreichischen Volksmedizin. Dazu wurde die VOLKSMED-Datenbank auf Indikationen im Bereich der Wundheilung durchsucht. Sieben weniger bekannte Pflanzengattungen wurden hinsichtlich ihres Status in der Literatur zur potentiellen Wundheilung untersucht (**Publikation I**). Für tiefergehende Untersuchungen im Rahmen dieser Arbeit wurde drei dieser fast vergessenen Gattungen ausgewählt.

Der Balsam der Gemeinen Fichte (*Picea abies* (L.) H.Karst.), traditionell mit Schweineschmalz zu einer Salbe verarbeitet, wurde auf seine phytochemische Zusammensetzung und seine *in vitro* re-epithelisierungsfördernden Effekte analysiert. Lignane und Diterpenharzsäuren wurden als die bioaktiven Hauptbestandteile identifiziert (**Publikation II**). Diterpenharzsäuren stellen die Hauptsubstanzen im Fichtenbalsam dar und können als Markerverbindungen für die Qualitätskontrolle verwendet werden. Daher wurde ein schnelles Protokoll für die überkritische Fluidchromatographie entwickelt, um diese Substanzen in verschiedenen Fichtenbalsamproben und -produkten zu trennen und zu quantifizieren (**Publikation III**). Des Weiteren verglich diese Arbeit die phytochemischen Profile und die re-epithelisierungsfördernden Aktivitäten von Fichtenbalsam, Fichtenharz, Lärchenbalsam und Schwarzkiefernharz. Entsprechende chromatographische Methoden wurden entwickelt. Lärchenbalsam führte zu signifikanten *in vitro*-Ergebnissen, und offenbarte die meisten noch unbekannten Substanzen (**Publikation IV**). Letztlich wurden verschiedene halbfeste Salbengrundlagen als potentielle Alternativen zum traditionellen Schweineschmalz analysiert. Das stabilste Vehikel, Wollwachs/Rizinusöl, wurde für Permeationsstudien mit Franz-Diffusionszellen ausgewählt und mit einem Schweineschmalz-basierten Produkt verglichen. Stärker polare Balsambestandteile zeigten höhere Permeationsraten aus Schweineschmalz als aus Wollwachs/Rizinusöl, wohingegen die apolaren Substanzen ein ähnliches Permeationsverhalten bei beiden Formulierungen aufwiesen (**Publikation V**).

Die ober- und unterirdischen Teile des Sanikels (*Sanicula europaea* L.) werden traditionell zur Behandlung von Wunden verwendet. Der Begriff „Radix Saniculae“ wird jedoch im österreichischen Dialekt auch für die quirlblättrige Zahnwurz (*Cardamine enneaphyllos* (L.) Crantz) verwendet. Daher wurden chromatographische Methoden entwickelt, um ihre Hauptinhaltsstoffe zu vergleichen. Neben Zuckern wurden im Sanikel

Hydroxyzimtsäurederivate und Triterpensaponine detektiert, in der Zahnwurz hingegen Glucosinolate (**Manuskript I**).

Frische Blätter des Weißkohls (*Brassica oleracea* var. *capitata* L. f. *alba*) werden traditionell als Wickel bei Wunden angewendet. Das Prinzip hinter dieser Tradition wurde mittels bioaktivitätsgeleiteter Fraktionierung und verschiedener *in vitro*-Tests untersucht, welche alle bei der Wundheilung nützliche Bioaktivitäten umfassten, nämlich Migration/Proliferation von Keratinozyten, Aktivierung von Nrf2- und Hemmung von NF-κB-Signalwegen). Die aktivsten Extrakte und Fraktionen wurden mit verschiedenen chromatographischen Techniken charakterisiert. Des Weiteren wurden die flüchtigen Hauptkomponenten analysiert (**Manuskript II**).

Die Ergebnisse dieser Dissertation untermauern die traditionelle Anwendung dieser wundheilenden Arzneipflanzen. Die etablierten chromatographischen Methoden dienen zur Qualitätskontrolle, um diese wundheilungsfördernden Traditionen in der modernen Zeit wiederzubeleben und die pharmazeutische Qualität zu sichern.

Table of Publications

This doctoral thesis is based on the following publications:

Publication	Title & Authors	Journal
Publication I	VOLKSMED Database: A Source for Forgotten Wound Healing Plants in Austrian Folk Medicine ***Eichenauer Elisabeth**, Saukel Johannes, Glasl Sabine*	*Planta Medica* 2024; 90: 498–511 10.1055/a-2225-7545
Publication II	Norway Spruce Balm: Phytochemical Composition and Ability to Enhance Re-epithelialization *In Vitro* *Goels Thomas, **Eichenauer Elisabeth**, Langeder Julia, Hoeller Franziska, Sykora, Christina, Tahir Ammar, Urban Ernst, Heiss Elke H., Saukel Johannes, Glasl Sabine*	*Planta Medica* 2020; 86: 1080–1088 10.1055/a-1141-0921
Publication III	Ultra High-Performance Supercritical Fluid Chromatography for the Quantitation of Diterpene Resin Acids in Norway Spruce Samples *Goels Thomas, **Eichenauer Elisabeth**, Langeder Julia, Aichner Georg F., Mauser Gregor, Amtmann Luisa, Grienke Ulrike, Glasl Sabine*	*Frontiers in Pharmacology* 2022; 13: 906411 10.3389/fphar.2022.906411
Publication IV	Exudates of *Picea abies*, *Pinus nigra*, and *Larix decidua*: Chromatographic Comparison and Pro-Migratory Effects on Keratinocytes In Vitro *Goels Thomas, **Eichenauer Elisabeth**, Tahir Ammar, Prochaska Paul, Hoeller Franziska, Heiss Elke H., Glasl Sabine*	*Plants* 2022; 11: 599 10.3390/plants11050599
Publication V	Spruce Balm-Based Semisolid Vehicles for Wound Healing: Effect of Excipients on Rheological Properties and Ex Vivo Skin Permeation ***Eichenauer Elisabeth**, Jozić Martina, Glasl Sabine, Klang Victoria*	*Pharmaceutics* 2023; 15: 1678 10.3390/pharmaceutics15061678

Manuscript I	"Radix Saniculae": Phytochemical Characterization and Potential Adulteration of an Austrian Traditional Wound-Healing Agent ***Eichenauer Elisabeth**, Christina Sykora, Karin Ortmayr, Glasl Sabine*	Submitted in November 2024
Manuscript II	White Cabbage as Wound-Healing Agent: Phytochemical Analysis and Bioactivity Screening ***Eichenauer Elisabeth**, Braunböck Barbara, Ortmayr Karin, Heiss Elke H., Glasl Sabine*	In preparation

List of Abbreviations

AMR	**A**nti**m**icrobial **R**esistance
AMS	**A**nti**m**icrobial **S**tewardship
AUC	**A**rea **U**nder the **C**urve
CP	**C**ommercial **P**roduct
DAD	**D**iode **A**rray **D**etector
DCM	**Dic**hloro**m**ethane
DHAA	**De**hydro**a**bietic **a**cid
DPPH	2,2-**Di**p**h**enyl-1-**P**icryl**h**ydrazine
DRAs	**D**iterpene **R**esin **A**cid**s**
EEA	**E**uropean **E**conomic **A**rea
ELSD	**E**vaporative **L**ight **S**cattering **D**etector
EMA	**E**uropean **M**edicines **A**gency
ESI	**E**lectro**s**pray **I**onization
FC	**F**lash **C**hromatography
GC	**G**as **C**hromatography
GNPS	**G**lobal **N**atural **P**roducts **S**ocial Molecular Networking
HMPC	**H**erbal **M**edicinal **P**roduct **C**ommittee
HPLC	**H**igh-**P**erformance **L**iquid **C**hromatography
LOD	**L**imit **o**f **D**etection
LOQ	**L**imit **o**f **Q**uantitation
MRSA	**M**ethicillin-**R**esistant ***S**taphylococcus **a**ureus*
MS	**M**ass **S**pectrometry
NF-κB	**N**uclear **F**actor **K**appa-Light-Chain-Enhancer of Activated **B** Cells
NPs	**N**atural **P**roduct**s**
Nrf2	**N**uclear Factor Erythroid 2-**R**elated **F**actor 2
NSB	**N**orway **S**pruce **B**alm
PDA	**P**hoto **D**iode **A**rray
ROS	**R**eactive **O**xygen **S**pecies
SFC	**S**upercritical **F**luid **C**hromatography
SPE	**S**olid **P**hase **E**xtraction
TLC	**T**hin **L**ayer **C**hromatography
TNF-α	**T**umor **N**ecrosis **F**actor **α**
UHPSFC	**U**ltra **H**igh-**P**erformance **S**upercritical Fluid **C**hromatography

Table of Contents

1. Introduction

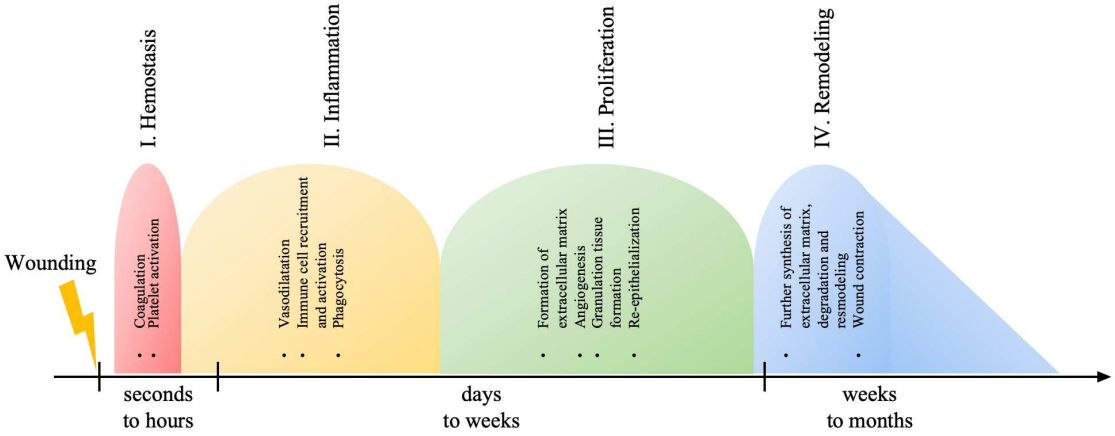
1.1 Wound Healing – Mechanism, Complications, and Management

The largest organ — the skin, entirely covers the human body. It is essential for defense, sensation, and regulation of body temperature. More precisely, the skin protects against physical, chemical, or thermal impacts and is a prerequisite for controlling temperature and fluid homeostasis and synthesizing Vitamin D [1,2]. The skin consists of various layers, with the epidermis as the outermost region. It is divided into several strata: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum germinativum. Keratinocytes represent the primary cell type found in the epidermis. Beneath the epidermis lays the dermis, which contains a collagen matrix formed by fibroblasts and blood vessels. The last layer of the skin is the hypodermis, or subcutis, consisting of superficial facia and loose adipose tissue. Throughout all three layers, appendages like hair follicles, sweat and sebaceous glands are located [3,4].

A wound arises whenever the integrity of the skin is disrupted, bringing to life a sophisticated cascade of healing processes run by various cell types and cytokines. This healing mechanism has four phases: 1. hemostasis, 2. inflammation, 3. proliferation, and 4. remodeling. The first phase, hemostasis, starts immediately after injury and leads to the rapid constriction of blood vessels and fibrin clot formation. The inflammatory phase begins shortly after the first phase, with the primary goal of preventing the invasion of microbes and removing debris. After about three days, the proliferative phase starts, resulting in the re-epithelialization of the wound, the formation of the extracellular matrix, and angiogenesis. Finally, the remodeling phase begins the first week after injury and may take several weeks. In this phase, the type III collagen is replaced by mature type I collagen, and wound contraction occurs [5,6]. The high number of various cell types, growth factors, and cytokines attest to the complexity of this mechanism (Figure 1.1, page 2).

This acute and physiological wound-healing process takes up to several weeks. However, different factors may lead to a delay in this expected time, leading to chronic wounds. These factors are multifaceted (e.g., infections with microbes, persistent inflammation, decreased angiogenesis, elevated oxidative stress) and often related to chronic diseases like diabetes and cardiovascular diseases. Therefore, the etiologies of chronic wounds are manifold [7,8]. However, the most common chronic wounds are pressure ulcers, vascular wounds (venous and arterial insufficiency ulcers), and diabetic ulcers. Elderly people are more likely to develop chronic wounds since a higher age increases the risk of chronic diseases, such as diabetes and cardiovascular problems, immobility, or poor nutritional sustenance [9]. It is

estimated that in 2045, over 783 million people will be affected by diabetes all over the world [10], of which 19% to 34% might develop diabetic foot ulcerations during their lifetime [11]. Furthermore, age-related changes in cell biology impair wound healing, such as delayed epithelialization and decreased levels of growth factors and extracellular matrix [9]. Hence, with the aging societies, the incidence of chronic wounds is rising worldwide [12].



	Main cell types	**Main growth factors and cytokines**
I. Hemostasis	Platelets	Platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), insulin growth factor (IGF)
II. Inflammation	Neutrophils Monocytes (Macrophages) Mast cells T cells	Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, IL-12, vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF)
III. Proliferation	Fibroblasts Endothelial cells Keratinocytes	VEGF, PDGF, fibroblast growth factor (FGF), TGF-β, EGF, Heparin-binding (HB)-EGF, fibroblast growth factor (FGF2), KGF, TNF-α, IL-1, IL-6
IV. Remodeling	Fibroblasts Macrophages	FGF, TGF-β

Figure 1.1 Overview of the wound-healing timeline with the main goals of each phase and a summary of principle cell types, essential growth factors, and cytokines involved (adapted from *[5,13–15]*).

Besides age and health status, the colonization of wounds by bacteria and fungi leads to problems in the physiological wound-healing mechanism [16]. Regarding bacteria, antimicrobial resistance (AMR) represents a global problem, even in wound healing. The misuse of antibiotics in wound management occurs due to unwarranted patient requests, missing diagnostic determination, clinical ignorance of antimicrobial stewardship (AMS), or even fear of insufficient healing results without antibiotics [17,18]. The most common bacteria colonizing chronic wounds in Europe are *Staphylococcus aureus* and *Pseudomonas aeruginosa*, followed by *Enterococcus* sp., such as *Enterococcus faecalis* [19,20]. According to the annual epidemiological report 2022 about the antimicrobial resistance in the EU/EEA,

all these microbes already exhibit resistance against various antibiotic agents, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [21].

Non-healing wounds influence the affected patients on several levels. Besides pain and the threat of the potential need for amputation (e.g., in patients with diabetic foot ulcers), chronic wounds impact sleep quality and lead to emotional stress and physical limitations. All these factors may result in social isolation and reduce the quality of life [12,22]. However, a study published in 2023 showed the importance of social support by family, friends, and professional wound care managers. This support was positively correlated with the patient's health-related quality of life and, therefore, should be highly acknowledged in treating chronic wounds [23].

Moreover, chronic wounds reveal economic consequences for national health systems. In high-income states, wound management accounts for approximately 2% to 4% of national healthcare budgets. However, the actual costs depend highly on the wound type. For example, diabetic foot ulcers in Europe accounted for an estimated 4-6 billion Euros per year in 2009. These numbers emphasize the need for optimizing wound management processes, such as reducing dressing change frequency, healing time, and complications such as infections [24–26].

Knowledge about the underlying etiology of a wound is essential for proper treatment. However, general assessment principles were created for managing any wound: TIME — an approach based on tissue (debridement of devitalized tissue), inflammation/infection (wound cleansing and antimicrobial agents), moisture (balanced moisture levels by appropriate dressings), and edges (maintenance of healthy edges to promote epithelial migration). This tool aids healthcare professionals in choosing the right therapy for an individual patient [9,27,28]. Depending on the wound type, standard wound dressings and therapies include alginate, honey, iodine, hydrocolloid, skin substitutes, growth factors, hyperbaric oxygen therapy, and negative pressure therapy. An ideal wound dressing suitable for all wound types is probably unrealizable. Still, ongoing interdisciplinary research in the design and material of wound dressings might lead to an optimization in wound care [9,29,30].

1.2 Medicinal Plants in Wound Healing

Natural products (NPs) are essential in modern medicine: approximately 66% of all small-molecule approved drugs are NPs or derived from NPs [31]. However, NPs cannot only be small molecules but also biologicals, such as insulin. 44% of all NPs originate in mammals (e.g., bovine or porcine origin), 26% in bacteria, and 12% in fungi. The remaining 25% are

derived from plants [32]. Plants have been known and used for their broad spectrum of phytochemicals as food sources and to treat diseases since the beginning of humanity [33]. Wound healing is one of these longstanding, traditional indications. In ancient Egypt, resin, especially myrrh, has been applied to wounds [34]. Nowadays, using plants and plant extracts in treating wounds is no less relevant. An essential aspect of applying plant extracts on wounds is the complex phytochemical composition: Plants produce a broad spectrum of secondary metabolites. These different compounds can lead to various bioactivities, such as antioxidant, anti-inflammatory, or antimicrobial effects. Therefore, plants and plant extracts containing hundreds of substances can lead to a multi-targeted wound-healing promotion [35–37]. For example, only the substance class of flavonoids demonstrates numerous beneficial effects in wound healing: increased re-epithelialization, accelerated wound contraction and angiogenesis, and antimicrobial activity [8]. Therefore, the wound-healing potential of plants and plant extracts cannot be explained by one effect alone but by the interaction of several bioactivities contributing to enhanced healing in different wound-healing phases.

Hypericum perforatum L. and *Calendula officinalis* L. are important wound-healing plants with a longstanding history of treating wounds [1]. *H. perforatum* (St. John's wort) contains several bioactive substance classes: phloroglucinols, naphthodianthrones, xanthones, flavonoids, phenylpropanoids, procyanidins, and essential oil. Due to this phytochemical composition, St. John's wort possesses antibacterial and antifungal properties against wound-colonizing microbes, such as *Candida albicans*, *Bacillus subtilis*, *Staphylococcus aureus*, or *Trichophyton rubrum*. Moreover, *H. perforatum* has antioxidant and anti-inflammatory properties. A range of *in vitro*, *in vivo,* and clinical studies has already been conducted to gain insights into the exact mechanisms of *H. perforatum* in wound healing. The results emphasize the potential of this traditionally used plant in treating wounds [38]. Moreover, the Herbal Medicinal Product Committee (HMPC) documented the traditional use of an oily extract, tinctures, or the comminuted herbal substance of St. John's wort in treating minor wounds in the respective European Union herbal monograph [39].

Pot marigold (*C. officinalis*) contains triterpenoids, flavonoids, carotenoids, coumarins, quinones, and essential oils. Its beneficial effect on wound healing can be traced back to its anti-inflammatory, antioxidant, antiedematous, and fibroblast-stimulating activity. Again, several *in vitro*, *in vivo,* and clinical trials have already shown the clinical effectiveness of this traditional agent as a wound-healing promoter [40]. The Austrian Pharmacopeia holds several monographs of pot marigold formulations: a fluid extract (Extractum Calendulae fluidum) and three different ointments (Unguentum Calendulae officinale I-III) [41]. Furthermore, in the respective European Union herbal monograph, the HMPC recommends

the traditional use of *C. officinalis* for treating minor skin inflammations and minor wounds [42].

A lesser-known example is birch (*Betula pendula* Roth/ *pubescens* Ehrh.). North American Indians already used birch bark to wrap their wounds. The wound healing-promoting activity of birch bark could be traced back to the pentacyclic triterpenoid betulin. An n-hexane extract was established, containing 87% betulin and 10% other triterpenes. This extract has shown *in vitro* wound-healing effects by boosting keratinocyte-re-epithelialization, anti-inflammatory activity, and accelerating the maturation of the skin barrier. These results were strengthened in a porcine *ex vivo* wound healing model using pig ears [43]. Finally, clinical trials were conducted, leading to the European Medicines Agency (EMA) approval of this betulin-enriched extract as an oleogel (Episalvan®) for treating partial-thickness skin wounds in 2016 [44,45]. However, this extract is also highly effective in treating epidermolysis bullosa, which led to the withdrawal of Episalvan® by the marketing authorization in the European Union in 2022 [46]. In the same year, EMA approved another gel containing the betulin-enriched extract (Filsuvez®) instead as an orphan medicine for the rare disease epidermolysis bullosa [47]. This means no birch bark product approved for wound healing is currently available. Nevertheless, the clinical evidence is strong enough that a birch bark product for wound healing might be back on the market in the future.

Birch, pot marigold, and St. John's wort are just three examples where the traditional use as wound-healing agents could be successfully investigated and validated. However, folk medicine might still hold a plethora of wound-healing applications that have been buried in oblivion. Investigating these forgotten traditions could lead to their revival as additional therapy options.

2. Aims

This thesis aimed to investigate traditional wound-healing applications of selected plants and natural products in Austrian folk medicine. For this purpose, the VOLKSMED database was screened for entries in the field of wound healing as a starting point, and literature published about lesser-known genera was reviewed. Three particularly interesting plants were chosen for further investigations: *Picea abies* (L.) H.Karst., *Sanicula europaea* L., and *Brassica oleracea* var. *capitata* L. f. *alba*.

Norway spruce balm, a resinous exudate of *Picea abies*, is traditionally processed with lard to form an ointment. Previous work analyzed the phytochemical composition and the positive effect on re-epithelialization of main substances *in vitro*. We aimed to quantitate the primary bioactive substance class: diterpene resin acids (DRAs). An ultra-high performance supercritical fluid chromatography (UHPSFC) protocol was developed to separate and quantify eight structurally similar DRAs. Furthermore, we compared Norway spruce balm's qualitative composition and *in vitro* re-epithelialization-boosting activity with other coniferous exudates traditionally used in wound healing. Therefore, appropriate methods for thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were established and applied, as well as the abovementioned UHPSFC method.

The traditional way of preparing a Norway spruce balm ointment relies on lard. To overcome patient rejection and rheological problems of this semi-solid vehicle, we aimed to find other appropriate ointment bases and investigate the permeation potential of important spruce balm constituents using Franz-diffusion cells. Applying another ointment base than lard can lead to the revival of this traditional application in modern medicine.

The aerial and underground parts of sanicle (*Sanicula europaea*) are traditionally used to treat wounds. However, in the Austrian vernacular language, the term "Radix Saniculae" is not only used for the underground parts of sanicle but also for those of drooping bittercress (*Cardamine enneaphyllos* (L.) Crantz), a different plant genus. We aimed to shed light on the use of this term for both plants and find potential similarities. Therefore, different chromatographic methods (TLC, HPLC, gas chromatography (GC)) were established and applied to characterize the main constituents in sanicle and drooping bittercress.

White cabbage (*Brassica oleracea* var. *capitata* f. *alba*) is one of the most essential vegetables worldwide. However, using fresh squeezed cabbage leaves as compresses in the treatment of wounds has yet to be investigated. Therefore, extracts were produced following this traditional application and fractionated. Different *in vitro* readouts, all assessing

beneficial effects in wound healing, guided this work to identify active extracts and fractions. Various chromatographic methods (TLC, HPLC, GC) were established to characterize the phytochemical composition of the most active fractions.

3. Wound Healing Plants in Austrian Folk Medicine (Publication I)

Traditional medicine is rich in valuable knowledge about applying plants and other materials for various diseases and conditions. In Austria, this knowledge was compiled in the VOLKSMED database, a comprehensive repository of traditional medicinal knowledge. For this publication, we presented this database's development, structure, and scope in depth for the first time. All entries in the field of wound healing were extensively analyzed and discussed. Eight lesser-known wound-healing genera (*Anthyllis vulneraria*, *Brassica* sp., *Gentiana* sp., *Abies alba*, *Larix decidua*, *Picea abies*, *Sambucus* sp., *Sanicula europaea*) were selected, and literature concerning their known *in vitro* and *in vivo* wound-healing bioactivities was reviewed. The results show this database's hidden potential and suggest that even the forgotten wound-healing plants should be investigated in future research to promote their revival in modern medicine.

The review article '*VOLSKMED Database: A Source for Forgotten Wound Healing Plants in Austrian Folk Medicine*' was accepted in November 2023 and published in **Planta Medica** in the special issue 'Celebrating the Austrian Pharmacognosy' dedicated to Prof. Rudolf Bauer, Prof. Chlodwig Franz, Prof. Brigitte Kopp, and Prof. Hermann Stuppner. This doctoral thesis used this review as a theoretical introduction to traditional Austrian wound-healing plants.

Contributions

I contributed by analyzing and interpreting the VOLSKMED database data, conducting a systematic literature search of chosen plants, discussing the results, and drafting the manuscript.

Key findings

- In traditional Austrian medicine, 111 plant genera from 53 families are used to treat wounds.
- *Hypericum* sp., *Arnica montana*, and *Calendula officinalis* are the most cited plants (Figure 3.1, page 10).
- Lesser-known wound-healing plants, such as *Gentiana* sp., *Brassica* sp., *Sambucus nigra,* and different Pinaceae members, have already been well-investigated in this field and justify their traditional use in treating wounds. However, the responsible phytochemicals and molecular mechanisms are often still unknown.

- Even though their Latin names indicate their healing potential, *Anthyllis vulneraria,* and *Sanicula europaea*, as well as other lesser-cited plants, such as *Sambucus racemosa*, have yet to be focused on in phytochemical analysis and bioactivity screening. Future research can substantiate their traditional application and may revive their use in wound treatment.

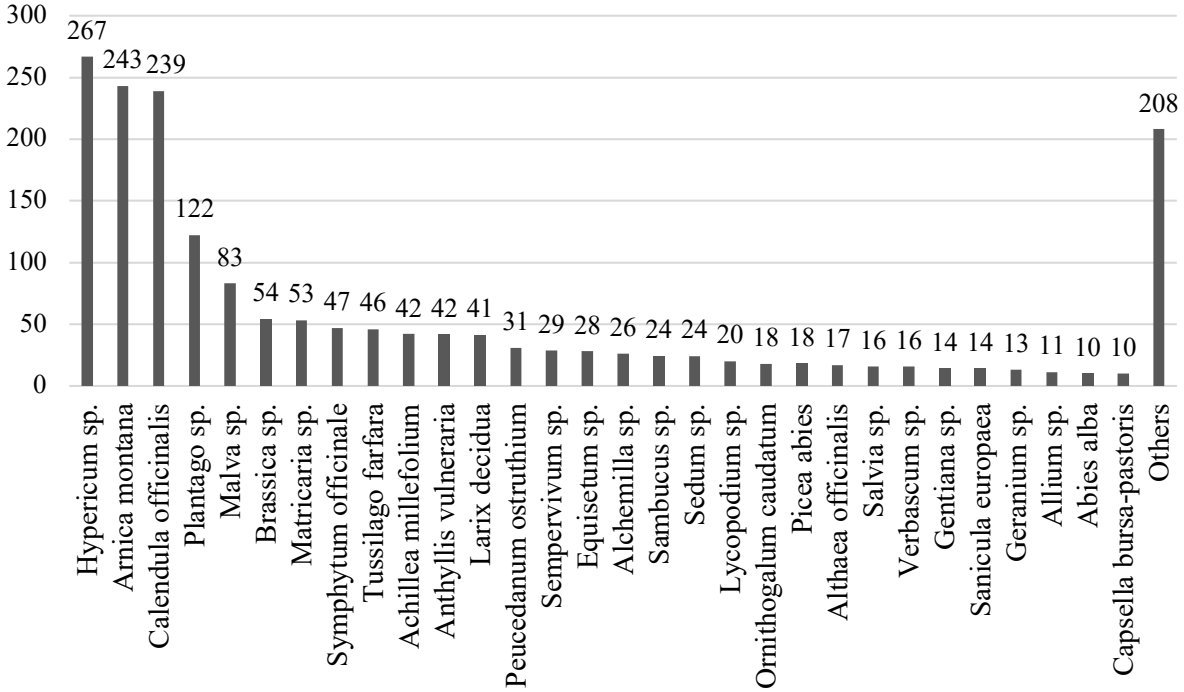


Figure 3.1 Traditional Austrian wound-healing plants with at least ten entries in the VOLKSMED database.

Concluding perspectives

The VOLSKMED database holds many hidden traditional applications of plants and other materials. In wound healing, promising data is available for the exudates of *Picea abies* and *Brassica oleracea* var. *capitata,* among others. The focus of a previous dissertation has already been Norway spruce balm [48]. Therefore, this doctoral thesis further processed this topic (chapter 4.1, page 25). Moreover, the wound-healing potential of *Brassica oleracea* var. *capitata* f. *alba* was chosen as a further research focus for this work (chapter 4.3, page 105). Even though past research on the wound-healing effects of *Sanicula europaea* was scarce, the risk of confusion with *Cardamine enneaphyllos* in the Austrian vernacular language made this plant another subject of phytochemical analysis in this thesis (chapter 4.2, page 87).

Thieme

VOLKSMED Database: A Source for Forgotten Wound Healing Plants in Austrian Folk Medicine#

Authors
Elisabeth Eichenauer[1,2], Johannes Saukel[1], Sabine Glasl[1]

Affiliations
1 Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna, Austria
2 Vienna Doctoral School of Pharmaceutical, Nutritional and Sport Sciences, University of Vienna, Austria

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Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

Correspondence
ao. Univ.-Prof. Mag. Dr. Sabine Glasl
Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna
Josef-Holaubek-Platz 2, 1090 Vienna, Austria
Phone: + 43 14 27 75 52 07
sabine.glasl@univie.ac.at

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ABSTRACT

The global increase in the incidence of wounds is concerning and fuels the search for new treatment options. The use of traditional medicinal plants in wound healing represents an appreciated available therapeutic possibility. This work introduces the VOLKSMED database, which contains plants and other materials used in Austrian folk medicine, either as monographs or mixtures. This work focuses on the monographs of the database. Concerning wound healing, *Hypericum* sp., *Arnica montana, Calendula officinalis, Plantago* sp., and *Malva* sp. are the most commonly used plants. The focus of this paper is set on selected lesser-known plants (*Abies alba, Anthyllis vulneraria, Brassica* sp., *Gentiana* sp., *Larix decidua, Picea abies, Sambucus* sp., *Sanicula europaea*) and their status quo in literature concerning wound healing. A systematic search using the databases SciFinder, SCOPUS, and PubMed yielded substantial evidence for the wound healing potential of *Brassica* sp., *Gentiana* sp., the Pinaceae *A. abies, L. decidua,* and *P. abies*, as well as *Sambucus nigra. In vivo* and clinical studies substantiate their use in Austrian folk medicine. According to the literature, especially *A. vulneraria, Sambucus racemosa,* and *S. europaea* would be worth investigating in-depth since data concerning their wound healing effects – even though scarce – are convincing. In conclusion, the VOLKSMED database contains promising opportunities for further treatment options in the field of wound healing. Future research should consider the listed plants to support their traditional use in Austrian folk medicine and possibly promote the implementation of old knowledge in modern medicine.

Introduction

The skin is the largest organ, covering the body entirely to protect the system from the intrusion of chemicals and pathogens from outside and the loss of water and electrolytes [1]. Whenever there is a disruption in the integrity of the skin, which means a wound occurs, a strictly regulated and incredibly complex mechanism of wound healing starts to repair the barrier function. During this mechanism, several growth factors, cytokines, and cell types are involved in four phases: hemostasis, inflammation, proliferation, and tissue remodeling. Different factors and comorbidities may impair the wound healing mechanism and lead to chronicity of the wound [2, 3]. A chronic wound is defined as a wound that does not heal within an anticipated time frame. There is no generally available definition of this time frame since the etiology, size, location, morbidity, and other variables of chronic wounds are fairly heterogeneous [3–5].

However, acute and chronic wounds occur with a prevalence of 3–4/1000 people, which equates to up to 2 million people only in the EU 27 [6]. The incidence of chronic wounds is often referred to as a silent epidemic since there is a global increase in chronic dis-

This work is dedicated to Professors Rudolf Bauer, Chlodwig Franz, Brigitte Kopp, and Hermann Stuppner for their invaluable contributions and commitment to Austrian Pharmacognosy.

eases and comorbidities leading to impaired wound healing such as diabetes, obesity, and vascular diseases, as well as antimicrobial resistance of microbes colonizing wounds, accompanied by an aging population [7–9]. Chronic wounds are associated with several individual and economic consequences. Patients generally suffer from a decreased quality of life due to pain and limitations in social contact and mobility as well as depression, and an enormous financial burden [10]. The latter also concerns the public healthcare systems. Approximately 2–4% of the European health expenditure has to be spent on the treatment of wounds. The treatment of a chronic wound in Europe accounts for up to €10000 per year [8, 11]. Only in Germany, the treatment of chronic wounds in 2023 was estimated to cost, overall, €10 billion per year [12].

Unfortunately, data on the situation in Austria is scarce. In 2015, a survey was conducted and resulted in the first realistic assessment of chronic wounds in the Austrian population. At that time point, 250000 people were suffering from at least one chronic wound, indicating a prevalence of up to 50 per 1000 citizens [13, 14].

Since the management of chronic wounds represents an increasing and significant problem worldwide, the necessity of alternative treatment options and research in this field is urgent [8]. One approach to heal wounds and prevent the chronicity of a wound is to use traditional medicinal plants. Plants and plant-derived substances have been used for centuries all over the world to treat and prevent various diseases [15]. Still, natural sources play an important role in the development of new drugs; either natural products derived directly from these sources, semisynthetic analogs, or synthetic compounds inspired by nature [16]. Medicinal plants can produce different secondary metabolites of various substance classes, and therefore, may possess numerous bioactivities. This feature is very beneficial in complex mechanisms such as wound healing. The power of plant extracts is given by their nature as multicomponent mixtures. In the case of skin lesions, they might hold antioxidant, anti-inflammatory, and antimicrobial activities as well as platelet aggregation inhibitory and immunomodulatory effects, which all contribute to wound healing [2, 16–19]. Therefore, using medicinal plants to treat wounds is reasonable and might hold some new, or forgotten, therapy options.

This review aims to give an overview of plants that are used in wound healing in Austrian folk medicine. Furthermore, chosen examples, which intentionally do not cover the most commonly used wound healing plants, are discussed in detail concerning their status quo in the literature about their wound healing effects. Hence, future research can concentrate on promising candidates to provide a treatment alternative to decrease the probability of chronicity of a wound.

The VOLKSMED Database

Traditional knowledge is often passed and preserved only by word of mouth without being laid down on paper. Therefore, important information about plants and their traditional use might be lost over the decades of transfer. To unite and preserve valuable knowledge about the traditional use of plants and other materials in Austrian folk medicine, the VOLKSMED database was compiled [20–22]. Between 1983 and 2004, diploma students studying pharmacy at the University of Vienna conducted their diploma thesis intending to gather information about Austrian folk medicine. For that purpose, the students interviewed people with traditional knowledge in their area of origin. These interviewed people were, on the one hand, pharmacists or physicians, but, on the other hand, people known to be well-educated in folk medicine, such as farmers or foresters. All the information gathered during the diploma theses was entered in the VOLKSMED database, either as monographs of single plants or other materials or as mixtures. This work focuses on the monographs of the database.

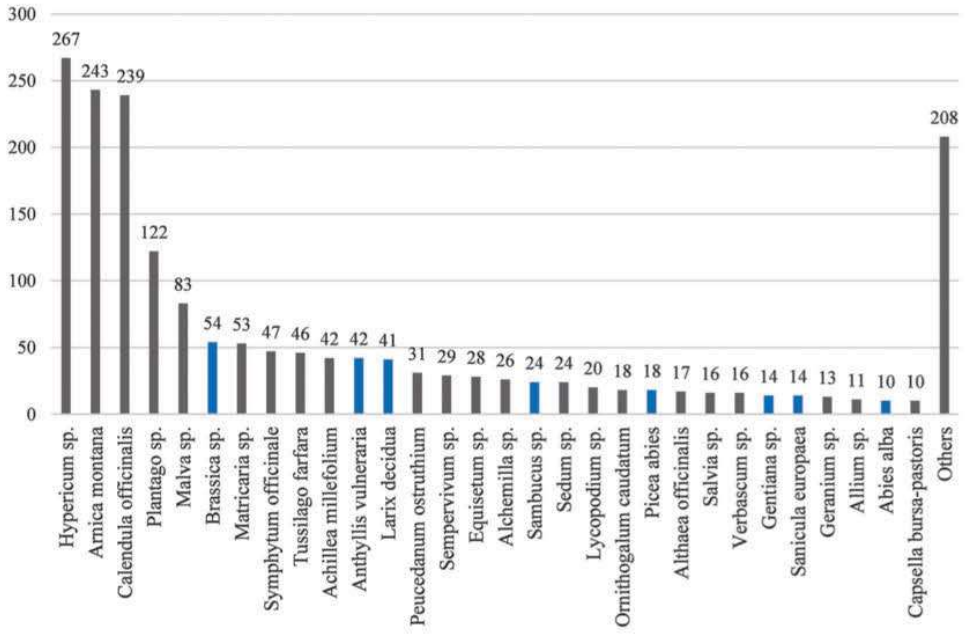
For the establishment of the monographs, so far, 46 diploma theses have been used, covering all parts of Austria, with some areas in South Tyrol as well as some regions in southeastern Bavaria/Germany (information on the respective diploma theses can be found as **Table 1S**, Supporting Information). In the monograph part of the database, overall, 2114 people were interviewed, resulting in 73681 "entries". The number of entries corresponds to the number of persons mentioning one particular piece of information. Similar information within one diploma work was combined to a "record", which resulted in 43348 records using these 46 diploma theses. The establishment of individual records depended on various parameters: (i) A new record was generated within every single diploma work that reflects the folk medicinal knowledge within one unique region; and (ii) A new record was generated in case slight differences in the extracting method, about preparation or application, were given. One record can be mentioned by more than one person interviewed within one diploma work. These records were composed as follows: identity number, number of the respective diploma thesis, genus (plants, animal, and mineral origin), plant genus (all other origins labeled "diverse"), family, Latin plant name (with genus and, if available, species, subspecies, or variety), part used, internal or external use, indication, preparation form, use of fresh or dry material, number of entries, and further information about preparation and application as well as the extracting agent (▶ **Fig. 1**). However, when comparing the use of plants within all studied Austrian regions, the number of entries was chosen as a suitable criterion.

The database contains valuable information but also reveals some drawbacks. For data compilation, no uniform questionnaire was used during the interviews, which may be regarded as a strength as well as a weakness. On the one hand, the biography or qualifications of the interviewed people were not recorded, only their identity and address. Therefore, it is not known, if a record was stated by a pharmacist or physician, which might indicate scientific evidence, or by a person without higher education. In any case, all interview partners were locally acknowledged as persons with profound folk medicinal knowledge. On the other hand, the records do not always contain all of the information as depicted in ▶ **Fig. 1**, especially about the use of fresh or dry material or further information about the application. Furthermore, in some records, only the plant genus is available without any further declaration of species, subspecies, or variety, since not all of the interviewed persons were botanically trained.

Nevertheless, the VOLKSMED database contains a huge amount of incredibly valuable information and knowledge about

ID	Diploma thesis	Genus (All)	Genus (Plants)	Family	Plant name	Part used	Internal/ external	Indication	Prep. form	Fresh/ dry	Entries	Further info	Extracting agent

▶ **Fig. 1** Registered information and structure of records in the VOLKSMED database.



▶ **Fig. 2** Traditional wound healing plants with more than 10 entries in the VOLKSMED database. Blue-marked plants are further discussed in this work.

Austrian folk medicine. The collected data is unique and can be used to gain insights into the traditional use of plants and other materials in Austria.

Wound Healing Plants in Austrian Folk Medicine

This paper aimed to review the VOLKSMED database concerning all kinds of wound healing. To simplify the enormous amounts of records, it was searched for all indications, including the key words "wounds" and "wound healing" (abrasions, burns, cuts, decubitus, ulcerous legs/feet, infected/nonhealing/purulent/slow-healing wounds). It has to be kept in mind that these key words are sub-indications of the indication "skin". While introducing the records into the database, some wound healing records might not have been linked to one of these sub-indications but were referred to the overall indication "skin", which was not considered for this review. Therefore, the listed records and entries in this work might not be complete.

However, in the field of "wounds" and "wound healing", the database contains 1037 records with 1945 entries, whereof 93 records with 119 entries are materials other than plants (fungi, animal, or mineral origin); however, plants cover 944 records with 1826 entries. The list contains 111 genera from 53 different families, with the following being most abundant: Asteraceae (16), Rosaceae (7), Apiaceae (6), Brassicaceae (5), Lamiaceae (5), and Pinaceae (4). All mentioned families and the respective count of genera are depicted in ▶ **Table 1**.

▶ **Fig. 2** depicts plants with more than 10 entries; the blue-colored examples were chosen arbitrarily and analyzed in-depth in this work. All plants with less than 10 entries can be found in **Table 2S**, Supporting Information.

▶ **Table 2** contains information about plants with more than 10 entries in the abovementioned indications in decreasing number of frequency of entries. It covers genera, species, and families, which were updated to the currently accepted names according to Plant of the Worlds online [23]. If older synonyms were used

▶ **Table 1** Families and respective number of genera found in the VOLKSMED database in wound healing indications.

Families	Genera per family
Asteraceae	16
Rosaceae	7
Apiaceae	6
Brassicaceae, Lamiaceae	5
Pinaceae	4
Boraginaceae, Fabaceae, Malvaceae, Poaceae, Polygonaceae	3
Amaranthaceae, Betulaceae, Crassulaceae, Gentianaceae, Iridaceae, Plantaginaceae, Primulaceae, Ranunculaceae, Solanaceae	2
Acoraceae, Amaryllidaceae, Araliaceae, Aristolochiaceae, Asphodelaceae, Cannabaceae, Cucurbitaceae, Cupressaceae, Cyperaceae, Equisetaceae, Ericaceae, Fagaceae, Geraniaceae, Hypericaceae, Juglandaceae, Liliaceae, Linaceae, Lycopodiaceae, Melanthiaceae, Papaveraceae, Polypodiaceae, Rubiaceae, Salicaceae, Sapindaceae, Scrophulariaceae, Taxaceae, Tropaeolaceae, Urticaceae, Verbenaceae, Viburnaceae, Violaceae, Vitaceae	1

in the database, the respective plant name is marked by a superscript, which covers the particular synonym in the footnote. Furthermore, the table contains information about the used plant part and the application form, as well as the usage of dry (d) or fresh (f) plant material, or both (df), if this information was available. The used part and the application form are listed according to their frequencies; in case of the same frequency, they are organized in alphabetic order.

Almost every part of plants is used in the treatment of wounds: flowers (37.2%), leaves (25.0%), the whole herb (21.7%), roots (6.9%), resin/balm (3.3%), fruits (1.3%), buds (1.2%), spores (1.0%), bark (0.66%), seeds (0.60%), bulb (0.49%), shoot tips (0.33%), cones (0.11%), rhizome (0.05%), stem (0.05%), stylus (0.05%), tuber (0.05%), and turnip (0.05%). The preparation methods are simple and reflect the possibilities people have commonly at home. The most frequent form of preparation is apolar formulations (33.8%), with slightly more entries for ointments (17.4%) than for oily preparations (16.4%). For ointments, plant parts are processed with fat, whereas for oily preparations, the plant parts are inserted into oil. In both cases, people are using oils and fats they have at home at the moment, mostly olive oil, linseed oil, lard, and butter, but also goose or rabbit fat. The use of plant parts without any extraction is the second most abundant form of preparation (31.5%). Parts of the plant are either directly applied (21.4%), or processed before application (10.1%), for exam-

▶ **Table 2** Traditional wound healing plants with more than 10 entries in the VOLKSMED database. The table contains currently accepted species. If the database contains synonyms, they are marked by superscripts and explained in the footnote. The application form indicates the usage of dry (d), fresh (f) plant material, or both (df), or the information is not available (−).

Genus	Species	Family	Part used	Application form	Entries
Hypericum	sp. *maculatum* Crantz *perforatum* L.	Hypericaceae	Herb	In oil (df), ointment (df), alcoholic extract (df), tea (d), herb directly on wounds (f)	267
			Flower	In oil (df), alcoholic extract (d), flower directly on wounds (d), ointment (df), tea (−)	
Arnica	*montana* L.	Asteraceae	Flower	Alcoholic extract (df), in oil (df), ointment (df), tea (as a bath) (d)	243
			Root	Alcoholic extract (−), ointment (df), tea (−)	
			Herb	Alcoholic extract (−)	
			Leaves	Leaves directly on wounds (df)	
Calendula	*officinalis* L.	Asteraceae	Flower	Ointment (df), alcoholic extract (as an embrocation) (df), in oil (df), tea (for rinsing, as a bath) (d), flower directly on wounds (as a compress) (−)	239
			Herb	Ointment (df), herb directly on wounds (as a compress) (−)	
Plantago	*lanceolata* L. *major* L. *media* L.	Plantaginaceae	Leaves	Juice from leaves or leaves directly on wounds (f), tea (as a bath) (df), ointment (f)	122
Malva	sp. *neglecta* Wallr. *pusilla* Sm. *sylvestris* L.	Malvaceae	Leaves	Tea (for rinsing, as a bath or compress) (df), leaves directly on wounds (f), ointment (df)	83
			Flower	Tea (for rinsing, as a bath or compress) (−)	
			Herb	Tea (for rinsing, as a bath or compress) (df), ointment (df)	

continued next page

▶ **Table 2** Traditional wound healing plants with more than 10 entries in the VOLKSMED database. The table contains currently accepted species. If the database contains synonyms, they are marked by superscripts and explained in the footnote. The application form indicates the usage of dry (d), fresh (f) plant material, or both (df), or the information is not available (–). *continued*

Genus	Species	Family	Part used	Application form	Entries
Brassica	*oleracea* var. *capitata* L. *oleracea* var. *sabauda* L. *oleracea* var. *sabellica* L.	Brassicaceae	Leaves	Leaves directly on wounds (f)	54
	rapa L.	Brassicaceae	Turnip	Turnip directly on wounds (f)	
Matricaria	*chamomilla*[1] L. *discoidea*[2] DC.	Asteraceae	Flower	Tea (for rinsing, as a bath or compress) (df), ointment (df), flower directly on wounds (as a compress) (df), in oil (–)	53
Symphytum	*officinale* L.	Boraginaceae	Root	Ointment (df), root directly on wounds (f), alcoholic extract (df)	47
			Leaves	Leaves directly on wounds (f), ointment (–)	
Tussilago	*farfara* L.	Asteraceae	Leaves	Leaves directly on wounds (f), tea (for rinsing) (–)	46
Achillea	*millefolium* L.	Asteraceae	Herb	Herb directly on wounds (as a compress) (df), tea (for rinsing, as a compress) (df), in oil (f), ointment (–)	42
			Leaves	Leaves directly on wounds (f)	
			Flower	Tea (d)	
Anthyllis	*vulneraria* L.	Fabaceae	Flower	Alcoholic extract (d), tea (for rinsing, as a bath) (d), ointment (d)	42
			Herb	Tea (for rinsing, as a bath or compress) (df), juice from leaves or leaves directly on wounds (f)	
			Leaves	Ointment (d), tea (d)	
Larix	*decidua* Mill.	Pinaceae	Resin/balm	Resin/balm directly on wounds (df), ointment (df), alcoholic extract (df)	41
Peucedanum	*ostruthium* (L.) W. D. J. Koch	Apiaceae	Root	Fumigation (d), root directly on wounds (d), tea (as a bath) (d), alcoholic extract (as an inunction) (–), ointment (d)	31
			Leaves	Leaves directly on wounds (as a compress) (f), ointment (–)	
Sempervivum	sp. *tectorum* L.	Crassulaceae	Leaves	Juice from leaves or leaves directly on wounds (f), ointment (d)	29
			Herb	Juice from herb directly on wounds (f)	
Equisetum	sp. *arvense* L. *telmateia* Ehrh.	Equisetaceae	Herb	Tea (for rinsing, as a bath or compress) (df), cold maceration in water (as a compress) (df), herb directly on wounds (as a compress) (df)	28
Alchemilla	sp. *alpina* L. *vulgaris* L.	Rosaceae	Herb	Tea (for rinsing, as a bath) (d), herb directly on wounds (as a compress) (f), ointment (–)	26
			Leaves	Leaves directly on wounds (df), tea (for rinsing, as a bath) (d)	
Sambucus	*nigra* L.	Viburnaceae	Leaves	Leaves directly on wounds (f)	24
			Bark	Bark directly on wounds (df)	
			Flower	Ointment (–)	
	racemosa L.	Viburnaceae	Fruits	Oil gained by boiling fruits in water applied on wounds (df), in oil (df)	
Sedum	*acre* L.	Crassulaceae	Leaves	Juice from leaves or leaves directly on wounds (f), alcoholic extract (–)	24
Hylotelephium	*maximum*[3] (L.) Holub				
telephium[4] (L.) H.Ohba					
Lycopodium	sp. *annotinum* L. *clavatum* L.	Lycopodiaceae	Spores	Spores directly on wounds as a powder (df)	20
Albuca	*bracteata*[5] (Thunb.) J. C.Manning & Goldblatt	Asparagaceae	Leaves	Juice from leaves or leaves directly on wounds (df), ointment (d)	18

continued next page

▶ **Table 2** Traditional wound healing plants with more than 10 entries in the VOLKSMED database. The table contains currently accepted species. If the database contains synonyms, they are marked by superscripts and explained in the footnote. The application form indicates the usage of dry (d), fresh (f) plant material, or both (df), or the information is not available (−). *continued*

Genus	Species	Family	Part used	Application form	Entries
Picea	*abies* (L.) H.Karst.	Pinaceae	Resin/balm	Resin/balm directly on wounds (df), ointment (df)	18
			Shoot tips	Tea (−)	
Althaea	*officinalis* L.	Malvaceae	Herb	Cold maceration in water (d), tea (for rinsing) (d)	17
			Root	Cold maceration in water (d), tea (for rinsing, as a bath or compress) (d)	
			Flower	Cold maceration in water (−), tea (as a bath or compress) (−)	
			Stem	Tea (as a bath) (−)	
Salvia	*glutinosa* L. *officinalis* L. *pratensis* L.	Lamiaceae	Leaves	Tea (for rinsing, as a bath or compress) (df), leaves directly on wounds (f), in oil (−), ointment (df)	16
			Herb	Tea (for rinsing, as a bath or compress) (−)	
Verbascum	sp. *densiflorum* Bertol. *phlomoides* L.	Scrophulariaceae	Flower	In oil (−), ointment (−), tea (for rinsing, as a bath) (df), alcoholic extract (df)	16
Gentiana	*lutea* L. *punctata* L.	Gentianaceae	Root	Tea (for rinsing, as a compress) (d), alcoholic extract (d)	14
			Leaves	Leaves directly on wounds (f)	
Sanicula	*europaea* L.	Apiaceae	Root	Ointment (d)	14
			Herb	Tea (for rinsing, as a bath) (−), herb directly on wounds (as a compress) (−)	
			Leaves	Leaves directly on wounds (d), ointment (−)	
Geranium	sp. *pratense* L. *robertianum* L.	Geraniaceae	Herb	Tea (for rinsing, as a bath or compress) (d), herb directly on wounds (f)	13
Allium	*cepa* L.	Amaryllidaceae	Bulb	Bulb directly on wounds (f), fried in olive oil and applied on wounds (f), ointment (−), tea (for rinsing) (f)	11
	sativum L.	Amaryllidaceae	Bulb	Alcoholic extract (f), bulb directly on wounds (f)	
	ursinum L.	Amaryllidaceae	Leaves	Juice from leaves or leaves directly on wounds (f), alcoholic extract (−)	
Abies	*alba* Mill.	Pinaceae	Resin	Bark with resin or resin directly on wounds (f), ointment (f)	10
			Needles	Tea (for rinsing) (df)	
Capsella	*bursa-pastoris* (L.) Medik.	Brassicaceae	Herb	Herb directly on wounds (df), tea (for rinsing, as a compress) (d), alcoholic extract (d), ointment (d)	10

[1] Matricaria recutita L; [2] Chamomilla suaveolens (Pursh) Rydb; [3] Sedum maximum (L.) Hoffm; [4] Sedum telephium L; [5] Ornithogalum caudatum Aiton

ple, squeezed and the juice applied, crushed, burnt, cut, or powdered. Of the entries, 18.4% belong to aqueous extracts, either as an infusion (17.7%) or cold maceration (0.7%). These extracts are used as a bath, for rinsing, or as a compress. Alcoholic extracts (15.9%) are made using schnapps (ethanol 40%) or spirits of wine (ethanol 96%). Furthermore, boiling plant parts in milk (0.16%) as well as extraction with vinegar (0.11%) are mentioned in the entries.

The most frequently used plants in Austrian folk medicine (*Hypericum* sp., *Arnica montana, Calendula officinalis, Plantago* sp., and *Malva* sp.) are well-known wound healing agents. They have already been intensively investigated in this field and discussed in numerous publications [1, 19, 24–29]. The next section focuses on lesser-known plants.

Known Wound Healing Properties of Selected Plants

Selected plants were analyzed in-depth with a focus on the current state of knowledge concerning their wound healing properties. English plant names were obtained from Plants of the World online [23], Wikispecies, [30], Medicinal Plant Name Services [31], or the Encyclopedia of Life [32]; German names were acquired from the Excursion Flora of Austria, Liechtenstein, and Switzerland [33]. The status quo in the literature about their wound healing effects was collected by a literature search using the databases SCOPUS, SciFinder, and PubMed. No time frame was set in the search and as key words, the Latin plant name and "wound healing" were used.

Anthyllis vulneraria

Anthyllis vulneraria L. (Engl. common kidney vetch; Ger. Echter Wundklee) belongs to the family Fabaceae. It is a perennial herb native throughout Europe and Northern Africa and is nowadays mostly used in cosmetics [34, 35]. In the VOLKSMED database, this plant has 42 entries. All aerial parts are mentioned as being used, mostly the flower, followed by the whole herb and the leaves. All of the plant parts are prepared as tea for rinsing or bathing the wound, but also used as a compress. Furthermore, an ethanolic extract is made of the flower and used mostly for cleansing the wound and as a compress. The preparation of an ointment is mentioned for the flower and leaves. Moreover, the fresh herb is either squeezed and the resulting juice is applied to the wound, or the herb is put directly on the wound as a compress.

The Latin name *A. vulneraria* already suggests the use of this plant in wound healing since the Latin word "vulnerare" means to wound. Already in medieval times, kidney vetch was used to treat wounds [36], however, this plant is meagerly investigated in the field of wound healing. The aerial parts of *A. vulneraria* contain different substance classes, such as saponins, flavonoids, phenolic acids, and tannins. Extracts of flowers and leaves exhibited significant antioxidant properties, whereby the total polyphenol and flavonoid content was higher in the flowers than in the leaves [37]. Csepregi et al. tested ethanolic and aqueous extracts of kidney vetch concerning several bioactivities that are beneficial in wound healing: antimicrobial, antioxidant, and pro-migratory effects. The 50% ethanolic extract showed moderate minimum inhibitory concentrations (MIC_{80}) against *Streptococcus pyogenes* and *Bacillus subtilis*, a weak antimicrobial activity on *Staphylococcus aureus*, as well as no effect at all on *Escherichia coli* and *Pseudomonas aeruginosa*. To determine the antioxidant properties of the extracts, different assays were conducted, leading to the lowest antioxidant capacity of all plants that were tested in this work, but still a good antioxidant effect. Furthermore, the pro-migratory activity of the extracts was tested on 3 T3 fibroblasts and HaCaT keratinocytes. A weak pro-migratory stimulation of the *A. vulneraria* extracts was detected on HaCaT keratinocytes, whereas no effect could be seen on fibroblasts [38]. These first findings emphasize the potential of *A. vulneraria* as a wound healing agent. Further research has to be conducted to gain more insights into the bioactivities of this plant.

Brassica sp.

Different species and varieties of the genus *Brassica* (Brassicaceae) are mentioned in the VOLKSMED database: *Brassica oleracea* var. *capitata* L. (Engl. cabbage; Ger. Weiß-Kraut/Rot-Kraut), *Brassica oleracea* var. *sabauda* L. (Engl. savoy cabbage; Ger. Kohl, Wirsing-Kohl), and *Brassica oleracea* var. *sabellica* L. (Engl. curly kale; Ger. Kraus-Kohl), as well as *Brassica rapa* L. (Engl. field mustard, turnip mustard; Ger. Rüben-Kohl, Rübsen). The database contains 54 entries of *Brassica* sp. All of the *B. oleracea* varieties are used the same way: the fresh leaves are used, either squeezed (often with a rolling pin) and applied to the wound or cooked to a soft consistency prior to application. For *B. rapa*, the turnip is mashed and applied as a wound healing agent.

Species from the Brassicaceae family are economically very valuable as edible plants, spice sources, or as feed plants all over the world. Due to their nutritional value, *Brassica* sp. are very well investigated, especially concerning their chemical composition, and are known to possess antitumor, antioxidant, antimicrobial, and anti-inflammatory effects [39–44]. But also, their potential and value as wound healing agents have already been analyzed.

Lysophosphatidic acid (LPA) is known to mediate cell proliferation and migration and, therefore, is used as a positive control in wound healing assays. Cabbage has a high activity of phospholipase D (PLD), an enzyme forming phosphatidic acid (PA) after tissue disruption like homogenization, cutting, or chewing. Therefore, cabbage leaves represent a rich source of PA, which is further hydrolyzed by phospholipase A_2 to LPA after ingestion in the gastrointestinal tract [45]. Tanaka et al. not only detected PA in homogenized cabbage leaves but also LPA [46]. Since phospholipase A_2 is also present in human skin [47], this enzyme might increase the content of LPA in topical applied squeezed cabbage leaves, which might be part of the wound healing activity of *B. oleracea* plants. In addition, pectin-type polysaccharides might be involved; different pectin fractions were extracted from white cabbage, kale, and red kale. All of the fractions showed complement-fixing activity and, therefore, may promote wound healing by stimulation of innate immunity [48, 49]. Moreover, bandages containing anthocyanin extracts gained from red cabbage are an easy-to-use, quick, and eco-friendly colorimetric indicator for pH changes in wound fluids, which is a helpful tool for monitoring the healing process. Additionally, an increasing concentration of anthocyanins in the bandage correlates with a higher antimicrobial activity [50–54].

Even *in vivo* studies are available: Hassanzadeh et al. reported the promoting effect of an aqueous *B. oleracea* extract applied as a cream on reepithelialization and contraction of burn wounds in Sprague-Dawley rats [55]. Rebolla et al. investigated the bioactivity of a *B. oleracea* var. *capitata* 10% glycolic extract on surgical skin wounds in Wistar rats. The results of this study revealed a predominance of mature type I collagen fibers in the treated group compared to the control group, and therefore an improved wound healing process [56]. These findings were confirmed by Sarandy et al., who also tested a *B. oleracea* var. *capitata* 10% glycolic extract balsam as well as an ointment on wounds in Wistar rats over 20 days. Both preparations led to a boost in cell proliferation and differentiation, and an initial increase in type III collagen fibers. The fast formation of granulation tissue as well as a higher

content of type I collagen than type III at the end of the treatment suggests the reconstruction of mature tissue [57]. Moreover, in another study, an ointment containing 10% glycolic extract of *B. oleracea* var. *capitata* was tested on wounds in Wistar rats. It resulted in an enhanced remodeling phase by faster substitution of type III collagen fibers for type I [58].

Nanoparticles (NPs) of silver, gold, or iron are known to promote wound healing and reveal antimicrobial activity. Different studies describe the use of *B. oleracea* var. *capitata* extracts for the successful synthesis of such NPs and demonstrate the antibacterial, antioxidant, anti-cancer, and *in vivo* wound healing potential of these NPs [59–62].

Broccoli (*B. oleracea* var. *italica* Plenck) is another variety not mentioned in the VOLKSMED database. Nevertheless, bioactive proteins and peptides isolated from the stems of broccoli were found to promote the proliferation of keratinocytes *in vitro* and, therefore, promote wound healing [63].

Taken together, different *Brassica* species repeatedly proved their traditional use as wound healing agents. Further research on these plants could lead to easily accessible, cheap, and commercially available preparations for wound healing.

Gentiana sp.

Gentiana lutea L. (Engl. yellow gentian; Ger. Gelb-Enzian) and *Gentiana punctata* L. (Engl. spotted gentian; Ger. Tüpfel-Enzian) are a class among the family of Gentianaceae. These perennial herbs are native throughout Europe and Western Asia [64, 65]. In Austrian folk medicine, gentian root is used as a tea for bathing and rinsing the wound or as an alcoholic extract. Only one entry contains the application of fresh leaves of *G. punctata* directly on cuts and wounds. Most publications considered for this review deal with *G. lutea*. *G. punctata* is only discussed by Pasdaran et al. [65]. However, since the chemical composition appears to be similar, the results found for *G. lutea* may be transferred to *G. punctata*.

The root of *G. lutea* is an authorized drug in the European Pharmacopeia ("Gentianae Radix") used to treat various digestive disorders but has also been used traditionally to treat wounds in several countries. Gentian is known to reveal antimicrobial, anti-inflammatory, and antioxidant effects, which are caused by different substance classes. The main constituents are secoiridoids, but also flavonoids, terpenoids, iridoids, and xanthones can be detected. The secoiridoid sweroside contributes to wound healing by an antioxidant effect and by increasing the formation of a procollagen complex. The terpenoid lupeol promotes reepithelialization, and the flavonoid isoorientin leads to an upregulation of the skin regenerative protein involucrin. Furthermore, the xanthone mangiferin has an impact on several inflammatory pathways as well as on immunohistochemical angiogenesis markers in wound healing [64–66]. The overall wound healing effect of gentian has also been investigated *in vivo* in mice and rats. After oral administration of alcohol and petrol ether extracts of *G. lutea* rhizomes, an increase in wound closure and breaking strength of the remodeled tissue, as well as a higher rate of granulation tissue with increment in fibroblasts and collagen fibers were reported [67]. The latter was also reported by Öztürk et al., who tested an ethanolic extract as well as three pure secoiridoids *in vitro* in cultured chicken embryonic fibroblasts. They indicate swertiamarine as the main bioactive secoiridoid [68]. Moreover, gentiopicroside alone was administered to rats with diabetic wounds, again resulting in an improvement in wound healing by the acceleration of collagen fiber synthesis [69].

Gentiana macrophylla Pall. is a *Gentiana* species native to Asia, mostly Mongolia, China, and Siberia, and is another species well investigated in the field of wound healing. The chemical composition seems to be similar to the European species [70, 71]. Yin et al. investigated a methanolic root extract and described an antioxidant activity as well as antibacterial effects against bacterial strains isolated from burn wounds [71]. Furthermore, Buyantogtokh et al. prepared a gel containing ethanolic extracts of *Calvatia gigantea* and *G. macrophylla* for the treatment of burn wounds. This gel increased the wound healing process significantly by promoting the migration of myofibroblasts, fibroblasts, and macrophages as well as reepithelialization [72].

Taken together, *Gentiana* species are well investigated in the field of wound healing, especially *G. lutea*. Since *G. macrophylla* appears to be an effective treatment alternative for burn wounds, it would be interesting to investigate if *G. lutea* reveals the same potential. Further *in vitro* and *in vivo* experiments concerning the wound healing mechanism of *Gentiana* species as well as clinical studies should be launched to push for the development of a pharmaceutical wound healing product.

Pinaceae members

Different plants belonging to the family Pinaceae are mentioned in the database: *Abies alba* Mill. (Engl. European silver fir; Ger. Edel-Tanne), *Larix decidua* Mill. (Engl. European larch; Ger. Europa-Lärche), as well as *Picea abies* (L.) H.Karst. (Engl. Norway spruce; Ger. Gewöhnlich-Fichte). Only the needles of *A. alba*, and the shoot tips of *P. abies*, which are prepared as tea for rinsing wounds, are different plant parts appearing in the VOLKSMED database. The other entries treat the resin or balm of these trees, and since their composition is expected to be resembling [73], they are discussed together in this section. These exudates are directly used on wounds as a compress or band-aid, or processed with lard to make an ointment.

The chemical composition of different parts of *A. alba, L. decidua,* and *P. abies* is well investigated [74–80]. The database contains the usage of needles and shoot tips of European silver fir and Norway spruce. Geana et al. investigated the phytochemical composition and bioactivities of 50% ethanolic extracts gained from the barks and needles of these two plants. Flavonoids, phenolic acids, and derivatives, as well as stilbenes, terpenes, lignans, and carboxylic acids, were detected in these extracts. The extract gained from *P. abies* needles exhibited a significant antibacterial effect against *P. aeruginosa, E. coli, Enterococcus faecalis*, and methicillin-resistant *S. aureus*, whereas the needle extract of *A. abies* only showed activity against *S. aureus* and *E. faecalis*, but with the lowest IC_{50} concentrations. Furthermore, *in silico* molecular docking experiments suggested the possible effect of selected phenolic compounds found in the extracts on the PI3K/AKT signaling pathway, which is linked to wound healing [75]. Moreover, a 40% ethanolic extract of European larch bark led to similar results. The extract revealed antimicrobial activity against *S. aureus, Klebsiella pneumoniae*, and *P. aeruginosa* [77]. Häsler Gunnarsdottir et al.

summarized the wound healing potential of different bark extracts from common European trees. Here, the main active compounds in larch bark were specified as flavonoids, lignans, and stilbenoids with antioxidant and antimicrobial activity, but no wound healing assays were available. Spruce bark revealed stilbenes, flavonoids, phenolic acids, tannins, and lignin as the main active substance classes, also with antioxidative and antimicrobial activity as well as results from an *in vivo* scratch assay using a human melanoma cell line [79]. Interestingly, the essential oils of four different *Abies* species, *Cedrus libani*, and *Picea orientalis* were found to hold wound healing potential *in vivo* on wounds in rats. Significant results were detected only for the essential oils from *C. libani* and *Abies cilicica* subsp. *cilicica* [81]. Moreover, a standardized extract of *Pinus pinaster* bark, another Pinaceae member, significantly promoted wound healing and reduced the development of scars in an *in vivo* study on female Sprague-Dawley rats, as well as showed antimicrobial activity [82]. Polycaprolactone/gelatin nanofibers containing extracts of *Pinus radiata* bark were developed for the application on wounds. These extracts revealed antibacterial activity against *S. aureus* and *E. coli* and a pro-migratory effect on HaCaT keratinocytes [83]. In addition, the essential oil gained from the cones and needles of different *Pinus* species showed *in vivo* wound healing potential in mice and rats [84–86].

According to the VOLKSMED database, Pinaceae exudates such as resins and balms are the main plant parts used in Austrian folk medicine. No publications on the exudates of *A. alba* concerning wound healing have been published. As mentioned above, the chemical composition of Pinaceae resins and balms appears to be very comparable. Therefore, the results found for other coniferous exudates might also apply to *A. alba* and would be an interesting research target for the future. Even though the Committee for Veterinary Medicinal Products from the European Medicines Agency approved the use of the resin of *L. decidua* for wound healing in animals due to its long history of safe topical application [87], only scarce data about its *in vitro* and *in vivo* activities is available. Substances contained in the resin of European larch contribute to an overall wound healing effect through anti-inflammatory and antimicrobial activity [74]. *In vitro*, the balm exhibits a pro-migratory effect on keratinocytes in a wound healing assay [73]. No *in vivo* studies concerning the wound healing effect of larch exudates have been conducted so far.

On the contrary, *P. abies* balm is a well-known wound healing agent. Different diterpenoid resin acids showed an *in vivo* pro-migratory effect on keratinocytes [76]. However, comparing the balms and resins of European larch, Norway spruce, and black pine in the respective wound healing assay on keratinocytes, only the balm of *L. decidua* revealed significant reepithelialization boosting effects [73]. The resin of Norway spruce is known to hold antimicrobial effects against different bacteria such as methicillin-resistant *S. aureus, B. subtilis,* or vancomycin-resistant *Enterococcus*, but also against fungi such as *Candida albicans, Trichophyton rubrum* or *Trichophyton mentagrophytes* [88–92]. Using ointments containing spruce balm or resin, different *in vivo* studies and clinical trials have already been conducted. Ointments of *P. abies* exudates were tested on castration wounds in piglets and showed higher rates of completely healed wounds and lower contamination of the wounds with fungi compared to the untreated group [93]. In 2005, the treatment of pressure ulcers in 37 patients with a 10% spruce resin salve resulted in significantly higher complete healing rates in the resin group compared to the control group (94 vs. 36%). Another study involving 23 patients with surgical wounds also showed the great wound healing potential of spruce resin salve, with a healing rate of 100% within a mean healing time of 43 days. However, it has to be kept in mind that an allergic reaction in the form of contact dermatitis is possible while using such natural resins [88, 94, 95]. This clinical evidence supported the admission of monographs on the balm and an ointment consisting of 20% balm in lard in the Austrian Pharmacopeia. Furthermore, a medicinal product with the same composition is available on the Austrian market. First experiments using other ointment bases than lard were conducted to develop a more stable, standardized, and animal-friendly formulation [96].

In summary, wound healing bioactivities of different Pinaceae members are well investigated. The balm of *P. abies* has proven its traditional use and is on a good way to be an alternative in the treatment of various wounds in everyday wound management. Since the composition of different balms and resins of Pinaceae trees is supposed to be similar, further research on other coniferous exudates would be promising.

Sambucus sp.

The VOLKSMED database contains *Sambucus nigra* L. (Engl. elder, black elder; Ger. Schwarz-Holunder) and *Sambucus racemosa* L. (Engl. red-berried elder; Ger. Rot-Holunder, Trauben-Holunder). In the literature, the genus *Sambucus* is attributed inconsistently to the families Viburnaceae, Adoxaceae, or Caprifoliaceae. However, according to Plants of the World online [23], *Sambucus* sp. belongs to the family Viburnaceae. The two species are used very differently. Leaves and bark, more precisely the green liber below the outermost bark of *S. nigra*, are applied directly on wounds, or the flowers are mixed with lard and applied as an ointment. Regarding *S. racemosa*, only the ripe red fruits are used traditionally. The fruits are either inserted in oil, or the fruits are boiled in water. During the boiling procedure, the oil contained in fruits separates and accumulates on the water surface due to the difference in density. After skimming this oil, it can be applied to wounds.

Black elder can be found all over the world, in Europe, North Africa, Asia, and America [97]. *S. nigra* is known to be used traditionally in different folk medicines as a wound healing agent. In the Balkan region, the bark of *S. nigra* is either extracted with olive oil for sunburns or boiled with buttermilk for thermal burns. Furthermore, the balm is mixed with fresh flowers for the treatment of all wounds [24]. The use of *S. nigra* flowers for wound healing is also reported in the folk medicine of the Maratea area, Italy, but there it is prepared as a decoction instead of an ointment [98]. In 2018, Jarić et al. already summarized the ethnobotanical and pharmacological characteristics of *S. nigra*. Anthocyanins, flavonoids, tannins, and triterpenes are active compounds found in different parts of the plant and bring forth antibacterial, antioxidant, astringent as well as anti-inflammatory activities. A soft extract from the leaves of *S. nigra* was tested *in vivo* on thermal skin burns in Wistar rats and led to a promotion in wound healing due to flavonoids and tannins. Furthermore, extracts of *S. nigra* berries and flowers revealed antimicrobial effects against *C. albicans, P. aerugi-*

nosa, Salmonella typhi, E. coli, and *B. subtilis* as well as *S. aureus* [24]. More recent publications confirmed this antimicrobial activity, especially against methicillin-resistant *S. aureus*, and suggested inhibition of bacterial efflux pumps as the mode of action [99]. Peptides found in the flowers of *S. nigra* are known to exhibit antimicrobial activity against gram-negative bacteria found in aquaculture [100], and therefore, might be responsible for these antibacterial properties. Popova et al. analyzed the effects of a cream containing extracts of *Pelargonium sidoides, S. nigra*, and *Hypericum perforatum* on different microbes, resulting in a high antimicrobial effect against *C. albicans, E. coli*, and *S. aureus* [101]. Concerning the positive effect of *S. nigra* on inflammation, aqueous and ethanolic black elder leaf extracts showed a high anti-inflammatory potential by inhibition of TNF-α and reactive oxygen species [102]. Furthermore, an aqueous black elder fruit extract and its ferment were investigated concerning their anti-inflammatory and antioxidant activity as well as their impact on fibroblasts and keratinocytes. Both extracts stimulated proliferation and metabolism in the two cell types, inhibited elastase and collagenase, and revealed a high antioxidant effect. Interestingly, the fermented extract exhibited a higher anti-inflammatory activity than the non-fermented extract, which could be explained by the increase in simple phenolics such as chlorogenic acid and protocatechuic acid during the fermentation process [103].

On the contrary, only very few publications about *S. racemosa* are available, and none of them concern the wound healing effects of its fatty oil. This species is native to North America and Europe and can be found in higher elevations [104–106]. The few publications available are about the chemical composition. The oldest literature (1902–1977) deals with the composition of the fatty oil of *S. racemosa* fruits [107–109]. Newer studies cover other substance classes such as glycoprotein lectins [110] and a neolignane glycoside [111] gained from the bark, as well as hydroxycinnamic acids and flavonoids in fruit extracts [112]. In 2020, Miljković et al. conducted the first assessment of the free radical scavenging activity of different *S. racemosa* fruit extracts using different solvents. All extracts were active in the DPPH assay, and the different activities were linked to the respective concentrations of phenolic compounds in each extract [113].

Moreover, a third *Sambucus* species, *Sambucus ebulus* L., has been investigated concerning its wound healing properties. An ointment containing 5% *S. ebulus* fruit extract led to an acceleration of wound healing in rats by reepithelialization, wound contraction, formation of granulation tissue and collagen, as well as revascularization. The authors only mentioned flavonoid glycosides as components found in *S. ebulus* fruits [114]. Therefore, a comparison of the composition of fruits of *S. ebulus* and *S. racemosa* could shed light on possible analogies in wound healing effects.

In summary, *S. nigra* is already well investigated concerning its antimicrobial effects, and the first *in vivo* study has already been conducted. The results suggest further investigations of this plant concerning its wound healing effects. For *S. racemosa*, first investigations in the direction of wound healing would be interesting to find evidence for its traditional use.

Sanicula europaea

Sanicula europaea L. (Engl. wood sanicle; Ger. Sanikel) belongs to the family Apiaceae and is a perennial plant found all over Europe [115]. In Austrian folk medicine, the roots of sanicle as well as the herb and leaves are used traditionally. The roots are traditionally processed into an ointment, and the herb is mostly prepared as tea and used for rinsing and bathing the wound. Furthermore, the fresh herb is applied as a compress after a bath. Leaves of sanicle are either prepared as an ointment, or dry leaves are applied directly onto the wound.

In Austria, only the herb of sanicle ("Herba Saniculae") is commercially available and also used in homeopathic preparations against rhinitis and sinusitis [116]. Interestingly, "Radix Saniculae" might also be linked to other plants (e.g., *Cardamine enneaphyllos, Polygonatum odoratum*) [33, 117–120] since they are all associated with the same Austrian vernacular names "Sanigl" or "Saunigl". Therefore, the use of "Radix Saniculae" has to be mentioned with caution in Austria, since the correct botanical source first must be identified. The entries discussed in this work refer to sanicle, *S. europaea*.

The Latin word "sanare" means "to heal", which is comprised in the Latin name *Sanicula europaea*, already indicating its use as a wound healing plant. The aerial parts of sanicle contain hydroxycinnamic acids, tannins, coumarins, and flavonoids [121] as well as triterpene saponins [122–124]. Interestingly, even though the use of sanicle in the field of wound healing has been reported several times [24, 115, 121, 122, 124–127], only a few publications are available concerning its detailed bioactivities in the treatment of wounds. *S. europaea* is known to possess antimicrobial as well as anti-inflammatory effects [22, 122, 128], which may contribute to an overall wound healing activity. Beggs et al. tested aqueous and methanolic extracts in an *in vitro* scratch assay on 3 T3 fibroblasts to investigate their potential to close this model wound over 20 h. In this assay, sanicle extracts did not lead to an increased wound closure but rather caused cytotoxic effects in the first hours of the experiments. The authors suggest that this cytotoxic activity may contribute positively to the wound healing process as an antibiotic to decrease wound infections [115]. Further *in vitro* tests on other cell lines than fibroblasts as well as investigations on the antibacterial potential of sanicle on wound-colonizing bacteria might lead to a better understanding of the wound healing activities of this plant.

Conclusion and Future Perspectives

This review presents the high value and application possibilities of the VOLKSMED database, a database containing monographs and mixtures of plants and other materials used in Austrian folk medicine. Concerning wound healing, the most abundant plants are well-known wound healing agents such as *Calendula officinalis* or *Hypericum* sp. However, the focus of this work was laid upon lesser-known or, in the meantime, forgotten plants. Eight genera were chosen at random for detailed discussion, keeping in mind that the VOLKSMED database holds many other possible candidates for future investigations. *A. vulneraria, S. racemosa,* and *S. europaea* appear to belong to the forgotten medicinal plants for wound healing. None or only very few publications about their

wound healing potential are available. Thus, these plants represent interesting opportunities for researchers to investigate their wound healing activities and support their traditional use in folk medicine. On the contrary, *Brassica* sp., *Gentiana* sp., Pinaceae members as well as *S. nigra* have already been well investigated concerning their wound healing effects, even with evidence from *in vivo* studies. Therefore, future research is instrumental for a better understanding of the underlying mechanisms as well as the development of new therapeutic products in the treatment of wounds, which definitely could have a place in modern medicine.

Supporting information

A table containing information about the used diploma theses as well as a table depicting traditional wound healing plants with less than 10 entries in the VOLKSMED database is available as Supporting Information.

Contributors' Statement

Design of the study: J. Saukel, S. Glasl; analysis and interpretation of the data: E. Eichenauer, J. Saukel; drafting the manuscript: E. Eichenauer, S. Glasl; critical revision of the manuscript: E. Eichenauer, S. Glasl, J. Saukel

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Conflict of Interest

The authors declare that they have no conflict of interest.

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4. Results

This chapter contains all original articles and manuscripts authored during this doctoral thesis. The publications are not chronological but grouped by plant: *Picea abies*, *Sanicula europaea*, and *Brassica oleracea* var. *capitata* f. *alba*. Each paper is briefly summarized, including a short introduction, my contributions, key findings, and concluding perspectives. All supplementary materials are in chapter E.A.3 (starting from page 143).

4.1 *Picea abies* (L.) H.Karst.

This work's main focus was on Norway spruce balm. *Picea abies* exudates are used not only in Austrian folk medicine but also in Scandinavia, especially by the Lapp people in northern Finland [49]. This tradition has already been investigated in terms of its antimicrobial and wound-healing properties in different *in vitro* and clinical studies [50]. However, this tradition differs from the Austrian tradition: in Finland, Norway spruce resin is used mainly with butter [51], whereas in Austria, the balm is traditionally used with lard. Since 2016, the Austrian Pharmacopeia has contained the monograph "Piceae abietis balsamum", mentioning explicitly the balm (sticky, kneadable, yellowish-orange to brownish color) and no resin (solid, not kneadable, sometimes drop-shaped parts) to be allowed for the use [41].

However, information about i) the main bioactive compounds in Norway spruce balm, ii) fast and efficient chromatographic methods for the analysis of diverse substance classes in Norway spruce balm, iii) similarities between Norway spruce balm and other coniferous exudates, as well as iv) using lard as the traditional ointment base are scarce.

4.1.1 Publication II

The balm's phytochemical composition and modes of action have not yet been entirely uncovered. The correlation between compounds and their bioactivity is crucial for a deeper understanding of the wound-healing potential of this traditional application. Therefore, we aimed to identify and structurally elucidate the main components responsible for the positive effect on re-epithelialization *in vitro* in HaCaT keratinocytes. For this purpose, bioactivity-guided fractionation was applied using various analytical techniques (Figure 4.1, page 26).

The original article '*Norway Spruce Balm: Phytochemical Composition and Ability to Enhance Re-epithelialization In Vitro*' was accepted in March 2020 and published in **Planta Medica** as part of the special issue dedicated to Prof. Wolfgang Kubelka.

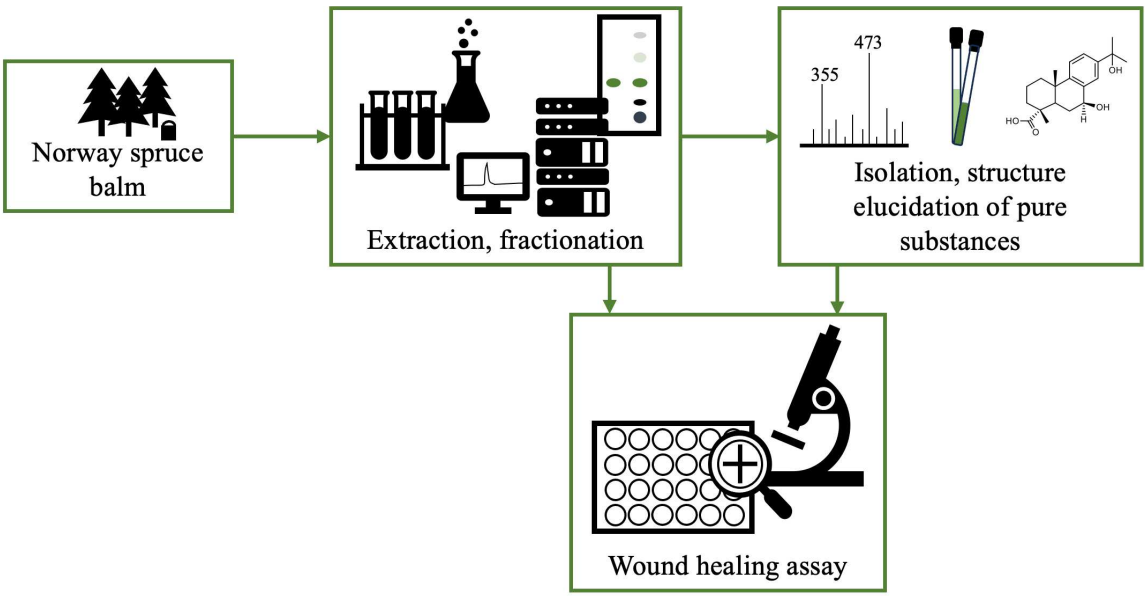

Figure 4.1 Workflow for the assessment of re-epithelialization-boosting constituents in Norway spruce balm.

Contributions

This original article was published before this doctoral thesis started. However, during my diploma thesis, I was already working on the isolation and structure elucidation of constituents found in Norway spruce balm. I contributed to this publication by doing phytochemical analyses and critically reviewing the manuscript. Therefore, this publication was added to this work to provide a better picture of the whole topic.

Key findings

- Several pure compounds from active fractions were isolated and structurally elucidated: the hydroxycinnamic acids p-coumaric acid, caffeic acid, and ferulic acid, the lignan pinoresinol, dehydroabietic acid, and four hydroxylated derivatives thereof.
- Furthermore, different further DRAs, such as isopimaric acid, pimaric acid, and neoabietic acid, were identified in an active subfraction.
- The lignan pinoresinol and different DRAs at concentrations of 1, 3, or 10 µM showed a significantly enhanced wound closure (Figure 4.2, page 27).
- Defined combinations of mono- and di-hydroxylated resin acids led to the highest re-epithelialization, up to 90% at a 10 µM concentration (Figure 4.2, page 27).

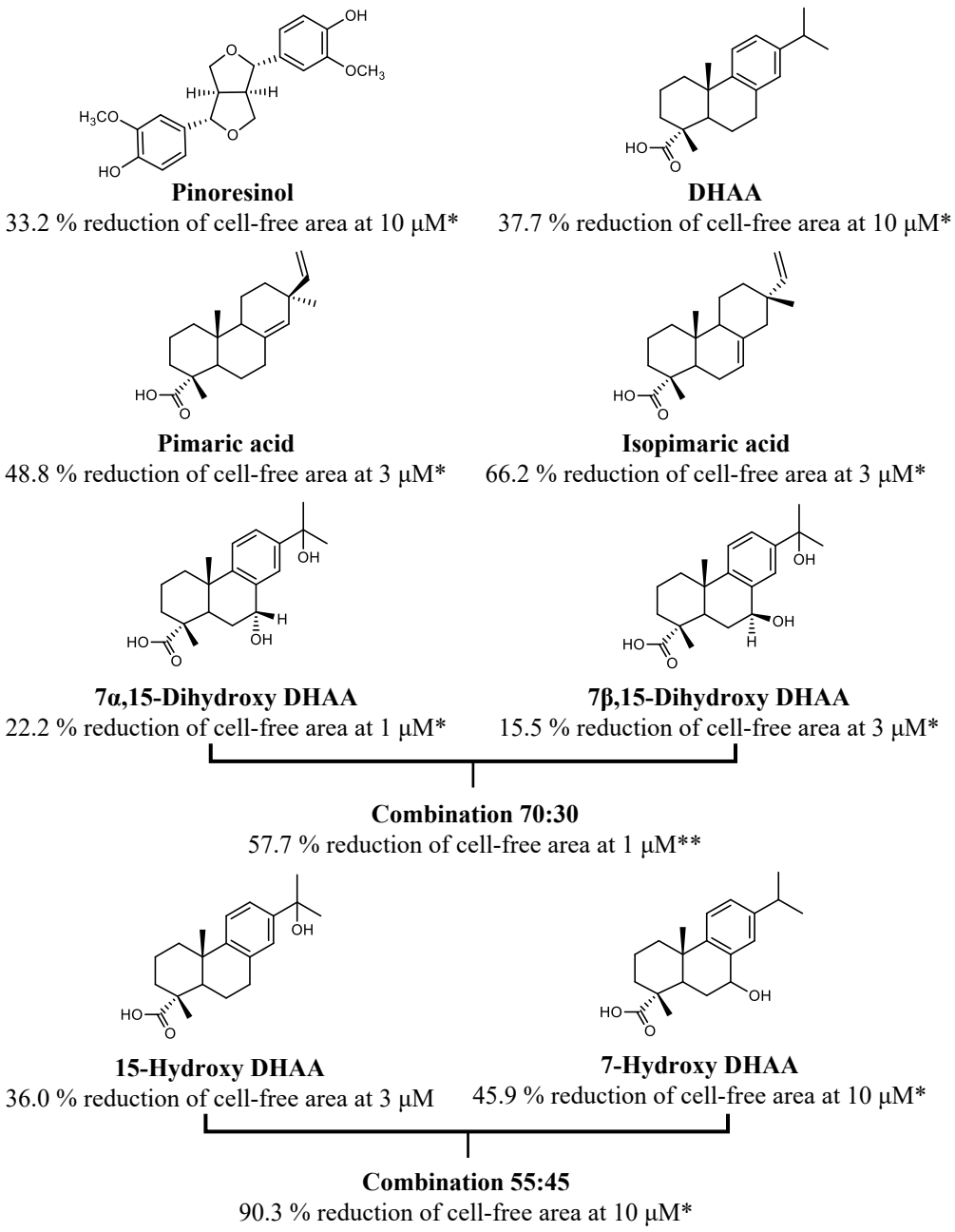

Figure 4.2 Substances in Norway spruce balm possessing the highest re-epithelialization boosting effect. DHAA = Dehydroabietic acid; * $p < 0.05$; ** $p < 0.01$

Concluding perspectives

This article led to a better understanding of Norway spruce balm's complex composition and insights into the wound-healing activities of single compounds. Several substances were identified in the active fractions, some of which have already been described in the callus resin of *Picea abies* [52]. However, these substances have never been directly linked to the wound-healing activity of the balm.

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Norway Spruce Balm: Phytochemical Composition and Ability to Enhance Re-epithelialization *In Vitro*#

Authors

Thomas Goels[1], Elisabeth Eichenauer[1], Julia Langeder[1], Franziska Hoeller[1], Christina Sykora[1], Ammar Tahir[1], Ernst Urban[2], Elke H. Heiss[1], Johannes Saukel[1], Sabine Glasl[1]

Affiliations

1 Department of Pharmacognosy, Faculty of Life Sciences, University of Vienna, Vienna, Austria
2 Department of Pharmaceutical Chemistry, Faculty of Life Sciences, University of Vienna, Vienna, Austria

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Correspondence

ao. Univ.-Prof. Mag. Dr. Sabine Glasl
Department of Pharmacognosy, Faculty of Life Sciences, University of Vienna
Althanstrasse 14, 1090 Vienna, Austria
Phone: + 43 1 4 27 75 52 07, Fax: + 43 1 42 77 85 52 07
sabine.glasl@univie.ac.at

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ABSTRACT

The balm of the Norway spruce (*Picea abies*) is a well-known traditional herbal medicine used to cure wounds. Even though clinical trials have confirmed its empirical use, the active constituents, their mode of action, and the exact composition of this natural product are still unknown. In this study, the balm was subjected to fractionated extraction and further purified employing flash chromatography, HPLC-PDA-ELSD, preparative and analytical TLC. Hydroxycinnamic acids (**1**–**3**), the lignan pinoresinol (**4**), four hydroxylated derivatives of dehydroabietic acid (DHAA) (**5**–**8**), and dehydroabietic acid (**9**) were isolated. Their structures were elucidated by LC-MS, 1D- and 2D-NMR. Four extracts, two commercially available resin acids–pimaric acid (**10**) and isopimaric acid (**11**)–and the isolated compounds were tested for increased re-epithelialization of cell-free areas in a human adult low calcium high temperature keratinocytes monolayer. Lysophosphatidic acid (10 µM) served as positive control and ranged between 100% and 150% rise in cell-covered area related to the vehicle control. Two extracts containing carboxylic acids and non-acidic apolar constituents, respectively, boosted wound closure by 47% and 36% at 10 and 3 µg/mL, respectively. Pinoresinol, DHAA, three of its hydroxylated derivatives, and pimaric and isopimaric acid as well as defined combinations of the hydroxylated DHAA derivatives led to a significantly enhanced wound closure by up to 90% at concentrations between 1 and 10 µM. Overall, lignans and diterpene resin acids, main constituents of Norway spruce balm, are able to increase migration or proliferation of keratinocytes *in vitro*. The presented data link the phytochemistry of this natural wound healing agent with boosted re-epithelialization.

Introduction

Management of chronic wounds is an increasing challenge for healthcare professionals due to aging populations, rising incidence of diabetic complications, or failure of available antibiotics [1]. Wounds can be classified based on various parameters, amongst them aetiology (*e.g.*, accidental, intentional, or consequential from other diseases), acuteness or chronicity, severity, the concerned skin layers and cell types etc. Wound healing is divided into four partially overlapping stages: (i) coagulation and hemostasis phase, (ii) inflammatory phase, (iii) proliferative phase, and (iv) remodelling phase. The first step after injury is the immediate stop of blood loss. The inflammation phase aims at neutralizing bacteria and foreign particles. In the proliferative phase, the focus is put on tissue repair and regeneration of the extracellular matrix. Re-epithelialization, one of the last steps in

Dedicated to emer. Univ.-Prof. Dr. Wolfgang Kubelka on behalf of his 85th birthday.

ABBREVIATIONS

DCM	dichloromethane
DHAA	dehydroabietic acid
HaCaT	keratinocytes human adult low calcium high temperature keratinocytes
LPA	lysophosphatidic acid
PSB	purified spruce balm
VC	vehicle control

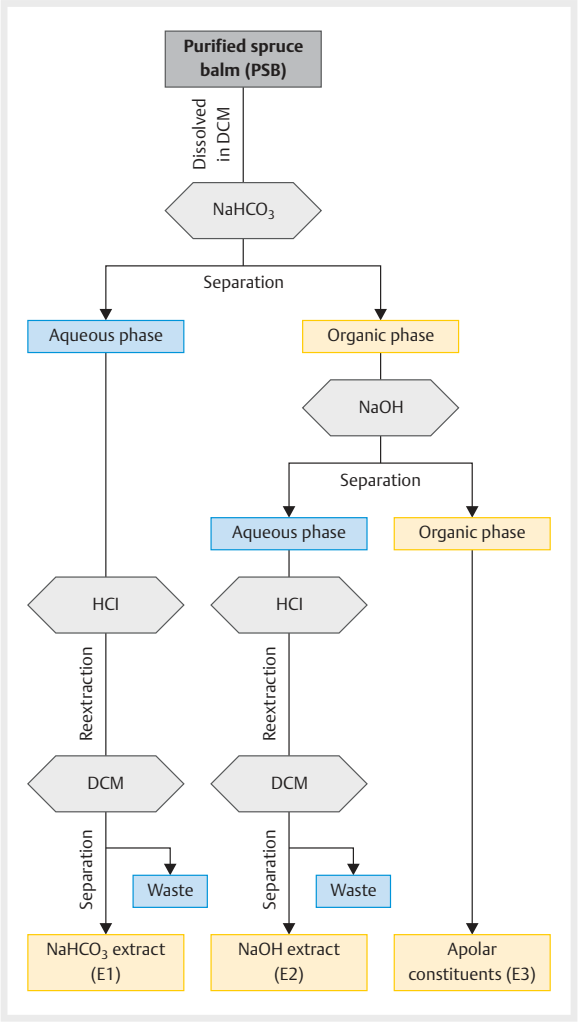
wound healing, starts from the healthy edges of the injured tissue. Mainly keratinocytes migrate across the wound site overgrowing only viable tissue in order to close the wound edges. In the remodelling phase the disorderly dispensed collagen is organized, newly built vessels are degraded, and the metabolic activity in the wound area decreases [2].

The resinous excretion of Norway spruce (*Picea abies* (L.) H. Karst., Pinaceae) [3] has a long-standing history in the treatment of skin ulcers and infected wounds [4]. Lipophilic preparations using butter or swine lard as ointment base were and still are known as an effective cure for acute and chronic wounds. A prospective, randomized, and controlled multicenter trial including 37 patients with pressure ulcers has already attested high wound-healing potential to Norway spruce balm [5]. In another clinical trial, 23 patients with complicated surgical wounds were successfully treated with resin salve [6]. Additionally, a strong antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE), *Streptococcus pyogenes*, and *S. agalactiae* in the agar diffusion test with a resin concentration of 1 % (w/v) was assessed [7]. At higher concentrations the resin also exerted an antimicrobial effect on *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, and *Pseudomonas aeruginosa* in the European Pharmacopoeia challenge test [8] as well as against dermatophytes (*Trichophyton rubrum*, *T. mentagrophytes*, *T. tonsurans*, *Microsporum canis*, *Epidermophyton floccosum*) [9]. These microbes are frequently associated with wound infections.

The chemical composition of Norway spruce balm is only partially known. It consists to a high extent of terpenoids, mainly resin acids that belong to the family of diterpenoids. Furthermore, it contains lignans, phenolic acids, and essential oil [10]. However, up to now, there has been no information about the correlation between phytochemical composition and wound healing activity or potential synergisms. In order to narrow this gap, pure compounds were isolated from the resin, and their structures were elucidated and tested for enhanced re-epithelialization in an *in vitro* wound healing assay using HaCaT keratinocytes.

Results and Discussion

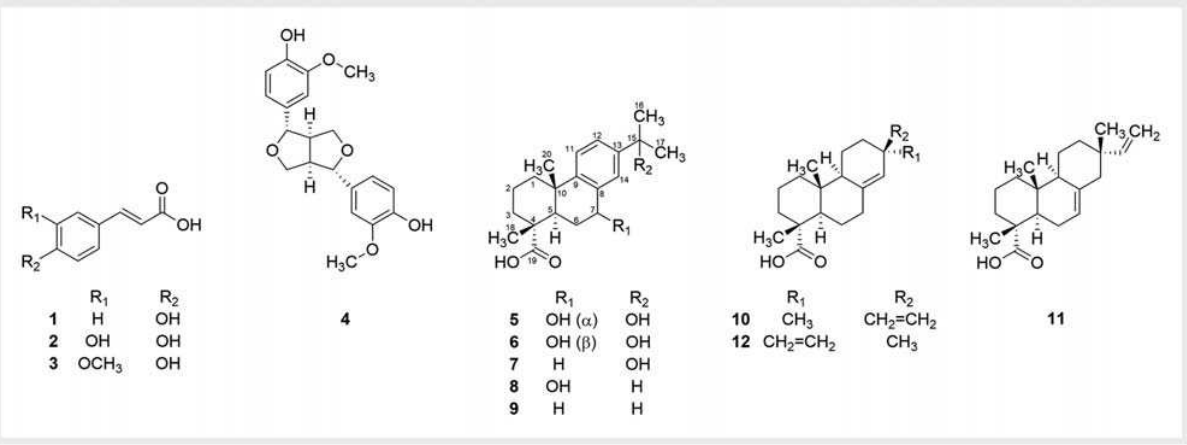
In traditional medicine, Norway spruce balm is processed by exposure to boiling water prior to use. Therefore, the crude balm was boiled in hot water as passed on from tradition. This process yielded the “purified spruce balm” (PSB) as product for therapeutic use on the one hand, and the “lyophilisate” as side product on the other hand. The PSB was extracted following the scheme of



▶ **Fig. 1** Scheme of the liquid-liquid-separation of purified spruce balm (PSB) and the resulting extracts (E1–3).

▶ **Fig. 1**, resulting in the $NaHCO_3$ extract, which contained carboxylic acids and water-soluble compounds, and the NaOH extract, which contained phenolic substances, and the apolar constituents (see section Material and Methods, extraction). The $NaHCO_3$ extract (2.0 g) was fractionated via flash chromatography and resulted in nine sub-fractions that were tested in the subsequent cell-based test system. Sub-fractions 6 and 8 exhibited significant activity and were processed further for the isolation of pure substances (▶ **Fig. 2**) via flash chromatography and preparative TLC.

Sub-fraction 1 was characterized by a high yield (153.4 mg) and by one main spot on TLC. The HPLC-chromatogram of this sub-fraction showed three dominant peaks and a high similarity to the HPLC-fingerprint of the lyophilisate. The three compounds (**1**–**3**) were isolated from the lyophilisate by column chromatography (see section Material and Methods, isolation). The identity of the three compounds was confirmed by co-chromatography and



▶ **Fig. 2** Chemical structures of compounds **1**–**12**.

UV-spectrum comparison with reference substances. They were identified as p-coumaric acid (**1**), caffeic acid (**2**), and ferulic acid (**3**). Sub-fraction 2 consisted mostly of pinoresinol (**4**), which was isolated by preparative TLC. Sub-fraction 5 contained the DHAA derivatives 7*α*,15-dihydroxy-DHAA and 7*β*,15-dihydroxy-DHAA, which were isolated by flash chromatography. Sub-fraction 6 contained 15-hydroxy-DHAA (**7**) and 7-hydroxy-DHAA (**8**). Substance **7** was isolated by flash chromatography and a subsequent purification step via preparative TLC. Substance **8** was isolated by preparative TLC. Sub-fraction 8 contained mostly resin acids without hydroxyl groups like abietic acid, neoabietic acid, pimaric acid, isopimaric acid, levopimaric acid, sandaracopimaric acid, palustric acid, and DHAA (**9**). They represent the main constituents in the balm. Their analytical separation is challenging due to the high chemical similarity. They appear as one single big spot on TLC (normal phase) and elute as one single peak in HPLC (reversed phase). The isolation of **9** was feasible via flash chromatography.

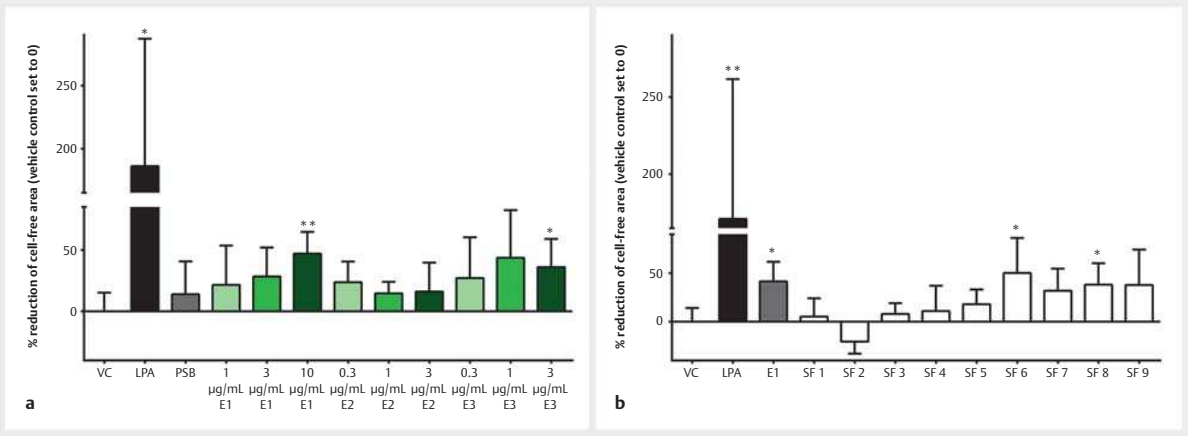
The structures of compounds **4**–**9** were elucidated by comparing their MS- and NMR-data to the literature. Physical, chromatographic, spectroscopic, and spectrometric data of compounds **1**–**9** are listed in the Supporting Information.

Purified Norway spruce balm and extracts thereof were tested in an *in vitro* HaCaT keratinocyte-based wound healing assay. The assay bases on the generation of a cell-free area in a monolayer of keratinocytes. The activity of a test sample is evaluated by measuring the recovered area after 24 h of sample exposure. The resulting value is expressed in % add-on related to the VC. Details of handling and calculation are given in section, Material and Methods, cell-based wound healing assay. Extracts were applied in concentrations from 0.3 up to 10 µg/mL and pure substances in the range of 0.3 to 5.8 µg/mL. These concentrations were chosen as they did not markedly impede HaCaT viability in previous experiments (data not shown). As positive control, we used 10 µM LPA, a substance known to boost migration in different cell-types [11].

The crude balm was treated according to traditional medicine yielding the PSB, the lyophilisate, and the essential oil (see section Material and Methods, plant material), of which all three were tested. The essential oil did not show any activity in the bioassay, and the lyophilisate exerted a pronounced cytotoxic effect at concentrations above 10 µg/mL (data not shown). Therefore, these two extracts were disregarded for further investigations. However, the findings might substantiate the traditional procedure to boil the balm before use.

The PSB showed a tendency towards an enhanced reduction of the cell-free area compared to the solvent control (see ▶ **Fig. 3a**). The extracts exhibited a more pronounced effect. Especially the $NaHCO_3$ extract at a concentration of 10 µg/mL (47.0%, p = 0.0071) and the apolar constituents at a concentration of 3 µg/mL (36.1%, p = 0.0394) led to a significantly enhanced gap closure. Therefore, the $NaHCO_3$ extract was chosen for further separation and analysis since it exhibited the highest potency in a concentration-dependent manner. Sub-fraction 1 contained the hydroxycinnamic acids, caffeic acid, p-coumaric acid, and ferulic acid. Their contribution to the growth or motility of keratinocytes seems to be limited, since their activity ranged at the level of the VC (see ▶ **Table 1**). Sub-fraction 2, tested concentration 10 µg/mL, provoked an antimigratory or antiproliferative effect. However, its main constituent pinoresinol (**4**) was able to increase gap closure concentration-dependently between 1 µM to 10 µM with significant activity at the highest concentration (see ▶ **Table 1**). This finding may be explained by masked pinoresinol activity in the fraction due to the presence of other constituents with in sum antimigratory action.

Sub-fraction **8** showed significant activity (see ▶ **Fig. 3b**). It represented the fraction with the highest yield and contained the main constituents of the balm, the non-hydroxylated resin acids **9**–**11**. DHAA (**9**), which was isolated from this fraction, pimaric acid (**10**), and isopimaric acid (**11**) (both purchased) were tested *in vitro*. The purity check revealed **11** to be a mixture of pimaric acid and sandaracopimaric acid (**12**) in a ratio of 80 : 15 (see Material and Methods). **9** significantly enhanced gap closure at every tested concentration but lacked concentration dependency. **10** and **11** in contrast showed a positive influence on re-epithe-



▶ **Fig. 3** Reduction of cell-free area (%) in the wound healing assay on HaCaT keratinocytes compared to the positive control lysophosphatidic acid (LPA). Note that the y-axis is discontinuous. n = ≥ 4 (see ▶ **Table 1**); student's t test, mean ± SEM, * $p < 0.05$; ** $p < 0.01$; **a** purified spruce balm (PSB, 3 µg/mL) and derived extracts E1 ($NaHCO_3$ extract), E2 (NaOH extract), E3 (apolar constituents), at concentrations 1, 3, and 10 µg/mL (see ▶ **Fig. 1**); **b** $NaHCO_3$ extract (E1) and derived sub-fractions 1–9 ($NaHCO_3$ SF1–9: SF1–9 (10 µg/mL).

lialization *in vitro* at 1 µM and 3 µM and a loss of activity at 10 µM. The latter observation may be due to already declining cell fitness at 10 µM as previous viability assays indicated cytotoxicity for 16.5 µM of **10**/**11**.

During further separation of sub-fraction 6, activity loss was observed with increasing purity. Sub-fraction 6 provoked an increased closure of the cell layer by 50.2% at a concentration of 3 µg/mL (see ▶ **Table 1**). But its pure constituents, 15-hydroxy-DHAA (**7**) and 7-hydroxy-DHAA (**8**), showed values of 26.4% and 45.9% at a concentration of 3.2 µg/mL (corresponds to 10 µM). In order to evaluate whether these compounds exert a cooperative effect, they were recombined according to their ratio in the mother fraction. The 55:45 mixture of **7** and **8** culminated in 90.3% increase in gap closure at 3.2 µg/mL, which exceeds the value of the mother fraction by far and indeed suggests a synergistic or additive effect for compounds **7** and **8**, which warrants further investigation.

Sub-fraction **5** provoked a boosted closure of the cell layer by 18.4% at a concentration of 3 µg/mL (see ▶ **Table 1**). The two substances isolated thereof revealed remarkable differences in activity measured at the same concentration (3.3 µg/mL corresponding to 10 µM). Whereas 7α,15-dihydroxy-DHAA (**5**) resulted in 19.8% wound closure at 10 µM, 7β,15-dihydroxy-DHAA (**6**) only achieved 1.9%. Two mixtures of both substances in the ratio of the mother fraction (70:30) and in the ratio 50:50 were prepared in order to evaluate whether cooperativity could be observed like for compounds **7** and **8**. Both mixtures were higher in activity than the pure compounds alone (see ▶ **Table 1**), with higher activity in the 70:30 than the 50:50 ratio. Since the only difference between **5** and **6** is the orientation of the hydroxyl-group at C-7, the stereochemistry in this position might be of relevance for the chosen readout.

Summarizing, the $NaHCO_3$ extract of spruce balm and its constituent pinoresinol (**4**) concentration-dependently led to an enhanced re-epithelialization of cell-free areas in keratinocyte monolayers *in vitro*. The two tested pimarane-type diterpenes pimaric acid (**10**) and isopimaric acid (**11**) showed a concentration-dependent increase at 1 and 3 µM followed by a decrease in activity at 10 µM. In contrast DHAA (**9**), an abietane-type diterpene, exhibited a constant, significant pro-epithelialization activity at all tested concentrations. Complementing our data, DHAA also overcame impeded migration of dermal fibroblasts in an *in vitro* model of diabetic wound healing [12]. The mono- and di-hydroxylated derivatives of **9** (**5**–**8**) displayed an activity increase compared to their mother fractions when recombined as single substances. This suggests an additive or synergistic effect of these compounds. The presented data show that representatives of two compound classes, lignans (**4**) and diterpene resin acids (**5**–**11**), contribute to increased migration and proliferation of keratinocytes upon exposure to spruce balm. These findings are an initial step in the comprehensive and laborious process from a traditional natural product to a rational phytotherapeutic. Such development starts with extraction optimization, and developing a pharmaceutical formulation, as well as toxicological and pharmacological safety testing, and it culminates in the examination of clinical efficacy, quality, and the mode of action [13]. Re-epithelialization is just a small cutout within the complex process of wound healing, including inflammation, angiogenesis, or granulation. Therefore, further (mechanistic) studies focusing on the (cooperative) influence of spruce balm constituents on other cell types such as immune cells, endothelial cells, or fibroblasts are required. The development of an adequate dermopharmaceutical formulation and the subsequent corroboration in appropriate *in vivo* or organ models are further challenges on the way to fully understand and exploit the wound healing potential of this natural product. The gained data, nevertheless, give a first insight into the correlation of the phytochemistry of spruce balm with one of the interdependent biochemical mechanisms of wound healing.

▶ **Table 1** Data from extracts, sub-fractions and pure compounds in the wound healing assay on HaCaT keratinocyte cells.

Extract/sub-fraction/substance	Concentration	% reduction of cell-free area	± SEM	p-value	n
Purified spruce balm (PSB)	3 µg/mL	13.9	13.4	0.4026	4
$NaHCO_3$ extract (E1)	1 µg/mL	21.6	16.0	0.2679	4
	3 µg/mL	28.5	11.8	0.0882	4
	10 µg/mL	47.0**	8.9	0.0071	4
NaOH extract (E2)	0.3 µg/mL	23.8	8.4	0.0814	4
	1 µg/mL	14.7	4.7	0.1521	4
	3 µg/mL	16.2	11.7	0.2908	4
Apolar constituents (E3)	0.3 µg/mL	27.2	16.6	0.1877	4
	1 µg/mL	43.6	19.4	0.0815	4
	3 µg/mL	36.1*	11.5	0.0394	4
$NaHCO_3$ extract sub-fraction 1	10 µg/mL	5.2	9.4	0.6748	4
$NaHCO_3$ extract sub-fraction 2	10 µg/mL	− 20.5	6.2	0.0721	4
$NaHCO_3$ extract sub-fraction 3	10 µg/mL	8.0	5.6	0.4051	4
$NaHCO_3$ extract sub-fraction 4	10 µg/mL	11.0	13.1	0.4866	4
$NaHCO_3$ extract sub-fraction 5	1 µg/mL	21.5*	5.0	0.0397	5
	3 µg/mL	18.4	4.0	0.0589	5
	10 µg/mL	18.0	5.1	0.0697	9
$NaHCO_3$ extract sub-fraction 6	1 µg/mL	20.7	6.8	0.1156	4
	3 µg/mL	50.2*	16.6	0.0376	4
	10 µg/mL	50.2*	12.8	0.0251	8
$NaHCO_3$ extract sub-fraction 7	10 µg/mL	31.9	11.4	0.0551	4
$NaHCO_3$ extract sub-fraction 8	10 µg/mL	38.3*	11.0	0.0262	4
$NaHCO_3$ extract sub-fraction 9	10 µg/mL	37.9	18.2	0.1002	4
p-coumaric acid (**1**)	3 µM	9.0	8.8	0.3989	3
	10 µM	− 1.4	10.0	0.8978	3
	30 µM	− 3.1	3.2	0.6745	3
Caffeic acid (**2**)	3 µM	7.2	12.7	0.5892	3
	10 µM	2.2	15.0	0.8801	3
	30 µM	− 10.3	17.3	0.5433	3
Ferulic acid (**3**)	3 µM	7.2	4.0	0.3708	3
	10 µM	1.1	4.5	0.8859	3
	30 µM	− 0.6	9.7	0.9563	3
(+)-pinoresinol (**4**)	1 µM	17.1	5.7	0.1559	3
	3 µM	24.4	8.6	0.0899	3
	10 µM	33.2*	8.5	0.0345	3
7α,15-dihydroxy-DHAA (**5**)	1 µM	22.2*	7.5	0.0329	3
	3 µM	12.4	8.6	0.1986	3
	10 µM	19.8	7.8	0.0521	3
7β,15-dihydroxy-DHAA(**6**)	1 µM	12.2	8.1	0.1872	3
	3 µM	15.5*	1.8	0.0185	3
	10 µM	1.9	2.8	0.7105	3
15-hydroxy-DHAA(**7**)	1 µM	27.5	19.4	0.2009	3
	3 µM	36.0	14.6	0.0650	3
	10 µM	26.4	6.5	0.0565	3

cont.

▶ **Table 1** *Continued*

Extract/sub-fraction/substance	Concentration	% reduction of cell-free area	± SEM	p-value	n
7-Hydroxy-DHAA (**8**)	1 µM	42.7*	15.6	0.0443	3
	3 µM	33.9	12.4	0.0588	3
	10 µM	45.9*	16.4	0.0388	3
DHAA (**9**)	1 µM	36.8*	5.6	0.0155	3
	3 µM	31.8*	7.0	0.0317	3
	10 µM	37.7*	3.1	0.0105	3
Pimaric acid (**10**)	1 µM	13.8	13.2	0.3782	3
	3 µM	48.8*	10.8	0.0126	3
	10 µM	− 18.9	4.2	0.1096	3
Isopimaric acid (**11**)	1 µM	34.9*	7.6	0.0180	3
	3 µM	66.2*	25.8	0.0341	3
	10 µM	14.2	14.6	0.3706	3
(**5**) + (**6**) (70 : 30)	1 µM	57.7**	12.2	0.0020	5
	3 µM	27.1*	9.5	0.0427	5
	10 µM	43.2*	14.2	0.0180	5
(**5**) + (**6**) (50 : 50)	1 µM	20.5	10.3	0.1235	5
	3 µM	29.1*	9.1	0.0293	5
	10 µM	35.6**	6.0	0.0043	5
(**7**) + (**8**) (55 : 45)	1 µM	50.7*	16.1	0.0332	4
	3 µM	41.9*	11.0	0.0256	4
	10 µM	90.3*	25.1	0.0147	4

* $p < 0.05$, ** $p < 0.01$

Material and Methods

Plant material

Spruce balm from *Picea abies* was identified and collected by Prof. Johannes Saukel (Department of Pharmacognosy, University of Vienna) in a forest near Sauerfeld, Lungau, Austria (47°07′5.0′′N; 13°52′50.0′′E) by scraping the balm off the tree trunk with a piece of tree bark. The crude material was obtained from multiple individuals in spring and in the summer 2016 and 2017. The harvested balm, constituting a few kilograms in amount, was collected in metal or glass containers and stored at − 20 °C upon further processing. The batches were assigned LU01 (harvest 2016) and LU02 (harvest 2017), and a stock of 10 g each was kept as reference. The samples are kept at the herbarium of the Department of Pharmacognosy, University of Vienna (WUP).

Following the traditional way of preparation, Norway spruce balm was subjected to a boiling step before extraction and further phytochemical investigation. Deionized water (2000 mL) was heated up to approximately 90 °C in a metal vessel. A metal sieve covered with a linen cloth, containing 609 g of the balm, was placed so that the balm was completely covered by water. The high temperature changed the consistency of the spruce balm into a viscose liquid and provoked single drops to pass through the linen tissue and sieve into the water. After temperature equalization, the PSB, which had gathered at the bottom of the vessel, was gained by decantation. Its yield amounted to 431 g. The supernatant was freeze-dried and yielded 12.8 g of the lyophilisate. In order to gain the essential oil for testing, a steam distillation of Norway spruce balm (10 g) was performed according to the European Pharmacopoeia yielding 1.2 mL of essential oil.

Chemicals

Ferulic acid (purity: > 98 %, determined by HPLC-ELSD) and p-coumaric acid (purity: > 98 %, determined by HPLC-ELSD) were obtained from Fluka; caffeic acid (purity: ≥ 99 %, determined by HPLC-ELSD) was supplied by Sigma-Aldrich. The two diterpene resin acids, pimaric acid and isopimaric acid, with specified purities were purchased from the companies given below. The purities were verified by GC-FID analysis in our lab calculating the peak areas according to the 100 % method. Isopimaric acid (**11**, lot: H0718, purchased from Santa Cruz Biotechnology, purity: > 99 %) was confirmed to reveal the value specified by the company. Pimaric acid (**10**, lot: QR13961, purchased from MP Biomedicals) was declared with a purity of 84 %. Our own GC-FID analysis showed an additional peak besides pimaric acid, which was tentatively assigned to sandaracopimaric acid (**12**). The ratio between these two compounds and additional impurities was determined to be 80 : 15 : 5.

General experimental procedures

Flash chromatography was performed on an Interchim puriFlash 4250 unit equipped with an integrated photo diode array (PDA) detector (detecting wavelengths at 200 nm and 254 nm, SCAN 200–600 nm) and a low temperature evaporative light scattering detector (ELSD). The sample was applied via dry load bound to pure silica gel (4-fold amount of silica), and the fractionation was guided automatically by the signal of the ELSD detector. The solvents of the mobile phase ran as binary gradient. Packed columns with silica or C18 material and a particle size of 15 µm were purchased from Interchim.

Analytical thin-layer chromatography was performed on Silica 60 F_{254} TLC plates (average particle size of 9.5 to 11.5 µm, aluminum sheets with fluorescence indicator, Merck). The samples were dissolved in acetone to a concentration of 2 mg/mL, and 2 µL were applied with a capillary to the TLC plate. For preparative thin-layer chromatography Silica gel 60 F_{254} glass plates (average particle size of 9.5 to 11.5 µm, glass plates with fluorescence indicator, Merck) were used. The samples were dissolved in acetone (2 mg/mL), and 180 µL were applied by a CAMAG Automatic TLC Sampler 4 (ATS4) in a length of 18 cm. After development, the compounds of interest were collected by scraping off the silica layer from the glass plate with a scalpel. Elution from the silica was performed using acetone. This procedure was repeated 8 times in order to gather an adequate amount for structure elucidation.

For HPLC analysis, a Shimadzu instrumentation (Shimadzu Degasser DGU-20A 5, Shimadzu Auto Sampler SIL-20AC HAT, Shimadzu Communications Bus Module CBM-20A, Shimadzu Liquid Chromatograph LC-20AD, Shimadzu Column Oven CTO-20AC, Shimadzu Diode Array Detector SPD-M20A, Shimadzu ELSD-LT, Shimadzu Lab Solutions Software) was employed. An RP18 column (LiChrospher 100 RP18e 5 µm, 250 mm × 4 mm) served as stationary phase. Elution of the analytes was realized using double distilled water + 0.1% formic acid (solvent A) and acetonitrile + 0.1% formic acid as mobile phase (solvent B). The gradient elution started with 20% solvent B rising to 95% solvent B within 37.5 min (rate 2%/min) at a flow of 1 mL/min.

The LC-MS instruments consisted of a liquid chromatography system with a CAD and a DAD detector (Dionex UltiMate 3000 RS Pump, Dionex UltiMate 3000 RS Autosampler, Dionex UltiMate 3000 RS Column Compartment, Dionex UltiMate 3000 Diode Array Detector, Dionex UltiMate 3000 Corona Ultra RS; Software: Dionex Chromeleon XPress) and a mass spectrometer (LC-MS: Bruker Daltonics HCT; LC-HRMS: Bruker Daltonics maXis HD-UHR-TOF; software: Bruker Esquire Control, Bruker HyStar 3.2, Bruker Compass Data Analysis). Electrospray ionization was performed for the ionization of the sample molecules. The particular analysis settings are depicted in the respective MS plots of the substances (see **Fig. 1S–6S**, Supporting Information). The LC instrumentation corresponded to the system described above.

A Bruker Avance 500 NMR spectrometer (UltraShield) combined with a CryoProbe Prodigy triple resonance inverse probe head for enhanced sensitivity was used for recording the NMR spectra of the pure substances. The resonance frequency for ^{1}H-NMR was 500.13 MHz and for ^{13}C-NMR 125.75 MHz. The measurements were performed in deuterated methanol or chloroform. Standard 1D and gradient-enhanced 2D experiments including double quantum filtered (DQF) HSQC, HMBC, and COSY were utilized as supplied by the manufacturer. The chemical shifts are referenced internally to the residual, non-deuterated solvent signal for methanol ^{1}H (δ 3.31 ppm) or chloroform ^{1}H (δ 7.26 ppm) and to the carbon signal of the solvent for methanol ^{13}C (δ 49.00 ppm) or chloroform ^{13}C (δ 77.00 ppm). Proton and carbon spectra as well as 2-dimensional experiments such as HSQC, HMBC, and COSY were recorded. Mestrelab Research's MestReNova 6.0.2.5475 was used to interpret the spectra. To verify the determined structures, the online tool CSEARCH-Robot-Referee was employed [14].

Extraction

The PSB was further purified by liquid-liquid-separation starting with 100 g which were dissolved in 150 mL DCM and partitioned 3 times against a saturated aqueous $NaHCO_3$ solution in order to enrich strong acids like carboxylic acids and water soluble substances. The collected aqueous phases were acidified with concentrated HCl to pH 3 and re-extracted with DCM, yielding 3.5 g of the $NaHCO_3$ extract (E1) after removal of the organic solvent. The organic phase that remained after the first liquid-liquid separation step was subsequently extracted with a 4% aqueous sodium hydroxide solution, with the aim to separate weak acids like phenols from potentially present bases and apolar compounds. The resulting aqueous phase was acidified with HCl to pH 3 and extracted with DCM, yielding 67.3 g of the NaOH extract (E2). The organic layer remaining after the 2 extraction steps was evaporated and yielded 16.4 g of the apolar constituents (E3). For better overview, a scheme of the fractionated extraction is given in ▶ **Fig. 1**.

Isolation

The lyophilisate (1.0 g) was fractionated via column chromatography (diameter 1.2 cm; length 30 cm) on silica gel (63.1 g) running 3 different mobile phases (300 mL each). All together 140 fractions with approximately 7 mL per fraction were collected: DCM (fractions 1–47), DCM/acetone/formic $acid_{conc.}$ 89.9 : 10 : 0.1 (fractions 48–94), and DCM/acetone/formic $acid_{conc.}$ 79.9 : 20 : 0.1 (fractions 95–140). **1** resulted from fraction 46–55 (19.2 mg), **2** resulted from fraction 64–68 (13.1 mg), and **3** resulted from fraction 35–37 (6.9 mg).

The $NaHCO_3$ extract was fractionated employing an Interchim C18 15 µm HQ 35 G column, double-distilled water (solvent A) and methanol (solvent B) as mobile phase in the following gradient system: 20% to 100% B (rate 2%/min), 100% B for 10 min, flow 15 mL/min. Fractions were automatically collected, guided by the ELSD signal and combined to 9 sub-fractions.

For the isolation of **4,** sub-fraction 2 (23.1 mg) was separated via preparative TLC using DCM/acetone/formic acid (80 : 18 : 2) as mobile phase. The process yielded 1.2 mg of **4** (purity 98.7%, determined by HPLC-ELSD). **5** and **6** were isolated from sub-fraction 5 (385.4 mg) by 4 consecutive flash chromatography separations on a Silica 15 µm HP 4 G column with DCM (solvent A) and acetone (solvent B) as mobile phase. The gradient system was as follows: 0% to 20% B within 40 min (rate 0.5%/min); 20% to 60% B within 40 min (rate 1%/min); 100% B for 10 min at a flow of

5 mL/min. 23 fractions were collected with **5** (purity 99.4%, determined by HPLC-ELSD) being present in fraction 20–23 with a yield of 1.0 mg and **6** (purity 97.4%, determined by HPLC-ELSD) in fraction 17–19 with 5.2 mg in amount. 15-hydroxy-DHAA (**7**) and 7-hydroxy-DHAA (**8**) were isolated from sub-fraction 6 (202.0 mg). For the isolation of compound **7,** a Silica 15 µm HP 4 G column was employed running the mobile phase DCM + 0.1% FA (solvent A) and acetone (solvent B). The gradient system was as follows: 4% isocratic for 40 min, 100% B for 10 min at a flow of 5 mL/min yielding 17 fractions. A subsequent preparative TLC purification step (DCM/acetone/formic acid 90 : 9 : 1) of fraction 9 from the preceding separation yielded 2.7 mg of **7** (purity 99.4%, determined by HPLC-ELSD). Substance **8** was isolated from sub-fraction 6 via preparative TLC with the mobile phase DCM/acetone/formic acid 94 : 5 : 1 yielding 1.8 mg (purity 88.0%, determined by HPLC-ELSD). **9** was isolated by flash chromatography from sub-fraction 8 (234.2 mg) on a C18 15 µm HP 6 G column with a mobile phase consisting of double distilled water (solvent A) and methanol (solvent B). The gradient system was as follows: 40% to 80% B (rate 2%/min), 80% to 100% B (rate 0.5%/min), 100% B for 10 min, flow 5 mL/min. The separation resulted in 40 fractions, fractions 25 and 26 contained 21.6 mg of DHAA (**9**) (purity 97.8%, determined by HPLC-ELSD).

Cell-based wound healing assay

Keratinocytes (HaCaT) were obtained from CLS (cell lines service) and used to simulate the epidermal skin layer. They were maintained in DMEM medium (high glucose, phenol-red free; Lonza) supplemented with 10% FBS (Gibco), 2 mM glutamine (Lonza), 100 U/mL benzylpenicillin (Lonza), 100 µg/mL streptomycin (Lonza) at 37 °C and 5% CO_2 in a humidified atmosphere. Cell mass and metabolic activity were determined using the crystal violet and the resazurin assay, respectively, and enabled assessment of non-cytotoxic concentrations of test samples prior to the wound healing assay.

To monitor the re-epithelialization enhancing effect of extracts and pure substances, a wound healing assay was adapted with modifications as published by Liang et al. [15]. For this, cross shaped plastic inserts (length: 13.10 mm; width: 0.88 mm) were placed in each well of a 24-well plate. A cell suspension (1 mL of a 1.5×10^5 cells/mL solution) was pipetted into each well, and the plate was incubated overnight (37 °C, 5% CO_2) to allow formation of a confluent monolayer interrupted by a cross of cell-free area. The next day inserts were removed (t = 0), media was aspirated, cells were washed with PBS, and different sample solutions (dissolved in DMSO BioUltra for molecular biology, Sigma Aldrich), diluted with medium containing 0.1% FBS to a final DMSO concentration of 1% were applied to the wells. LPA (Santa Cruz Biotechnology) served as positive control. Photos of the cell-free gaps at the starting time (t = 0) and after 24 h (t = 24) were taken. The cell-free area of the respective gaps were analyzed by Fiji, which is an image processing package containing a distribution of ImageJ (Version 1.52i) combined with various plugins [16]. Additionally the ImageJ macro Wound Healing Tool [17] was used for the automated analysis of each gap (variance filter radius: 24; threshold: 2–4; radius open: 4).

Two wells were tested per sample, and from each well 4 images were taken (see **Fig. 7S**, Supporting Information) so that finally a total of 8 cell gaps per sample were recorded. The cell-free area at time point t24 is subtracted from the cell-free area at time point t0, which gives a measure for the obtained wound closure within 24 h. Since the camera takes pictures in a landscape format (image resolution: 4608 × 3456 pixel (aspect ratio: 1.33), the recorded 4 areas are inequal (**Fig. 7S, 8S**, Supporting Information). For this reason the calculations of the values resulting from the horizontal (**Fig. 7S**: 1, 3, 5, 7; Supporting Information) and vertical (**Fig. 7S**: 2, 4, 6, 8; Supporting Information) pictures were performed separately according to the formula given below: the area difference of the vertical sample was divided by the mean area difference of all vertical VC values. The horizontal values were processed the same way. Subtraction of the value 1 sets the VC to the value 0; multiplication with 100 indicates the reduction of the cell-free area in % normalized to the VC. The final mean value of the respective sample is calculated from these normalized values.

$$\left(\frac{\text{area t0}_{\text{sample}} - \text{area t24}_{\text{sample}}}{\text{area t0}_{\text{VC}} - \text{area t24}_{\text{VC}}} - 1\right) \times 100$$

Statistics

The experiments were conducted in at least 3 independent biological replicates with 8 technical replicates. Calculations were performed with Excel, and for the statistical analysis Graphpad Prism 6.01 (GraphPad Software, Inc.) was used. The student's t-test was performed to compare 2 groups, and significance was defined as $p < 0.05$.

Supporting Information

Compound data, MS spectra of the isolated substances and analysis settings, a scheme of the image sections as well as sample images from the bioassay are available as Supporting Information.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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4.1.2 Publication III

As one of the main findings of the previous article (see chapter 4.1.1, page 25), DRAs were revealed as major bioactive constituents in Norway spruce balm. Besides different mono- and di-hydroxylated DRAs, eight non-hydroxylated DRAs pose analytical challenges due to low polarity and structural similarity: conventional liquid chromatography techniques often fail to reach sufficient separation of these substances, while gas chromatography requires time- and labor-intensive derivatization prior to analysis. Therefore, we aimed to establish a sustainable, straightforward, short, and efficient separation method using UHPSFC coupled with electrospray ionization-mass spectrometry (ESI-MS). This method was used to quantitate the DRAs in several spruce balm samples and commercial products (Figure 4.3).

The original article '*Ultra High-Performance Supercritical Fluid Chromatography for the Quantitation of Diterpene Resin Acids in Norway Spruce Samples*' was accepted in May 2022 and published in **Frontiers in Pharmacology**.

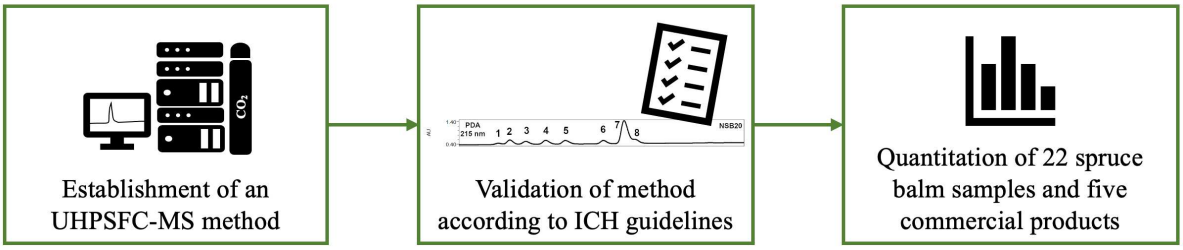

Figure 4.3 Workflow of the quantitation of DRAs in several spruce balm samples and commercial products using UFPSFC-MS.

Contributions

My contributions included executing phytochemical experiments, data analysis, and drafting and critically reviewing the manuscript.

Key findings

- DRAs were separated by establishing a UHPSFC protocol using a Torus 2-Picolylamin column, supercritical carbon dioxide (CO_2), and ethanol as the mobile phase in less than 20 minutes of analysis. A photo diode array (PDA) detector and a mass spectrometer were utilized for detection.
- The resulting method aligned with the ICH Q2 (R1) guidelines in terms of limit of detection (LOD), limit of quantitation (LOQ), linearity, specificity, accuracy, repeatability, and intermediate precision.

- 22 spruce balm samples harvested in different regions at different time points were analyzed, and the revealed content of DRAs ranged from 8 to 47 % was revealed (Figure 4.4).
- Even balm samples taken from one tree but at different wounding sites showed varying contents of DRAs between 15-31 % DRAs (Figure 4.4).
- The phytochemical composition of two balm-based Austrian commercial products resembled the Austrian raw balm samples. However, the content and composition of three Finnish resin-based products differed significantly (Figure 4.4).

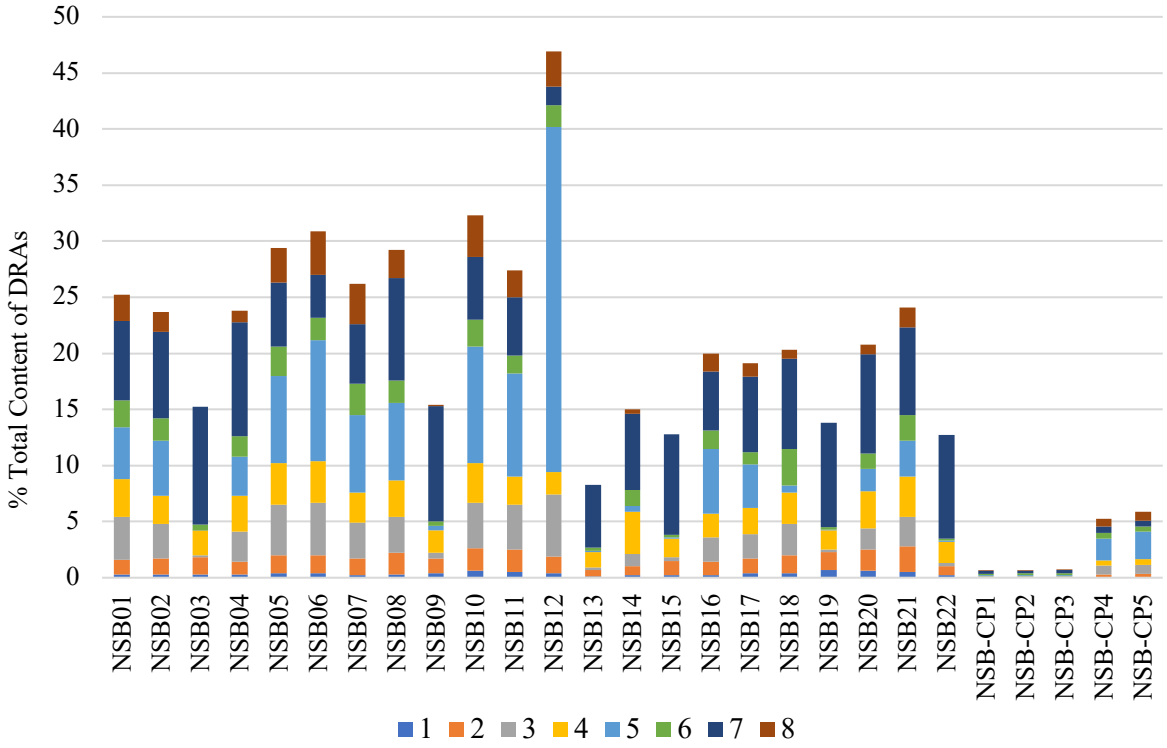

Figure 4.4 Total content of DRAs in Norway spruce balm (NSB) samples NSB01-22 and in commercial products (CP) NSB-CP1-5. **1** = pimaric acid, **2** = sandaracopimaric acid, **3** = palustric acid, **4** = isopimaric acid, **5** = levopimaric acid, **6** = abietic acid, **7** = dehydroabietic acid, **8** = neoabietic acid.

Concluding perspectives

The established method provides a rapid and efficient separation of structurally similar DRAs. Even though the total content of DRAs varied widely between the 22 different balm samples, levopimaric acid or dehydroabietic acid consistently remained the main component. Holmbom et al. [52] have already found these two substances as main constituents of *Picea abies* oleoresin. The immense differences in the contents of the Finnish and Austrian CPs might be due to the use of different exudates, final concentrations in the product, or ointment bases with associated matrix effects.

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ORIGINAL RESEARCH
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Ultra High-Performance Supercritical Fluid Chromatography for the Quantitation of Diterpene Resin Acids in Norway Spruce Samples

Thomas Goels[1,2], *Elisabeth Eichenauer*[1,2], *Julia Langeder*[1,2], *Georg F. Aichner*[1], *Gregor Mauser*[1], *Luisa Amtmann*[1], *Ulrike Grienke*[1†] and *Sabine Glasl*[1*†]

[1]Division of Pharmacognosy, Department of Pharmaceutical Sciences, Faculty of Life Sciences, University of Vienna, Vienna, Austria, [2]Vienna Doctoral School of Pharmaceutical, Nutritional and Sport Sciences, University of Vienna, Vienna, Austria

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Federal University of Minas Gerais, Brazil

***Correspondence:**
Sabine Glasl
sabine.glasl@univie.ac.at

†ORCID:
Ulrike Grienke
orcid.org/0000-0003-0305-9270
Sabine Glasl
orcid.org/0000-0001-9091-6263

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Picea abies (L.) H. Karst. (Pinaceae) is native to Northern, Central and Eastern Europe. The fast-growing tree reaches up to 50 m in height, has modest nutritional requirements and depends on sufficient water supply. The conifer, commonly called Norway spruce, produces exudates which are traditionally used to treat skin wounds in Northern European countries. Major bioactive constituents of the conifer oleoresin are diterpene resin acids (DRAs) of the abietane and the pimarane type. To assure consistent pharmaceutical quality of Norway spruce balm and commercial products thereof, an analytical method for the quantitation of DRAs is the prerequisite. However, high structural similarity among DRAs and their poor UV absorption makes chromatographic separation and detection challenging: Conventional liquid chromatography systems often fail to achieve sufficient separation, moreover, they are not sustainable. Gas chromatography on the other hand requires time-consuming derivatization prior to unacceptably long analyses (>60 min). These drawbacks prompted the development of the first validated supercritical fluid-based protocol for the separation and quantitation of eight DRAs, i.e., pimaric acid (**1**), sandaracopimaric acid (**2**), palustric acid (**3**), isopimaric acid (**4**), levopimaric acid (**5**), abietic acid (**6**), dehydroabietic acid (**7**), and neoabietic acid (**8**). By using an ultra high-performance supercritical fluid chromatography (UHPSFC) device hyphenated to a quadrupole mass detector, the DRAs were separated in less than 20 min on a Torus 2-Picolylamin (2-PIC) column (3.0 mm × 100 mm; 1.7 µm particle size) applying supercritical CO_2 and ethanol as mobile phase. Regarding selectivity, accuracy (recovery rates: 87–108%), intermediate precision (between 6.6 and 11.1%), and linearity ($R^2 \geq 0.99$; linear between 0.75 µg/ml and 2.5 mg/ml), results were obtained in line with ICH guidelines. The lowest limit of detection (LOD) was at 0.75 µg/ml (**7**) and the lowest limit of quantitation (LOQ) at 2 µg/ml (**8**). As application examples, 22 Norway spruce balm samples and five commercial products were analyzed. The here presented protocol not only simplifies and shortens the analytical workflow, but also reduces the amount of organic solvent waste by about two thirds compared to conventional liquid chromatographic set-ups. These advantages qualify this fast and efficient method as an ideal tool for an environmentally friendly quality control of traditionally used wound-healing Norway spruce balm products.

Keywords: *Picea abies*, Pinaceae, diterpene resin acids, quantitation, ultra high-performance supercritical fluid chromatography

1 INTRODUCTION

Coniferous trees produce highly viscous and adhesive exudates, in response to mechanical injuries, which provide protection against microbial infection and water loss (Holmbom et al., 2008; Kopaczyk et al., 2020). These exudates are complex mixtures consisting mainly of diterpene resin acids (DRAs), essential oil, lignans, and phenol carbonic acids (Keeling and Bohlmann, 2006; Holmbom et al., 2008; Metsämuuronen and Sirén, 2019; Kopaczyk et al., 2020). Fresh excretions are rich in essential oil, exhibiting paste-like and ductile characteristics with an aromatic odour. These fresh exudates are defined pharmaceutically as "balms" synonymously named "oleoresins" or "soft resins", whereas "resins" result after evaporation of the essential oil, yielding non-volatile, brittle, and amorphous products soluble in nonpolar solvents (Burger, 1998). Coniferous tree balms have a long tradition in the treatment of infected and chronic wounds. The Ancient Greeks and Romans used coniferous excretions for wound dressings and hemostasis (Schmitz, 1998; Schnabl, 2001), and they have been continually used since the Middle Ages for their reputed curative effects. In Nordic countries, where the trees are in wide occurrence, ointments prepared from Norway spruce exudates have been among the most popular folk remedies for centuries, handed down from lumberjacks and others, who applied spruce excretions directly onto wounds (Pohl-Sennhauser, 1996). The application areas described, include infected wounds, sores, pressure ulcers, punctured abscesses, suppurating burns, onychomycosis and paronychia (Jokinen and Sipponen, 2016). Norway spruce balm and a lard-based ointment of the balm are monographed in the Austrian Pharmacopoeia (Österreichische Arzneibuchkommission, 2021). However, this monograph lacks precise parameters for quality control such as R_f-values for marker compounds in TLC-fingerprints, or requirements of minimum contents for any class of compounds. DRAs play a major role in the wound healing activity of these natural products (Goels et al., 2020). For abietic acid (**6**), an angiogenic and migratory activity in human umbilical vein vascular endothelial cells (HUVECs) is proposed. The induced angiogenesis is associated with the upregulation of ERK and p38 expression, resulting in elevated cell migration and tube formation (Park et al., 2017). Dehydroabietic acid (**7**) was shown to reverse the tumour necrosis factor-α (TNF-α)/forkhead box O1 (FOXO1) and transforming growth factor-β1 (TGF-β1)/Smads mediated decrease in diabetic wound healing in human adult dermal fibroblasts (Wang et al., 2014). DRAs are therefore highly suitable as marker compounds for quality assessment of raw tree exudates (Goels et al., 2022). Efficient GC-MS based quantitation methods have already been described in the literature (Latorre et al., 2003; Holmbom et al., 2008; Alicandri et al., 2021). However, in addition to long analysis times (>60 min), this chromatographic technique requires sample derivatization prior to analysis in order to achieve volatility of the analytes. Although several authors focused on using HPLC with either UV- or MS-detection, their published chromatographic methods did not achieve sufficient separation of DRAs (Latorre et al., 2003; Kersten et al., 2006; Nilsson et al., 2008). Hence, the aim of this study was to overcome these drawbacks by developing a protocol using ultra-high performance supercritical fluid chromatography (UHPSFC) coupled with electrospray ionization mass spectrometry (ESI-MS) for the separation and detection of eight representative DRAs of the abietane and the pimarane type (**Figure 1**), pimaric acid (**1**), sandaracopimaric acid (**2**), palustric acid (**3**), isopimaric acid (**4**), levopimaric acid (**5**), abietic acid (**6**), dehydroabietic acid (**7**), and neoabietic acid (**8**). The eight specified diterpene acids possess high structural similarity, and thus exhibit highly similar chromatographic behaviour. The DRAs **1**–**6** and **8** have a molecular weight of 302 g/mol and differ solely in the position of one double bond and the side chain in position 13. DRA **7** has an additional double bond, resulting in an aromatic ring and a molecular weight of 300 g/mol.

The determining factors for the selection of an analytical setup were the properties of the targeted analytes (e.g., volatility, polarity etc.) and the availability of a suitable instrument. The high complexity and varying composition of samples from natural sources (e.g., due to different growth conditions) also presented a challenge. In this context, GC and HPLC are favoured techniques resulting from their broad availability and well-known chromatographic features. However, since the first application of a supercritical fluid in a chromatographic setting in the 1960s, supercritical-fluid chromatography (SFC) has evolved into a valuable technique for the separation and analysis of natural products. As a result of technical advances and the increased availability of SFC instruments, the current amount of analysable compound classes has broadened and is no longer limited to non-polar constituents. The combination of comparatively rapid and highly efficient separation with simple sample preparation and mild separation conditions, are significant advantages of this technology. The application of supercritical CO_2 enables the analysis of thermolabile and hydrolysable constituents. A reduced environmental impact due to shortened run times and the omission of organic solvents as mobile phase, is an additional benefit. Today, SFC and the advanced ultra-high performance SFC (UHPSFC) constitute a versatile technology providing high separation efficiency, access to a broad range of compound classes, with various stationary phase chemistries available and high compatibility with numerous detectors (Schretter et al., 2020; Ganzera and Zwerger, 2021; Langeder and Grienke, 2021). For these reasons, this first UHPSFC method for the quantitative analysis of DRAs is preferable to the previously suggested techniques. Direct application to the UHPSFC

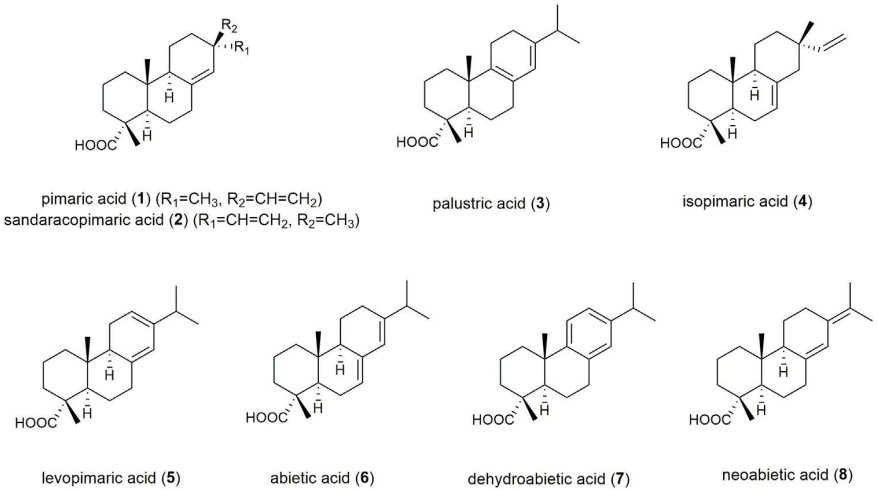

FIGURE 1 | Structures of quantitated highly similar DRA congeners.

system provides a big advantage over laborious sample preparation methods such as silylation for GC, which costs time and represents an additional source of errors and non-reproducibility. Compared to HPLC, UHPSFC is superior in separation efficiency and organic solvent waste production.

The UHPSFC method developed was validated according to the ICH guidelines and proved to be suitable for the quantitation of DRAs in raw plant material (balms) and commercial products prepared from spruce balms.

2 MATERIALS AND METHODS

2.1 Chemicals and Standard Compounds

Dehydroabietic acid (**7**) was purchased from TCI chemicals with a declared purity less than 70%. Prior to validation it was purified *via* flash chromatography using a "Puriflash Column 15 C_{18} HP 6G - 6.0 g" as stationary phase and a linear gradient of methanol and water starting at 40% methanol with a rate of 2% per min. Dehydroabietic acid eluted at 33.1 min, its purity was determined *via* GC-FID to be 99.3%. All other resin acids were purchased from various suppliers and additionally checked for their purity by GC-FID: Neoabietic acid (**8**) (batch number: C1518, Santa Cruz Biotechnology; declared purity: ≥99%, confirmed *via* GC-FID: 99.0%); pimaric acid (**1**) (batch number: QR13961, MP Biomedicals; declared purity: 84%, confirmed *via* GC-FID: 84.6%); isopimaric acid (**4**) (batch number: H0718, Santa Cruz Biotechnology; declared purity: ≥99%, confirmed *via* GC-FID: 98.3%); palustric acid (**3**) (batch number: 3-TAH-178-1, TRC Canada; declared purity: 96%); levopimaric acid (**5**) (batch number: G2618, Santa Cruz Biotechnology; declared purity: 99.87%, confirmed *via* GC-FID: 46.9%); abietic acid (**6**) (batch number: X25C012, Alfa Aesar; declared purity: >90%, confirmed *via* GC-FID: 74.1%). For the identification of sandaracopimaric acid, sandarac resin was analyzed *via* UHPSFC-ESI-MS (**Supplementary Figure S1**) and GC-FID (**Supplementary Figures S2, S3**), since the pure substance was not commercially available. This resin was obtained from *Tetraclinis articulate* (supplier: Die Kräuterdrogerie) and contains sandaracopimaric acid as one of the most abundant components (Sugimoto et al., 2006; Kononenko et al., 2017). Sandaracopimaric acid corresponds to compound **2** in the Norway spruce balm samples (NSB). Compounds **7** and **8** were used as external standards and quantitated directly, whereas compounds **1**–**6** were calculated as compound **8** due to their structural similarities.

2.2 Samples and Sample Preparation

All samples analyzed in this work are listed in **Supplementary Table S1** indicating batch number, date of harvest, harvest location, and expiration date. As a reference, 1 g of each sample is stored at −20°C at the Division of Pharmacognosy, University of Vienna. Norway spruce balm samples were either collected from various locations at different timepoints in Austria or were obtained from an in-house repository (**Supplementary Table S1**). The trees from which the samples were harvested were identified by Thomas Goels, Sabine Glasl or the in-house botanist Johannes Saukel. NSB01-NSB08 constitute samples from one single tree taken on the same day, but at eight different lesion sites. For NSB09-NSB22 different individuals were harvested. The samples were stored at −20°C prior to further processing. Before analysis, each sample was dissolved in acetone and filtered to remove cellular debris and other foreign material (e.g., insects, tree needles, pieces of bark). After removing the acetone under reduced pressure, the samples were re-dissolved in a mixture of *n*-hexane and isopropanol (50 + 50) at a concentration of 2 mg/ml. Two commercial products containing Norway spruce balm at concentrations of 10% (NSB-CP1, NSB-CP2 and NSB-CP3) and 20% (NSB-CP4 and NSB-CP5) were analyzed. The samples varied in batch numbers and storage conditions.

(**Supplementary Table S1**). Both preparations were dissolved at a concentration of 10 mg/ml. For NSB-CP4 and NSB-CP5 a mixture of *n*-hexane and isopropanol (50 + 50) and for NSB-CP1, NSB-CP2 and NSB-CP3 pure hexane was used as solvent. All reference compounds were dissolved in a mixture of *n*-hexane and isopropanol (50 + 50) at varying concentrations. For each solution (balm, commercial product, reference), 1 µl was applied to the UHPSFC-MS system.

2.3 Analytical Method

The UHPSFC instrument Acquity UPC^2 (ultra-performance convergence chromatography) from Waters was used, which consists of a sample manager (sample temperature: 8°C), convergence manager, binary solvent manager (flow: 1 ml/min), isocratic solvent manager (10 mM ammonium formate in MeOH-H_2O (95 + 5); flow: 0.6 ml/min) and a column manager (column temperature: 40°C). A Waters Acquity QDa single quadrupole mass detector (mass range 150–700 Da, cone voltage positive scan 15 V, negative scan 30 V, capillary voltage positive 0.8 kV, negative 0.8 kV) was used. The mass chromatograms used for the integration of peak areas were deduced from the negative ionization mode. A Torus 2-Picolylamin (2-PIC) column (3.0 mm × 100.0 mm, 1.7 µm) served as stationary phase. The mobile phase consisted of supercritical CO_2 with ethanol as co-solvent. The separation was achieved by applying a gradient of the co-solvent starting from 0 to 3% ethanol within 8 min (hold for 2 min) and then to 5.5% within 5 min. A final washing step (50% ethanol for 1 min and 0% ethanol for 1 min) marked the end of the gradient.

2.4 Method Validation and Quantitation

In a first step, the main DRAs in the samples were identified by co-chromatography with commercially available references. Solutions of pimaric acid (**1**), palustric acid (**3**), isopimaric acid (**4**), levopimaric acid (**5**), abietic acid (**6**), dehydroabietic acid (**7**), and neoabietic acid (**8**) were prepared in a mixture of *n*-hexane and isopropanol (50 + 50) at a concentration of 0.75 mg/ml each. Sandarac resin was prepared the same way to identify sandaracopimaric acid (**2**). The method was validated in accordance with the ICH Q2 (R1) guideline ("Validation of analytical procedures") (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), 1995) using dehydroabietic acid (**7**) and neoabietic acid (**8**) as external standards and sample NSB22 as herbal material. The choice of **7** was made due to its aromatic structure with a molecular weight of 300 g/mol, **8** represented the abietic-type acid with a molecular weight of 302 g/mol. The linearity was determined by preparing solutions of the standards in *n*-hexane and isopropanol (50 + 50) at nine different concentrations (7.5, 2.5, 0.75, 0.25, 0.075, 0.025, 0.0075, 0.0025, and 0.00075 mg/ml, **Supplementary Tables S2, S3** and **Supplementary Figures S4, S5**). A sample of 1 µl was injected into the chromatographic system. The specificity was examined comparing the retention times and *m/z* values of the pure compounds in a concentration of 0.75 mg/ml to the respective substance in sample NSB22 (2 mg/ml, **Supplementary Table S4**). The accuracy of the method was determined by spiking sample NSB22 (2 mg/ml) with three different concentrations of **7** (0.183, 0.244 and 0.305 mg/ml, **Supplementary Table S5**) and **8** (0.0013, 0.0017, 0.0022 mg/ml, **Supplementary Table S6**). For the precision experiments three independent solutions of sample NSB22 at three different concentrations (0.625, 1.25, and 1.875 mg/ml) were prepared. Mean values, standard deviation and relative standard deviations of three independently prepared sample solutions were calculated for each of the eight diterpene acids to measure their reproducibility (**Supplementary Table S7**). The intermediate precision was assessed by injection of the highest concentration (1.875 mg/ml) in triplicates on three consecutive days (**Supplementary Tables S8, S9**). For LOD and LOQ the values of the noise were recorded as peak heights at ten different time points in an analysis of a solvent blank and calculated as a mean value (**Supplementary Tables S10–S13**).

The contents of all diterpene acids, except for dehydroabietic acid, in the samples were related to neoabietic acid and calculated according to following formula:

$$\%\ \text{analyte} = \frac{amount_{standard} \times AUC_{analyte} \times 100}{AUC_{standard} \times weight_{sample}}$$

3 RESULTS AND DISCUSSION

3.1 Sample Preparation

Since Norway spruce balm is a conifer exudate without cellular components, no conventional extraction method was necessary. The crude balm had only to be separated from foreign material (e.g., tree bark particles), which was accomplished by a dissolution and filtering step. Different solvents (heptane, hexane, ethyl acetate, methanol, ethanol, acetone, chloroform, dichloromethane) were tested, with acetone and dichloromethane found to be the most efficient solvents. Acetone was selected on the basis of low toxicity, ease of waste management and low adverse environmental impact.

3.2 Method Development

An enriched extract containing the DRAs **1**–**8** was prepared for the optimisation of the UHPSFC analysis. Method development was accomplished in three steps: 1) rapid column and co-solvent screening with a generic gradient, 2) defined gradient optimization with a specified stationary phase chemistry and co-solvent, and 3) detailed optimization regarding flow rate, column temperature, back pressure, and mass detector parameters including make-up solvents for ionization. As an initial step, eight columns with different stationary phase chemistries were tested for their separation capacity, by employing a generic gradient from 0 to 50% co-solvent (methanol). Out of the eight stationary phases tested (Waters Torus series: 1-Aminoanthracene (1-AA, 1.7 µm), 2-Picoylamine (2-PIC, 1.7 µm), Diethylamine (DEA, 1.7 µm), High-density Diol (DIOL, 1.7 µm); Waters Vidiris series: Bridged-Ethylene-Hybrid (BEH, 1.7 µm), Bridged-Ethylene-Hybrid 2-Ethylpyridine (BEH 2-EP, 1.7 µm), Charged-

TABLE 1 | Optimized UHPSFC method settings.

Parameter		Value	
Injection volume		1 µl	
Sample temperature		8°C	
Co-solvent A		CO_2	
Co-solvent B		Ethanol	
Gradient	Time (min)	%A	%B
	0	100.0	0.0
	8	97.0	3.0
	10	97.0	3.0
	15	94.5	5.5
Stationary phase		Torus 2-Picolylamin (3.0 mm × 100.0 mm, 1.7 µm)	
Column temperature		40°C	
Flow rate		1.00 ml/min (BSM) 0.60 ml/min (ISM)	
Detection		QDa (neg. mode)	
Make-up solvent		10 mM ammonium formate in MeOH-H_2O (95 + 5)	
Back-pressure		2000 psi	

Surface-Hybrid Fluoro-Phenyl (CSH FP, 1.7 µm), Silica 2-Ethylpyridine (Silica 2-EP, 5 µm); dimension for each column 3.0 × 100 mm), the 2-Picoylamine column showed the highest potential. In a subsequent step, four co-solvents (methanol, ethanol, isopropyl alcohol, acetonitrile) were screened with CO_2 on the 2-PIC column for the best suitability. Ethanol achieved a satisfactory separation and was used for further gradient optimization. Starting with a linear gradient from 0% to 50% ethanol, different variations were examined. The final gradient is depicted in **Table 1**. As a last step the effect of varying the flow rate, the temperature of the column, the backpressure and the use of different make-up solvents for ionization was analyzed. For an improved ionization for mass detection of the QDa detector, a make-up solvent is necessary. Of the three employed make-up solvents (95% MeOH + 5% H_2O with 10 mM ammonium formate; 95% MeOH + 5% H_2O with 10 mM ammonium acetate; 99% MeOH + 1% H_2O with 0.1% formic acid) the mixture with ammonium formate showed the best results. The variation of the other named parameters had no advantageous effect on the peak shape or resolution and were therefore kept to the values listed in **Table 1**.

Figure 2 illustrates the analysis of the balm sample NSB20 and the available reference substances (**1**–**8**) with the final chromatographic method. A UHPSFC-MS and GC-FID chromatogram of sandarac resin containing mainly compound

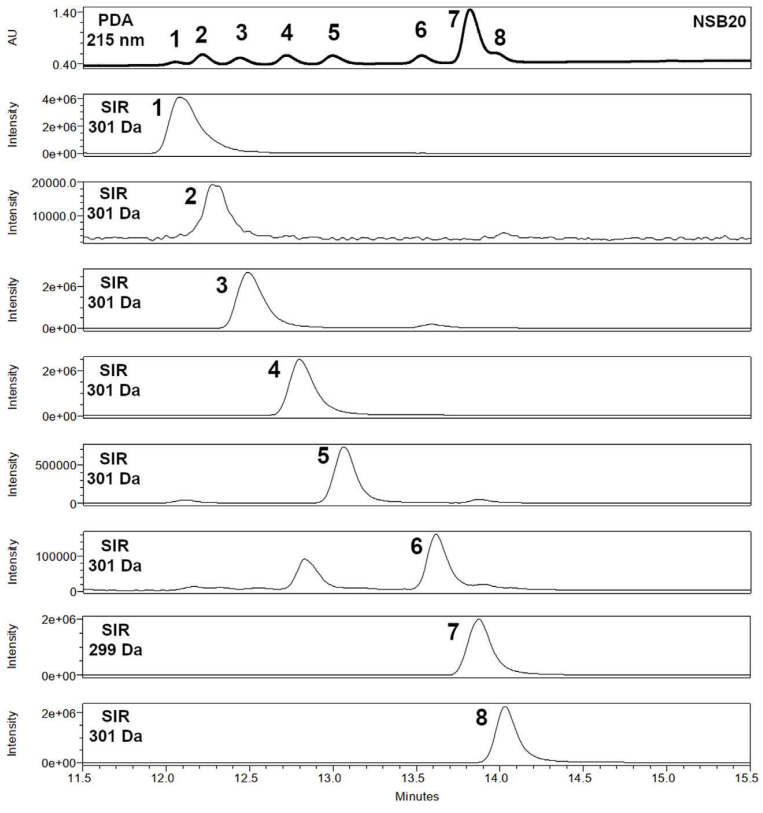

FIGURE 2 | Detailed section (RT 11.5–15.5 min) of the PDA chromatogram of spruce balm sample NSB20 (at 215 nm) and UHPSFC-ESI-MS Extracted Ion Chromatograms (negative mode, 299 and 301 Da) of diterpene resin acids **1**–**8** with optimized parameters on a 2-PIC column (3.0 mm × 100.0 mm, 1.7 µm).

TABLE 2 | Validation results.

	7	8
Regression equation	y = 1E+08x−63000	y = 1E+08x−70000
R^2	0.9992	0.9966
Linearity	0.00075 and 2.5 mg/ml	0.00075 and 2.5 mg/ml
LOD	0.00075 mg/ml	0.00100 mg/ml
LOQ	0.0025 mg/ml	0.0020 mg/ml
Accuracy		
Low spike amount	97.0% (±0.1% RSD)	104.1% (±4.5% RSD)
Medium spike amount	93.1% (±0.3% RSD)	105.6% (±8.0% RSD)
High spike amount	86.6% (±1.7% RSD)	107.8% (±4.9% RSD)
Precision		
Repeatability	0.625 mg/ml: 0.7 RSD	0.625 mg/ml: 6.4 RSD
	1.250 mg/ml: 0.9 RSD	1.250 mg/ml: 8.2 RSD
	1.875 mg/ml: 1.8 RSD	1.875 mg/ml: 3.8 RSD
Intermediate Precision	1.875 mg/ml: 10.5 RSD	1.875 mg/ml: 11.1 RSD
Specificity	−5.3 and +6.1 s	

2 in comparison to the balm sample NSB22 is available in the supporting material (**Supplementary Figures S1, S2**).

3.3 Method Validation

To ensure the suitability of the generated protocol for the quantitation of compounds **1**–**8** in Norway spruce samples using mass detection, a validation according to ICH guidelines (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), 1995) was performed. The raw data from the validation is listed in the supporting information under **Section 3**. Results shown in **Table 2** including evaluation of linearity, limit of detection (LOD), limit of quantitation (LOQ), as well as precision and accuracy are in accordance with the ICH recommendations.

3.4 Application to Balm Samples and Commercial Products

From the mass chromatograms recorded in the negative mode the ion chromatograms at m/z = 299 Da and m/z = 301 Da were extracted and used for integration of peak areas. The identification of the compounds was performed by comparing retention times and mass spectra with the references. Compounds **7** and **8** were directly quantitated, whereas compounds **1**–**6** were calculated as neoabietic acid (compound **8**).

Figure 3 and **Supplementary Table S14** show the quantitative composition of the investigated samples and confirm a substantial variation of the total DRA content in the raw balms ranging between 8 and 47%. Even samples harvested from one single individual on the same day from different injury sites (NSB01-08) show heterogeneity (15–31%). The main constituents in all samples are **5** and **7** (**Supplementary Table S14**), which is in accordance with the literature where samples of "*Picea abies* oleoresin" originating from Southern Finland had been analyzed by GC-FID (Holmbom et al., 2008). The two samples of the commercial product NSB-CP4 and NSB-CP5 are coherent. These samples were produced from Norway spruce balms originating from Lungau/Austria. Their composition approximatively matches the analyzed balms which had all been harvested in Austria. In contrast, the composition of the medical device NSB-CP1, NSB-CP2, and NSB-CP3 clearly differs in content

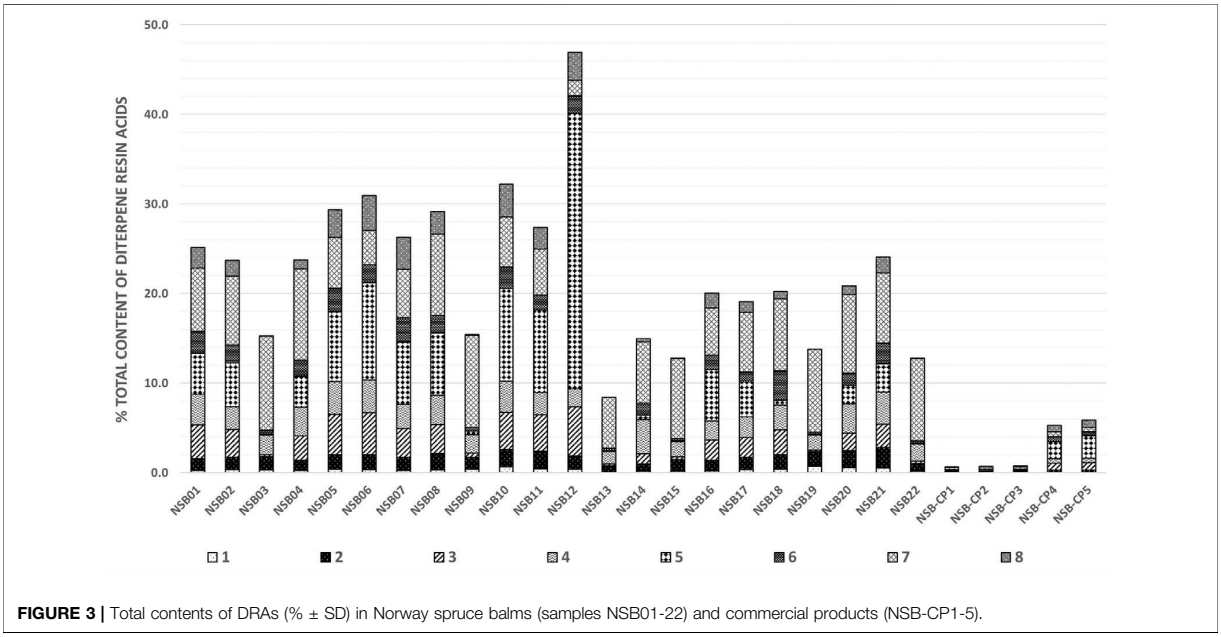

FIGURE 3 | Total contents of DRAs (% ± SD) in Norway spruce balms (samples NSB01-22) and commercial products (NSB-CP1-5).

and composition. The product is produced in Finland and claims to contain "resin" of Norway spruce. Use of the resin instead of the balm could be one explanation for these differences. Matrix effects could be another reason for unexplainable high or low values. The influence of co-eluting components, e.g., from the ointment base or other biological material on the ionization efficiency has not been investigated in this paper but needs to be evaluated in a next step.

4 CONCLUSION

UHPSFC was used to separate and quantitatively determine the main DRAs in *Picea abies*. To the best of our knowledge, this is the first time that UHPSFC has been used as a separation technique for spruce balms and commercial products containing such exudates. The method presented provides high selectivity and achieves separation of structurally similar DRAs where conventional RP-HPLC fails. Even though previously-employed GC analyses had shown better separation, they were disadvantageous with regard to sample preparation (e.g., derivatization) and duration of analysis (>60 min). With a run time of 17 min the optimised UHPSFC method is clearly superior to GC at acceptable separation performance. The minimized consumption of organic solvent and the resulting reduced ecological impact together with simple sample preparation, are significant advantages of this technology. Up until now there has been only an organoleptic examination and a TLC analysis required according to the monograph of the Austrian Pharmacopoeia (Österreichische Arzneibuchkommission, 2021). The method presented allows for the determination of DRAs in pharmaceutical matrices like Vaseline (NSB-CP1, NSB-CP2, and NSB-CP3) and lard (NSB-CP4 and NSB-CP5). Thus, it represents a basic tool for quality control providing the differentiation of balms from different species of the Pinaceae family (Goels et al., 2022). Moreover, it may serve as quantitation method, e.g., in *ex vivo* permeation experiments revealing information about the ability of DRAs to permeate skin into an acceptor medium. Such analyses require preceding spiking experiments to evaluate, whether matrix effects of compounds from biological material influence the detector response or whether such effects may be disregarded.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TG, EE, JL, GFA, GM, and LA performed the experiments. TG, EE, and JL analyzed the data. TG and EE prepared a draft of the manuscript. UG contributed to the preparation of the manuscript and gave advice regarding the set-up of the UHPSFC workflow. SG designed and supervised the study, while contributing to all stages of the experimental procedures and the preparation of the manuscript. All the authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.906411/full#supplementary-material

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Conflict of Interest: 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.

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4.1.3 Publication IV

Both previous publications shed light on the phytochemical composition of Norway spruce balm (see chapters 4.1.1, page 25, and 4.1.2, page 37). However, besides the resin and balm of *Picea abies*, those of other trees from the family Pinaceae, such as the balm of *Larix decidua* Mill. and the resin of *Pinus nigra* J.F.Arnold, are traditionally used in wound treatment as well.

We aimed to compare and differentiate the phytochemical composition of these various coniferous exudates. Therefore, different chromatographic methods were established and applied: TLC, HPLC coupled with a diode array detector (DAD), and the UHPSFC-ESIMS method published in the previous article (see chapter 4.1.2, page 37). Furthermore, the exudates were investigated *in vitro* for their potential to enhance re-epithelialization in an assay using HaCaT keratinocytes. The well-known wound-healing plants birch, pot marigold, Manuka honey, and St. John's wort served as herbal positive controls (Figure 4.5).

The original article '*Exudated of Picea abies, Pinus nigra, and Larix decidua: Chromatographic Comparison and Pro-Migratory Effects on Keratinocytes In Vitro*' was accepted in February 2022 and published in **Plants**.

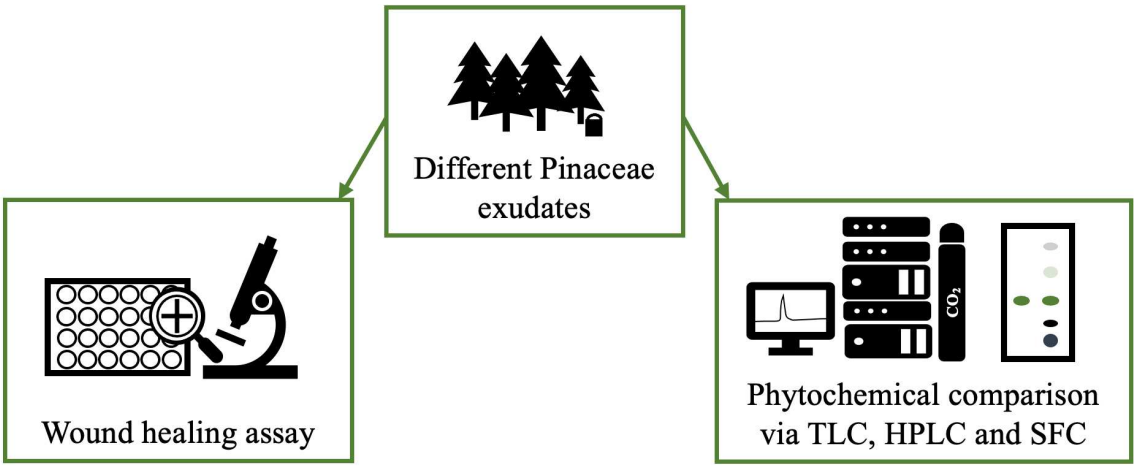

Figure 4.5 Workflow of the comparison of phytochemical composition and *in vitro* re-epithelialization-boosting potential of balms and resins of *Larix decidua*, *Pinus nigra*, and *Picea abies*.

Contributions

I contributed to establishing chromatographic methods, performed the phytochemical analyses, interpreted the resulting data, and drafted and critically reviewed the manuscript.

Key findings

- The established TLC method allowed a rapid fingerprint comparison of multiple exudates and pure substances. Only the fingerprints of the resin and balm of *Picea abies* appeared highly similar.
- An HPLC-DAD method was developed to analyze all substance classes present in the exudates, enabling a more detailed examination. More polar substances were detected in the Norway spruce exudates, whereas larch balm and black pine resin contained more non-polar peaks (Figure 4.6).
- The abovementioned UHPSFC-MS method was employed to analyze the seven structurally similar DRAs, which were entirely contained exclusively in *Picea abies* balm and *Pinus nigra* resin (Figure 4.6).
- The exudates of *Picea abies* and *Pinus nigra* showed low to moderate reproducible effects in the *in vitro* wound healing assay but without statistical significance. Only the balm of *Larix decidua* exhibited a statistically significant promotion of re-epithelialization at 3 µg/mL (Figure 4.6).
- The herbal positive controls *Betula pendula* Roth, *Calendula officinalis* L., and Manuka honey significantly accelerated the reduction of the cell-free gap. In contrast, *Hypericum perforatum* L. led to adverse effects at 10 µg/mL.

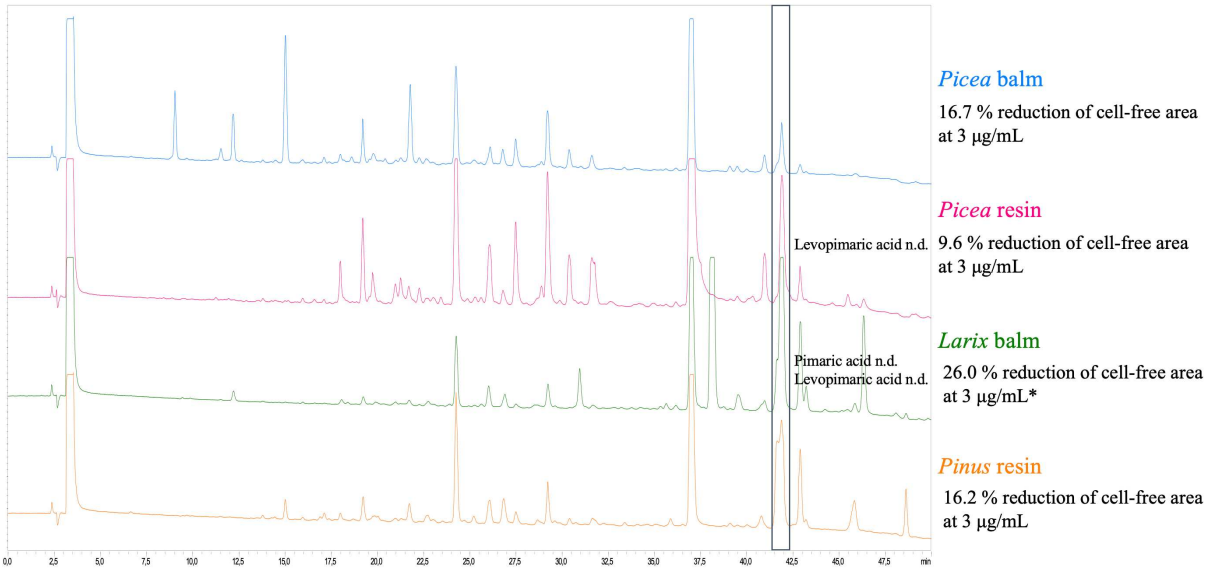

Figure 4.6 HPLC-DAD chromatograms of each Pinaceae exudate at 190 nm. The marked peak contains seven DRAs (pimaric acid, sandaracopimaric acid, palustric acid, isopimaric acid, levopimaric acid, abietic acid, and neoabietic acid), which had to be analyzed via supercritical fluid chromatography (SFC) (not detected (n.d.) DRAs are quoted). Moreover, the reduction of cell-free area (in %) for each extract at 3 µg/mL is listed (* $p < 0.05$).

Concluding perspectives

The only statistically significant reduction of cell-free area in the *in vitro* wound healing assay was achieved by larch balm at 3 µg/mL, even though higher concentrations led to cytotoxic effects. As larch balm revealed numerous differences in the composition compared to Norway spruce balm, the identity and quantity of substances in larch balm could be essential for this wound-healing activity. However, the established methods represent necessary tools for quality control of Pinaceae exudates, not only for crude starting materials but also for processed commercial products and the detection of possible adulteration with other exudates.

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Exudates of *Picea abies*, *Pinus nigra*, and *Larix decidua*: Chromatographic Comparison and Pro-Migratory Effects on Keratinocytes In Vitro

Thomas Goels, Elisabeth Eichenauer, Ammar Tahir, Paul Prochaska, Franziska Hoeller, Elke H. Heiß and Sabine Glasl *

Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna, Althanstraße 14, 1090 Vienna, Austria; thomas.goels@univie.ac.at (T.G.); elisabeth.eichenauer@univie.ac.at (E.E.); ammar.tahir@univie.ac.at (A.T.); a1009982@unet.univie.ac.at (P.P.); franziska.hoeller@univie.ac.at (F.H.); elke.heiss@univie.ac.at (E.H.H.)

* Correspondence: sabine.glasl@univie.ac.at; Tel.: +43-1-4277-55207

Abstract: Balms and resins of *Picea abies*, *Larix decidua*, and *Pinus nigra* are traditionally used to treat wounds. Three chromatographic techniques differing in separation capacity and technical demands were employed to distinguish among these plant exudates. A TLC method was established for fingerprint comparison, providing a quick overview of a large number of samples at low cost. HPLC-DAD (RP18) and UHPSFC-DAD (Torus 2-Picolylamin), hyphenated to ESI-MS, represented orthogonal chromatographic systems with high separation performance. The developed methods allow for the separation and detection of major and minor constituents belonging to different compound classes (phenyl carboxylic acids, lignans, diterpene resin acids). The qualitative compositions of the diterpene resin acids, the main compounds in the exudates, were comparable in all three genera. Differences were detected in the distribution of hydroxylated diterpene resin acids, pinoresinol, and hydroxycinnamic acids. The three tested chromatographic systems with varying demands on lab equipment offer appropriate tools for the quality assessment of *Picea abies*, *Larix decidua*, and *Pinus nigra*. The extracts were furthermore tested at three different concentrations (10 µg/mL, 3 µg/mL, and 1 µg/mL) for boosted re-epithelialization, a crucial step in the wound-healing process, in an in vitro HaCaT keratinocyte-based scratch assay. Lysophosphatidic acid (LPA, 10 µM) and extracts of several medicinal plants well known for their wound-healing properties (birch, marigold, St. John's wort, manuka honey) were used as positive controls. *Picea abies* and *Pinus nigra* showed concentration dependency; significant activity was measured for *Larix decidua* at 3 µg/mL.

Keywords: Pinaceae exudates; *Picea abies* balm; *Picea abies* resin; *Pinus nigra* resin; *Larix decidua* balm; fingerprint comparison; in vitro re-epithelialization

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1. Introduction

Skin diseases are globally increasing [1]. The Global Burden of Disease study, based on health data from over 195 countries, reported skin and subcutaneous diseases to have grown from 493 million patients in 2005 to 605 million in 2015 [2]. Impaired wound healing occurs as co-morbidity of obesity, diabetes, autoimmune disease, bedsore, etc., burdens health care systems worldwide with tremendous costs [3–6]. There is a critical need for advanced wound management and additional efficient and broadly accessible therapies. One viable approach would be to make use of traditional knowledge about "wound healing" and provide scientific evidence for the benefits of traditional remedies [7,8]. In this line, the excretion products of different members of the Pinaceae family have been used for centuries in traditional European medicine to treat wounds. This study focused on *Picea abies*, *Pinus nigra*, and *Larix decidua*, as they play an important role in Austrian folk medicine [9–11] with their balms (kneadable, sticky mixtures of resins and essential oil [12]) and brittle resins

(which do not contain essential oil [12]) processed to lipophilic ointments for acute, chronic, and infected wounds. Scientific research and literature comparing both the wound-healing properties and the phytochemical composition of the exudates of *Picea abies*, *Pinus nigra*, and *Larix decidua* are relatively scarce. Whereas there are studies about Norway spruce balm [13–15], data on European larch and black pine are still missing. Norway spruce balm consists of hydroxycinnamic acids such as ferulic acid, lignans such as pinoresinol, and hydroxylated and non-hydroxylated diterpene resin acids as main compounds [13] (see Figure 1). A monograph of this balm was included in the Austrian pharmacopoeia in 2016, followed by a monograph for a lard-based salve in 2019. The monograph envisions a simple TLC check for Norway spruce balm, but lacks sufficient analytical parameters for quality control. The first aim of this paper was to establish chromatographic methods at three different sophistication levels to facilitate analysis in simply-equipped as well as high-tech laboratories. An improved TLC method was developed including R_f-values and assignment of prominent bands for correct identification. HPLC-DAD and UHPSFC-DAD-MS methods enabled the separation of highly similar structures such as diterpene resin acids with only minor structural differences (Figure 1). Secondly, based on these techniques, the resins of *Picea abies* and *Pinus nigra* and the balms of *Picea abies* and *Larix decidua* were compared. Thirdly, referring to the potential wound-healing claim, the balms/resins were tested in an HaCaT keratinocyte based in vitro assay for enhanced re-epithelization. Lysophosphatidic acid (LPA, 10 µM) and extracts of several medicinal plants well known for their wound-healing properties (birch [16,17], marigold [18–20], St. John´s wort [21,22], manuka honey [23,24]) served as references in these experiments.

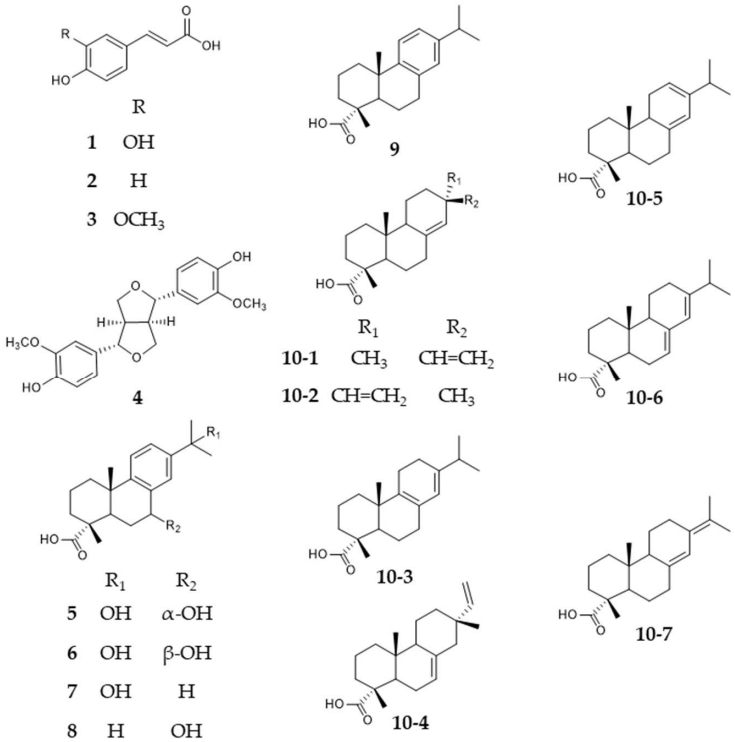


Figure 1. Hydroxycinnamic acids (**1**–**3**), lignans (**4**), and diterpene resin acids (**5**–**8**, **9** (300 Da), **10-1**–**10-7** (302 Da)) contained in Norway spruce balm. **1**—caffeic acid, **2**—p-coumaric acid, **3**—ferulic acid, **4**—pinoresinol, **5**—7α,15-dihydroxydehydroabietic acid, **6**—7β,15-dihydroxydehydroabietic acid, **7**—15-hydroxydehydroabietic acid, **8**—7-hydroxydehydroabietic acid, **9**—dehydroabietic acid, **10-1**—pimaric acid, **10-2**—sandaracopimaric acid, **10-3**—palustric acid, **10-4**—isopimaric acid, **10-5**—levopimaric acid, **10-6**—abietic acid, **10-7**—neoabietic acid.

2. Results

2.1. Chromatographic Comparison of Pinaceae Exudates

As already mentioned, the exudates of Pinaceae trees are complex mixtures of different substance classes. The first aim was to establish a simple and cheap TLC method, as well as high performance techniques achieving the separation of the structurally highly-related diterpene resin acids. The latter involves resin acids of the abietic- and pimaric-types sharing the same molecular weight of 302 Da and differ only in the position of one double bond and a side chain (compounds **10-1**–**10-7**, Figure 1). Hydroxylated resin acids (**5**–**8**) and dehydroabietic acid (**9**) are easier to separate due to their greater difference in structure and molecular weight [13]. The obtained fingerprints should facilitate comparison and differentiation of the exudates on a qualitative level.

2.1.1. Thin-Layer Chromatography (TLC)

TLC is a planar chromatography technique and provides a time- and cost-effective phytochemical fingerprint of multiple samples at the same time [25]. It should allow the comparison of the qualitative composition of the three exudates. We analyzed the extracts together with selected representatives of different substance classes present in resins and balms of Pinaceae as references i.e., ferulic acid (**3**, hydroxycinnamic acid), pinoresinol (**4**, lignan), dehydroabietic acid (**9**), and neoabietic acid (**10-7**, diterpene resin acids). Figure 2 shows the resulting TLC plate after derivatization with anisaldehyde/sulphuric acid solution.

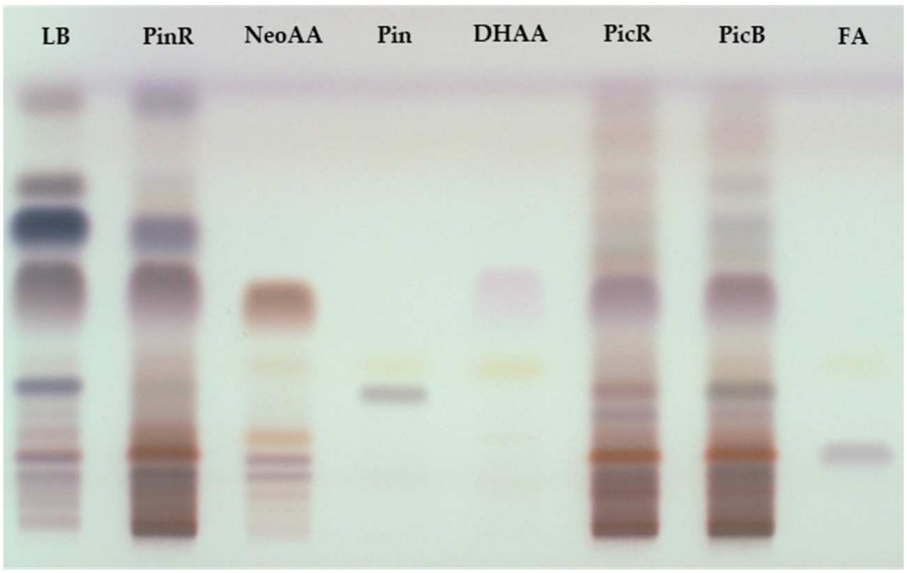
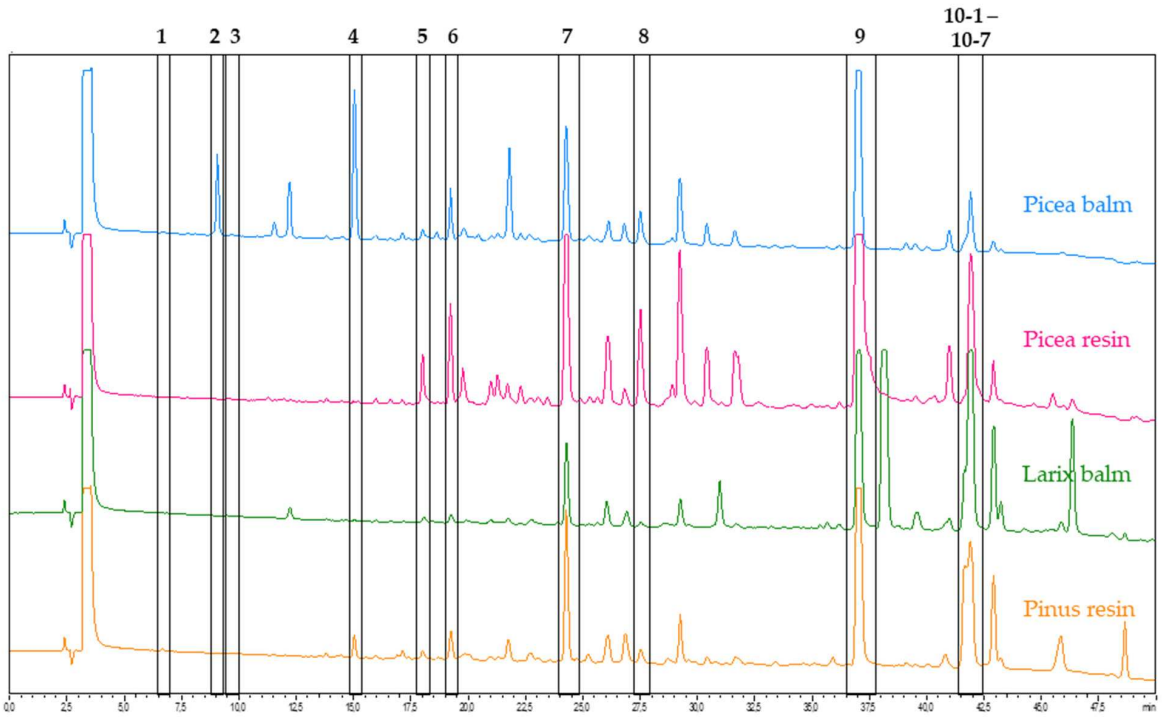


Figure 2. TLC comparison of Pinaceae exudates and reference substances after derivatization with anisaldehyde/sulphuric acid in daylight (stationary phase: silica 60 F_{254}; mobile phase: chloroform-methanol-trifluoroacetic acid (97 + 3 + 0.1)).

The balm and resin of *Picea abies* revealed comparable fingerprints on the TLC plate; the resin of *Pinus nigra* showed a prominent additional band at R_f 0.7. The most significant differences at R_f 0.7–0.8 and R_f 0.2–0.3 appeared in the balm of *Larix decidua*. No separation was achieved for the resin acids. Neoabietic acid **10-7** and dehydroabietic acid **9**, shown as representative standards in Figure 2, and all other resin acids (**10-1**–**10-6**, data not shown) appeared as one single large spot at R_f 0.5.

2.1.2. High-Performance Liquid Chromatography-Diode Array Detection/Mass Spectrometry (HPLC-DAD/MS)

Subsequent to planar normal phase TLC, a high-performance liquid chromatography (HPLC) method with a reversed phase RP18 column was developed. By means of co-chromatography with pure reference substances, it was possible to assign the most prominent peaks. In Figure 3, the HPLC-DAD chromatogram (detection at 190 nm) of each exudate is depicted, including the annotations of the used reference substances. Rough semi-quantitative estimations were deduced from the obtained peak sizes.



1	Caffeic acid	**10-1**	Pimaric acid
2	p-Coumaric acid	**10-2**	Sandaracopimaric acid
3	Ferulic acid	**10-3**	Palustric acid
4	Pinoresinol	**10-4**	Isopimaric acid
5	7α,15-Dihydroxydehydroabietic acid	**10-5**	Levopimaric acid
6	7β,15-Dihydroxydehydroabietic acid	**10-6**	Abietic acid
7	15-Hydroxydehydroabietic acid	**10-7**	Neoabietic acid
8	7-Hydroxydehydroabietic acid		
9	Dehydroabietic acid		

Figure 3. HPLC-DAD chromatograms of each Pinaceae exudate at 190 nm (stationary phase: RP18e; mobile phase: water + 0.1% formic acid, acetonitrile + 0.1% formic acid); the peaks were assigned by co-chromatography with the pure reference substances.

The hydroxycinnamic acids (**1**–**3**) caffeic acid, p-coumaric acid, and ferulic acid were minor constituents of the analyzed samples: only p-coumaric acid was detected and it appeared solely in the balm of *Picea abies*. The lignan pinoresinol (**4**) was present only in Norway spruce balm and, to a considerably lesser extent, in black pine resin. The four hydroxylated resin acids (**5**–**8**), 7α,15-dihydroxydehydroabietic acid, 7β,15-dihydroxydehydroabietic acid, 15-hydroxydehydroabietic acid, and 7-hydroxydehydroabietic acid, were detected in all exudate samples. The peak of any of the four hydroxylated resin acids was lowest in the balm of *Larix decidua*. Dehydroabietic acid (**9**) was present in all samples, and eluted approximately five minutes before all other diterpene resin acids with a molecular weight of 302 Da. The other acids (**10-1**–**10-7**) eluted together as one peak. Even the use of different gradients of the mobile phase could not achieve separation of the resin acids with a molecular weight of 302 Da by HPLC-DAD with RP18 as a stationary phase.

Making use of an alternative UHPLC instrumentation, hyphenated to ESI-TOF-MS, using a Kinetex RP18 column with 2.6 µm particle size and mixtures of acetonitrile and methanol as organic components of the mobile phase confirmed the HPLC-DAD results. Unknown compounds were characterized by m/z values and sum formulas (see Table S1). Experiments were conducted to differentiate the overlapping resin acids **10-1**–**10-7**: Different MS parameters were adjusted to potentially provoke differences in the fragmentation patterns, but none of the attempts led to any successful annotation of compounds **10-1**–**10-7**.

2.1.3. Ultra-High-Performance Supercritical Fluid Chromatography-Mass Spectrometry (UHPSFC-MS)

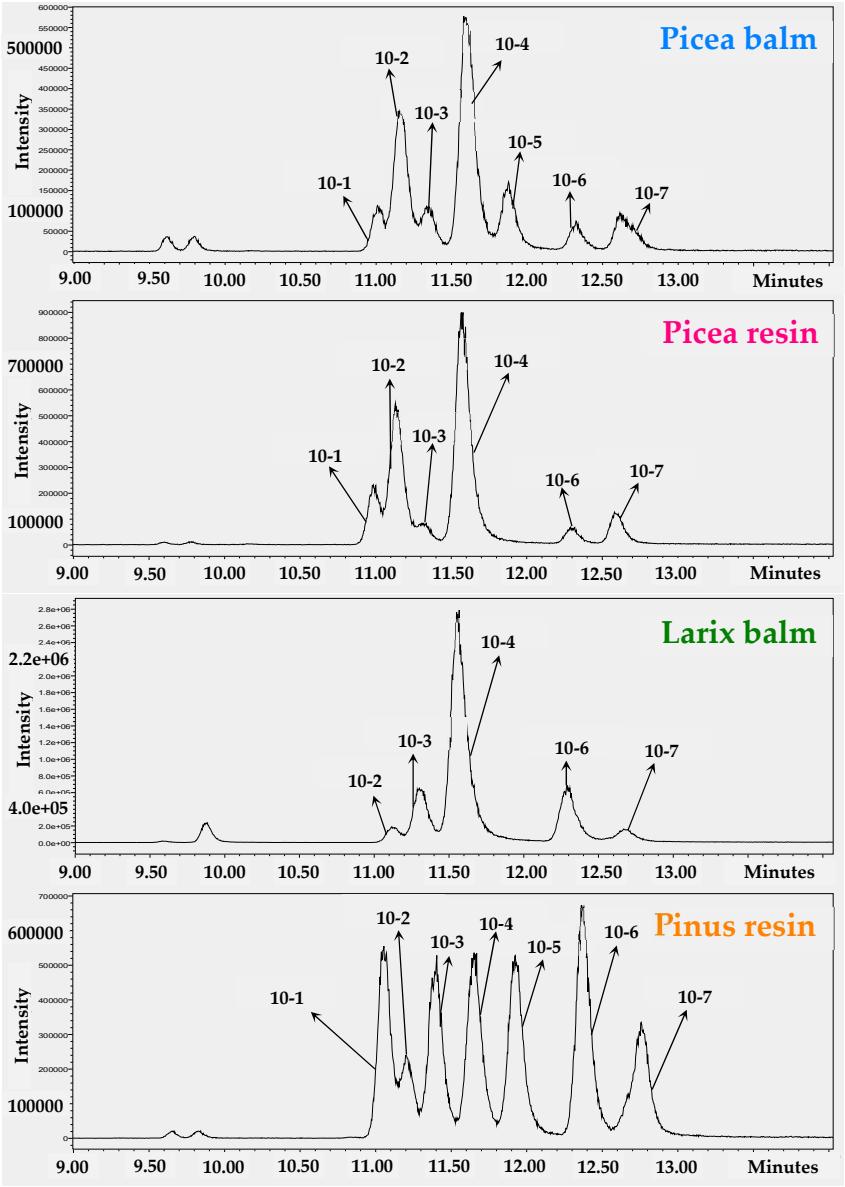
Supercritical CO_2 used as a mobile phase provides a highly lipophilic solvent and is widely used in the analysis of nonpolar plant constituents. By adding organic modifiers, the dissolving capacity and the elution of more polar substances can be adjusted, which makes ultra-high-performance supercritical fluid chromatography (UHPSFC) a potent technique in the analysis of plant extracts containing compounds with a wide range of polarities [26].

Using a Torus 2-Picolylamin separation column and ethanol as modifier, it was possible to separate the diterpene resin acids with a molecular weight of 302 Da. The mass spectra acquired in the negative mode were used and the ion chromatograms at 301 Da were extracted. Figure 4 shows the respective ion chromatograms at 301 Da of each exudate.

The four analyzed samples differed in the relative content of the various diterpene resin acids. Only the balm of *Picea abies* and the resin of *Pinus nigra* contained all of the seven resin acids, although in different quantities. Levopimaric acid (**10-5**) was not detected in Norway spruce resin and larch balm. Furthermore, pimaric acid (**10-1**) has not been detected in larch balm.

2.2. Adaption of the Bio-Assay

In the next step, the plant exudates were tested in vitro for enhanced re-epithelialization of a gap in a monolayer of human adult low-calcium high-temperature (HaCaT) keratinocytes, as published previously [13,27]. Positive controls included lysophosphatidic acid and extracts of birch, marigold, St. John´s wort, and manuka honey—all well known for their wound-healing properties. The handling of the assay demanded adaptions regarding the solubility of the extracts and the creation of the cell-free gap, in particular.



10-1 Pimaric acid
10-2 Sandaracopimaric acid
10-3 Palustric acid
10-4 Isopimaric acid
10-5 Levopimaric acid
10-6 Abietic acid
10-7 Neoabietic acid

Figure 4. Ion chromatograms at 301 Da in negative mode of the Pinaceae exudates gained by UHPSFC-MS (stationary phase: Torus 2-Picolylamin; mobile phase: CO_2, ethanol); the peaks were assigned by co-chromatography with the pure reference substances.

2.2.1. Lipophilic Extracts in Aqueous Cell Systems

The application of cell-based assays in aqueous medium as in vitro models for skin biology often causes solubility to be a bottleneck for tests of lipophilic extracts or compounds, and requires for compromises in the experimental setup. In order to maximize the solubility of the nonpolar conifer extracts, the solubilizers Tween 80 (0.05%–5%), isopropyl myristate (3 and 5%), Poloxamer 188 (0.01–10%), and soy lecithin (1–10%) were tested. They were dissolved in water at the given concentration ranges. The stock solutions of the extracts were prepared in DMSO (100 mg/mL) and diluted with the respective solubilizer-containing solutions. However, the substances were either unable to solubilize the nonpolar extracts (isopropyl myristate, Poloxamer, soy lecithin) or appeared to be toxic for the cells at the tested concentrations. Tween 80 caused cell lysis after 24 h of exposure. Consequently, the use of solubilizers was avoided to rule out any impact on the cells other than from the extracts. All extracts were dissolved in DMSO (stock solutions) and diluted with medium, with the exception of Manuka honey, which was directly dissolved in medium. Thus, the test concentrations needed to be selected so that they did not yield any obvious precipitates or lipophilic aggregates when added to the culture medium (1–10 µg/mL).

2.2.2. Generation of Cell Gap

The establishment of uniform cell gaps emerged as a challenge during the first experiments. The scratch assays followed the protocol published by Liang et al. [27], and scratches were initially introduced using a 200 µL pipette tip, which is frequently described in literature [28,29]. This procedure requires extreme caution, as one wants to disrupt only the cell layer by evenly sized scratches, but not to damage the coating of the cell culture vial (which would hinder the attached cells from migrating or proliferating). Various alternatives to the classical "pipette tip scratch" were sought that could guarantee reproducible sizes of the cell free area and intact surface coating at the same time. Inserts in the form of silicone cell spacers from Ibidi performed well, but were for single use only. Attempts for re-use failed, since the high temperature during sterilization caused increased adhesiveness of the silicone and again, destruction of coating during removal. Finally, the use of cross-shaped commercially available plastic inserts from LUX® tools was successful. They were re-usable and weighted down with small metal weights to allow formation cell-free gaps in a growing cell monolayer, with acceptable variation in size with concurrent intact coating.

2.3. Results of Tested Extracts in the Bio-Assay

All plant extracts exhibited a tendency to enhance gap closure in the keratinocyte monolayer compared to solvent control cells, which can most likely be attributed to increased cell proliferation or migration.

2.3.1. *Larix decidua*, *Picea abies*, *Pinus nigra*

The extracts of *Picea abies* and *Pinus nigra* elicited a moderate and concentration-dependent decrease in cell-free area, but statistical significance (see Table 1). The balm of *Larix decidua* boosted re-epithelialization at 3 µg/mL (+26.0%, statistically significant, see Figure 5) but resulted in negative values at 10 µg/ missed mL (see Table 1). This points to cytotoxic/cytostatic effects at the higher concentration, which could be confirmed in the resazurin conversion and crystal violet assay (data not shown).

Table 1. Reduction of cell-free area (%) provoked by the plant extracts of *Larix decidua*, *Picea abies*, and *Pinus nigra* in the "wound healing assay" on HaCaT keratinocyte cells; given percentages are mean values (n = 3); * statistically significant ($p < 0.05$).

Plant (Extraction Solvent)	Concentration [µg/mL]	% Reduction of Cell-Free Area	±SEM	*p* Value
Larix decidua balm (acetone)	1	4.2	2.7	0.6472
	3	26.0 *	2.2	0.0294
	10	−4.1	14.1	0.8352
Picea abies balm (acetone)	1	−5.6	3.4	0.6556
	3	16.7	6.9	0.2503
	10	−2.0	6.4	0.9142
Picea abies resin (acetone)	1	5.9	14.2	0.7610
	3	9.6	0.9	0.5265
	10	47.6	17.2	0.0815
Pinus nigra resin (acetone)	1	10.9	8.7	0.5202
	3	16.2	6.7	0.332
	10	38.7	17.4	0.137

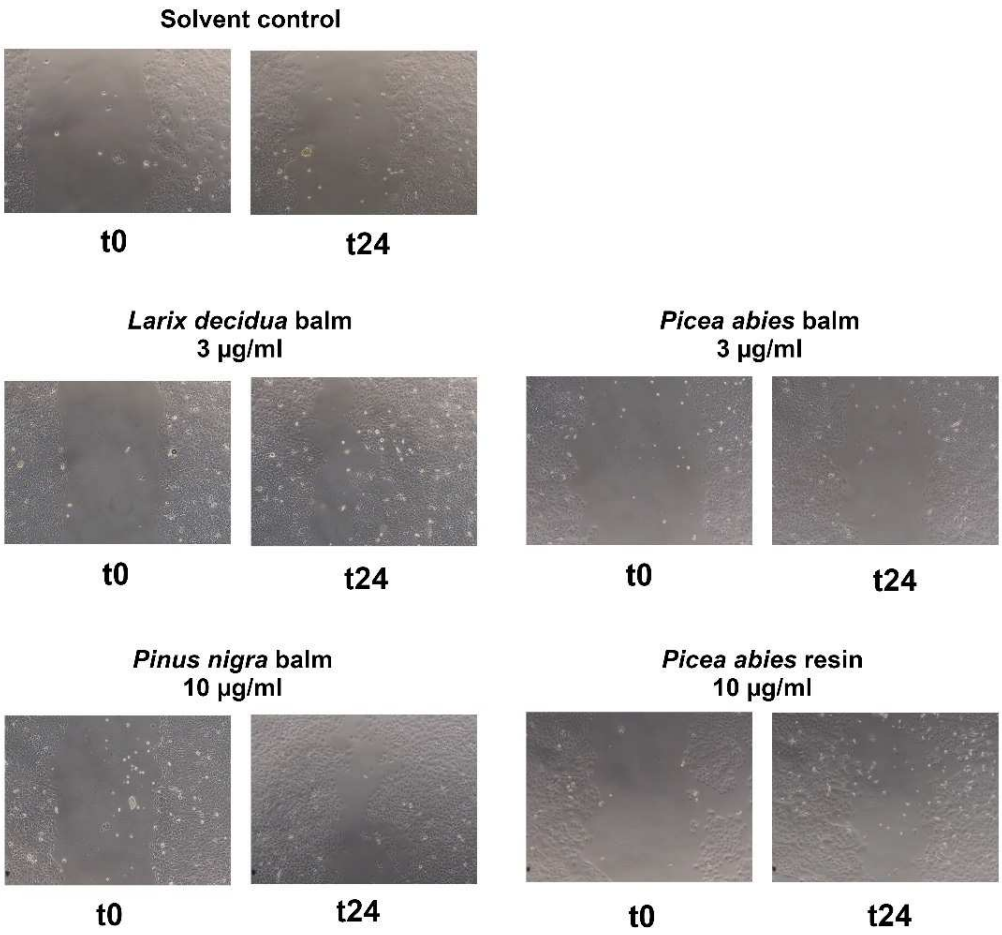


Figure 5. Effects of conifer resins and balms at different concentrations on scratch wound healing of HaCaT cells at timepoint t0 and after 24 h (t24).

2.3.2. Herbal Positive Controls

In order to better rank the activity of the tested exudates, we also ran the assay with other traditionally used wound-healing agents. *Betula pendula* (10 µg/mL: +41.6%), *Calendula officinalis* (1 µg/mL: +62.1%), and *Leptospermum scoparium* honey (Manuka honey) (10 µg/mL: +42.0%) demonstrated statistically significant differences to the vehicle control (see Figure 6). The extract of *Hypericum perforatum* showed moderate activity on keratinocytes (see Table 2). Concentration-dependency was shown for birch and Manuka honey.

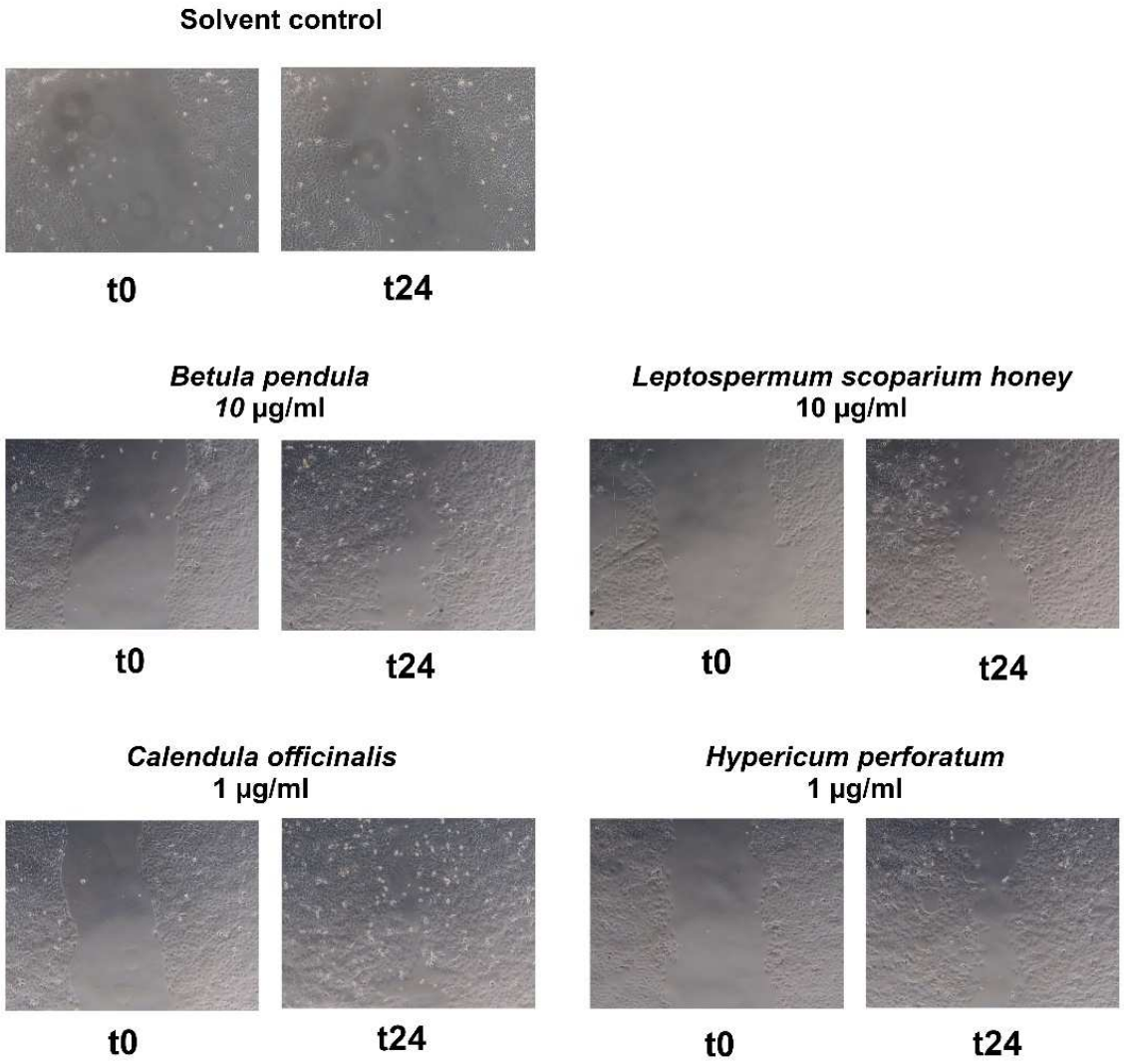


Figure 6. Effects of herbal positive controls at different concentrations on scratch wound healing of HaCaT cells at timepoint t0 and after 24 h (t24).

The extract of St. John's wort did not show significant activity on keratinocytes, even though this plant is known as one of the most potent medicinal plants to cure burns. It even revealed negative values at 10 µg/mL (see Table 2). This might be due to the different spectrum of constituents in the tested aqueous alcoholic extract (70%) compared to the traditionally prepared oily extract, or due to targets in the wound-healing process other than re-epithelialization. Marigold showed good activity, but exhibited an unexpected

concentration-dependent decrease in activity. Assessment of HaCaT metabolic activity and cell mass after treatment with the extracts indicated an inhibitory impact of the *Calendula officinalis* tincture on cell viability (data not shown). This may provide a possible explanation for the low performance of the plant extract, especially at higher concentrations.

Table 2. Reduction of cell-free area (%) provoked by the herbal positive controls in the "wound healing assay" on HaCaT keratinocyte cells; given percentages are mean values (n = 3); * statistically significant ($p < 0.05$); ** statistically significant ($p < 0.01$).

Plant (Extraction Solvent)	Concentration [µg/mL]	% Reduction of Cell-Free Area	±SEM	*p* Value
Betula pendula (96% EtOH)	1	18.3	12.9	0.2757
	3	20.0	16.7	0.3037
	10	41.6 *	11.3	0.0313
Calendula officinalis (70% EtOH)	1	62.1 *	16.0	0.0121
	3	54.9	31.7	0.1073
	10	35.0	18.9	0.1125
Hypericum perforatum (70% EtOH)	1	33.1	22.9	0.2072
	3	27.3	9.5	0.1244
	10	−12.4	20.2	0.6249
Honey from *Leptospermum scoparium* (H_2O)	1	4.9	8.9	0.7724
	3	23.1	10.1	0.2181
	10	42.0 **	12.5	0.0087

3. Discussion

The balms of *Picea abies* and *Larix decidua* and the resins of *Picea abies* and *Pinus nigra* were investigated side by side for their phytochemical composition by three different developed analytical methods and for their ability to enhance re-epithelialization in vitro. This study complements and significantly contributes to the currently existing picture in this area of research, as most studies on the phytochemical composition and wound-healing properties of conifer balms have been conducted with *Picea abies* [13–15]. The essential oil of cones and needles of various *Pinus* species [30,31] and extracts from the bark of *Pinus brutia* [32,33] also showed promising in vivo activity with regard to wound healing. However, the excretions of *Pinus nigra* and *Larix decidua* have been barely scientifically studied regarding their wound-healing effect. Moreover, the bioactive constituents have rarely been identified and the (relative) composition of the bark extracts remained largely unknown, which leaves a knowledge gap in research on the wound-healing properties and constituents of different *Pinus* species. In addition, GC [34,35] and HPLC [36,37] are well-established, whereas SFC protocols for these exudates have not been published so far.

The balms and resins were analyzed via three chromatographic techniques and compared semi-quantitatively regarding their composition. Detailed descriptions of patterns and differences are discussed in Sections 2.1.1–2.1.3. The value of TLC is represented by the comparison of chemical fingerprints and the throughput of a high number of samples within a short time. The TLC pattern of *Larix* clearly differed from the other exudates by three additional bands with different colors. *Pinus* and *Picea* resembled each other, but could also be distinguished by one additional prominent band occurring only in *Pinus*. The two high-performance techniques outperformed TLC regarding separation power and provided additional structural information about single compounds. The chromatograms obtained by HPLC-DAD covered various substance classes and revealed more or less minor differences between the exudates with the exception of *Larix*. The separation of the highly similar resin acids required employment of UHPSFC-MS. The relative amounts of the resin acids varied, and again showed *Larix* as easy to distinguish from *Pinus* and *Picea*. In parallel, an in vitro assay was performed to evaluate the proliferation- and migration-enhancing potential of the exudates in keratinocytes. Tested extracts showed moderate activity, and balm and resin extracts performed comparably. The only significant activity

was seen with *Larix decidua* at a concentration of 3 µg/mL, which boosted gap closure by 26%, which was more potent than birch extract at the same concentration. Other conifer extracts showed partly good and concentration-dependent mean values, but also high standard deviations. The latter may still be caused by solubility issues (despite the lack of obvious precipitates). Moreover, wound healing is a complex process that involves various cell types and signaling pathways, and is usually divided in four phases. Blood clotting (Stage 1: hemostasis) occurs momentarily after an injury. Immune cells then infiltrate the wound site and cause a local inflammation (Stage 2: inflammation). In the proliferation phase (Stage 3: proliferation), keratinocytes and fibroblasts fill the wound crater with cells by migrating to the injured site and engaging in increased cell division. In this phase, new blood vessels and extracellular matrices also are formed. Maturation marks the final step of wound healing (Stage 4: remodeling), during which collagen is remodeled and scars are formed [38]. Therefore, although keratinocytes play an important role in Stages 3 and 4, and their proliferation and migration are popular readouts for assessing wound-healing properties, this assay can only capture a limited set of affected targets and may miss many others. The extracts should therefore be subjected to complementary in vitro or even in vivo models in the future.

Concerning the link between constituents and observed bioactivity, the content of hydroxylated and non-hydroxylated resin acids seems to correlate with enhanced re-epithelialization. In line with this assumption, pimaric acid (**10-1**), isopimaric acid (**10-4**), dehydroabietic acid (**9**), and combinations of isolated hydroxylated diterpene resin acids (**7** and **8**) have already displayed a significant effect in the employed bio-assay [13]. Moreover, relevant signaling pathways for wound healing are affected by abietic and dehydroabietic acid, including the TNF-α/forkhead box O1 (FOXO1), transforming growth factor-β1 (TGF-β1)/Smads, or MAPK (ERK and p38) pathways [39,40]. High concentrations of isopimaric acid may, however, counteract re-epithelialization (as seen with 10 µg/mL of the *Larix* extract or in [13]). As the extract of *Larix* was the only one to show significant bioactivity in this study, its composition might be of particular interest. As seen in Figure 4, it is characterized by a unique pattern of diterpene resin acids, in which isopimaric acid is predominant over the other acids, which are either present in minor amounts or completely missing. Moreover, in the HPLC-DAD and TLC analyses, *Larix* showed additional, unidentified peaks (e.g., peak at 38 min: m/z 312 ($C_{21}H_{28}O_2$), and 46.0 min: m/z 388 ($C_{24}H_{36}O_4$); see Table S1) and exclusive regions at Rf 0.2–0.3 and 0.7–0.8, respectively, that could be of importance for bioactivity. Future research must concentrate on unambiguous and detailed dissection of active principles and underlying mode of action.

4. Materials and Methods

4.1. Plant Material

The spruce balm, which has a kneadable texture and a whitish to brown color, was collected from Norway spruce (*Picea abies* (L.) H. Karst., Pinaceae, engl. Norway spruce, ger. Gewöhnlich-Fichte) in a forest near Sauerfeld, Lungau, Austria (47°07′15.0″ N, 13°52′50.0″ E). It was identified by Prof. Johannes Saukel, Division of Pharmacognosy, University of Vienna (BN. LU01). Spruce resin, which in contrast to the balm, has a typically amber color and a very sticky consistency, was obtained by removing it from spruce wood boards originating from trees grown in eastern Austria. Larch resin (*Larix decidua* Mill., Pinaceae, engl. European Larch, ger. Europa-Lärche) from Carinthia was purchased as a commercial product (BN. 124101, Schusser OG). Black pine resin (*Pinus nigra* J. F. Arnold, Pinaceae, engl. European black pine, ger. (Österreichische) Schwarzföhre) was collected in 2016 at Parapluieberg in Perchtoldsdorf, Lower Austria (48°07′02.6″ N, 16°14′00.6″ E) and identified by Prof. Johannes Saukel, Division of Pharmacognosy, University of Vienna (BN. PinNig01).

Birch bark (*Betula pendula* Roth, Betulaceae, engl. Silver Birch, ger. Hänge-Birke) was supplied by Kottas Pharma GmbH (BN. KLA30556). St. John's wort (*Hypericum perforatum* L., Hypericaceae, engl. perforate St. John's-wort, ger. Echt-Johanniskraut) and

marigold (*Calendula officinalis* L., Asteraceae, engl. pot marigold, ger. Garten-Ringelblume) were purchased as tinctures in a local pharmacy (Tinctura Hyperici (70% ethanol) 1:5, BN. 20,171,400 FA003026, Gatt-Koller; Tinctura Calendulae (70% ethanol) 1:5, BN. 15063711, Caelo). MediHoney (BN. 1734, Dermasciences) containing pure manuka honey (honey of *Leptospermum scoparium* J. R. Forst. & G. Forst., Myrtaceae, engl. Manuka, ger. Südseemyrte) without any pharmaceutical additives was bought in a local pharmacy.

Scientific plant names were evaluated by using Medicinal Plant Names Services [41]. German names correspond to the excursion flora of Austria, Liechtenstein, and Switzerland [42], and English names were acquired from Wikispecies [43].

4.2. Preparation of Extracts

4.2.1. *Picea abies*, *Larix decidua*, and *Pinus nigra*

As indicated in the monograph of the Austrian Pharmacopeia [44] for Norway spruce balm, the exudates of all three conifers were dissolved in acetone. Solid particles were filtered and the solvent was evaporated to give the corresponding extracts for the stock solutions in DMSO.

4.2.2. *Betula pendula*

A betulin-enriched birch bark extract was prepared according to Kuznetsova et al. In brief, 10.0 g of birch bark were suspended in 300 mL ethanol 96% in a round bottom flask. Twenty percent aqueous KOH solution (14 g in 70 mL) was added and the whole solution was heated to 80 °C in a water bath for 8 h. The hot mixture was filtered and left for crystallization overnight in the fridge. 544.6 mg of betulin-enriched extract were gained [45] and used for the preparation of the stock solutions in DMSO.

4.2.3. Hypericum Perforatum and Calendula Officinalis

The solvent of the respective tinctures was evaporated, and the residues were used for the preparation of stock solutions in DMSO.

4.2.4. Manuka Honey

The honey of *Leptospermum scoparium* was weighed directly in an Eppendorf tube and dissolved with medium, since it was poorly soluble in DMSO. This solution was freshly prepared for every experiment.

4.3. Chromatographic Methods

The chemical fingerprints and the resin acid compositions of *Picea abies*, *Pinus nigra*, and *Larix decidua* were investigated via TLC, HPLC-DAD/MS, and UHPSFC-MS.

4.3.1. Thin-Layer Chromatography (TLC)

For analytical TLC experiments, Silica 60 F_{254} TLC plates (average particle size 9.5 to 11.5 µm, aluminum sheets with fluorescence indicator, Merck KGaA, Darmstadt, Germany) were used. The samples of Pinaceae exudates were dissolved in acetone to a concentration of 10 mg/mL. As reference substances for the substance class of diterpene resin acids, neoabietic acid and dehydroabietic acid were used (both dissolved in acetone, neoabietic acid to a concentration of 0.5 mg/mL, dehydroabietic acid to 1 mg/mL). Pinoresinol (as representative of lignans) was dissolved in methanol to a concentration of 1 mg/mL; ferulic acid (as representative of hydroxycinnamic acids) was also dissolved in methanol to a final concentration of 2.5 mg/mL. Five microliters of each sample solution were applied by a CAMAG Automatic TLC Sampler 4 (ATS4). Development was done in the CAMAG Automatic Developing Chamber (ADC2) with chloroform-methanol-trifluoroacetic acid (97 + 3 + 0.1) as mobile phase. The CAMAG TLC Visualizer was used to interpret and photograph the developed and dried plates under white light and at wavelengths 254 nm and 366 nm. The constituents showed improved visibility after derivatization with

anisaldehyde/sulphuric acid solution, which was applied with the help of the CAMAG Chromatogram Immersion Device (III) (CAMAG, Muttenz, Switzerland).

4.3.2. High-Performance Liquid Chromatography-Diode Array Detection/Mass Spectrometry (HPLC-DAD/MS)

For HPLC analysis, two different instrumentations were used. For detection via diode array detector, Shimadzu instrumentation (Shimadzu Degasser DGU-20A 5, Shimadzu Auto Sampler SIL-20AC HAT, Shimadzu Communications Bus Module CBM-20A, Shimadzu Liquid Chromatograph LC-20AD, Shimadzu Column Oven CTO-20AC, Shimadzu Diode Array Detector SPD-M20A, Shimadzu Lab Solutions Software, Shimadzu, Kyoto, Japan) was employed. An RP18 column (LiCrospher 100 RP18e, 5 µm, 250 mm × 4 mm, Merck KGaA, Darmstadt, Germany) was used as stationary phase. Two solvents served as the mobile phase: double distilled water + 0.1% formic acid (solvent A) and acetonitrile + 0.1% formic acid (solvent B). Elution of the analytes was done with a gradient elution, starting with 15% solvent B rising to 95% solvent B within 45 min (rate 1.77%/min) at a flow of 1 mL/min.

For detection via mass spectrometer, an ultra-high-performance liquid chromatography instrumentation (EXIONLC AD SYSTEM (AB Sciex, Darmstadt, Germany)) was used. It was equipped with a reversed-phase RP18 column (Kinetex; 2.1 mm × 15 cm, 2.6 µm, RP18 100 Å, Phenomenex, Aschaffenburg, Germany). Mobile phase A (H_2O/FA, 100:0.02) and mobile phase B (ACN/MeOH/FA, 80:20:0.02) were degassed prior to their usage. A 45 min binary gradient with flow rate set to 350 µL/min was applied as follows: 0−2 min, 5% mobile phase B; 2−30 min, 5−85% mobile phase B; 30–40 min, 95% mobile phase B; 40–45 min re-equilibration with 5% mobile phase B). Five microliters of each sample (1 mg/mL) were injected followed by a blank injection to ensure proper column washing and equilibration. Mass spectrometric detection was performed using the turbo ion source ESI X500 QTOF mass spectrometer (AB Sciex, Darmstadt, Germany), 500 °C heater temperature, ion source gas1 set to 30 psi, ion source gas2 set to 30 psi, curtain gas set to 45 psi. −4.5 KV/+5.0 KV spray voltages were applied to achieve negative/positive ion mode ionization. TOFMS scans were performed with an m/z range from 50 to 1500. TOF MS/MS scans of the three most abundant ions were fragmented at 35 V CES (collision energy spread), 0.1 accumulation time, and −80 V declustering potential.

4.3.3. Ultra-High-Performance Supercritical Fluid Chromatography-Mass Spectrometry (UHPSFC-MS)

For the analysis of the diterpene resin acid composition, high-performance supercritical fluid chromatography coupled with mass spectrometry was utilized. An Acquity ultra-performance convergence chromatography system (UPC^2) from Waters was used as instrumentation, consisting of a sample manager (sample temperature: 8 °C), convergence manager, binary solvent manager (flow: 1 mL/min), isocratic solvent manager (10 mM ammonium formate in MeOH-H_2O (95 + 5); flow: 0.6 mL/min), and a column manager (column temperature: 40 °C). For detection, a Waters Acquity QDa single quadrupole mass detector (Waters Corporation, Milford, United States) was used with a mass range of 150–700 Da (capillary voltage positive 0.8 kV, negative 0.8 kV, cone voltage positive scan 15 V, negative scan 30 V). The mass spectra used for identification of the peaks were derived from the negative mode. As stationary phase, a Torus 2-Picolylamin (2-PIC) column (3.0 mm × 100.0 mm, 1.7 µm) was used. Supercritical CO_2 and ethanol as co-solvents served as the mobile phase. A gradient system of the co-solvent starting from 0% to 3% ethanol within 8 min (hold for 2 min) and then to 5.5% within 5 min was applied. The end of the gradient was indicated by a final washing step (50% ethanol for 1 min and 0% ethanol for 1 min).

4.4. Bio-Assay

For the stock solutions, each extract was re-dissolved in DMSO to a concentration of 1 mg/mL. Further dilutions were prepared using DMSO except for Manuka honey,

which was diluted with medium instead of DMSO. The resulting sample solutions (0.3 mg/mL and 0.1 mg/mL) were further diluted 1:100 with medium to the respective final concentrations of 10 µg/mL, 3 µg/mL, and 1 µg/mL.

HaCaT cells (CLS; Eppelheim Deutschland) were maintained in DMEM medium (high glucose, phenol-red free; Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (Gibco, Dreieich, Germany), 2 mM glutamine (Lonza), 100 U/mL benzylpenicillin (Lonza), 100 µg/mL streptomycin (Lonza) at 37 °C and 5% CO_2 in a humidified atmosphere. DMSO (BioUltra for molecular biology) was supplied by Sigma Aldrich. LPA was purchased from Santa Cruz Biotechnology, Heidelberg, Germany.

The resazurin assay and a subsequent crystal violet assay were performed prior to the "wound healing assay" to assess metabolic activity and cell mass, respectively, which allowed deductions of cytotoxicity and tolerable concentrations of tested extracts [46,47].

Re-epithelialization was examined in an in vitro assay on HaCaT keratinocytes. This so-called "wound healing assay" was adapted from Liang et al. [27] (see Section 2.2.2 Induction of cell gap). In brief, cell spacers (length: 13.10 mm; width: 0.88 mm) made of plastic available in any hardware store (LUX® tools, Wermelskirchen, Germany) were inserted into the wells of a 24-well plate. A suspension of HaCaT keratinocytes (1 mL of a 1.5 × 105 cells/mL solution) was pipetted into each well and the plate was incubated overnight (37 °C, 5% CO_2). The cell spacers were removed (=t0), media was aspirated off, and debris and detached cells were removed by washing with PBS. Solutions of the plant extracts, dissolved in DMSO (BioUltra for molecular biology, Sigma Aldrich) and diluted with medium containing 0.1% fetal bovine serum to a final concentration of 1% DMSO, were applied to the wells. Figure 7 gives an overview of the workflow. As a positive control, lysophosphatidic acid (LPA, Santa Cruz Biotechnology, Heidelberg, Germany) was applied; 1% DMSO in medium containing 0.1% fetal bovine serum served as a vehicle control. Photos of the cell free gaps at the starting time (t0) and after 24 h (t24) were taken (Olympus camera PEN Lite E-PL7, Hamburg, Germany). To evaluate the cell free area of the respective gaps, Fiji, which is an image processing package containing a distribution of ImageJ combined with various Plugins [48], was utilized. For an automated analysis of each gap, the ImageJ macro "Wound Healing Tool" [49] was added to the software (variance filter radius: 24, threshold: 2–4; radius open: 4).

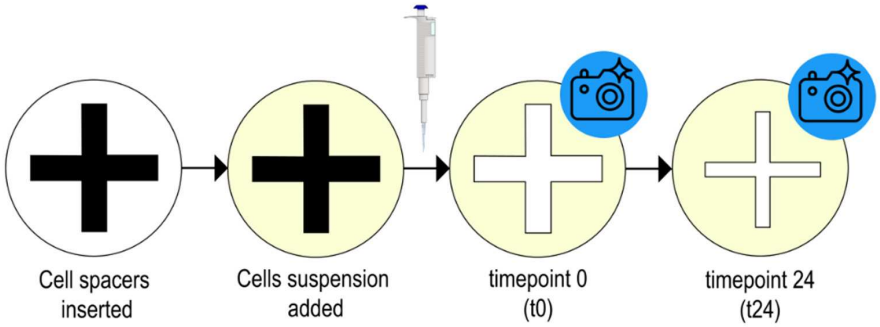


Figure 7. Workflow of the scratch assay on HaCaT keratinocytes.

Two wells were tested per sample and from each well four images were taken so that, finally, a total of eight cell gaps per sample were recorded. The cell-free area at time point t24 was subtracted from the cell-free area at time point t0, which gives a measure for the obtained wound closure within 24 h. Since the camera takes pictures in a landscape format (image resolution: 4608 × 3456-pixel, aspect ratio: 1.33), the recorded four areas are inequal. For this reason, the calculations of the values resulting from the horizontal and vertical pictures were performed separately according to the formula given below: The area difference of the "vertical sample" was divided by the mean area difference of all "vertical vehicle control (VC) values". The "horizontal values" were processed the same

way. Subtraction of the value "1" sets the vehicle control to the value "0"; multiplication with "100" indicates the reduction of the cell-free area in percentage normalized to the vehicle control. The final mean value of the respective sample was calculated from these normalized values [13].

$$\left(\frac{\text{Area t0}_{\text{sample}} - \text{area t24}_{\text{sample}}}{\text{area t0}_{\text{VC}} - \text{area t24}_{\text{VC}}} - 1\right) \times 100 \quad (1)$$

Statistics

The experiments were conducted at least in three independent biological replicates with eight technical replicates. Calculations were performed with Excel and for the statistical analysis, GraphPad Prism 6.01 (GraphPad Software, Inc., San Diego, United States) was used. Student's *t* test was performed to compare the two groups; significance was defined as $p < 0.05$.

5. Conclusions

With this study, we provide three methods (TLC, HPLC-DAD/MS and UHPSFC-DAD/MS) for the differential analysis of exudates of three species from the Pinaceae family (*Larix*, *Pinus*, and *Picea*), and thus an important tool for the quality control of starting materials on the one hand, and for processed medicinal products on the other hand. A validated method for the quantitation of diterpene resin acids via UHPSFC-MS is under way.

All investigated balm and resin extracts could be shown to reproducibly enhance re-epithelialization in vitro, comparably to the reference extracts (birch, medicinal honey). The effect of the Pinaceae exudates may be in part assigned to diterpene resin acids. The role of hydroxylated resin acids (e.g., 15-hydroxydehydroabietic acid and 7-hydroxydehydroabietic acid) and yet unknown compounds of the conifer exudates, together with the extensive pharmacological characterization of active principles, are a promising starting point for future research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11050599/s1, Table S1: Summary of the qualitative chromatographic comparison.

Author Contributions: Conceptualization, S.G. and T.G.; methodology, T.G. and E.E.; data curation, T.G., E.E., A.T., P.P. and F.H.; resources, S.G. and E.H.H.; writing—original draft preparation, T.G. and E.E.; writing—review and editing, S.G. and E.H.H.; supervision, S.G. and E.H.H. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All figures and tables in this manuscript are original and unpublished anywhere else.

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Conflicts of Interest: The authors declare no conflict of interest.

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4.1.4 Publication V

According to traditional Austrian medicine, Norway spruce balm is processed with lard to form a wound-healing ointment. The respective monograph in the Austrian Pharmacopeia and a commercial product on the Austrian market rely on this traditional form of preparation. However, an ointment base other than lard should be considered regarding animal welfare, consumer needs, standardized quality, and stability. Hence, we aimed to develop other formulations and tested them for storage stability at different temperatures. Furthermore, the first *ex vivo* experiments using Franz-type diffusion cells and subsequent HPLC-DAD analyses were conducted to evaluate the permeation potential of selected substances through the skin (Figure 4.7).

The original article '*Spruce Balm-Based Semisolid Vehicles for Wound Healing: Effect of Excipients on Rheological Properties and Ex Vivo Skin Permeation*' was accepted in June 2023 and published in **Pharmaceutics**.

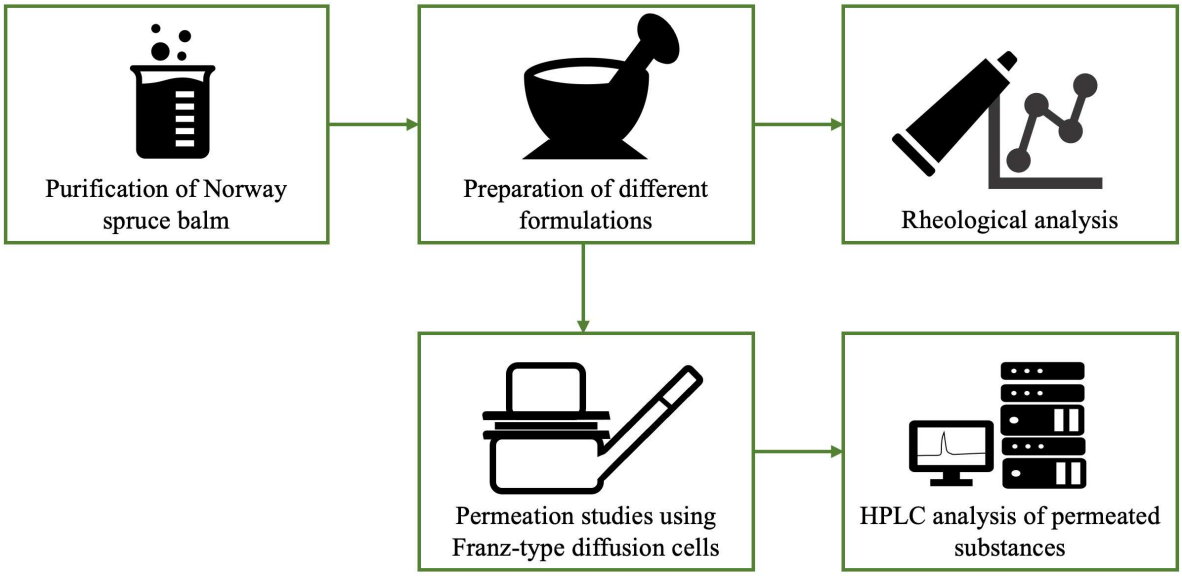

Figure 4.7 Workflow of the development and rheological characterization of different spruce balm formulations and the analysis of the permeation potential of selected substances.

Contributions

My contributions included conceptualizing the study, developing the HPLC method, conducting the phytochemical experiments (sample preparation and HPLC analyses), interpreting the data, and drafting and critically reviewing the manuscript.

Key findings

- Incorporation of Norway spruce balm (NSB) into petrolatum and paraffin oil requires further additives. Using wool wax/castor oil, wool wax/water, and

Ultrabas®/castor oil as ointment bases led to homogenous formulations. NSB in wool wax/castor oil revealed the most homogenous and rheologically stable formulation.

- NSB in wool wax/castor oil was selected for permeation experiments in Franz-diffusion cells and compared to the lard-based commercial product (Figure 4.8 a).
- An aliquot of the acceptor medium was taken after 2, 4, 6, 8, 24, 28, and 32 h, analyzed using HPLC-DAD, and the peak areas (area under the curve (AUC)) of chosen substances were compared (Figure 4.8 a).
- For the more polar substances, lard significantly facilitated permeation compared to wool wax/castor oil. The apolar substances showed comparable permeation behavior from both ointments (Figure 4.8 a).
- The commercial sample and the newly prepared formulation contained different NSB batches. Therefore, the differences in permeation behavior might either be explained by the different ointment bases used or by the different NSB batches in the two formulations. The analysis of different NSB samples showed highly heterogeneous amounts of different substance classes (Figure 4.8 b).

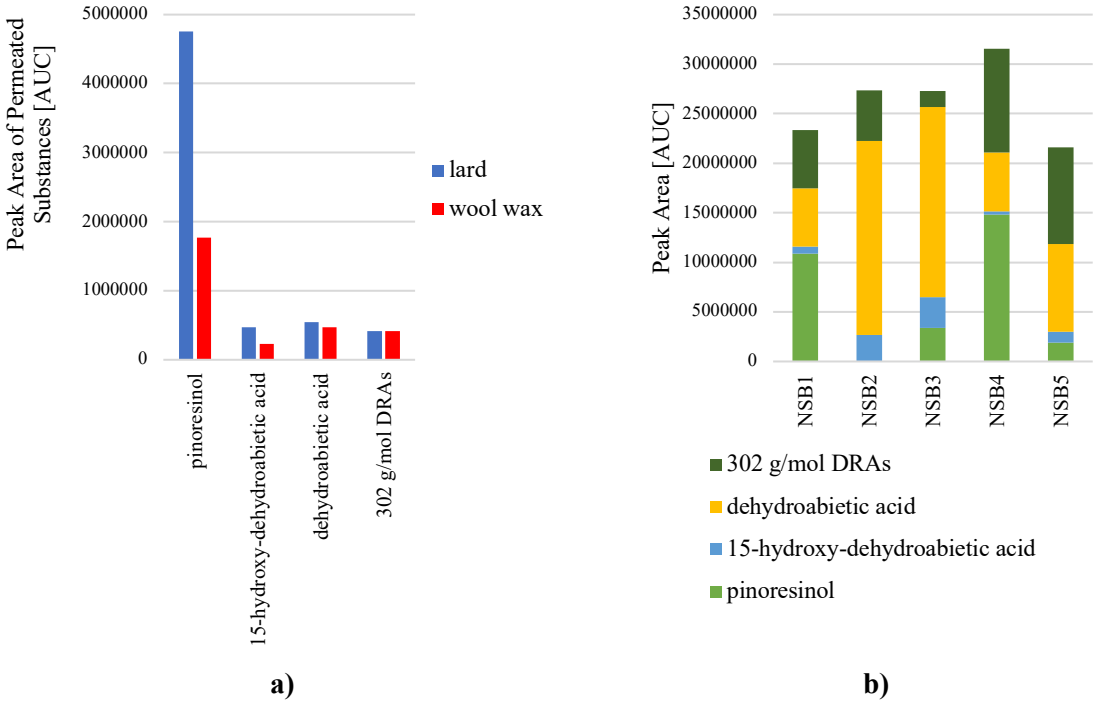

Figure 4.8 Mean peak areas (AUC) of **a)** permeated substances after 24 h from the wool wax and lard-based formulations to the acceptor medium after nine permeation experiments, and **b)** substances of interest in five different Norway spruce balm (NSB) samples after three injections. Figures containing standard deviations are depicted in the publication.

Concluding perspectives

The permeation experiments need to be repeated using formulations prepared with the same spruce balm batch to unambiguously unmask the actual vehicle's impact on permeation rates. Furthermore, an appropriate internal standard for the HPLC methods will be established for the absolute quantitation of permeated substances. Nonetheless, this article investigated the permeation potential of Norway spruce balm constituents for the first time, showing that permeation actually takes place. Wool wax/castor oil represents a rheologically stable alternative to lard. Implementing a more appealing ointment base than lard might promote the use of Norway spruce balm as an alternative therapy option in wound healing by a broader collective of patients.

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Spruce Balm-Based Semisolid Vehicles for Wound Healing: Effect of Excipients on Rheological Properties and Ex Vivo Skin Permeation

Elisabeth Eichenauer [1,2], Martina Jozić [1], Sabine Glasl [1] and Victoria Klang [3,*]

[1] Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna, Josef-Holaubek-Platz 2, 1090 Vienna, Austria
[2] Vienna Doctoral School of Pharmaceutical, Nutritional and Sport Sciences, University of Vienna, Josef-Holaubek-Platz 2, 1090 Vienna, Austria
[3] Division of Pharmaceutical Technology and Biopharmaceutics, Department of Pharmaceutical Sciences, University of Vienna, Josef-Holaubek-Platz 2, 1090 Vienna, Austria
* Correspondence: victoria.klang@univie.ac.at; Tel.: +43-1427755403

Abstract: The treatment of chronic wounds, an important issue with the growing elderly population, is increasingly hindered by antibiotic resistance. Alternative wound care approaches involve the use of traditional plant-derived remedies, such as purified spruce balm (PSB), with antimicrobial effects and the promotion of cell proliferation. However, spruce balm is difficult to formulate due to its stickiness and high viscosity; dermal products with satisfying technological properties and the scientific literature on this topic are scarce. Thus, the aim of the present work was to develop and rheologically characterize a range of PSB-based dermal formulations with different hydrophilic/lipophilic compositions. Mono- and biphasic semisolid formulations based on different compounds (petrolatum, paraffin oil, wool wax, castor oil, and water) were developed and characterized by their organoleptic and rheological measurements. A chromatographic method of analysis was established, and skin permeation data were collected for pivotal compounds. The results showed that the dynamic viscosity ranged from 10 to 70 Pas at 10/s for the different shear-thinning systems. The best formulation properties were observed for water-free wool wax/castor oil systems with 20% *w*/*w* PSB followed by different water-in-oil cream systems. Skin permeation through porcine skin was observed for different PSB compounds (e.g., pinoresinol, dehydroabietic acid, and 15-hydroxy-dehydroabietic acid) using Franz-type diffusion cells. The permeation potential of wool wax/castor oil- and lard-based formulations was shown for all the analyzed substance classes. The varying content of pivotal compounds in different PSB batches collected at different timepoints from different spruce individuals might have contributed to observed differences in vehicle performance.

Keywords: Norway spruce balm; *Picea abies*; rheology; semisolid; skin permeation; storage stability; wound healing ointment

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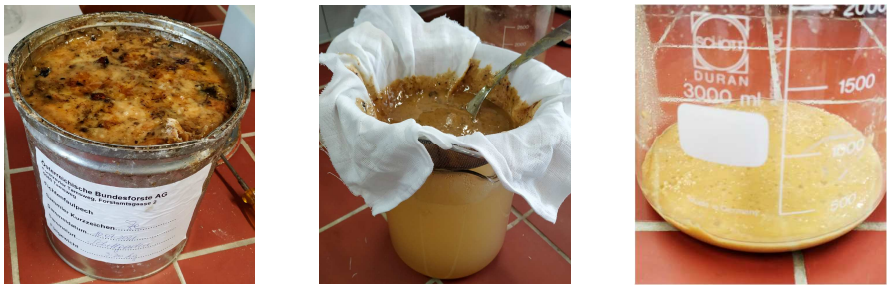
1. Introduction

Wound healing is a complex mechanism involving many different cell types, growth factors, and cytokines. Hence, plenty of different factors may impair this wound healing process, especially chronic diseases, such as diabetes and obesity, but also acute infections and the process of aging. Approximately one billion people are affected by chronic wounds globally, with an increasing incidence due to the rising occurrence of antimicrobial resistance, metabolic syndrome, and a higher lifespan with accompanying comorbidities. Chronic wounds compromise patients' quality of life and represent a monetary burden for healthcare systems worldwide [1,2]. Therefore, additional therapy options are urgently needed. Plants and extracts thereof have been used for centuries as traditional medicines and are indispensable in the treatment of various diseases and in the development of

new drugs. They often contain several different compound classes, which can provide multitargeted effects. Especially in complex mechanisms such as wound healing, the presence of different substances with diverse activities, such as substances with antioxidant, anti-inflammatory, or antimicrobial properties, is highly beneficial [1].

The Norway spruce (*Picea abies* (L.) H. Karst.) [3] produces, like other members of the family Pinaceae, resinous excretions for protection from pathogens such as bacteria and fungi [4]. The exudates can be divided into balms, the fresh, sticky, and kneadable excretions with a high content of essential oil, and resins, the brittle, nonvolatile excretions that do not contain essential oil and that harden after outflow [5]. The balms and resins of *Picea abies* have been used for centuries in the treatment of infected acute or chronic wounds and burns, but also onychomycosis. Traditionally, an ointment made from exudates processed with lard or butter was applied to the affected area [4]. In Austria, mostly the balm of the Norway spruce has had a long-standing tradition in folk medicine. After collecting the balm from tree trunks, impurities such as bark, needles, and insects can be detected (Figure 1a). Therefore, a purification process has to be conducted prior to further usage. For this purpose, the balm is placed in a linen cloth and is boiled in water (Figure 1b). The high temperature changes the balm's consistency to a viscous liquid and permits it to pass through the linen tissue into the water, whereas the solid impurities remain in the cloth. After cooling down, the purified spruce balm (PSB) gathers at the bottom due to its higher density (Figure 1c), and the water can be removed through decantation [6–9]. After this purification step, the balm can be used to produce semisolid products such as ointments. Since 2016, the Austrian Pharmacopeia has contained a monograph entitled "Piceae abietis balsamum". In 2019, the monograph "Unguentum balsami Piceae Abietis officinale", an ointment consisting of 80 g of pig lard and 20 g of Norway spruce balm, was added [10].



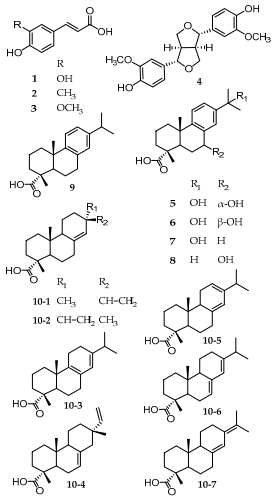
(**a**) (**b**) (**c**)

Figure 1. Purification process of Norway spruce balm: (**a**) Crude balm after collection from tree trunks, including impurities such as bark, needles, and insects. (**b**) The balm is placed in a linen cloth, which is then tied up and placed in boiling water. The balm becomes a viscous liquid and flows into the water; the impurities remain in the linen cloth. (**c**) After cooling down, the purified spruce balm (PSB) sinks to the bottom, and the water can be drained.

The wound healing potential of Norway spruce balm has already been extensively investigated. A summary of the in vitro effects of PSB compounds that are relevant for wound healing applications is shown in Table 1. Wounds are often colonized by bacteria and fungi that impair the healing mechanism. The balm exhibits an antimicrobial effect against such microbes, such as methicillin-resistant *Staphylococcus aureus* and the dermatophyte *Trichophyton rubrum*. Two clinical trials with patients suffering from pressure ulcers or complicated surgical wounds successfully confirmed this wound healing potential in vivo [4,11–15]. In order to gain insight into the underlying mechanisms and substances responsible for this effect, the chemical composition of the balm has been investigated. The substance classes contained in Norway spruce balm are hydroxycinnamic acids, such as ferulic acid and caffeic acid; lignans, such as pinoresinol; and essential oil, which is mostly removed in the boiling step in the traditional method of preparation. The main

compounds are different resin acids, which belong to the class of diterpene resin acids (DRAs). These acids can be hydroxylated or nonhydroxylated. Seven of these acids have the same molecular weight of 302 g/mol and only differ in the position of a double bond or in their stereochemistry [9,16–18]. Table 1 displays the most important substances identified in PSB and their bioactivities, which possibly contribute to the overall wound healing effect.

Table 1. Main compounds of purified spruce balm (PSB), respective substance classes, and biological effects that may contribute to the overall wound healing properties.



	Substance	Structure class	Effects contributing to wound healing
1 **2** **3**	Caffeic acid p-Coumaric acid Ferulic acid	Hydroxy-cinnamic acids	antioxidant, antimicrobial, and anti-inflammatory effects [19]
4	Pinoresinol	Lignans	antifungal [20], antioxidant, anti-inflammatory [21], and re-epithelialization boosting effects [9]
5	7α,15-Dihydroxy-dehydroabietic acid	Hydroxylated resin acids	re-epithelialization boosting [9], antimicrobial [22], and anti-inflammatory effects [23]
6	7β,15-Dihydroxy-dehydroabietic acid		
7	15-Hydroxy-dehydroabietic acid		
8	7-Hydroxy-dehydroabietic acid		
9	Dehydroabietic acid	Diterpene resin acids (300 Da)	re-epithelialization boosting [9], anti-inflammatory [23], and antimicrobial effects [24]
10-1 **10-2** **10-3** **10-4** **10-5** **10-6** **10-7**	Pimaric acid Sandaracopimaric acid Palustric acid Isopimaric acid Levopimaric acid Abietic acid Neoabietic acid	Diterpene resin acids (302 Da)	re-epithelialization boosting [9], antimicrobial [24–27], anti-inflammatory [28–30], and antioxidant effects [31]

The different substance classes cover a large range of polarity. Diterpene resin acids, which form a major part of PSB, are apolar, whereas hydroxycinnamic acids and lignans possess more polar properties. Traditionally, the balm is processed with butter or lard, which results in very lipophilic formulations. The available PSB-containing ointment on the market follows this traditional preparation and is based on lard (Picea-Salbe®, Sico Pharma GmbH, Grafenwörth, Austria). Very little is known, however, about other options to produce semisolid vehicles, e.g., those with different polarities, optimized rheological properties, and storage stability. The potential to produce multiphase systems such as emulsions has not been explored yet, although different technologies would be a definite asset to targeting different wound healing applications. Moreover, different patient groups could be addressed with a larger portfolio of spruce-balm-based semisolid vehicles, as lard is not considered to be an ideal raw material in terms of standardized quality, chemical stability, or patient compliance.

Thus, the aim of this work was to develop and rheologically characterize a range of PSB-based dermal formulations with different hydrophilic/lipophilic compositions. Mono- and biphasic semisolid formulations based on different compounds (petrolatum, paraffin

oil, wool wax, castor oil, and water) were developed and characterized by their organoleptic and rheological measurements. To our knowledge, the impact of PSB in semisolid vehicles on their rheological properties and storage stability has not been reported yet. Furthermore, permeation studies using an ex vivo porcine ear model [32,33] were conducted using the most promising formulation as well as the commercially available product Picea-Salbe® (Sico Pharma GmbH, Grafenwörth, Austria). So far, there are no published data at all on the permeation potential of different PSB compounds. Therefore, the HPLC-DAD method was applied for the relative quantification of pivotal PSB compounds during diffusion cell studies to confirm and compare their permeation potential. The scope of this study was thus a first nonquantitative evaluation of the permeation of PSB compounds ex vivo; however, the exact quantification of skin permeation is planned to be performed in follow-up studies.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Material

Spruce balm from *Picea abies* (L.) H. Karst. was collected by the Österreichische Bundesforste AG (ÖBf, Austrian federal forests) in a forest in Lungau, Salzburg, Austria, in July 2021 and was provided by Sico Pharma GmbH, Grafenwörth, Austria. The batch was assigned the name PSB5 and was stored in a metal container at 4 °C.

Since the traditional method of preparation includes the step of boiling Norway spruce balm, 342 g of balm was sealed in a linen cloth and was placed in a beaker containing 2000 mL of deionized water heated up to approximately 90 °C. After boiling the balm for about one hour and letting the water cool down, the purified spruce balm (PSB) gathered at the bottom of the beaker, the water was removed through decantation, and the residue was dried and yielded 187 g of PSB.

2.1.2. Other Materials

The commercial product Picea-Salbe® (containing 20% *w*/*w* Norway spruce balm in lard, L200408) was kindly provided by Sico Pharma GmbH, Grafenwörth, Austria. Aerosil® (KNC1914J14), paraffin oil PHQ (KNC0029J20), castor oil PHQ (KNC0504J21), petrolatum PHQ (KNC1132J19), and wool wax PHQ (KNC0113J20) were obtained from Herba Chemosan Apotheker AG, Vienna, Austria. Ultrabas® (YY04P1H) was purchased from Bayer Austria GmbH, Vienna, Austria.

2.2. Preparation of Formulations

Different formulations were developed to incorporate 20% *w*/*w* of PSB. The respective compositions are given in Table 2. PSB was melted together with a lipophilic base in a water bath at 80 °C and was stirred afterward until the formulation was cool. If requested, distilled water was incorporated stepwise in small portions. For the preparation of an oleogel with colloidal silica (Aerosil®), disperse powder was incorporated stepwise in a PSB/castor oil mixture in small portions. Using Ultrabas® as ointment base, PSB was melted with castor oil, and the resulting cool homogenate was mixed stepwise with Ultrabas®. All ointments were produced in triplicate and were stored in gel tubes under light protection. Each formulation was divided in half to give two batches, which were stored either at room temperature (23 °C) or in the refrigerator (8 °C) to assess the effect of different storage temperatures.

Table 2. Composition in % *w*/*w* of the prepared ointments with 20% *w*/*w* PSB. All formulations were produced in triplicate (n = 3).

Formulation	Composition	% *w*/*w*
petrolatum/paraffin	petrolatum	60
	paraffin oil	20
	PSB	20

Table 2. *Cont.*

Formulation	Composition	% *w*/*w*
wool wax/castor oil	wool wax	60
	castor oil	20
	PSB	20
wool wax/water	wool wax	60
	distilled water	20
	PSB	20
Ultrabas/castor oil	Ultrabas®	73.33
	castor oil	6.66
	PSB	20
Aerosil/castor oil	Aerosil®	7
	castor oil	73
	PSB	20

2.3. Rheological Characterization and Stability

The rheological properties of the produced semisolid ointments were analyzed with a modular compact rheometer (MCR 302 with viscotherm VT 2 thermostatic control system, Anton Paar GmbH, Graz, Austria). Data were analyzed using the RheoCompass Professional® 1.26 software (Anton Paar GmbH, Graz, Austria). Flow curves were recorded at room temperature of 23 ± 0.2 °C with a cone–plate measuring system (CP-25, diameter of 25 mm, angle of 2°) using ~1.0 g of the respective vehicle. Dynamic viscosity η was determined based on dependence of shear rate at 1 to 100 s^{-1}. For stability monitoring at different storage temperatures, respectively, three samples of each formulation type were filled in gel tubes after production. For initial assessment, all ointments were stored under light protection at 23 °C for 24 h to allow for equilibrium and were then characterized. Subsequently, the samples were either stored at 23 °C (room temperature, n = 3 per type) or 8 °C (refrigerated storage, n = 3 per type) and were analyzed in regular intervals using the same parameters over six months. Cooled samples were allowed to acclimatize for six hours at 23 °C before each measurement.

2.4. Skin Permeation

2.4.1. Diffusion Cell Setup

Skin permeation studies were performed using dermatomed porcine ear skin and static Franz-type diffusion cells (PermeGear, Hellertown, PA, USA, permeation area of 0.95 cm^2, acceptor volume of 2 mL). Porcine ears were obtained after sacrifice from a local abattoir (Johann Gantner GmbH, Gemeinde Hollabrunn, Austria, male and female animals at six months of age). The ears were cleaned under running water and were dried and stored at −18 °C until use.

Fresh vehicles were prepared 48 h before the start of the experiments. Skin from the dorsal side of the ears was cut to a thickness of 400 µm with a dermatome (Aesculap GA 630 DBP, Aesculap AG, Tuttlingen, Germany). Hair was removed with scissors. The skin was cut into 1 × 1 cm pieces and was clamped between donor and acceptor chamber. The acceptor medium consisted of a mixture of phosphate-buffered saline and ethanol (60:40% v/v) to ensure sink conditions. The phosphate-buffered saline was prepared as described in the Pharmacopoeia Europaea (0.012 M, pH of 7.4, 0.238% w/w sodium monohydrogen phosphate, 0.019% w/w potassium dihydrogen phosphate, and 0.8% w/w sodium chloride). Before starting the experiments, the transepidermal water loss (TEWL) was measured with a closed-chamber device and a coupling adapter (Aquaflux® AF200, Biox Systems Ltd., London, UK) to ensure consistent skin quality and barrier function.

2.4.2. Skin Permeation of Bioactive Substances

The ointments were applied to the donor chambers at infinite dose conditions (500 mg of formulation). The donor compartment and sampling arm were occluded with Parafilm to prevent evaporation of sample or acceptor fluid. The diffusion cells were kept in a water bath at 32 °C to simulate skin surface temperature. Magnetic stirring was employed to avoid concentration layers within the acceptor fluid. Cells were regularly checked for air bubbles. After predefined intervals (2, 4, 6, 8, 24, 28, and 32 h), an aliquot of 200 µL of acceptor medium was taken for analysis and was replaced with fresh acceptor medium at 32 °C.

The most suitable formulation was chosen for analysis of permeation potential as well as the commercial product Picea-Salbe®. Three individual permeation experiments with $n = 3$ cells were performed for formulations and controls. The final results depict mean values ± SDs of $n = 9$ cells per formulation. As controls, the respective ointment bases without PSB as well as the plain acceptor medium alone were used.

2.5. High-Performance Liquid Chromatography

For the analysis of samples drawn from Franz diffusion cells, a Shimadzu HPLC instrumentation with diode array detection (SHIMADZU Autosampler SIL-20AC HAT, SHIMADZU Column Oven CTO-20AC, SHIMADZU Communications Bus Module CBM-20A, SHIMADZU Degasser DGU-20A5, SHIMADZU Diode Array Detector SPD-M20A, SHIMADZU Liquid Chromatograph LC-20AD, SHIMADZU LabSolutions Software 5.81, Shimadzu, Kyoto, Japan) was used. A reversed-phase C18 column (LiChrospher 100 RP18e, 5 µm, 250 × 4 mm, Merck KGaA, Darmstadt, Germany) was employed as stationary phase. Double-distilled water + 0.1% formic acid (solvent A) and acetonitrile + 0.1% formic acid (solvent B) served as mobile phase, and gradient elution at 1 mL/min flow rate was applied as follows: 15% solvent B rose to 95% solvent B within 45 min (rate of 1.77%/min) followed by 5 min washing step at 98% solvent B and re-equilibration for 10 min with 15% solvent B. Samples drawn from Franz diffusion cells were centrifuged, and 10 µL were injected into the system. PSB samples were dissolved in acetone to a final concentration of 5 mg/mL, and 2 µL were analyzed. Since the different substance classes exhibit different absorption maxima but can be seen at 190 nm, all chromatograms were analyzed at 190 nm. Peak areas (AUC) were obtained using the SHIMADZU LabSolutions Software 5.81 (Shimadzu, Kyoto, Japan) and were further utilized for interpretation of data.

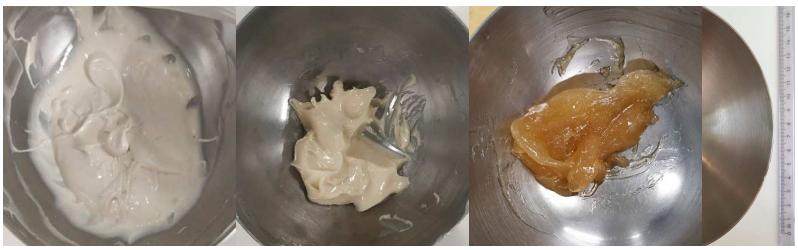
2.6. Statistics

Results are expressed as statistical means with standard deviations. Data analysis was performed using GraphPad Prism 9.4.1 software (GraphPad Software, San Diego, CA, USA). Parametric data were analyzed using Student's *t*-test or ANOVA and Tukey's post hoc test. Nonparametric data were analyzed using the Mann–Whitney test or Kruskal–Wallis test with Dunn's multiple comparison test as post hoc test. Statistical significance was expressed by minimum of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3. Results

3.1. Preparation and Organoleptic Appearance of Formulations

By using different ointment bases, 20% *w*/*w* PSB-containing ointments with varying hydrophilic/lipophilic compositions were prepared. The incorporation of PSB into petrolatum and paraffin oil led to the caking of the balm, suggesting the need for further additives in hydrocarbon-based PSB ointments. This formulation was excluded from further experiments. Preparation with wool wax and castor oil/water, Ultrabas® and castor oil, and colloidal silica (Aerosil®) and castor oil resulted in dermal formulations with homogenous optical properties and a whiteish to brownish appearance (Figure 2).

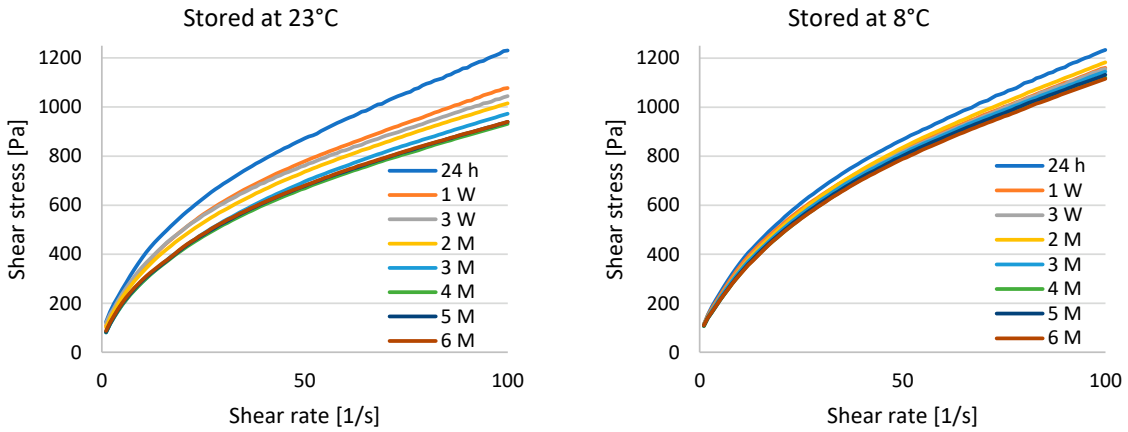


(**a**) (**b**) (**c**) (**d**)

Figure 2. Optical appearance of different formulations: (**a**) wool wax/castor oil; (**b**) Ultrabas/castor oil; (**c**) Aerosil/castor oil. All formulations were prepared using a smooth mortar ((**d**) upper diameter of 12 cm) and pestle.

3.2. Rheological Properties and Stability

Flow curves were established for all the developed vehicles by plotting the shear stress τ against the shear rate γ. The flow curves of the preparation of wool wax/castor oil are depicted in Figure 3; the flow curves of wool wax/water and Ultrabas/castor oil can be found in Supplementary Figures S1 and S2. The formulation of Aerosil/castor oil was excluded for methodological reasons after its initial assessment (it had a paste-like consistency due to its high powder content; the product was deemed unsuitable for use on open wounds).



(**a**) (**b**)

Figure 3. Flow curves of (**a**) wool wax/castor oil stored at room temperature (23 °C) and (**b**) wool wax/castor oil stored in the refrigerator (8 °C). The colored curves are means of n = 3 samples analyzed at room temperature (23 °C) at a shear rate of 1–100 s^{-1}. SDs are omitted for better visualization. The refrigerated samples were left to acclimate for six hours at 23 °C before each measurement. Abbreviations: h = hour/s, W = week/s, and M = month/s.

Furthermore, viscosity curves were established by plotting the dynamic viscosity η against the shear rate γ. The dynamic viscosity in Pas at a shear rate of 10 s^{-1} was monitored during storage at two different conditions (room temperature of 23 °C and refrigerated temperature of 8 °C) and served to compare the rheological storage stability of the different formulations (Figure 4).

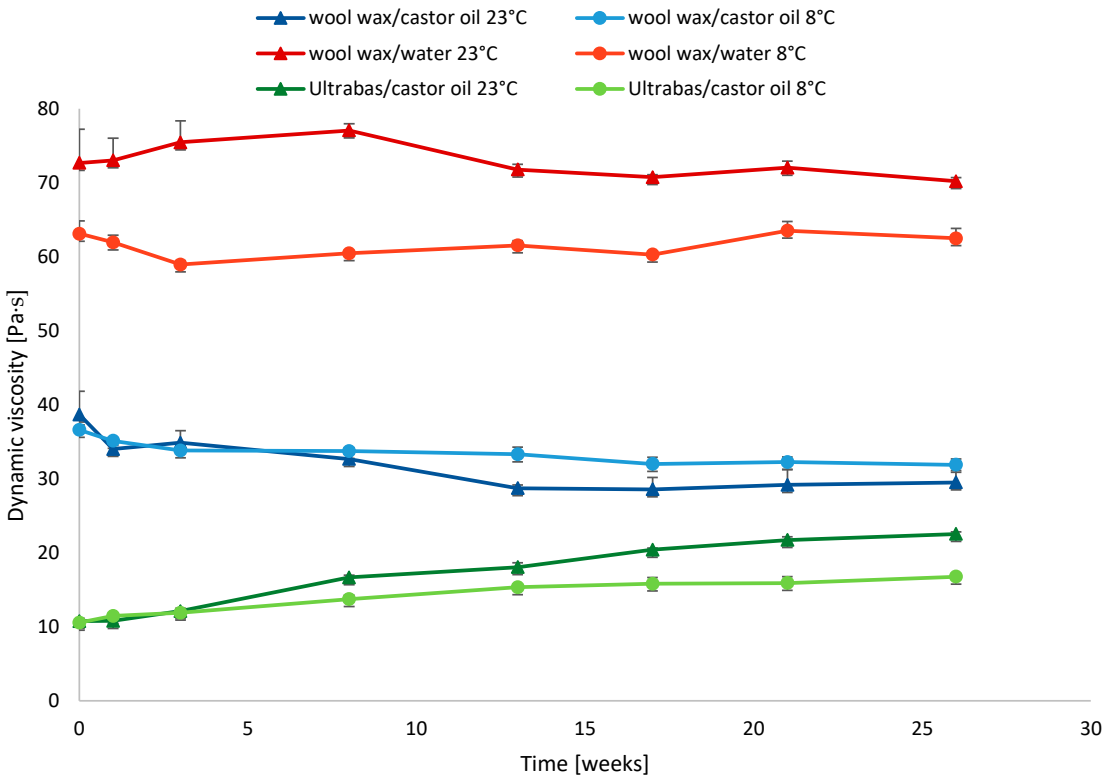


Figure 4. Effect of storage time and temperature on dynamic viscosity (η, in Pas) of semisolid vehicles with 20% *w*/*w* PSB at a shear rate of 10 s^{-1}. Values are means ± SDs of $n = 3$ samples.

The initial dynamic viscosity was calculated for all the samples of the three formulations independent from the temperature, and it was highest for the wool wax/water formulation followed by wool wax/castor oil and finally Ultrabas/castor oil (68.05 ± 5.88 > 37.64 ± 2.38 > 10.63 ± 0.35 Pas at 10 s^{-1}, $p < 0.0001$, $n = 6$).

The formulation of wool wax/castor oil visually exhibited the most stable formulation properties and storage stability. After an initial structural stabilization period over the first week, the dynamic viscosity only showed a slow decrease over a storage period of six months (Figure 4). The flow curves were highly reproducible for wool wax/castor oil (Figure 3); no visual signs of the structural rearrangement or physical separation of PSB from the vehicle were observed. As can be derived from the flow curves (Figure 3), the dynamic viscosity decreased with increasing shear rates. This corresponds to pseudoplastic, i.e., shear-thinning, flow behavior. After six months, the dynamic viscosity of the refrigerated product generally remained at a higher value than that of the product that was stored at room temperature (Figure 4). At 23 °C, the dynamic viscosity at 10 s^{-1} changed from the initial 36.68 ± 3.15 Pas to 29.52 ± 1.63 Pas after six months ($p < 0.05$). At 8 °C, the values changed from the initial 36.60 ± 0.67 Pas to 31.90 ± 0.78 Pas after six months ($p < 0.01$). This, however, was owed to the reorganization phase within week 1 after which only minor changes were observed, especially with respect to cooled storage.

The multiphase system based on wool wax/water showed the first signs of visual phase separation within the first weeks of storage. The flow curves generally showed pseudoplastic behavior, but they showed less consistent shear thinning during measurement (Supplementary Figure S1) than the wool wax/castor oil system. A thickening effect during storage was visible due to a loss of water, which was more pronounced for the formulations stored at room temperature. Although the measured values of the dynamic viscosity at 10 s^{-1} remained largely unaffected after six months for both the formulations

stored at 23 °C (72.68 ± 4.57 vs. 71.19 ± 1.99 Pas, $p > 0.05$) and at 8 °C (63.43 ± 1.58 vs. 62.49 ± 1.38 Pas, $p > 0.05$), the homogeneity of the formulations was obviously impaired, and the reproducibility between the batches was limited under lab-scale compounding conditions. The observed separation of water was a disruptive factor for the rheological measurements, and it could be deduced from the fluctuating values of the dynamic viscosity at 10 s^{-1} during rheological monitoring during storage (Figure 4).

For the Ultrabas/castor oil formulation, a clear tendency towards an increasing dynamic viscosity was observed during storage (Figure 4). As for the wool wax/water system, the flow curves indicated pseudoplastic flow behavior, but with strong irregularities during measurement (Supplementary Figure S2). Since the water content of Ultrabas® is lower than that of the wool wax/water-based product, more homogeneous formulation properties were visually observed. Nonetheless, a slow separation of water from the main structure and internal structural changes could be deduced from rheological monitoring, leading to an apparent increased dynamic viscosity. This effect was more pronounced for the formulation stored at room temperature after six months. The dynamic viscosity at 10 s^{-1} at 23 °C increased from 10.78 ± 0.38 to 23.15 ± 1.22 Pas ($p < 0.001$). At 8 °C, the increase still amounted to a change from 10.48 ± 0.28 to 16.38 ± 0.81 Pas ($p < 0.01$).

In summary, the wool wax/castor oil-based formulation exhibited the most homogeneous formulation properties and the highest rheological stability. Thus, this vehicle was selected for evaluation against a marketed product in skin diffusion studies.

3.3. Solubility and Choice of Acceptor Medium

Diffusion cell studies were conducted to probe for the permeation potential of important PSB compounds through intact porcine ear skin in an ex vivo setup. Focus was placed on four important groups of PSB compounds: pinoresinol (**1**), 15-hydroxy-dehydroabietic acid (**2**), dehydroabietic acid (**3**), and DRAs as summarized by the content of seven diterpene resin acids with a molecular weight of 302 g/mol (**4**) (Figure 5).

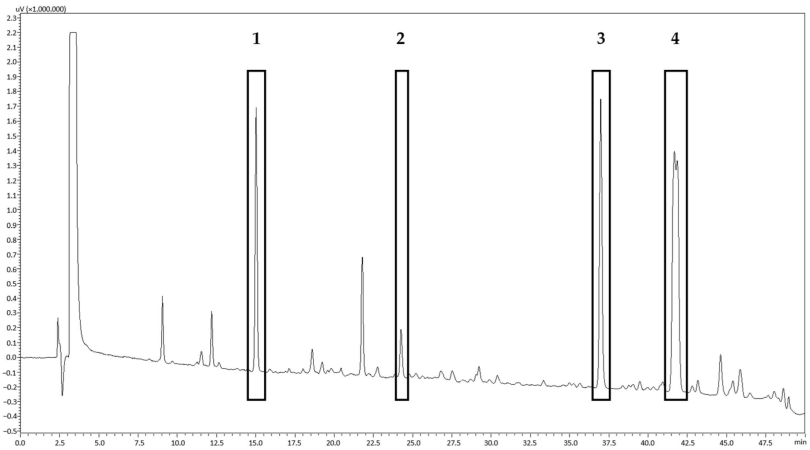


Figure 5. HPLC-DAD chromatogram of PSB (batch PSB5) at 190 nm (balm dissolved in acetone, final concentration of 5 mg/mL, 2 µL were injected); stationary phase was RP18e, and mobile phase was water + 0.1% formic acid and acetonitrile + 0.1% formic acid. The peaks of substances, which were chosen for analysis of their permeation potential, were assigned through co-chromatography with pure reference substances. Peak 1 is pinoresinol, Peak 2 is 15-hydroxy-dehydroabietic acid, and Peak 3 is dehydroabietic acid. Peak 4 contains 7 different DRAs, which all share the same molecular weight of 302 g/mol and cannot be separated using this method due to their structural similarities.

Since the majority of these compounds is apolar in nature and since their solubility in an aqueous phosphate buffer as the standard acceptor medium is thus limited, two different solvents were tested: 50 mg of PSB were dissolved in each acceptor medium (either ethanol or propylene glycol in phosphate-buffered saline (40 + 60)), was shaken for

24 h at 30 °C, and was centrifuged afterwards, and 5 µL were analyzed through HPLC-DAD. The obtained AUCs can be found in Supplementary Figure S3. Since a larger amount of substances, especially the more polar pinoresinol and 15-hydroxy-dehydroabietic acid, was detected in the acceptor medium containing ethanol, the solubility of the substances was higher in ethanol than in propylene glycol, and, therefore, ethanol + phosphate-buffer saline (40 + 60) was used as the acceptor medium.

Furthermore, permeation experiments with the same setup as those with the PSB-containing ointments were conducted using no ointment or only the ointment base (lard or wool wax/castor oil) in order to identify possible overlapping peaks in the respective HPLC chromatograms deriving from variables other than PSB itself. Since no additional peaks other than the expected peaks were detected, it was assumed that the detected mass permeation of these compounds was not adulterated by other factors (Supplementary Figure S4).

3.4. Skin Permeation of PSB Compounds

Skin permeation experiments were conducted through the application of the wool wax/castor oil-based formulation and the lard-based Picea-Salbe®. The PSB compounds were analyzed over 32 h of experiment time ex vivo. The relative quantification of mass permeation was possible based on the obtained AUC values (Figure 6, measured at the timepoint of 32 h). The cumulative permeated amounts of pinoresinol, 15-hydroxy-dehydroabietic acid, dehydroabietic acid, and the DRAs were established using the obtained AUCs and were plotted against the experiment time for the comparison of the two tested products (Figure 7). For pinoresinol (Figure 7a), significantly higher total permeated amounts were observed from the lard ointment than from the wool wax/castor oil ointment ($p < 0.001$). For 15-hydroxy-dehydroabietic acid (Figure 7b), a similar trend was observed despite large standard deviations in the data set (still, p was < 0.05). For dehydroabietic acid and the DRAs (Figure 7c,d), the total permeated amounts were highly comparable from the two different ointments ($p > 0.05$ in both cases).

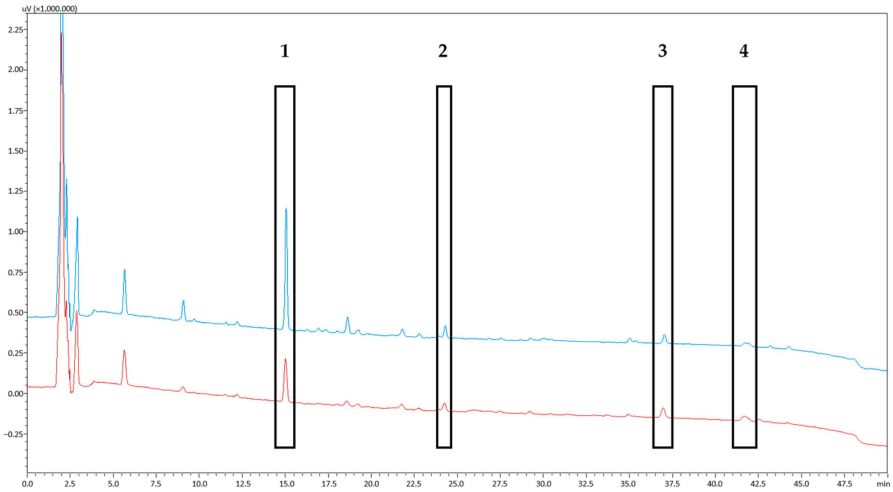


Figure 6. HPLC-DAD chromatograms (190 nm) of samples drawn after 32 h of skin permeation experiments of 20% PSB in lard (blue) and 20% PSB in wool wax/castor oil (red). The assigned peaks mark the substances, which were analyzed in depth (pinoresinol (**1**), 15-hydroxy-dehydroabietic acid (**2**), dehydroabietic acid (**3**), and 302 g/mol DRAs (**4**)).

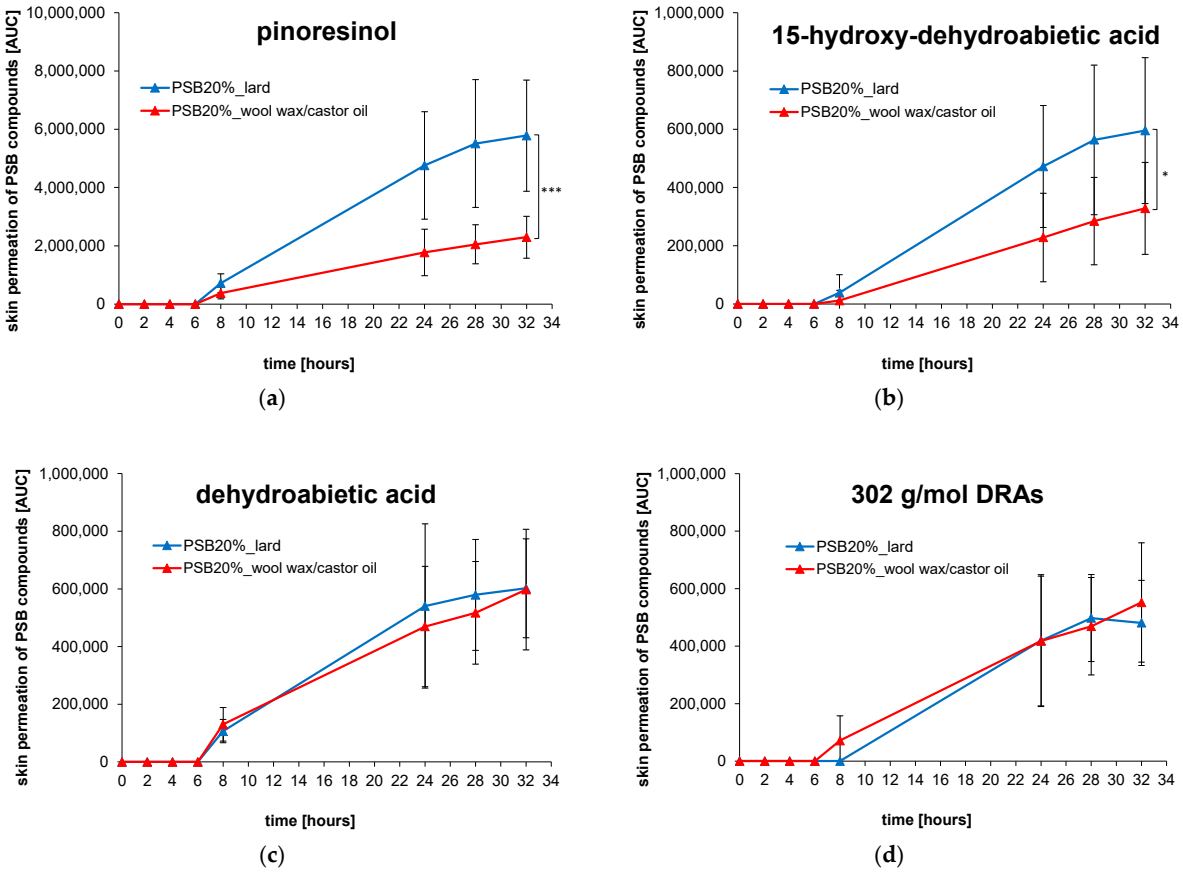


Figure 7. Skin permeation of PSB compounds (pinoresinol (**a**), 15-hydroxy-dehydroabietic acid (**b**), dehydroabietic acid (**c**), and 302 g/mol DRAs (**d**)) from different ointments. Data represent means of $n = 9$ cells ± SDs. Statistically significant differences between formulations are marked for AUC values after 32 h with * for $p < 0.05$, and *** for $p < 0.001$.

In addition, the steady-state flux in AUC/cm^2/h was calculated through linear regression for the individual cells after the respective lag time (6 or 8 h). The mean relative mass flux of the different PSB compounds was calculated through linear regression to compare the two vehicles (Table 3); since no absolute quantification was yet possible, the mass flux is termed "relative" in this context. The results confirmed a significantly higher mass flux for pinoresinol and 15-hydroxy-dehydroabietic acid from the lard-based ointment and a highly similar mass flux for dehydroabietic acid and the DRAs from the two ointments.

Table 3. Mean relative mass flux in AUC/cm^2/h ± SDs of the different PSB compounds (** $p < 0.01$, *** $p < 0.001$).

PSB Compound	Vehicle	Mean Flux Based on AUC/cm^2/h	Enhancement Factor	Sign.	Mean R^2
pinoresinol	wool wax/castor oil lard	87.258 ± 27.553 243.842 ± 93.731	2.8	***	0.973 ± 0.030 0.989 ± 0.015
15-hydroxy-dehydroabietic acid	wool wax/castor oil lard	13.606 ± 6.196 25.558 ± 9.673	1.8	**	0.985 ± 0.014 0.988 ± 0.010
dehydroabietic acid	wool wax/castor oil lard	21.848 ± 7.791 24.041 ± 7.099	1.1	n.s.	0.964 ± 0.026 0.985 ± 0.012

Table 3. *Cont.*

PSB Compound	Vehicle	Mean Flux Based on AUC/cm^2/h	Enhancement Factor	Sign.	Mean R^2
302 g/mol DRAs	wool wax/castor oil lard	21.797 ± 7.567 22.847 ± 7.299	1.1	n.s.	0.985 ± 0.023 0.960 ± 0.035

When comparing the two products, it has to be kept in mind that different plant materials were used for their preparation. For the wool wax/castor oil-based formulation, PSB5 with a known composition was used (Figure 5). The commercially available product Picea-Salbe®, however, consists of a Norway spruce balm with an unknown composition. The detected differences in permeation between the two products may, on the one hand, result from the different solubilities of the compounds in their respective underlying ointment base. On the other hand, they may be due to the varying contents of the substances in the different spruce balm samples. In order to show the heterogeneity of Norway spruce balms, five different batches were analyzed via HPLC-DAD (Figure 8). These batches were collected at different timepoints and in different areas around Austria (Supplementary Table S1, PSB5 was used for the permeation experiments in wool wax/castor oil), and they demonstrate the highly varying relative contents of the substances of interest.

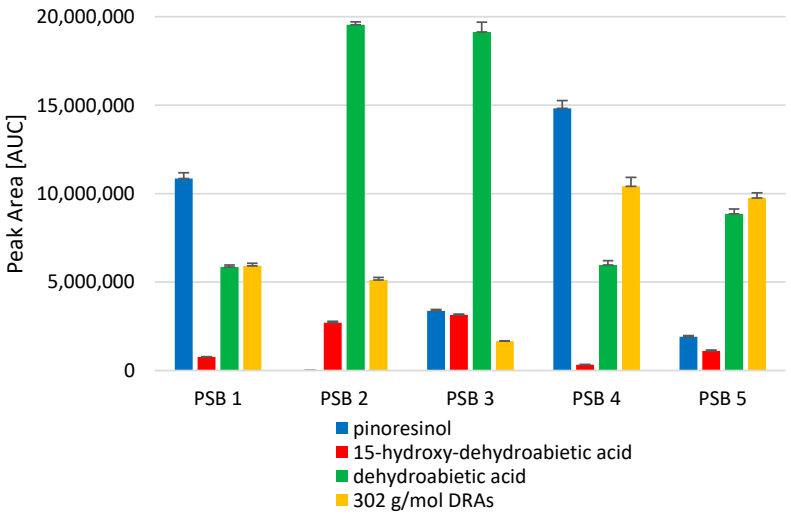


Figure 8. Relative content (AUC) ± SDs of substances in five different PSB batches (means of $n = 3$ injections); batches were collected in different areas and at different timepoints around Austria.

4. Discussion

4.1. Preparation of PSB Formulations and Storage Stability

The successful incorporation of PSB into semisolid dermal vehicles is challenging due to the large diversity of phytochemicals present in the balm and the peculiar rheological behavior of the balm itself after harvesting. Both the monograph in the Austrian Pharmacopeia dealing with PSB ointments as well as the commercial product Picea-Salbe® rely on a very traditional production method: the purified spruce balm is either mixed with lard or butter after heating, and stirred until a homogeneous mixture at room temperature is obtained. However, these animal fats are not commonly available in pharmaceutical quality and/or are not sufficiently standardized, thus not meeting today's technological standards. As a result, the physicochemical formulation parameters of the produced semisolid vehicles as well as patient compliance may vary. To overcome these issues, alternative ointment bases should be explored for the delivery of PSB in wound healing applications.

Interestingly, however, lard itself has been shown to exhibit anti-inflammatory properties [34] and might, therefore, contribute to an overall wound healing effect. An in vivo study on the healing of castration wounds in piglets compared lard alone to a PSB-/lard-based ointment. In this study, the use of a pure ointment base did not lead to the reduction of proinflammatory factors or faster wound healing [35]. Thus, other ointment bases should be considered as a replacement.

In Finland, a medical product containing the resin of *Picea abies* is available, which is based on petrolatum, paraffin oil, and further additives for stabilization. A simple prototype formulation was developed at the beginning of this study using this formulation approach but was unsuccessful and was thus excluded. In further studies, a more complex formulation based on hydrocarbon-based products will be explored as an alternative to lard.

Wool wax was chosen as an ointment base due to its lipophilic texture and high emulsifying properties. Castor oil was added for a smoother texture and easier manual preparation. The rheological analysis of the preparation of wool wax/castor oil showed that the formulation required approximately one week to reach its quiescent structure. Over six months, the refrigerated product showed a higher rheological stability than the product stored at room temperature. As the most promising vehicle, the wool wax/castor oil ointment was selected as a candidate for the first diffusion cell experiments using porcine ear skin to evaluate the skin permeation potential of the PSB compounds ex vivo.

Wool wax was combined with water instead of castor oil in order to create a more hydrophilic vehicle, a semisolid oil-in-water cream stabilized by the nonionic wool wax surfactants. It should be noted that the presence of 20% w/w water might lead to increased microbial contamination during storage. Spruce balm incorporated in an apolar vehicle is considered to possess sufficient antimicrobial activity for storage over six months, as indicated by the Austrian Pharmacopoeia. However, this should be investigated in future studies for multiphase vehicles to ensure the safety of the products.

Ultrabas® is a manufactured ointment base that is popular in the pharmaceutical compounding of dermatological products in Austria. From a technological viewpoint, it is a water-in-oil emulsion based on hydrocarbons, distilled water, and nonionic surfactants. Since it should not be heated during its formulation processes, castor oil was used to facilitate the manual incorporation of PSB in this study.

Both of the multiphase vehicles containing an additional water phase, the wool wax/water system and the Ultrabas/castor oil system, showed decreased rheological stability due to a slow separation of water from the main ointment structure. This effect was more pronounced for creams based on wool wax/water, where a fluctuating dynamic viscosity was observed during storage. For Ultrabas/castor oil systems, a steady increase in the dynamic viscosity showed a slower but consistent separation of water from the main cream structure during storage, leading to the thickening of the remaining cream system.

It is known that water-in-oil emulsions show technological incompatibilities with phenolic entities. The presence of phenolic compounds leads to a concentration-dependent destabilization of the emulsion system through H-bonding with nonionic surfactants. As a result, the separation of water, coagulation, and phase separation may occur. Several substances found in Norway spruce balm, such as pinoresinol or several hydroxycinnamic acids, exhibit phenolic structures and might lead to this undesired physicochemical interaction in the cream formulations. Consequently, storage stability is decreased.

Finally, an oleogel preparation based on colloidal silica (Aerosil®) was developed. The incorporation of PSB at 20% w/w into the gel led to a homogenous product. Colloidal silica should not be used on open wounds, but it might be an alternative in the treatment of dry skin or fissures. This approach will be taken up in separate studies since a more thorough rheological examination is required for the highly viscous product.

4.2. Permeation of Bioactive Substances

Two ointments (wool wax/castor oil and Picea-Salbe®) were investigated concerning the permeation potential of the bioactive substances (pinoresinol, 15-hydroxy-dehydroabietic acid, dehydroabietic acid, and the 302 g/mol DRAs). First, the samples drawn from diffusion cells containing only the respective ointment base or no ointment at all were analyzed in order to prove the absence of detected peaks with the same retention time as the wanted PSB components.

Pinoresinol and dehydroabietic acid were already detectable in all the samples after 8 h, whereas 15-hydroxy-dehydroabietic acid and the 302 g/mol DRAs could often only be detected after 24 h. This might be due to the original content in the ointment. The analysis of the different PSB samples showed that the relative content of the respective substances varied in the different batches. Not only the area of collection, but also the date of collection seemed to have an impact on the content of the compounds. This fact underlines the importance of different analytical methods for quality control [16]. Since it is not known which PSB was used for the medical device Picea-Salbe®, the differences in the permeation studies may be due to the variety in the content found in the different balms.

On the other hand, the differences may be due to the varying polarities of the substances (pinoresinol XLogP3-AA: 2.3; 15-hydroxy-dehydroabietic acid XLogP3-AA: 4.2; dehydroabietic acid XLogP3-AA: 5.6; sandaracopimaric acid as the representative of the 302 g/mol DRAs XLogP3-AA: 5.5) and, therefore, their solubility in the two different vehicles (the ointment basis and acceptor medium). Pinoresinol and 15-hydroxy-dehydroabietic acid exhibit smaller logP values than the DRAs and are therefore more likely to permeate from a lipophilic ointment into a more hydrophilic acceptor medium. The next step will be the absolute quantification of the components in crude PSB as well as in the acceptor medium after permeation experiments using an internal standard.

In a follow-up study it will be clarified whether the two different vehicles are responsible for the differences found in the release of the substances using the same batch of PSB, or whether they approximate and the varying content in the balm itself is responsible for these differences. In the latter case, it has to be excluded that lard is crucial for the wound healing effect of the traditional PSB ointment. If so, wool wax/castor oil represents a more standardized, stable, and animal-friendly alternative to lard in the monography of the Austrian Pharmacopeia as well as in the preparation of medical devices.

4.3. Implications for the Successful Formulation of Semisolid Products with Plant-Based Materials

The present study shows that it is of the utmost importance for an informed formulation design of plant-based products to consider the presence of phenolic compounds as well as charged compounds and surface-active substances, especially in multiphase systems. Technological incompatibilities due to ionic interactions or H-bonding are dependent on the total concentration of individual plant compounds and may not lead to the immediate phase separation of emulsion systems. However, masked incompatibilities may ultimately promote physical destabilization and thus impair storage stability as well.

5. Conclusions

A wool wax/castor oil formulation was found to be a suitable alternative to traditional lard as a semisolid base for the production of Norway spruce balm ointments (20% *w*/*w* PSB). Visual and rheological monitoring confirmed a satisfying physical stability over six months. Refrigerated storage at 8 °C led to the best results, especially in the case of the other tested cream formulations. Ex vivo experiments showed the permeation potential of the bioactive PSB compounds from the newly developed semisolid formulation and the commercial reference product Picea-Salbe®. As the next step, the impact of the vehicle composition will be investigated using the same PSB batch, and the absolute quantification of the permeated substances will be targeted.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/pharmaceutics15061678/s1: Figure S1: Flow curves of (a) wool wax/water stored at room temperature (23°C) and (b) wool wax/water stored in the refrigerator (8 °C). The colored curves are means of $n = 3$ formulations analyzed at room temperature (23 °C) at a shear rate of 1–100 s^{-1}. SDs are omitted for better visualization. The refrigerated samples were left to acclimate for six hours at 23 °C before each measurement. Abbreviations: h = hour/s, W = week/s, and M = month/s. Figure S2: Flow curves of (a) Ultrabas/castor oil stored at room temperature (23 °C) and (b) Ultrabas/castor oil stored in the refrigerator (8 °C). The colored curves are means of $n = 3$ formulations analyzed at room temperature (23 °C) at a shear rate of 1–100 s^{-1}. SDs are omitted for better visualization. The refrigerated samples were left to acclimate for six hours at 23 °C before each measurement. Abbreviations: h = hour/s, W = week/s, and M = month/s. Figure S3: Comparison of the relative solubility of PSB compounds in different acceptor media. AUCs were obtained after dissolving an identical surplus amount of purified spruce balm (PSB) in either phosphate-buffered saline/ethanol at 60:40% v/v (blue bars) or phosphate-buffered saline/propylene glycol at 60:40% v/v (red bars). Figure S4: HPLC-DAD chromatograms of samples drawn from permeation experiments after 32 h using no ointment (red), lard without PSB (green), and wool wax/castor oil without PSB (blue) at 190 nm. Table S1: Batch numbers (PSB), internal assignment (FP), date of harvest, and harvest location of analyzed PSB batches.

Author Contributions: Conceptualization, E.E., S.G. and V.K.; methodology, E.E., M.J. and V.K.; resources, S.G. and V.K.; data curation and analysis, E.E., M.J. and V.K.; writing—original draft preparation, E.E. and V.K.; writing—review and editing, S.G. and V.K.; visualization, E.E. and V.K.; supervision, S.G. and V.K. All authors have read and agreed to the published version of the manuscript.

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4.2 *Sanicula europaea* L.

4.2.1 Manuscript I

The aerial parts of sanicle, "Herba Saniculae", are used for treating respiratory tract catarrhs. Furthermore, folk medicine reports the use of Herba Saniculae for the indications of gastric hemorrhages and wounds [53]. Interestingly, in Austrian folk medicine, the aerial parts as well as the underground parts, referred to as "Radix Saniculae", serve as wound-healing agents [54]. However, in the Austrian vernacular language, the term "Radix Saniculae" is not only used for the underground parts of *Sanicula europaea* L. but also for those of *Cardamine enneaphyllos* (L.) Crantz [55]. To clarify the use of the term "Radix Saniculae" in the context of wound healing, we aimed to investigate the phytochemical composition of Herba Saniculae (the aerial parts of *Sanicula europaea*), Radix Saniculae (the underground parts of *Sanicula europaea*), and Radix Cardamines enneaphyllos (the roots and rhizomes of *Cardamine enneaphyllos*). Therefore, different chromatographic methods were developed: TLC, HPLC, and GC. These methods were applied to characterize and identify the main constituent in each plant part (see Figure 4.9).

The original article '*"Radix Saniculae": Phytochemical Characterization and Potential Adulteration of an Austrian Traditional Wound-Healing Agent*' was submitted for publication in **Plants** in November 2024.

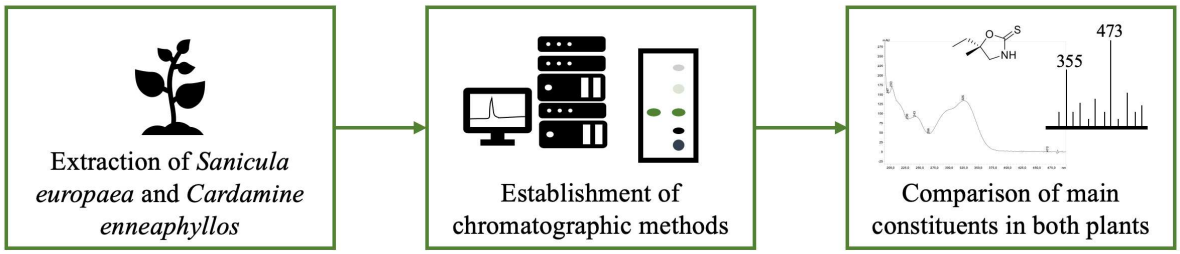

Figure 4.9 Workflow of the phytochemical comparison of sanicle and drooping bittercress using various chromatographic techniques.

Contributions

I contributed by conceptualizing the study, developing all chromatographic methods, performing phytochemical experiments, interpreting data, and drafting the manuscript.

Key findings

- Human blood was used to visualize triterpene saponins in Herba and Radix Saniculae based on the hemolysis of erythrocytes on a developed TLC plate.

- Using a C18 polar advantage column as the stationary phase, the established HPLC method, hyphenated with a DAD and an evaporative light scattering detector (ELSD), was able to show significant differences in the composition between the two plants (Figure 4.10).
- All three extracts (Herba Saniculae, Radix Saniculae, and Radix Cardamines enneaphyllos) contain high amounts of saccharides, such as fructose, glucose, and sucrose (Figure 4.10).
- With the aid of MS, the major compound classes in both parts of *Sanicula europaea* were found to be hydroxycinnamic acid derivatives, with rosmarinic acid as the main component, as well as triterpene saponins (Figure 4.10).
- In contrast, Radix Cardamines enneaphyllos contains the glucosinolates glucosisymbrin and glucocleomin. Using GC-MS, their breakdown products, sisymbrin and cleomin, were detected (Figure 4.10).

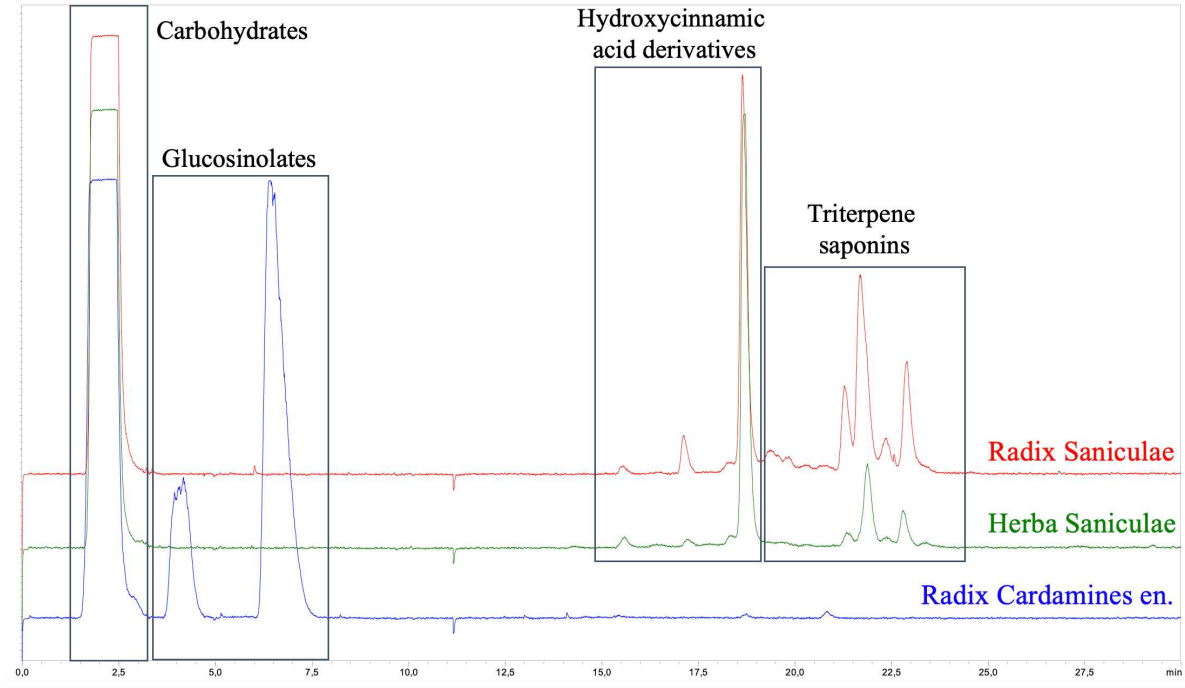

Figure 4.10 HPLC-ELSD chromatograms of the three investigated extracts. The main substance classes are marked with rectangles in the chromatogram.

Concluding perspectives

Both plants contain high levels of saccharides. The composition of the aerial parts of *Sanicula europaea* is similar to that of the underground parts. Therefore, similar effects can be expected, as Herba Saniculae has already been investigated in an *in vitro* assay regarding its wound-healing potential [56]. However, to explore the wound-healing effects and responsible substances of *Sanicula europaea* and *Cardamines enneaphyllos*, future research

should focus on the isolation, structure elucidation, and biological testing of the main components found in both plants.

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"Radix Saniculae": Phytochemical Characterization and Potential Adulteration of an Austrian Traditional Wound-Healing Agent

Elisabeth Eichenauer[1,2], Christina Sykora[1], Karin Ortmayr[1] and Sabine Glasl[1,*]

[1] Department of Pharmaceutical Sciences, Division of Pharmacognosy, University of Vienna, Josef-Holaubek-Platz 2, 1090 Vienna, Austria
[2] Vienna Doctoral School of Pharmaceutical, Nutritional and Sport Sciences, University of Vienna, Josef-Holaubek- Platz 2, 1090 Vienna, Austria
* Correspondence: sabine.glasl@univie.ac.at; Tel.: +43-1-4277-55207

Abstract: The aerial parts (Herba Saniculae) and the underground parts (Radix Saniculae) of *Sanicula europaea* (sanicle) have been used traditionally in Austrian folk medicine to treat wounds. Interestingly, in the Austrian vernacular, "Radix Saniculae" can also refer to the underground parts of *Cardamine enneaphyllos*. This ambiguity can lead to mistakes in using these two plants and, importantly, adulterations. The present work aims to shed light on using Radix Saniculae as a wound-healing agent. Thus, the main components in the aerial and the scarcely investigated underground parts of *Sanicula europaea* were identified and compared to the underground parts of *Cardamine enneaphyllos*. For this purpose, different analytical techniques were employed: TLC, HPLC-DAD/ELSD, UHPLC-ESI-MS, and GC-MS. The main components in both *Sanicula* and *Cardamine* plant extract were saccharides. Both parts of *Sanicula europaea* showed similar compositions: hydroxycinnamic acid derivatives and triterpene saponins. In contrast, the underground parts of *Cardamine enneaphyllos* contain two glucosinolates and their breakdown products. These findings suggest the same wound-healing activity for the underground parts of *Sanicula europaea* as was already found for its aerial parts. The glucosinolates detected in *Cardamine enneaphyllos* substantiate its use in wound healing. Nevertheless, the presented analytical methods allow easy discovery of adulterations.

Keywords: Austrian traditional medicine; wound-healing plants; *Sanicula europaea*; *Cardamine enneaphyllos*; phytochemical composition

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1. Introduction

The treatment of acute and chronic wounds benefits from multicomponent mixtures such as plant extracts, which have been used for hundreds of years in traditional medicine. The broad spectrum of plant substance classes present in extracts of a single plant leads to a multi-targeted wound healing promotion via different pathways and mechanisms. With minimal side effects, good effectiveness, ubiquitous availability, and low costs, medicinal plants represent promising and necessary treatment alternatives to prevent wounds from becoming chronic [1–4].

In Austrian folk medicine, different plants have been used for centuries to treat wounds. The valuable knowledge about traditionally used plants in Austria was compiled in the so-called VOLKSMED database [5]. Using the indication "wound healing" as a filter in this database, the most frequently referred plants are *Hypericum* sp. (*H. maculatum* and *H. perforatum*), *Arnica montana,* and *Calendula officinalis*. One of the lesser-cited plants is *Sanicula europaea* L., wood sanicle (German: Sanikel), which has not yet been well investigated concerning its wound healing potential [5,6]. Sanicle belongs to the family of

Apiaceae and is native to Europe and northern Africa [7]. The aerial parts, as well as the underground parts, are mentioned in the VOLKSMED database for medicinal use. The rhizome and/or roots are traditionally prepared as an ointment using lard or butter or are extracted with oil and applied onto the wounds. The herb and/or leaves are primarily prepared as a tea to rinse and bath wounds, put directly onto the wounds, or as an ointment using lard [5]. In Austria, the herb of sanicle can be purchased in pharmacies, whereas the underground parts are not commercially available. The plant is used traditionally not only for wound healing but also in the treatment of gastrointestinal hemorrhages, catarrhs, and other lung diseases [8,9]. Rosmarinic acid, a hydroxycinnamic acid, was found to be one of the main components in the herb of sanicle [8,10]. Besides hydroxycinnamic acids, flavonoids, saccharides, tannins, coumarins, essential oil, and triterpene saponins are described in the aerial parts [8,10–12]. The herb of *Sanicula europaea* is known to possess anti-inflammatory, antioxidant, antiviral, and antimicrobial activities [10,13]. Although in an *in vitro* cell assay using 3T3 fibroblasts, an extract of the herb of sanicle showed no enhancement of wound closure, it was revealed to be beneficial as an antibiotic and debridement boosting agent [14]. In contrast, the roots of sanicle have not been well investigated to date and have only been tested once in a study by Vogl et al. [15], exhibiting an *in vitro* anti-inflammatory effect.

Considering the Austrian vernacular language, another plant could be meant by the German name "Sanikel", namely drooping bittercress (Brassicaceae) [16] (*Cardamine enneaphyllos* (L.) Crantz, old synonym *Dentaria enneaphyllos* L.; German: Neunblatt-Zahnwurz) [7]. Several different vernacular names from Austria and Bavaria, Germany, for drooping bittercress can be found in literature, all closely resembling the German name of *Sanicula europaea* "Sanikel": "Sanigl" [17], "Saunigl" [18–20], „Schanikel"[21], "Scharnikel" [21,22], „Scharniggl" [23], „Sanikl, Saunickel or Sauigl" [24] (Figure 1).

(**a**) (**b**) (**c**)

Figure 1. (**a**) Leaves of *Sanicula europaea* L. (Engl. wood sanicle, German: Sanikel); (**b**) Examples of Austrian vernacular names of drooping bittercress from literature (Fischer (2002) [19], Ricek (1981) [18], Smola (1958) [22], Vogl (1904) [24]); (**c**) Aerial parts of *Cardamine enneaphyllos* (L.) Crantz (old synonym *Dentaria enneaphyllos* L., Engl. drooping bittercress, German: Neunblatt-Zahnwurz).

Therefore, these vernacular names may lead to misinterpretation of the correct botanical source. Scientific reports from the early twentieth century describe the problem of the misleading name "Radix Saniculi/Saniculae" for the rhizome and roots of *Cardamine enneaphyllos*. In 1904, "Radix Saniculi" from drooping bittercress was equaled with "Radix Saniculae" from wood sanicle, leading to intense microscopic and first phytochemical investigations [23,24]. Even the Austrian field flora by Fischer et al. [17] states that "Radix Saniculae" is derived from *Cardamine enneaphyllos* instead of *Sanicula europaea*. Interestingly, the roots and rhizomes of drooping bittercress are also mentioned in the

VOLKSMED database as wound-healing agents. They are prepared as an ointment with lard or butter, or the powdered roots are directly applied to wounds [5]. In 1737, the external use of drooping bittercress for wound healing was described by Nicolas Lémery [25]. Furthermore, this plant has been used to treat colic or dysentery [20]. However, the phytochemical composition of *Cardamine enneaphyllos* has not been well investigated so far. Schultz and Wagner were the first authors to describe the presence of glucosinolates in the aerial parts of the plant [26]. In 1985, Jurenitsch et al. [20] published the identification of two substances isolated from the underground parts of drooping bittercress, namely cleomin and sisymbrin, and their genuine glucosinolates glucocleomin and glucosisymbrin.

The phytochemical composition of Herba Saniculae and some bioactivities related to wound healing are already known [8–14]. Therefore, this work's first aim was to compare the aerial and underground parts of *Sanicula europaea* to find differences and similarities in their phytochemical composition. Since the preparation method of the underground parts of *Sanicula europaea* in the VOLKSMED database resembles the preparation method of the roots and rhizomes of *Cardamine enneaphyllos*, the second aim of this work was to compare the composition of these two different plants, implying the development of respective chromatographic systems. The data gained is essential for quality control and for detecting potential adulterations. Furthermore, once the main constituents of all extracts were known, a literature search was done to identify the possible effects of these compounds contributing to a wound-healing effect. These results should shed light on the topical use of Radix Saniculae in Austrian folk medicine.

2. Results

2.1. Thin-Layer Chromatography (TLC)

A first comparison of all methanolic extracts was done using TLC. In the more apolar system A (Figure S1), Radix and Herba Saniculae exhibited similar light spots, with more intensive red fluorescent chlorophyll spots in the aerial than in the underground parts. Radix Cardamines enneaphyllos required a more polar system since the sample components remained at the starting line, similar to a large fraction of unresolved compounds in both sanicle extracts. Using the more polar system B (Figure S2), the same two spots could be found in all three extracts after derivatization with anisaldehyde/sulfuric acid: a dark green spot at Rf 0.31 and another just below, at Rf 0.23. Using pure substances, these spots were identified as disaccharides such as sucrose (Rf 0.23) and monosaccharides such as glucose and fructose (Rf 0.31). Furthermore, the extracts of *Sanicula europaea* showed similar purple spots at Rf 0.57 and Rf 0.62 after derivatization with anisaldehyde, indicating terpenoids [27]. Since it is known that sanicle contains saponins [8], another derivatization method was conducted to verify the suspicion that these purple spots were saponins. Therefore, the characteristic hemolytic activity of saponins [28] was tested using erythrocytes in human blood to make the respective spots on the TLC plate visible (Figure 2). After applying blood gelatine onto the TLC plate and incubating for 4 to 6 hours, the saponins could be seen as whitish spots on the red blood gelatine. This experiment confirmed that the purple spots after derivatization originated from saponins and that this substance class is present in both aerial and underground parts of sanicle. In contrast, in the extract of *Cardamine enneaphyllos*, no spots other than those of the carbohydrates were detected using this mobile phase system and anisaldehyde/sulfuric acid as derivatization reagent. Therefore, another chromatographic technique had to be used to find other constituents.

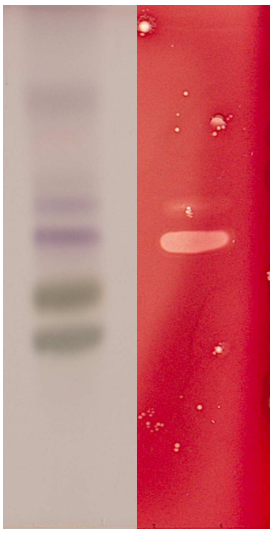


Figure 2. TLC of the extract Herba Saniculae in system B derivatized with anisaldehyde (left) and blood gelatin (right).

2.2. High-Performance Liquid Chromatography - Diode Array Detection/Evaporative Light Scattering Detector (HPLC-DAD/ELSD) and Ultra High-Performance Liquid Chromatography - Electrospray Ionization Mass Spectrometry (UHPLC-ESIMS)

The next step for further characterization and identification of main components or compound classes was an analysis via HPLC-DAD/ELSD. An HPLC method using a C18 Polar Advantage column was developed, providing broad selectivity for compounds ranging from nonpolar to moderately polar. Moreover, two different detectors were used to maximize the information gain: a diode array detector to record the UV spectra of different substances and a universal ELSD to deduce semi-quantitative information about the content of various components. From the ELSD trace, only the main components of each extract were chosen for further analysis, characterization, and putative identification (Figure 3). The TLC experiments have already suggested that all extracts contained large amounts of saccharides, which did not interact with the C18 Polar Advantage stationary phase. Consistently, the HPLC-ELSD chromatogram showed a large signal corresponding to compounds that elute in the dead volume as peak **1**, most likely different sugars. Besides these carbohydrates, the methanolic extract of *Cardamine enneaphyllos* contained only two more peaks of interest, peak **2** at Rt 4.18 min and peak **3** at Rt 6.14 min. A completely different pattern could be found for both sanicle extracts, where aerial and underground parts showed a qualitatively similar pattern. Peaks **2**, **3,** and **4** occurred as minor components, even though **3** appeared in a higher concentration in the underground than in the aerial parts. Peak **5** was the main component in Radix and Herba Saniculae extracts. In the later-eluting fraction, peaks **6-11** were detected in Radix Saniculae and peaks **6-10** in Herba Saniculae. These peaks seemed similar or related in both plant parts; however, they had a higher content in the underground than in the aerial parts.

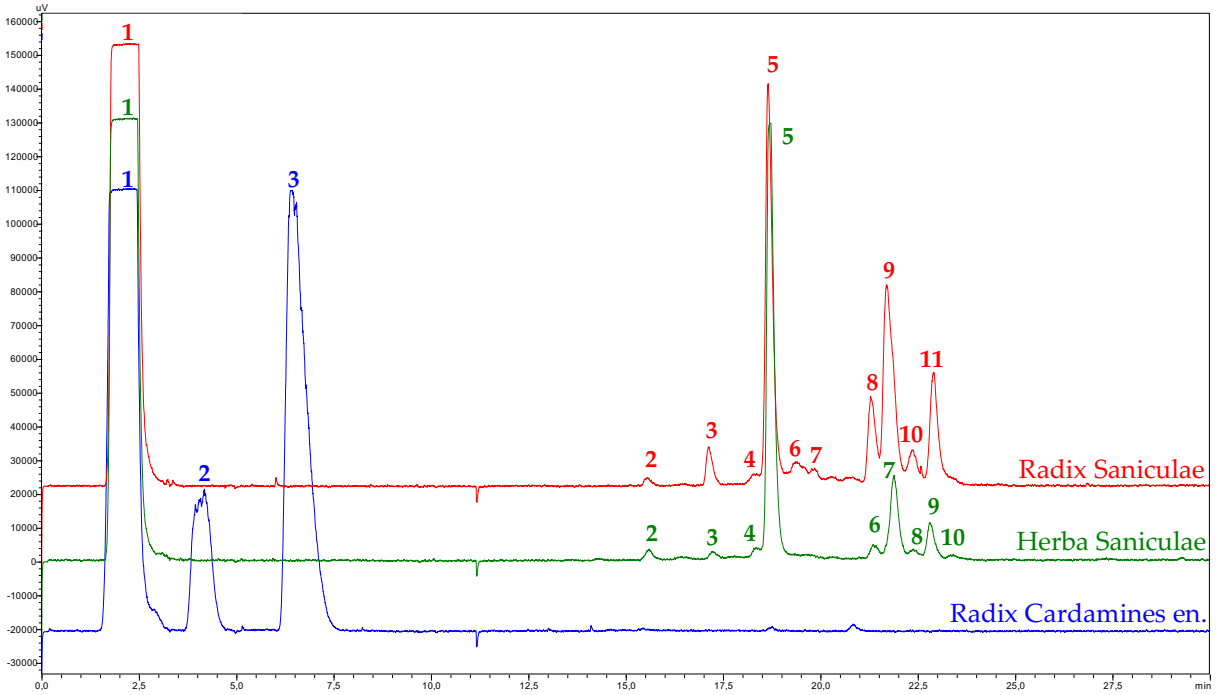


Figure 3. HPLC-ELSD chromatogram of all methanolic extracts (stationary phase: C18 Polar Advantage; mobile phase: water + 0.1% formic acid, acetonitrile + 0.1% formic acid).

Mass spectrometry (UHPLC-ESIMS) was chosen as a further analytical tool to gain more information about the major components detected by HPLC-ELSD. Of note, although the same method was used for the UHPLC as for the HPLC analyses, the retention times slightly deviated due to the different instrumentations used (UHPLC-CAD chromatograms see Figure S1-S3). However, the peak pattern from the UHPLC-CAD corresponded to the respective HPLC-ELSD chromatograms in all three extracts, confirming identical chromatographic performance. A mass spectrum was extracted for each prominent peak mentioned above, and mass signals were searched for corresponding to already known constituents in sanicle or related family members. Annotated compounds were identified by co-chromatography with pure reference substances. A summary of the characterization of the main components labeled by numbers in the HPLC-ELSD chromatogram (Figure 3) of each extract can be found in Tables 1 and 2 for the extracts of *Sanicula europaea* and Table 3 for the extract of *Cardamine enneaphyllos* (respective UV and mass spectra see Tables S1-S3). In cases where pure substances for final identification were unavailable, the detected constituents are given as belonging to a "compound class" or are indicated as "putatively assigned" (Table 1-3), implying that further fractionation and purification of components for structure elucidation must be done in future work.

Table 1. Tentative annotation of main components and compound classes found in Radix Saniculae using HPLC-DAD/ELSD and UHPLC-ESIMS.

Peak Nr.[1]	Rt. [min][1]	UV max [nm][1]	*m/z* [neg.]	*m/z* [pos.]	Putatively assigned compound / compound class
1	2.29	nd	341.31 $[M-H]^-$, 387.27 $[M+FA-H]^-$, 683.42 $[2M-H]^-$, 729.20 $[2M+FA-H]^-$, 1025.27 $[3M-H]^-$, 1071.15 $[3M+FA-H]^-$	365.37 $[M+Na]^+$, 381.30 $[M+K]^+$, 707.37 $[2M+Na]^+$, 723.15 $[2M+K]^+$	Sucrose[2] (sugars)

2	15.55	203, 243, 326	191.15 $[M-H]^-$ Quinic acid, 353.27 $[M-H]^-$, 707.34 $[2M-H]^-$, 1061.12 $[3M-H]^-$	355.24 $[M+H]^+$, 377.32 $[M+Na]^+$	Chlorogenic acid[2]
3	17.12	200, 329	161.09 $[M-H]^-$ Glucopyranose, 359.36 $[M-H]^-$ Rosmarinic acid, 521.40 $[M-H]^-$, 1043.39 $[2M-H]^-$	163.05 $[M+H]^+$ Glucopyranose, 361.11 $[M+H]^+$ Rosmarinic acid, 523.16 $[M+H]^+$, 721.12 $[2M+H]^+$ Rosmarinic acid, 1045.06 $[2M+H]^+$	4-O-ß-D-glucopyranosyl rosmarinic acid (or another rosmarinic acid derivative)
4	18.36	203, 241, 326	353.33 [M-Caffeoyl-H]$^-$, 515.35 $[M-H]^-$	499.38 $[M-H_2O+H]^+$, 517.20 $[M+H]^+$	3,4-Dicaffeoylquinic acid[2]
5	18.65	196, 212, 329	359.29 $[M-H]^-$, 719.32 $[2M-H]^-$, 1079.05 $[3M-H]^-$	361.14 $[M+H]^+$, 721.13 $[2M+H]^+$, 1081.07 $[3M+H]^+$	Rosmarinic acid[2]
6	19.37	202, 328	1099.87 $[M-H]^-$	1101.49 $[M+H]^+$	Saponins
7	19.84	203, 323	925.83 $[M-H]^-$	927.48 $[M+H]^+$	Saponins
8	21.29	203, 329	1099.91 $[M-H]^-$	1101.37 $[M+H]^+$	Saponins
9	21.69	205	967.93 $[M-H]^-$	969.42 $[M+H]^+$	Saponins
10	22.35	203, 322	969.86 $[M-H]^-$	971.17 $[M+H]^+$	Saponins
11	22.91	205	909.88 $[M-H]^-$	911.42 $[M+H]^+$	Saponins

[1] Derived from the HPLC-DAD/ELSD experiments; [2] Identified using pure reference substance, other substances only tentatively assigned

Table 2. Tentative annotation of main components and compound classes found in Herba Saniculae using HPLC-DAD/ELSD and UHPLC-ESIMS.

Peak Nr.[1]	**Rt. [min]**[1]	**UV max [nm]**[1]	***m/z* [neg.]**	***m/z* [pos.]**	**Putatively assigned compound/ compound class**
1	2.29	nd	341.31 $[M-H]^-$, 387.25 $[M+FA-H]^-$, 683.41 $[2M-H]^-$, 729.20 $[2M+FA-H]^-$, 1025.23 $[3M-H]^-$, 1071.20 $[3M+FA-H]^-$	365.36 $[M+Na]^+$, 381.28 $[M+K]^+$, 707.21 $[2M+Na]^+$, 723.13 $[2M+K]^+$	Sucrose[2] (sugars)
2	15.60	202, 242, 325	191.11 $[M-H]^-$ Quinic acid, 353.29 $[M-H]^-$, 707.39 $[2M-H]^-$, 1061.03 $[3M-H]^-$	355.24 $[M+H]^+$, 377.30 $[M+Na]^+$	Chlorogenic acid[2]
3	17.24	200, 283, 324	161.08 $[M-H]^-$ Glucopyranose, 359.31 $[M-H]^-$ Rosmarinic acid, 521.39 $[M-H_2O-H]^-$, 539.34 $[M-H]^-$	163.06 $[M+H]^+$ Glucopyranose, 361.22 $[M+H]^+$ Rosmarinic acid, 541.30 $[M+H]^+$	Rosmarinic acid derivative
4	18.35	201, 244, 328	353.29 [M-Caffeoyl-H]$^-$, 515.36 $[M-H]^-$	499.36 $[M-H_2O+H]^+$, 517.15 $[M+H]^+$	3,4-Dicaffeoylquinic acid[2]
5	18.70	196, 211, 329	359.28 $[M-H]^-$, 719.27 $[2M-H]^-$, 1079.04 $[3M-H]^-$	361.12 $[M+H]^+$, 721.09 $[2M+H]^+$, 1081.09 $[3M+H]^+$	Rosmarinic acid[2]
6	21.35	203, 323	1099.89 $[M-H]^-$	1101.26 $[M+H]^+$	Saponins
7	21.89	201	1101.91 $[M-H]^-$	1085.38 $[M-H_2O+H]^+$, 1103.34 $[M-+H]^+$	Saponins
8	22.37	203, 315	969.83 $[M-H]^-$	953.16 $[M-H_2O+H]^+$, 971.15 $[M+H]^+$	Saponins
9	22.90	196	1001.89 $[M-H]^-$	1003.35 $[M+H]^+$	Saponins
10	23.41	202, 322	969.85 $[M-H]^-$	971.42 $[M+H]^+$	Saponins

[1] Derived from the HPLC-DAD/ELSD experiments; [2] Identified using pure reference substance, other substances only tentatively assigned

As the prior analyses suggested, the first eluting peak **1** consisted of saccharides such as sucrose. They showed no interaction with the stationary phase and rushed through the system after injection in the dead volume. In both sanicle extracts, these sugars

represented the main components. Besides these carbohydrates, the extracts of aerial parts and the underground parts contained two main substance classes: hydroxycinnamic acid derivatives (peaks **2**-**5**) and saponins (peaks **6**-**11**). The main component in both extracts was peak **5**, which could be identified as rosmarinic acid (Figure 4). Furthermore, peak **2** consisted of chlorogenic acid in Herba and Radix Saniculae (Figure 4). A literature search using SciFinder® suggested 4-O-ß-D-glucopyranosyl rosmarinic acid with a molecular weight of 522 g/mol as a possible substance detected as peak **3** in Radix Saniculae. Consistent with this suggestion, signals were detected at m/z values of 521.40 $[M-H]^-$ and 523.16 $[M+H]^+$, indicating a molecule with the matching molecular weight. Furthermore, signals matching the m/z values of rosmarinic acid and glucopyranose were detected at the same retention time, likely originating from hydrolysis during the ionization process in MS analysis. In Herba Saniculae, peak **3** seemed to correspond to a rosmarinic acid derivative, as again, the *m*/*z* values of rosmarinic acid and glucopyranose were detected. However, this derivative appeared to have a molecular weight of 540 g/mol according to the *m*/*z* values observed in positive and negative mode, respectively, suggesting a hydroxylated rosmarinic acid derivative. Again, further fractionation, purification, and structure elucidation of the substance corresponding to peak **3** must be done to identify the respective rosmarinic acid derivative. Peak **4** in both extracts was identified as 3,4-dicaffeoylquinic acid.

As already observed in TLC analysis, the other substance class contained in both parts of sanicle was triterpene saponins. In both extracts, substances with a molecular weight of 1100 g/mol were detected, likely corresponding to one or more already published saponins like saniculoside N or saniculasaponin III [10,29]. The other detected masses in sanicle were also likely saponins due to their molecular weight (910 g/mol, 926 g/mol, 968 g/mol, 970 g/mol, and 1102 g/mol) and their presence in the purple, hemolytic spot. However, they could not be assigned, necessitating further fractionation, purification, and structure elucidation for final identification.

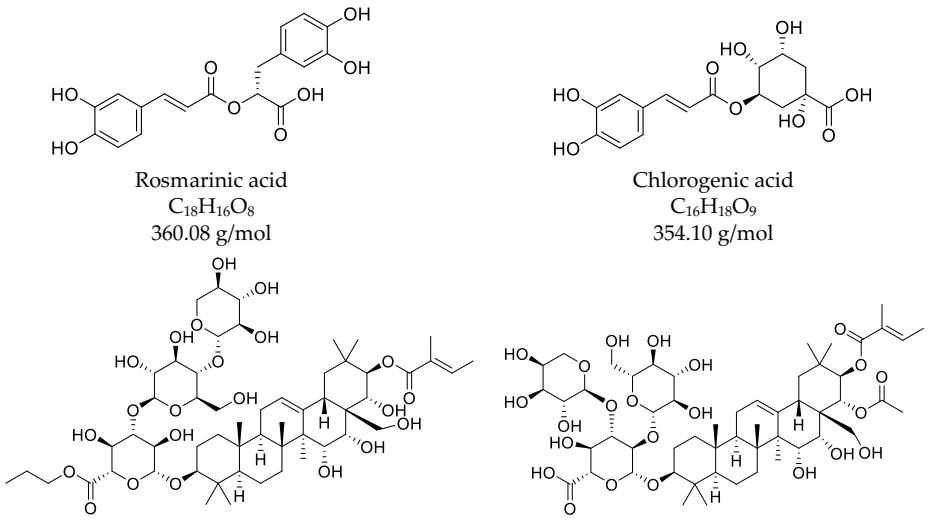


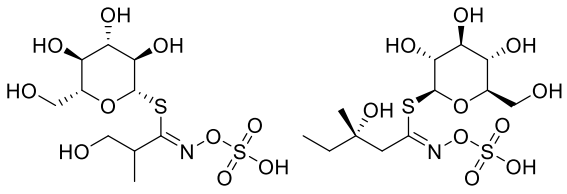
Figure 4. Structural and molecular formulas, and monoisotopic masses of the hydroxycinnamic acid derivatives rosmarinic acid (upper left) and chlorogenic acid (upper right), and the saponins saniuloside N (lower left) and saniculasaponin III (lower right).

Table 3. Tentative annotation of main components and compound classes found in Radix Cardamines en. using HPLC-DAD/ELSD and UHPLC-ESIMS.

Peak Nr.[1]	Rt. [min][1]	UV max [nm][1]	*m/z* [neg.]	*m/z* [pos.]	Putatively assigned compound / compound class
1	2.29	nd	341.33 [M-H]$^-$, 387.27 [M+FA-H]$^-$, 683.45 [2M-H]$^-$, 729.23 [2M+FA-H]$^-$, 1025.26 [3M-H]$^-$, 1071.23 [3M+FA-H]$^-$	365.35 [M+Na]$^+$, 381.32 [M+K]$^+$, 707.37 [2M+Na]$^+$, 723.19 [2M+K]$^+$	Sucrose[2] (sugars)
2	4.18	196	376.26 [M-H]$^-$, 753.17 [2M-H]$^-$, 1129.94 [3M-H]$^-$	378.24 [M+H]$^+$, 755.02 [2M+H]$^+$, 1132.05 [3M+H]$^+$	Glucosisymbrin (glucosinolate)
3	6.41	230	404.31 [M-H]$^-$, 809.20 [2M-H]$^-$, 1214.00 [3M-H]$^-$	406.25 [M+H]$^+$, 811.07 [2M+H]$^+$, 1216.04 [3M+H]$^+$	Glucocleomin (glucosinolate)

[1] Derived from the HPLC-DAD/ELSD experiments; [2] Identified using pure reference substance, other substances only tentatively assigned

Besides the already known carbohydrates in peak **1**, two main components were putatively found in the underground parts of *Cardamine enneaphyllos*: the glucosinolates glucosisymbrin as peak **2** and glucocleomin as peak **3** (Figure 5). The detected molecular ion peaks (*m/z* 376.26 [M-H]$^-$ and 378.24 [M+H]$^+$ for glucosisymbrin; *m/z* 404.31 [M-H]$^-$ and 406.25 [M+H]$^+$ for glucocleomin) fitted with the information on these glucosinolates published by Jurenitsch et al. in 1985 [20]. To verify the presence of glucosisymbrin and glucocleomin in the extract, GC-MS was used to search for their breakdown products, sisymbrin and cleomin.



Glucosisymbrin
$C_{10}H_{19}NO_{10}S_2$
377.05 g/mol

Glucocleomin
$C_{12}H_{23}NO_{10}S_2$
405.08 g/mol

Figure 5. Structural and molecular formulas and monoisotopic masses of the glucosinolates glucosisymbrin (left) and glucocleomin (right).

2.3 Gas Chromatography-Mass Spectrometry (GC-MS)

UHPLC-ESIMS analysis showed the possible presence of the glucosinolates glucosisymbrin and glucocleomin. Therefore, a GC-MS analysis was done with a dichloromethane (DCM) extract of the fresh, cut underground parts. In intact plant tissue, glucosinolates are separated from enzymes called myrosinase. Whenever the plant cells are destroyed, for example, by chewing or cutting, the enzyme hydrolyzes the glucosinolates, which leads to different breakdown products, such as isothiocyanates, thiocyanates, or nitriles [30]. These products can be easily analyzed using gas chromatography. Therefore, identifying the breakdown products (sisymbrin and cleomin) was crucial for determining the genuine glucosinolates.

The total ion chromatogram is depicted in Figure 6a, sisymbrin eluted at 25.72 min, cleomin at 28.46 min. The mass spectra found for sisymbrin and cleomin matched those recorded by Jurenitsch et al. [20]: Sisymbrin shows a base peak at *m/z* 117 and a fragment at *m/z* 102, which corresponds to the cleavage of a methyl group (Figure 6b). Cleomin has a base peak at *m/z* 145, and eliminating methyl and an ethyl group leads to fragments at *m/z* 130 and *m/z* 116 (Figure 6c).

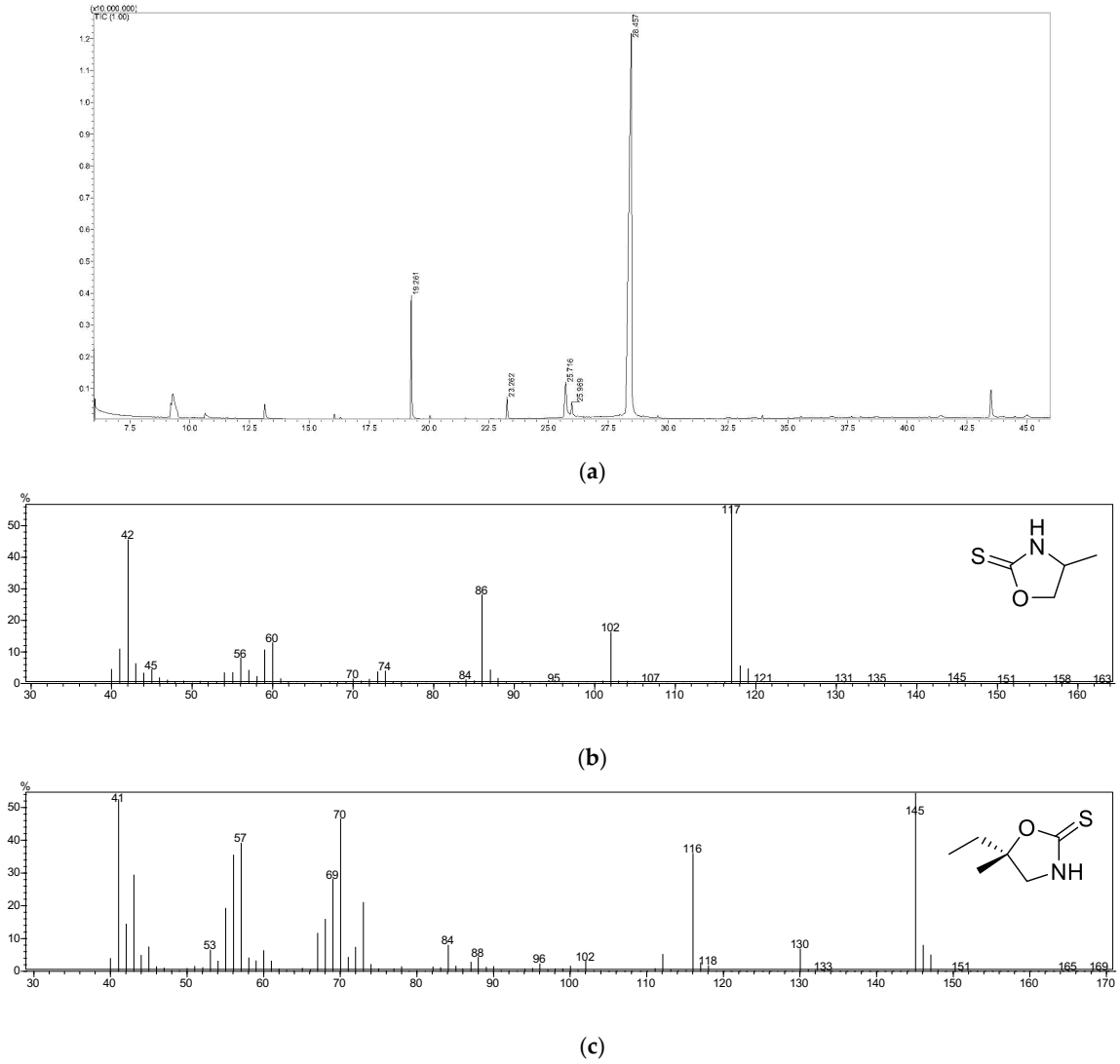


Figure 6. (**a**) Total ion chromatogram of the GC-MS analysis of the DCM extract of the fresh, cut underground parts of *Cardamine enneaphyllos* (sisymbrin at 25.72 min, cleomin at 28.46 min). Mass spectra and structural formula of (**b**) sisymbrin, and (**c**) cleomin.

3. Discussion

The aim of this work was the phytochemical characterization of methanolic extracts of Radix and Herba Saniculae, as well as Radix Cardamines enneaphyllos. All three extracts contained compounds with a potential bioactivity related to wound healing, albeit that they partially belong to different compound classes and act through different mechanisms. Commonalities in the chemical composition suggest similar bioactivities related to wound healing. The herb of *Sanicula europaea* has been tested in an *in vitro* wound-healing assay on fibroblasts. In this assay, sanicle showed cytotoxic effects on cells along the wound margin and, therefore, might promote wound healing due to wound debridement and as an antibiotic agent. However, the responsible substances have not been investigated in this study [14].

The only chemical similarity between all three extracts was the high amount of carbohydrates, such as sucrose, glucose, and fructose. Sugars are well known for their wound-healing potential, e.g., as medicinal honey. An osmotic gradient is created when

high sugar concentrations are applied to a wound. This leads to a fluid flow from subdermal tissue, which cleans the wound from debris and microorganisms. Furthermore, sugars have a low water activity, inhibiting the growth of bacteria in the wound [31]. High carbohydrate contents might, therefore, equip the extracts with antibacterial and debriding properties.

Besides these carbohydrates, the aerial and underground parts of sanicle are composed very similarly. Two main substance classes were identified: hydroxycinnamic acid derivatives, with rosmarinic acid as the main component in both plant parts, and triterpene saponins. Rosmarinic acid and chlorogenic acid were already described as constituents of *Sanicula europaea* [8,11], whereas 4-O-β-D-glucopyranosyl rosmarinic acid has only been found in *S. lamelligera* [32]. 3,4-Dicaffeoylquinic acid, or derivatives with other substitution patterns, have not yet been identified in the genus *Sanicula*, but in other genera in the family of Apiaceae, such as *Peucedanum ostruthium*, which is also used as wound-healing agent in traditional medicine [33]. The detected hydroxycinnamic acid derivatives possess antibacterial, antioxidative, and anti-inflammatory activities, which benefit wound healing [3,34–38]. Küba et al. [39] compared the *in vitro* wound-healing effect of a 10% rosmarinic acid cream to a 5% dexpanthenol group on wounds in Wistar albino rats. This study resulted in a significantly higher wound size reduction in the rosmarinic acid group than in the dexpanthenol and control group. An ointment containing chlorogenic acid accelerated wound healing by proliferative, antibacterial, and antioxidant effects in infected wounds in diabetic rats [38]. Moreover, Chen et al. [40] showed an *in vitro* wound healing boosting effect of a 1% chlorogenic acid ointment due to increased collagen synthesis and antioxidant activity on excision wounds in Wistar rats.

The second substance class found in the aerial parts and in the roots and rhizomes of *Sanicula europaea* was triterpene saponins. Molecular ion peaks at *m*/*z* 1099 $[M-H]^-$ and 1101 $[M+H]^+$ were detected in both parts of sanicle, indicating a saponin with a molecular weight of 1100 g/mol. Saniculoside N, a triterpene saponin glycoside with anti-HIV activity, has already been isolated from the aerial parts of *Sanicula europaea* [10] and, therefore, might be one of the detected molecular ion peaks found in this work. Moreover, several other saponins with a molecular weight of 1100 g/mol have been identified in another *Sanicula* species, namely in *S. elata* var. *chinensis*: saniculasaponin II, III, and IV [29]. The other detected saponins could not be assigned to any published substances in the genus *Sanicula*. Nevertheless, the closely related genus *Eryngium* in the family Apiaceae contains triterpene saponins, including saniculasaponin II and III [41]. Various saponins were already published for different *Eryngium* species, some of which might match the detected $[M-H]^-$ peaks at *m*/*z* 925, 928, and 910 in this work [42]. These saponins are structurally closely related to those already published for *Sanicula*, with barrigenol A_1 or R_1 as aglycon [41–44]. Therefore, the saponins in *Eryngium* and *Sanicula* are derivatives of oleanane-type pentacyclic triterpenes. Barrigenol-like triterpenoids are known to exhibit anti-inflammatory and antimicrobial activity [45]. Oleanane-type triterpene saponins like glycyrrhizin have already been investigated in different *in vitro* wound healing assays, which underline the potential of this substance class as a wound-healing agent [46]. However, the presented phytochemical characterization of extracts facilitates further isolation and structure elucidation to identify hydroxycinnamic acids and saponins as the next step.

Furthermore, the use of the roots and rhizomes of *Cardamine enneaphyllos* instead of *Sanicula europaea* in Austrian folk medicine was investigated. On the one hand, the Austrian vernacular name "Saunigl" might be derived from the German word for hedgehog, "Igel" due to the quill-like appendages found on the rhizome of drooping bittercress [18]. Therefore, the morphological appearance and colloquial language might have been the reason for the misleading use of the term "Radix Saniculae". On the other hand, the chemical composition of the underground parts of *Cardamine enneaphyllos* has not yet been intensively investigated. Jurenitsch et al. [20] isolated glucosinolates and their breakdown products while hunting for alkaloids. However, members of the Brassicaceae family also contain hydroxycinnamic acids and flavonoids, which were already described for *Sanicula*

europaea [8,47]. Therefore, not only the vernacular term but also commonalities in the phytochemical composition and wound-healing effects could explain the use of different botanical sources for "Radix Saniculae".

Yet, comparing the phytochemical composition, it became clear that only the high sugar content was similar between these two plants. However, even though carbohydrates are beneficial in wound healing, as mentioned above, the VOLKSMED database cites fat- or oil-based preparations for the use of "Radix Saniculae". Therefore, the role of sugars in these wound-healing applications might be negligible. But even if carbohydrates play a minor part in the wound-healing effect of Radix Cardamines enneaphyllos, the other detected substances also reveal promising wound-healing activities. Glucosinolate breakdown products are well known for their anticancer effect but also possess potent antioxidant and antimicrobial activities, which play an essential role in wound healing [48]. The hydrolytic product cleomin has been investigated regarding its antinociceptive bioactivity mediated by muscarinic and $GABA_B$ receptors [49]. Literature on the bioactivities of sisymbrin is missing. However, examining the effects of these compounds is crucial for substantiating the use of drooping bittercress in wound healing and will be anticipated in future work.

4. Materials and Methods

4.1 Plant material and extraction

Herba Saniculae was supplied by KOTTAS PHARMA GmbH (200 g, W20201762). The dried roots of sanicle were also provided by KOTTAS PHARMA GmbH (wild harvesting in Slovakia, Season 2022/23). The rhizomes and roots of *Cardamine enneaphyllos* were collected in April 2024 at Guglzipf, Berndorf, Lower Austria, Austria (47,93998°N, 16,11343°E) and identified by Elisabeth Eichenauer. Altogether, 134.1 g of underground parts were collected. The roots and rhizomes were ridden from dirt using a brush. 15.2 g were directly stored at -20°C for GC-MS analysis. The rest of the underground parts were washed with water to eliminate the remaining dirt, broken into smaller (1-3 cm long) pieces, and put into a rack dryer at 35°C for 48 h. The dried rhizomes yielded 29.1 g, which corresponds to a drying loss of 75.6%.

The underground parts of *Sanicula europaea* and *Cardamine enneaphyllos* were comminuted using an IKA Werke mill, whereas the aerial parts of *Sanicula europaea* were already purchased as cut herbal drug. 10.0 g of each plant part was suspended in 100 mL methanol (MeOH) and extracted for approximately 2 h at room temperature on a shaker. The resulting extracts were filtered and evaporated. The extracts were re-dissolved in MeOH to final concentrations of 10 mg/mL (TLC) or 5 mg/mL (HPLC/MS) for analysis via different chromatographic techniques. For comparison of the extracts via different chromatographic techniques, the extracts of *Sanicula europaea* were called "Radix Saniculae" for the underground parts and "Herba Saniculae" for the aerial parts, and the extract of the roots and rhizomes of *Cardamine enneaphyllos* got the name "Radix Cardamines enneaphyllos".

For GC-MS analysis, the fresh rhizomes of *Cardamine enneaphyllos* were used. After collection, they were stored at -20°C. To activate the enzyme myrosinase by the destruction of cellular integrity and gain the volatile degradation products of glucosinolates, 5 g of the rhizome were cut into small pieces with a knife and extracted with 50 mL dichloromethane (DCM) for approximately 1 h on a shaker at room temperature. After drying with anhydrous sodium sulfate and filtering the extract, the solvent was evaporated. The resulting extract was re-dissolved in 100 µL DCM, centrifuged, and diluted 1:50 with DCM before GC-MS analysis. All solvents used for extraction and chromatographic analysis were bought as analytical-grade solvents from VWR GmbH (Vienna, Austria).

4.2 Thin-Layer Chromatography (TLC)

For TLC experiments, Silica 60 F254 TLC plates (average particle size 9.5 to 11.5 µm, aluminum sheets with fluorescence indicator, Merck KGaA, Darmstadt, Germany) were used as stationary phase. 10 µL of each extract (10 mg/mL in MeOH) was applied. Two

different mobile phase systems were used: System A consisting of DCM-MeOH (9+1), and System B consisting of DCM-MeOH-10% formic acid (8+5+1). The CAMAG TLC Visualizer was used to photograph and interpret the developed and dried plates under white light and at wavelengths 254 nm and 366 nm. For improved visibility of constituents, the plates were derivatized with anisaldehyde/sulfuric acid, which was applied using the CAMAG Chromatogram Immersion Device (III) (CAMAG, Muttenz, Switzerland).

To detect hemolytic triterpene saponins, TLC plates were derivatized with blood gelatin. 5 gelatin sheets (approx. 9 g) of "Dr. Oetker Gelatine" (A07756/ 03) were left to swell in water for 30 minutes. Afterward, the sheets were removed from the water, squeezed, and blended in 115 mL phosphate buffer pH 7.4 as described in the Pharmacopoeia Europaea (250 mL KH_2PO_4-solution (0.2 mol/L) + 393.4 mL NaOH-solution (0.1 mol/L)). This mixture was heated to 40°C until the gelatin was completely dissolved. After that, 4 mL of human blood was added to 50 mL buffer gelatin. Finally, the blood gelatin suspension was filtered through cotton before approximately 5 mL was applied using a graduated pipette onto the developed TLC plate, which was framed with adhesive tape to form a tray. The suspension was distributed equally on the plate and left for 4-6 hours of development time. The final plates were photographed and compared to plated derivatized with anisaldehyde/sulfuric acid to identify the hemolytic spots and their corresponding behavior after reaction with anisaldehyde.

4.3 High-Performance Liquid Chromatography - Diode Array Detection/Evaporative Light Scattering Detector (HPLC-DAD/ELSD)

A Shimadzu instrumentation (Degasser DGU-20A 5, Auto Sampler SIL-20AC HAT, Liquid Chromatograph LC-20AD, Column Oven CTO-20AC, Diode Array Detector SPD-M20A, Low Temperature Evaporative Light Scattering Detector ELSD-LT, Communications Bus Module CBM-20A, Lab Solutions Software, Shimadzu, Kyoto, Japan) was used for HPLC analysis. An RP18 polar advantage column (Acclaim™ Polar Advantage II, C18, 3 µm, 150 mm x 2.1 mm) was used as the stationary phase, and the mobile phase was composed of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Gradient elution was utilized at a flow rate of 0.3 ml/min as follows: 0-5 min a plateau at 5% B, 5-8 min from 5 to 20% B, 8-13 min from 20 to 45% B, 13-25 min 45-70% B, followed by 5 minutes at 98% B and 10 minutes of re-equilibration at 5% B. As detectors a DAD as well as an ELSD were used. 5 µL of each extract dissolved to a final concentration of 5 mg/mL in MeOH were injected.

4.4 Gas Chromatography - Mass Spectrometry (GC-MS)

For the analysis of volatile compounds in the underground parts of *Cardamine enneaphyllos*, a Shimadzu instrumentation (GC-2010, coupled with a GCMS-QP2010 single quadrupole and an AOC-20s Autosampler; GCMS Solutions Software 4.11) was employed. As stationary phase, an OPTIMA-5 capillary column (Macherey-Nagel™, 50 m x 0.25 mm x 0.5 µm, Item number: 726099.50) was used, and the carrier gas was helium (ALPHAGAZ™ 1 He purity > 99.99%; flow rate 1.14 mL/min). After splitless injection of 2 µL of samples at 270°C, a temperature gradient was applied as follows: from 50°C to 270°C with 6°C/min, followed by an isothermic plateau at 270°C for 10 min. The scan area for the EI-QP (ion source temperature 250°C) was set to 40-500 m/z. For the identification of substances, the spectra were analyzed using the GCMS Solutions Software, and compared with library databases (Wiley 229, NIST 27, 147) and already published GC-MS spectra.

4.5 Ultra High-Performance Liquid Chromatography – Electrospray Ionization Mass Spectrometry (UHPLC-ESIMS)

An LTQ XL ion trap mass spectrometer was coupled to a Dionex UltiMate 3000 system (Thermo Fisher Scientific, CA) and used to further characterize the main components. For separation via UHPLC, the same method and parameters as for HPLC-DAD/ELSD experiments were employed. A HESI source (300°C heater temperature, 40/10/1 arb. units

for the sheath, aux, and sweep gases, respectively, and 3.5 kV spray voltage at 275°C capillary temperature) was used to achieve negative and positive ion mode ionization. The scans were performed with a *m*/*z* range from 100 to 1500 in positive mode and 110 to 2000 in negative mode. For data acquisition and evaluation, the Xcalibur software was utilized.

5. Conclusion

To conclude, the entries found for "Radix Saniculae" in the VOLKDMED database cannot be attributed to the correct botanical source with certainty. The underground parts of *Sanicula europaea* contain the same major substance classes as the aerial parts, which substantiates the traditional use of this plant part due to the already-known wound-healing effects of Herba Saniculae. However, using *Cardamine enneaphyllos* instead of sanicle due to vernacular names still holds beneficial properties in wound healing. Since "Radix Saniculae" is still used as a wound-healing agent in Austria, especially in rural areas, the established TLC and HPLC methods deliver a fast and easy way to determine the botanical source. Future research will concentrate on the isolation and structure elucidation of principal components and bioactivity testing to further explain the traditional use of these two plants in wound healing.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: TLC comparison of the extracts in system A after derivatization with anisaldehyde (left) at visible light, and without derivatization at 365 nm (right). Figure S2: TLC comparison of the extracts in system B after derivatization with anisaldehyde (left) at visible light, and without derivatization at 365 nm (right). Figure S3: UHPLC-ESIMS base peak chromatogram in positive mode (upper), in negative mode (middle) and CAD chromatogram (lower) of Radix Saniculae. Figure S4: UHPLC-ESIMS base peak chromatogram in positive mode (upper), in negative mode (middle) and CAD chromatogram (lower) of Herba Saniculae. Figure S5: UHPLC-ESIMS base peak chromatogram in positive mode (upper), in negative mode (middle) and CAD chromatogram (lower) of Radix Cardamines enneaphyllos. Table S1: UV spectra and mass spectra (negative mode upper spectrum, positive mode lower spectrum) of main peaks found in the extracts of Radix Saniculae. Table S2: UV spectra and mass spectra (negative mode upper spectrum, positive mode lower spectrum) of main peaks found in the extracts of Herba Saniculae. Table S3: UV spectra and mass spectra (negative mode upper spectrum, positive mode lower spectrum) of main peaks found in the extracts of Radix Cardamines enneaphyllos

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4.3 *Brassica oleracea* var. *capitata* L. f. *alba*

4.3.1 Manuscript II

White cabbage is not only a fundamental food source worldwide. It has also been used medicinally for centuries to treat rheumatic diseases, as a laxative, or in cancer prophylaxis [57]. Furthermore, fresh leaves are used to treat wounds [54,58]. This tradition has not yet been investigated regarding possible modes of action and responsible phytochemicals. Therefore, extracts were prepared according to the traditional application, characterized using bioactivity-guided fractionation, and subjected to *in vitro* bioassays monitoring the capability of enhancing keratinocyte growth or motility, as well as altering NF-κB and Nrf2-dependent reporter gene expression (see Figure 4.11).

The original manuscript '*White Cabbage as Wound-Healing Agent: Phytochemical Analysis and Bioactivity Screening*' is still in preparation. MS data need additional in-depth analysis for the final annotation of principal components. For this purpose, the generated MS/MS data will be analyzed using databases and tools such as MS-DIAL and Global Natural Products Social Molecular Networking (GNPS). Once the manuscript is finalized, it will be submitted to the **Journal of Ethnopharmacology**.

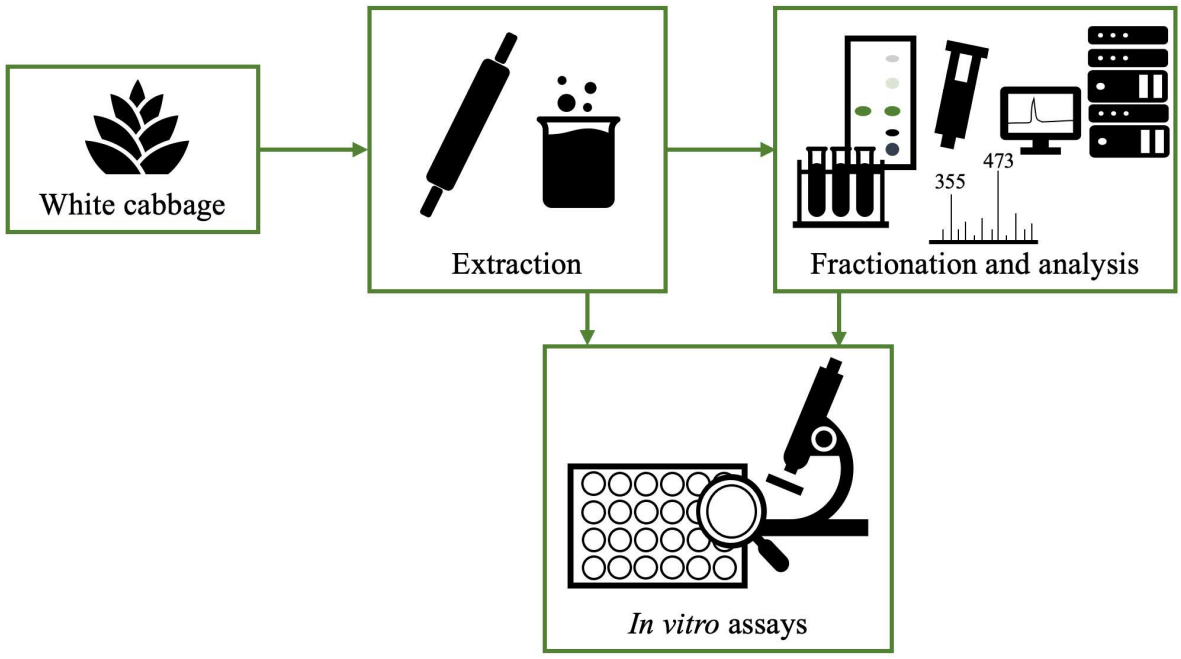


Figure 4.11 Workflow of the extraction according to the traditional application and subsequent bioactivity-guided fractionation of cabbage leaves.

Contributions

My contributions included conceptualizing the study, establishing chromatographic methods, performing phytochemical and keratinocyte-based cell experiments, interpreting data, and drafting the manuscript.

Key findings

- An aqueous and a dichloromethane (DCM) extract were prepared, as the polarity of these solvents reflects the conditions found in wounds. Crushing the leaves with a rolling pin before extraction affected the volatile compounds but did not influence the nonvolatile ones.
- Different chromatographic methods (solid phase extraction (SPE), flash chromatography (FC), TLC, HPLC-DAD/ELSD, GC-MS) were established to fractionate, analyze and characterize the extracts and respective fractions.
- The aqueous extract and a purified fraction thereof led to a significant increase in *in vitro* re-epithelialization. In contrast, the DCM extract and its fractions were significantly active in inducing the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and inhibiting the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transactivation activity upon tumor necrosis factor α (TNF-α)-stimulation.
- A fresh DCM extract was analyzed via GC-MS to identify the main volatile compounds, such as allyl isothiocyanate and cyclopropyl isothiocyanate.

Concluding perspectives

Various bioactivities, such as enhanced keratinocyte growth or motility, Nrf2 activation, and NF-κB transactivation inhibition, seem to support the wound-healing potential of fresh cabbage leaves. These effects can be attributed to a variety of phytochemicals. These results represent another example of the invaluable potential that multicomponent mixtures exhibit in wound healing due to their multitarget activities [59]. Future research will focus on the identification and structure elucidation of the most active components. A deeper understanding of the underlying mechanisms will help to revive this cost-effective, straightforward, and widely accessible wound-healing tradition.

White Cabbage as Wound-Healing Agent: Phytochemical Analysis and Bioactivity Screening

Elisabeth Eichenauer[a,b], Barbara Braunböck[a], Karin Ortmayr[a], Elke H. Heiss[a], Sabine Glasl[a,*]

[a] Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna, Josef-Holaubek-Platz 2, 1090 Vienna, Austria
[b] Vienna Doctoral School of Pharmaceutical, Nutritional and Sport Sciences, University of Vienna, Josef-Holaubek-Platz 2, 1090 Vienna, Austria
* Correspondence: sabine.glasl@univie.ac.at; Phone: +43-1-4277-55207

Abstract
Ethnopharmacological relevance: White cabbage is a widely used cruciferous vegetable for nutritional and medicinal reasons. It has been well investigated, also in the field of wound healing. However, applying fresh cabbage leaves as compresses remains underexplored. This study aims to investigate the phytochemical composition and wound-healing bioactivities associated with this traditional application.
Materials and methods: Aqueous and dichloromethane extracts of white cabbage leaves were prepared, fractionated, and analyzed using numerous chromatographic techniques (FC, SPE, TLC, HPLC, LC-MS, GC-MS). All extracts and fractions were analyzed in a series of *in vitro* assays covering bioactivities relevant to wound healing: re-epithelialization of keratinocytes, activation of Nrf2- and inhibition of NF-κB signaling.
Results: The aqueous extract and a derived purified fraction thereof enhanced *in vitro* re-epithelialization in a keratinocyte monolayer, reducing the cell-free area by 50% at 10 µg/mL in a scratch assay. The DCM extract and purified fractions (10 µg/mL) significantly activated Nrf2-dependent transcription, increasing it up to 9-fold of the control, and inhibited the TNF-α-stimulated NF-κB pathway by up to 98% in respective reporter gene assays. Moreover, the main identified volatile compounds in a fresh cabbage extract were different isothiocyanates and nitriles.
Conclusion: These results support the use of fresh cabbage leaves on wounds as their bioactive components promote keratinocyte growth or motility and modulate inflammatory or redox signaling pathways. Further investigations regarding the phytochemical profile and mechanisms of action will help revive this traditional application.

Keywords
Brassica oleracea var. *capitata* f. *alba*, White Cabbage, Wound Healing, Traditional Medicine, Phytochemistry, *In Vitro* Bioactivities

1. Introduction

Cruciferous vegetables, which belong to the family of Brassicaceae, have been a fundamental part of the human diet and have been cultivated worldwide for thousands of years. Today, the nutritional importance of these plants persists, with Brassicaceae crops including horseradish (*Armoracia rusticana*), cress (*Lepidium sativum*), and turnip (*Brassica rapa*). Among these, *Brassica oleracea* is probably the most important species, including cabbage (var. *capitata*),

broccoli (var. *italica*), and cauliflower (var. *botrytis*) (Al-Shehbaz, 2011; Zayed et al., 2023). In addition to their value as food sources, cruciferous vegetables are also cultivated for their medicinal properties, offering various health benefits, such as decreased risk of different cancer types or prevention of neurodegenerative and cardiovascular diseases (Björkman et al., 2011; Koss-Mikołajczyk et al., 2019).

One of the most important cruciferous vegetables as an affordable and commonly available source of potential health-promoting phytochemicals is white cabbage (*Brassica oleracea* var. *capitata* f. *alba*). Consumed raw, fermented, or cooked, white cabbage also holds medicinal potential: Cabbage juice has been traditionally used as a laxative, even dating back to ancient Egypt. Moreover, white cabbage has been used for treating headaches, rheumatism, and hoarseness, as well as for wound healing. Due to its economic significance, the phytochemical composition and *in vitro* bioactivities of white cabbage have already been well studied – the main substance classes found in *Brassica oleracea* var. *capitata* f. *alba* include primary metabolites (sugars and proteins), glucosinolates and their breakdown products, flavonoids, carotenoids, vitamins, and phenolic acids. These phytochemicals contribute to its diverse bioactivities, including anticancer, antioxidant, anti-inflammatory, antimicrobial, hypoglycemic, and hypolipidemic effects (Arrais et al., 2022; Šamec et al., 2017; Zayed et al., 2023).

The application of fresh cabbage leaves as bandages or compresses, the so-called "Kohlwickel", is a longstanding practice in Austrian folk medicine to treat wounds. Typically, the fresh leaves are squeezed, often with a rolling pin, before application to the affected area. Softening the leaves by cooking prior to use has also been reported (Eichenauer et al., 2024). Different species and varieties of *Brassica* sp., including white cabbage, kale, and red kale, have already been studied for their wound-healing properties. They are rich in pectin-type polysaccharides, which possess complement-fixing activity, possibly enhancing wound healing by boosting the innate immune system (Samuelsen et al., 2007; Westereng et al., 2006). Furthermore, cruciferous vegetables display high phospholipase D (PLD) activity, leading to phosphatidic acid (PA) production after tissue disruption, for example, by cutting, chewing, or homogenization. The enzyme phospholipase A_2 catalyzes the hydrolyzation of PA to lysophosphatidic acid (LPA), a phospholipid mediator known to promote the proliferation and migration of various cell types. This mechanism applies to the anti-ulcer effect observed after intake of cut cabbage leaves in damaged digestive tissue (Tanaka et al., 2009; Urikura et al., 2012). Since different phospholipase A_2 isoforms are found in human skin (Murakami et al., 2018), squeezed cabbage might also increase dermal LPA production, contributing to enhanced wound healing.

Several studies have already investigated the *in vivo* wound-healing effect of cabbage (*Brassica oleracea* var. *capitata*) and came to similar results: Topical application of 10% *Brassica oleracea* var. *capitata* glycolic extract to skin wounds accelerated wound healing in Wistar rats by enhanced collagen maturation (Gonçalves et al., 2013; Rebolla et al., 2013; Sarandy et al., 2015). Furthermore, a cream containing an aqueous extract of *Brassica oleracea* promoted the re-epithelialization and contraction of burn wounds in Sprague Dawley rats (Hassanzadeh et al., 2013). Recently, Miranda et al. tested ointments of 10% or 20% ethanolic *Brassica oleracea* var. *capitata* extract in third-degree burn wounds in Wistar rats. The ointments sped up

extracellular matrix formation, increased cell and blood vessel counts, and promoted the activity of antioxidant enzymes (Miranda et al., 2024).
However, the use of fresh cabbage leaves for wounds, their bioactivities, and responsible bioactive substances have yet to be deeply investigated. Therefore, this work addresses this gap by preparing extracts based on the traditional application method: Fresh leaves were squeezed using a rolling pin, incubated overnight, cut into small pieces, and extracted with water or dichloromethane. The resulting extracts were then tested in three different *in vitro* assays for their antioxidant, anti-inflammatory, and re-epithelialization-promoting effects, all contributing to wound healing (Ambrozova et al., 2017; Suarez-Arnedo et al., 2020). Bioactivity-guided fractionation allowed phytochemical characterization of the extracts, enhancing our understanding of the traditional application of fresh cabbage leaves on wounds.

2. Materials and Methods

2.1 Plant Material and Extraction

Seven heads of *Brassica oleracea* var. *capitata* f. *alba* were purchased from different local markets. They were all cultivated and harvested in Austria. The extraction method was based on the traditional way of applying the leaves as compresses: Fresh cabbage leaves were crushed using a rolling pin, put into a box, and incubated overnight in an incubator (Lucky Reptile Thermoelectric Cooler & Warmer) at 32-35°C. The next day, the leaves were defatted for 1-2 minutes in a hexane bath, cut into small pieces (approximately 1x1 cm), and extracted in a ratio of 1:5 with either water or dichloromethane (DCM) for 2 h on an orbital shaker. Afterward, the extract was filtered using a Büchner funnel. The DCM was evaporated using a rotary evaporator (yield: 0.052% of fresh weight (FW)), and the water was removed via lyophilization (yield: 4.06% of FW). Furthermore, dichloromethane extracts without incubation were prepared. For this purpose, fresh cabbage leaves were directly defatted, cut, and extracted with DCM, as mentioned above.
Accelerated solvent extraction (ASE) was done using a Dionex ASE 350 instrumentation (Thermo Scientific™). The fresh leaves were prepared the same way as mentioned above, but after cutting them into small pieces, they were filled into ASE extraction tubes. Different methods (standard, flow, and pressure) were used to extract the tubes with DCM. The temperature was generally set to 40°C, but the pressure method was also conducted at 60°C. The resulting extracts were dried using anhydrous sodium sulfate, and DCM was evaporated using a rotary evaporator (yield: 0.046% of FW).
A fresh DCM extract was prepared for GC-MS analysis: 20 g of leaves were crushed using a rolling pin, and 200 mL of DCM was directly added to the crushed leaves without cutting them. The mixture was extracted on a shaker for 60 min, filtered over vacuum, and dried with anhydrous sodium sulfate. Finally, the solvent was evaporated using a rotary evaporator. This fresh extract was redissolved in DCM and diluted 1:100 for the injection. All solvents used for extraction and chromatographic analysis were purchased as analytical-grade solvents from VWR GmbH (Vienna, Austria).

2.2 Chromatographic Methods

2.2.1 Thin Layer Chromatography (TLC)

TLC was used to compare fingerprints of extracts and fractions. Silica gel 60 F_{254} aluminum plates with an average particle size of 9.5 to 11.5 µm (Merck KGaA, Darmstadt, Germany)

were used as stationary phase for TLC experiments. The samples were dissolved in the appropriate solvent (MeOH, water, MeOH/water mixtures, DCM) and applied using a glass capillary. For more polar substances, DCM-methanol (MeOH)-1% formic acid (FA) (8+5+1) was used as the mobile phase. In contrast, DCM-MeOH (9+1) was used to analyze more apolar extracts and fractions. For interpretation, photos of the developed and dried TLC plates were taken under white light and at wavelengths 254 nm and 366 nm using the CAMAG TLC Visualizer (CAMAG, Muttenz, Switzerland). The plates were derivatized with an anisaldehyde/sulfuric acid reagent in the CAMAG Chromatogram Immersion Device (III) for improved visibility of substances.

2.2.2 Solid Phase Extraction (SPE)

Both the aqueous and the DCM extract were fractionated using SPE. A Supelco SPE chamber (Supelco® Visiprep SPE Vacuum Manifold DL) was chosen for both fractionations. C8 cartridges (VARIAN® Bond elute RP6, 6CC) were used to fractionate the aqueous extract. MeOH was used to condition the stationary phase, followed by equilibration to starting conditions with water. The extract was dissolved in water (10 mg/ 100 µL) and applied to the cartridge (200 µL were applied, 20 mg of extract W). For elution, a gradient was used: First, the cartridge was eluted three times with 6 mL of the mobile phase, beginning with water, then 50% MeOH, and lastly with MeOH. A rate of approximately one drop/sec was used for elution. Several SPE experiments were conducted to increase the overall yield, and the resulting collected fractions were combined to form three final fractions, for which a rotary evaporator was used to remove the solvents (yield in % of extract W: W_H_2O 95.2%, W_50% 2.7%, and W_MeOH 1.6%).

To fractionate the DCM extract, C18 cartridges (VARIAN® Bond elute, RP18, 6CC or 12CC) with a volume of 6 ml or 12 ml were used. First, the stationary phase was conditioned with MeOH and then equilibrated with water to the starting conditions. The cuticular waxes had to be removed prior to the fractionation. For this purpose, the extract was suspended in MeOH using an ultrasonic bath. The insoluble waxes were separated from the soluble part by centrifugation. This soluble part was then applied to the cartridges (20 mg in 200 µL on 6CC cartridges and 40 mg in 200 µL on 12CC cartridges) and eluted using a gradient. First, the cartridge was filled three times with 6 mL or 12 mL, respectively, of water, followed by 15% MeOH, 40% MeOH, 70% MeOH, and pure MeOH. The fractions were eluted at a rate of approximately one drop/sec and collected in the SPE chamber. Again, several SPE experiments were done, the resulting collected fractions were combined and yielded in the final five fractions, from which DCM was removed by evaporation using a rotary evaporator (yield in % of extract D: D_H_2O 14.62%, D_15% 14.62%, D_40%:14.85%, D_70% 14.62%, and D_MeOH 14.62%).

2.2.3 Flash Chromatography (FC)

Flash chromatography was applied further to SPE to gain higher yields of the aqueous extract fractions. It was performed on an Interchim Puriflash 4250 instrument equipped with a photodiode array (PDA) detector and an evaporative light scattering detector (ELSD). A phenyl column (Puriflash PF-15PHC4-F0004) was used as the stationary phase. The aqueous extract was applied via dry load, which means that the extract was bound on twice the amount of pure silica gel 60. Water and MeOH served as mobile phases and were run as a binary gradient: 0-30 min a plateau of pure water, 30-75 min from 0 to 95% MeOH, followed by a 15 min plateau

at 95% MeOH. Fractionation was guided automatically through the ELSD signal. After checking their composition via TLC, these fractions were combined according to their phytochemical composition, resulting in the SPE fractions W_H_2O, W_50%, and W_MeOH (yield in % of extract W: W_H_2O 94.4%, W_50% 1.37%, and W_MeOH 0.43%).

2.2.4 High-Performance Liquid Chromatography (HPLC) - Diode Array Detector (DAD)/ Evaporative Light Scattering Detector (ELSD)

The HPLC analyses were performed on a Shimadzu instrumentation (Degasser DGU-20A 5, Liquid Chromatograph LC-20AD, Auto Sampler SIL-20AC HAT, Column Oven CTO-20AC, Communications Bus Module CBM-20A, Lab Solutions Software, Shimadzu, Kyoto, Japan) equipped with a PDA detector (Diode Array Detector SPD-M20A, Shimadzu, Kyoto, Japan) and an ELSD (Low Temperature Evaporative Light Scattering Detector ELSD-LT). Reversed-phase C18 material was chosen as the stationary phase (LiChrosphere® 100 RP 18e 5 µm, 250 x 4mm, flow rate 1mL/min; or Acclaim™ 120 RP C18 3µm, 150 x 2.1 mm, flow rate 0.3 mL/min). Water + 0.1% formic acid (A), and acetonitrile + 0.1% formic acid (B) were used as mobile phase, and gradient elution was employed as follows: 0-40 min from 5% to 75% B, 40-45 min a plateau at 98% B, and 10 minutes of re-equilibration at 5% B. A volume of 5 µL or 10 µL, respectively, was injected into the smaller and larger columns.

2.2.5 Gas Chromatography (GC) - Electron Impact Mass Spectrometry (EI-MS)

A Shimadzu GC-MS instrumentation (GC-2010, coupled with a GCMS-QP2010 single quadrupole and an AOC-20s Autosampler; GCMS Solutions Software 4.11) was used for the analysis of volatile compounds in a fresh DCM extract of cabbage leaves. An OPTIMA-5 capillary column (Macherey-Nagel™, 50 m x 0.25 mm x 0.5 µm, Item number 726099.50) was used as the stationary phase. Helium (ALPHAGAZ™ 1 He, purity > 99.99%; flow rate 1.14 mL/min) was applied as the carrier gas. 2 µL of the respective sample were injected via splitless injection at 270°C. The separation of substances was achieved using a temperature gradient from 50°C to 270°C with an increase of 6°C/min and a 10 min isothermic plateau at 270°C. For detection, an electron impact ionization (EI)-single quadrupole (QP) with the scan area set to 40-500 m/z was used (ion source temperature 250°C). GCMS Solutions Software was used to analyze spectra. Furthermore, a comparison with library databases (Wiley 229, NIST 27, 147) was done. These databases were applied, and available pure substances were used for co-chromatography to identify substances. A semi-quantitative comparison of the content of certain substances in the different extracts was done by comparing the respective peak areas.

2.2.6 Ultra High-Performance Liquid Chromatography (UHPLC) - Electrospray Ionization Mass Spectrometry (ESI-MS)

The LC-MS instrumentation used in this work consisted of an UltiMate 3000 LC system equipped with a CAD and a DAD detector (Dionex UltiMate 3000 RS Pump, Dionex UltiMate 3000 RS Autosampler, Dionex UltiMate 3000 RS Column Compartment, Dionex UltiMate 3000 Diode Array Detector, Dionex UltiMate 3000 Corona Ultra RS; Software: Dionex Chromeleon XPress) hyphenated with a mass spectrometer (Thermo Scientific™ LTQ XL™ Linear Ion Trap Mass Spectrometer). The same LC method and parameters were applied for separation as for the HPLC experiments. Ionization of substances was achieved using an electrospray ionization (ESI) source (300°C heater temperature, 40/10/1 arb. units for the sheath, aux, and sweep gases, respectively, and 3.5 kV spray voltage at 275°C capillary

temperature). A m/z range from 100 to 1500 in positive mode and 110 to 2000 in negative mode was chosen for the scans. MS and MS/MS experiments were conducted. The Xcalibur software was used to acquire and evaluate data.

2.3 In Vitro Assays

2.3.1 Re-epithelialization-enhancing effect on keratinocytes

HaCaT keratinocytes (CLS Cell Lines Service GmbH, Eppelheim, Germany) were used as *in vitro* surrogate for the epidermal skin layer. They were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland), supplemented with 10% fetal bovine serum (FBS), streptomycin (100 µg/mL), benzylpenicillin (100 U/mL), and glutamine (2mM). Cell mass and metabolic activity were assessed using the crystal violet and resazurin assay, respectively, to determine non-cytotoxic concentrations of tested samples, as reported before (Czekanska, 2011; Feoktistova et al., 2016).

The wound healing assay was conducted as described by Goels et al. (2022). Briefly, after placing cross-shaped plastic cell spacers (LUX® tools, Wermelskirchen, Germany) into the wells of a 24-well plate, 1 mL of a HaCaT keratinocyte suspension (1.5 x 10^5 cells/mL) was added to each well, and the plates were incubated overnight. The next day, the cell spacers were removed, resulting in a confluent monolayer of cells disrupted by the cell-free area of the spacers. The cells were washed with PBS, and sample solutions in the desired concentrations were added. All samples were prepared as stock solutions in DMSO and diluted with DMEM containing 0.1% fetal bovine serum (FBS) to the final concentrations containing 1% DMSO. Lysophosphatidic acid (LPA) at 10 µM was the positive control, and 1% DMSO in DMEM containing 0.1% FBS served as vehicle control. Immediately after applying all test samples to the cells, photos were taken at the starting time (= t0), as well as after 24 h of incubation (= t24) using an Olympus camera (PEN Lite E-PL7, Hamburg, Germany). The image processing tool ImageJ with the macro "Wound Healing Tool" was used to assess the wound-healing activity as the reduction of the cell-free area (Schneider et al., 2012; Suarez-Arnedo et al., 2020). Each sample was tested in two wells in an experiment. Four photos were taken from each well, resulting in eight pictures per sample. For evaluation, the cell-free area at t0 was subtracted from the one at t24, resulting in the reduction of the cell-free area in 24 h. The area difference was then divided by the mean area difference of all vehicle control values. Subtraction of 1 and multiplication with 100 led to the reduction of cell-free area in % normalized to the vehicle control. These normalized values were used to calculate the final mean value of the respective sample.

$$\%\, reduction\ of\ cell-free\ area = \left(\frac{area\ t0_{Sample} - area\ t24_{sample}}{area\ t0_{VC} - area\ t24_{VC}} - 1\right) x\ 100$$

2.3.2 Nrf2 (ARE) dependent luciferase reporter gene assay

Stably transfected cells with HepG2-ARE-Luc (Signosis, Santa Clara, CA, USA) were cultivated at 37 °C and 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland), supplemented with benzylpenicillin (100 U/mL), streptomycin (100 µg/mL), glutamine (2 mM) and 10% fetal bovine serum (FBS).

Prior to seeding the cells for the assay, they were stained for 1 h in serum-free medium supplemented with 2 µM Cell Tracker Green CMFDA (Invitrogen, Waltham, MA, USA), a fluorescent probe used as an indicator for viable cell number. Cells were then plated in 96-well plates (2 × 104 cells/well) and incubated overnight. The following day, cells were treated with

test samples at the desired concentrations with respective solvent controls. 0.1% DMSO was usually used as a negative control, and the isothiocyanate iberin (5 µM) or the triterpene derivative CDDO-Im (10 nM; 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole) were used as positive controls with a well-known capacity of inducing Nrf2 signaling. After incubation for 16 h to 24 h, cells were lysed with a luciferase lysis buffer (Promega, Madison, WI, USA;), and the luminescence of the firefly luciferase and the fluorescence of the Cell Tracker Green CMFDA were measured by a Tecan Spark® microplate reader (Männedorf, Switzerland).

For quantification of induced Nrf2 activity by the test samples, the luciferase-derived signal from the Nrf2-dependent reporter gene was normalized by the fluorescence signal derived from Cell Tracker Green CMFDA to account for potential differences in cell number. Obtained values were referred to that of the solvent control, which was set as 1.

2.3.3 NF-κB-dependent luciferase reporter gene assay

HEK293 cells stably transfected with a NF-κB luciferase reporter gene (Panomics, RC0014) were cultivated at 37 °C and 5% CO2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) with 100 U/mL benzylpenicillin, 100 µg/mL streptomycin, 2 mM glutamine, and 10% fetal bovine serum (FBS).

Before seeding for the assay, cells were again stained for 1 h in serum-free medium supplemented with 2 µM Cell Tracker Green CMFDA (Invitrogen), a fluorescent probe used as an indicator for viable cell number. Cells were then plated in 96-well plates (4x104 cells/well). After 16-24 h, cells were pre-treated with test samples at the desired concentration. Parthenolide, 10 µM was used as the positive control, and 0.1% DMSO was usually used as a negative control. After 30 minutes, cells were stimulated with 2 ng/mL TNF-α for 4 h. Cells were then lysed with a luciferase lysis buffer (Promega), and the luminescence of the firefly luciferase and the fluorescence of the Cell Tracker Green CMFDA were measured by a Tecan Spark® microplate reader (Männedorf, Switzerland).

For quantification of TNF-induced NF-κB activity, the luciferase-derived signal from the NF-κB reporter was normalized by the fluorescence signal derived from Cell Tracker Green CMFDA to account for differences in cell number. Obtained values were referred to the solvent control, which was defined as 100% induction. Potential cytotoxicity of the test samples was excluded upon comparable Cell Tracker Green CMFDA fluorescence values between solvent-treated cells and cells treated with test samples.

2.3.4 Statistics

The re-epithelialization-boosting experiments were conducted in three independent biological replicates with eight technical replicates. The significance of this assay was defined as * $p < 0.05$ (Student's t-test). Both reporter gene assays were performed in at least three independent biological replicates. In these assays, significance was defined as ****$p < 0.0001$ (One-way ANOVA with Dunnett's post hoc test vs vehicle control). Statistical analysis was done using GraphPad Prism 9.

3. Results

3.1 Extraction

This work investigated the wound-healing potential of the traditional application of cabbage leaves as compresses. Traditionally, the fresh leaves of *Brassica oleracea* var. *capitata* f. *alba*

are crushed using a rolling pin, applied to the respective body part, and fixed using a bandage or something similar. This compress is left on the respective wound overnight (Droz et al., 1960; Eichenauer et al., 2024). Therefore, the extraction method was chosen as described in section 2.1: The fresh leaves were squeezed by a rolling pin and stored in an incubator at 32-35°C overnight to simulate the surface temperature of the human body. The next day, the leaves were cut into small pieces and extracted with either water or DCM. These solvents were chosen due to the differing polarities present in wounds. Both existing milieus, the aqueous exudate of an open wound, and parts of intact skin imply that polar as well as apolar substances might be part of the wound-healing effects of white cabbage. Polar substances were extracted with water, leading to the aqueous extract W (4.06 g/100 g fresh weight (FW)). In contrast, dichloromethane was used to extract the apolar substances. The yield of this dichloromethane extract D was immensely lower (0.0518 g/100 g FW), which can be explained by the high water content in fresh cabbage leaves (over 90%) (Luo et al., 2021). Accelerated solvent extraction (ASE) was chosen as an attempt to increase the yield of DCM extract. Different methods (standard, flow, pressure) and temperatures (40°C, 60°C) were tried. However, ASE did not lead to a higher yield of the DCM extract (0.0456 g/100g FW). Furthermore, we investigated the effect of treating the fresh leaves with a rolling pin to eventually save time during the extraction process with the loss of overnight incubation. For this purpose, leaves of the same cabbage heads were extracted with or without incubation. HPLC and TLC analysis of the non-volatile compounds revealed that all three extracted cabbage heads differed in the quantitative content of main components but showed similar qualitative composition. Likewise, incubating the crushed leaves before the extraction only led to different contents of specific components, but no additional substances were detected (see Figure S1).

3.2 Bioactivity-guided fractionation and phytochemical characterization of active fractions

Both extracts were subjected to *in vitro* assays, and the more active extract underwent bioactivity-guided fractionation. The resulting fractions were tested in the same assays, and the most active fractions were characterized phytochemically.

3.2.1 Aqueous extract W and subfractions

To evaluate the potential of the extracts to promote re-epithelialization, they were subjected to a keratinocyte-based *in vitro* assay, in which an initially cell-free gap is re-covered by cells over time. The aqueous extract W showed significant ($p < 0.05$) enhancement of re-epithelialization, reducing the cell-free area by 49.4% at 10 µg/mL compared to the vehicle control (set to 0). In contrast, the DCM extract D only reduced the cell-free area by 4.6% at the same concentration and was considered inactive in this assay. Lysophosphatidic acid (LPA) was used as the positive control. As the reduction of cell-free area of the extracts and fractions was compared to the vehicle control, which was set to 0, the positive control LPA showed a reduction of over 300% (Figure 1 and Table 1). Due to its superior performance, the aqueous extract W was selected for fractionation. SPE, with manual application of a gradient of water, 50% methanol, and methanol, resulted in three fractions (W_H_2O, W_50%, and W_MeOH, TLC see Figure S2). The weight distribution of these fractions revealed that the extract W consists mainly of the aqueous fraction (W_H_2O 95.2% of W, W_50% 2.7% of W, and W_MeOH 1.6% of W). Upon re-testing the fractions in the *in vitro* assay, they all showed similar effects to the parent extract W, with a reduction of the cell-free gap by around 50%.

However, only the aqueous fraction W_H_2O showed statistically significant results at the two tested concentrations, 3 µg/mL ($p < 0.05$) and 10 µg/mL ($p < 0.01$), as depicted in Figure 1 and Table 1. Thus, this fraction was selected for further phytochemical characterization.

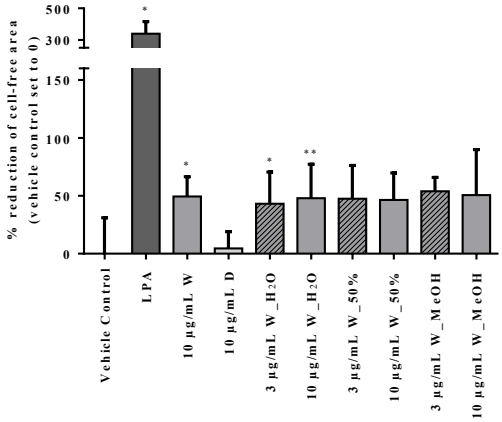


Figure 1 Reduction of cell-free area (%) on HaCaT keratinocytes of tested extracts and subfractions at 3 or 10 µg/mL, compared to the positive control lysophosphatidic acid (LPA) at 10 µM. The vehicle control (1% DMSO in 0.1% DMEM) was set to zero. Note that the y-axis is discontinuous; bars represent the mean ± SEM of three independent experiments, n = 3; * $p < 0.05$, ** $p < 0.01$ (Student's t-test).

Table 1 Data of the aqueous extract (W), the dichloromethane extract (D), and the subfractions of W (methanolic W_MeOH, 50% methanolic W_50%, and aqueous W_H_2O) from the wound healing assay on HaCaT keratinocytes (n = 3).

Extract/Fraction	Concentration	% reduction of cell-free area	±SEM	p-value
W	10 µg/mL	49.4*	9.8	0.0433
D	10 µg/mL	4.6	8.3	0.8522
W_H_2O	3µg/mL	43.1*	15.8	0.0362
	10 µg/mL	47.9**	17.0	0.0023
W_50%	3µg/mL	47.4	17.7	0.1422
	10 µg/mL	46.4	13.5	0.1185
W_MeOH	3µg/mL	53.7	6.9	0.1380
	10 µg/mL	50.6	22.7	0.0934

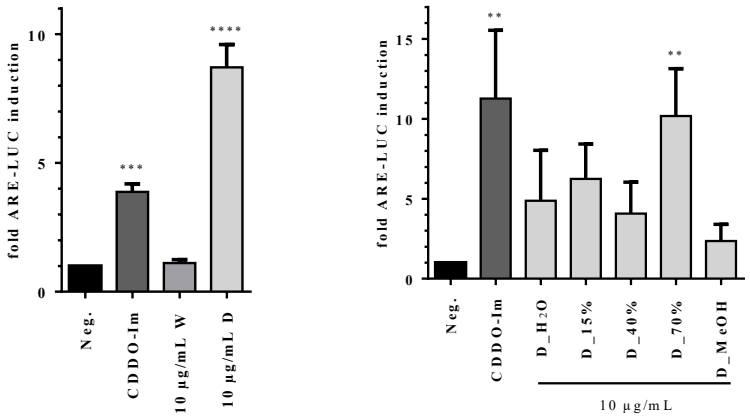
* $p < 0.05$, ** $p < 0.01$

The fraction W_H_2O, representing over 95% of the extract W, consists of various sugars. Previous studies have identified glucose, fructose, and sucrose as the main carbohydrates in white cabbage (Wennberg et al., 2006). Therefore, these sugars were chosen as reference substances for different chromatographic techniques, such as TLC and HPLC-DAD. Their presence was confirmed in fraction W_H_2O, but further sugars may be present in the fraction as well.

3.2.2 DCM extract D and subfractions

Although the DCM extract was inactive in the *in vitro* re-epithelialization assay, it was still tested for other bioactivities known to enhance wound healing, i.e., reduction of oxidative stress

and pro-inflammatory signaling. For this, we employed reporter gene assays for activation of the transcription factor Nrf2, the primary regulator of the cellular antioxidant defense, and inhibition of NF-κB, a crucial inflammatory transcription factor. The DCM extract D at a concentration of 10 µg/mL elicited a significant ($p < 0.001$) 9-fold activation of the Nrf2-dependent luminescence in the ARE-LUC reporter gene assay (Figure 2a) and was more active than the used positive control (CDDO-Im, 10 nM, 4-fold activation). The induction was concentration-dependent (see Figure S4a). In contrast, the aqueous extract W could not activate the Nrf2 response and was deemed inactive in this assay. Based on its superior activity, the DCM extract D was fractionated using SPE, with a manual gradient of water, 15% methanol, 40% methanol, 70% methanol, and pure methanol. The five obtained fractions (D_H_2O, D_15%, D_40%, D_70%, and D_MeOH, TLC-analysis see Figure S3, and yields see chapter 2.2.2) were re-tested in the Nrf2 (ARE)-dependent reporter gene assay. The 70% methanolic fraction D_70% exhibited the highest induction of Nrf2 activity (see Figure 2b and Figure S4b).



a) **b)**

Figure 2 Screening of Nrf2 pathway activation in HepG2-ARE/Nrf2-luc cells of different extracts and subfractions. The negative control was DMSO 0.1%, and the positive control was CDDO-Im at 10 nM. Bars represent the mean ± SD of three independent experiments, n= 3, ****$p < 0.0001$ (One-way ANOVA with Dunnett's post hoc test vs. vehicle control). **a)** Activity of the aqueous (W) and dichloromethane extract (D) at 10 µg/mL. **b)** Activity of the subfractions of D (methanolic D_MeOH, 70% methanolic D_70%, 40% methanolic D_40%, 15% methanolic D_15%, aqueous D_H_2O) at 10 µg/mL.

The very same extracts and fractions were also tested for inhibition of the TNF-α-stimulated NF-κB pathway in a respective reporter gene assay. Again, the DCM extract D was more active than the aqueous extract W at 10 µg/mL, leading to a significant ($p < 0.0001$) 75% inhibition of the NF-κB-dependent luminescence compared to the control cells. The positive control parthenolide (10 µM) even resulted in an inhibition of over 90% (Figure 3). Interestingly, experiments using lower concentrations of extract D showed significant activation of the NF-κB-dependent luminescence at concentrations below 5 µg/mL (see Figure S5a). After re-testing the subfractions of the extract D in this assay, the 70% methanolic fraction D_70% showed significant bioactivity with around 45% inhibition of the TNF-α-stimulated NF-κB signal. The most active fractions were the 15% and 40% methanolic fractions D_15% and D_40% (see Figure 3b), with concentration-dependent inhibitions of up to 98% at 10 µg/mL (Figure S5b).

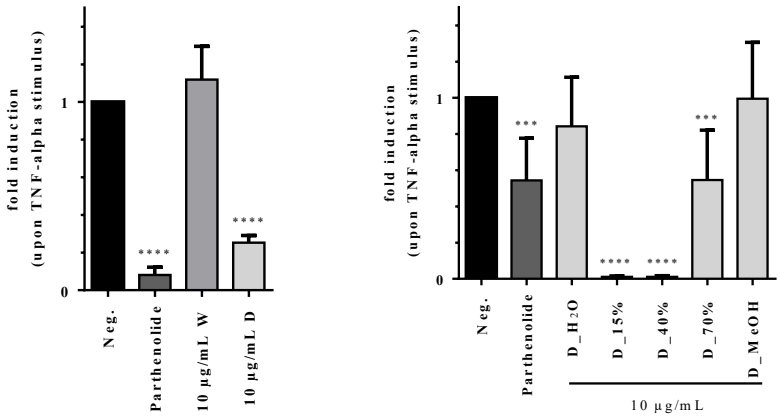


Figure 3 Screening of inhibition of the NF-κB transactivation activity in TNF-α-stimulated HEK-293/NF-κB-luc cells of different extracts and fractions. The negative control was DMSO 0.1%, and the positive control was parthenolide at 10 µM. After 30 min of pre-treatment with the samples, the cells were stimulated with TNF-α (2 ng/ml). Bars represent the mean ± SD of three independent experiments, n= 3, ****p < 0.0001 (One-way ANOVA with Dunnett's post hoc test vs. vehicle control). **a)** Activity of the aqueous (W) and dichloromethane extract (D) at 10 µg/mL. **b)** Activity of the subfractions of D (methanolic D_MeOH, 70% methanolic D_70%, 40% methanolic D_40%, 15% methanolic D_15%, aqueous D_H_2O) at 10 µg/mL.

The three most active subfractions, D_15%, D_40%, and D_70%, were phytochemically characterized using different chromatographic techniques, primarily focusing on the main compounds according to the HPLC-ELSD signal. The HPLC-ELSD chromatograms of these fractions and mass spectra of the main compounds can be found in the supplementary material (Figure S6 and Table S1). The characterization of these main components is summarized in Table 2, containing retention time (Rt.) and UV max from HPLC-DAD experiments and *m/z* values from UHPLC-MS analysis.

Table 2 Characterization of main compounds found in the active fractions D_15%, D_40%, and D_70% using HPLC-DAD and UHPLC-ESIMS ('-' indicates that MS data is not precise due to poor ionization).

	Peak Nr.	**Rt. [min]**	**UV max [nm]**	**m/z [neg.]**	**m/z [pos.]**
D_15%	1	7.237	206 237	283.15 $[M-H]^-$	285.20 $[M+H]^+$ 307.25 $[M+Na]^+$
	2	8.804	204 237	297.17 $[M-H]^-$ 343.05 $[M+FA-H]^-$	299.24 $[M+H]^+$ 321.25 $[M+Na]^+$
	3	9.881	207 236	311.21 $[M-H]^-$ 357.06 $[M+FA-H]^-$	313.28 $[M+H]^+$ 335.27 $[M+Na]^+$
	4	15.807	196	-	113.98 $[M+H]^+$
	5	22.683	287	128.03 $[M-H]^-$	130.04 $[M+H]^+$
D_40%	6	8.823	207 237	297.17 $[M-H]^-$ 343.06 $[M+FA-H]^-$	299.25 $[M+H]^+$ 321.26 $[M+Na]^+$
	7	9.898	206 237	311.20 $[M-H]^-$ 357.06 $[M+FA-H]^-$	313.29 $[M+H]^+$ 335.25 $[M+Na]^+$

	8	15.864	196 238	-	113.97 $[M+H]^+$
	9	18.649	206 226 260	424.12 $[M-H]^-$	426.32 $[M+H]^+$
	10	20.425	204 225 258	591.22 $[M-H]^-$	-
	11	22.701	286	128.05 $[M-H]^-$	130.05 $[M+H]^+$
	12	24.289	196 211 271	-	-
D_70%	13	24.377	286	327.37 $[M-H]^-$	329.23 $[M+H]^+$,
	14	35.542	281	-	-
	15	36.260	206 272	291.31 $[M-H]^-$	293.37 $[M+H]^+$ 315.38 $[M+Na]^+$

Since many different phytochemicals in white cabbage have already been described, MS/MS data is needed to identify the main components of each extract using tools such as MS-Dial and GNPS. However, the detected molecular weights of these main components were foremost subjected to CAS SciFinder® to gain first insights into possible substance classes. According to SciFinder®, peak **1** in D_15% might be a fatty acid, such as stearic acid (284.48 g/mol), an isoflavone, such as biochanin A (284.26 g/mol), or a flavone, such as acacetin (284.26 g/mol). Peak **2** and peak **6** reveal the same molecular weight of 298 g/mol, again indicating the potential presence of fatty acids, such as 17-methyl-octadecanoic acid (298.50 g/mol). Due to the similar UV maxima, peaks **3** and **7** might also correspond to a fatty acid derivative with a molecular weight of 312 g/mol. Peak **4** and **8**, revealing a molecular weight of 113 g/mol, could be artificial products gained by MS fragmentation of genuine glucosinolates, such as 3-butenyl isothiocyanate (113.18 g/mol). The same is possible for peaks **5** and **11**, corresponding to another glucosinolate breakdown product such as 5-(methylthio)pentanenitrile (129.22 g/mol). Peak **9** showed a molecular weight of 425 g/mol, which might be a genuine glucosinolate such as glucosinalbin (425.43 g/mol). With a molecular weight of 592 g/mol for peak **10**, hydroxycinnamic acid derivatives, such as 1,2-disinapoylglucose (592.55 g/mol), might also be present in the active fraction D_40%. Peak **13** could correspond to cabbage identification factor 2 (328.35 g/mol), a substance located on the surface of cabbage leaves. For the final identification of all corresponding substances, further MS experiments and annotation tools are needed. However, fatty acids, flavonoids, glucosinolates, breakdown products thereof, and hydroxycinnamic acid derivatives are conceivable to confer anti-inflammatory and anti-oxidant potential to white cabbage.

3.3 Phytochemical investigation of volatile compounds

Members of the Brassicaceae family are known for their high content of glucosinolates and their breakdown products, such as isothiocyanates and nitriles (Avato and Argentieri, 2015). These breakdown products are formed whenever the cells are disrupted, and the enzyme myrosinase, stored separately from glucosinolates in intact cells, encounters and hydrolyzes these substances to create various breakdown products (Arora, 2024). To investigate these

volatile breakdown products, a fresh DCM extract of cabbage leaves was produced after crushing the leaves with a rolling pin. GC-MS analysis was conducted to identify the main components found in the extract (total ion chromatogram Figure 4). The generated spectra were compared with those found in databases (Wiley 229, NIST 27, 147); the results with more than 75% similarity are depicted in Table 3. Mass spectra can be found in the supplementary material (Table S2). Pure substances were available for allyl isothiocyanate (AITC) and 3-butenyl isothiocyanate (3-BITC), which were used for final identification in the DCM extract via mass spectra, retention time, and co-chromatography.

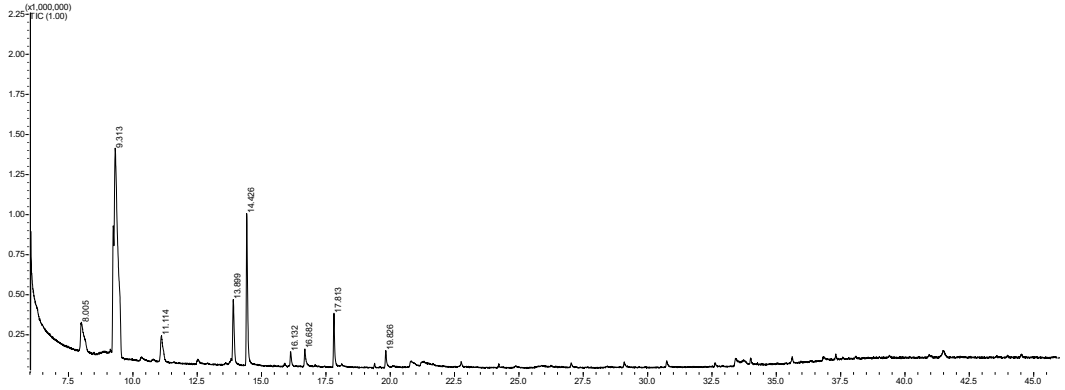


Figure 4 Total ion chromatogram (TIC) of the fresh cabbage extract. MS spectra were compared with databases (WILEY, NIST), and identified peaks were marked with numbers. The peaks at 8.01 and 9.31 are the solvent dichloromethane and its stabilizer tetrachloroethylene.

Table 3 Compounds in the fresh cabbage extract identified by databases (WILEY, NIST) with a similarity score of at least 75%.

Peak Nr.	Retention time [min]	Compound name (Abbreviation)	Molecular weight [g/mol]	Similarity [%]
1	11.11	Allyl isothiocyanate (AITC)	99	81
2	13.90	3-Butenyl isothiocyanate (3-BITC)	113	88
3	14.43	Cyclopropyl isothiocyanate (CPITC)	99	78
4	16.13	S-Methyl methanethiosulfonate (MMTS)	126	84
5	16.69	4-(Methylthio)butylnitrile (4-MTBN)	115	89
6	17.81	Thiiranepropanenitrile (TPN)	113	77
7	19.83	5-(Methylthio)pentanenitrile (5-MTPN)	129	83

4. Discussion

The traditional application of squeezed cabbage leaves exposes wounds to various pro-healing substances of different polarities and different bioactivities. The aqueous extract W and its aqueous fraction W_H_2O significantly boosted the re-epithelialization of a gap in a keratinocyte monolayer *in vitro*. Both samples consisted mainly of sugars, such as fructose, glucose, and sucrose. Glucose was already readily available for the cultivated keratinocytes as the used DMEM medium contains 4500 mg/L glucose. Moreover, high glucose concentrations have been shown to inhibit human epidermal keratinocyte proliferation *in vitro*. Therefore, hyperglycemia may directly contribute to delayed epithelialization of wounds in diabetic patients (Terashi et al., 2005). Hence, glucose does not appear to be an obvious principle that

can explain the observed enhanced gap closure with extract W or fraction W_H_2O *in vitro*. However, short-term local application of glucose, as done with white cabbage leaves, might relieve the competition for fuel between the different cell types in *in vivo* wounds. In addition, sugars are antimicrobial due to their high osmotic activity, leading to inhibited bacterial growth and increased lymph outflow. Also, medical honey is mainly composed of sugars and already has a safe position in wound management in clinical practice (Jodidio and Schwartz, 2024; Mizzi et al., 2020). Furthermore, the active extract W and fraction W_H_2O do not only contain glucose but also other saccharides, such as fructose and sucrose. Sucrose has been shown to enhance autophagy in keratinocytes (Chen et al., 2016), which in turn is essential for keratinocyte migration and proliferation, as well as activation of dermal fibroblasts (Qiang et al., 2021). Moreover, a sucrose-containing paste increased collagen synthesis of fibroblasts and the urokinase-type plasminogen activation (u-PA) secretion of keratinocytes, with the latter favoring keratinocyte migration and activation of latent collagenase (Nakao et al., 2006). Hence, sugars may take a good share in the wound-healing activity of squeezed white cabbage leaves.

Reporter gene assays revealed the DCM extract and its fractions D_15%, D_40%, and D_70% to be significantly active in the activation of Nrf2- and the inhibition of the TNF-α-stimulated NF-κB pathway. Both transcription factors, Nrf2 and NF-κB, can modulate the wound-healing process. Nrf2 upregulation during wound healing protects cells against excessive amounts of reactive oxygen species (ROS) in inflamed tissue. Hence, activating Nrf2 by exogenous inducers such as phytochemicals leads to positive effects by boosting the cellular antioxidant defense against ROS and other oxidative and inflammatory stressors (Ambrozova et al., 2017; Süntar et al., 2021). The most potent Nrf2-activating fraction was D_70%. However, the responsible substance is yet to be uncovered. So far, the detected molecular weight could be cabbage identification factor 2, a compound located on the leaf surface that stimulates the oviposition of the cabbage root fly (De Jong et al., 2000; Hurter et al., 1999). However, possible medicinal bioactivities have not yet been investigated. Therefore, the final identification of the active substance(s) in fraction D_70% is crucial.

The role of NF-κB in wound healing is complex. On the one hand, an activated NF-κB pathway ensures an innate immune reaction after tissue injury and protection against pathogens. Furthermore, NF-κB exerts proliferative effects, which are beneficial in wound healing in terms of keratinocyte proliferation and migration and, therefore, re-epithelialization. On the other hand, impaired wound healing is often accompanied and caused by unresolved inflammation. In diabetic wounds, for example, chronic inflammation occurs due to overly activation of the NF-κB pathway by different factors, such as hyperglycemia or oxidative stress, and delays wound healing. Thus, inhibition of NF-κB activity through phytochemicals can enhance the healing process of such wounds (Ambrozova et al., 2017; Yadav et al., 2024). The most active fractions inhibiting the TNF-α-stimulated NF-κB pathway in our study were D_15% and D_40%, both with inhibition of almost 98% at 10 µg/mL. Their phytochemical compositions share some similarities, which might be the key to this anti-inflammatory activity. Both fractions show peaks corresponding to substances with molecular weights 298 g/mol, 312 g/mol, 113 g/mol, and 129 g/mol. The first two might correspond to fatty acids, which are constituents of the cuticular wax (Zhu et al., 2019) and have already been investigated in wound healing due to their anti-inflammatory potential (Jara et al., 2020). The latter two peaks, with

molecular weights of 113 g/mol and 129 g/mol, could correspond to the volatile glucosinolate breakdown products such as 3-butenyl isothiocyanate and 5-(methylthio)pentanenitrile, which have been detected in this work in a fresh cabbage extract via GC-MS. However, this fresh cabbage extract has not been used for the *in* vitro assays, but extract D, in which no volatile compounds were found after resolving in DCM and injecting it into the GS-MS. Therefore, the measured anti-inflammatory and antioxidant effects of extract D were not caused by volatile glucosinolate breakdown products, such as isothiocyanates. Thus, further analysis of the genuine glucosinolates is needed. Possible degradation products might arise under extraction and storage conditions prior to LC-MS analysis. These genuine glucosinolates have to be investigated and correlated to the constituents detected in the respective fractions.
Nevertheless, the volatile compounds must be present in the cabbage compress since the traditional use of fresh leaves involves squeezing them with a rolling pin. Therefore, a fresh DCM extract was prepared and analyzed using GC-MS. The main identified compounds include isothiocyanates (allyl isothiocyanate, 3-butenyl isothiocyanate, cyclopropyl isothiocyanate), nitriles (4-(methylthio)butylnitrile, thiiranepropanenitrile, 5-(methylthio)pentanenitrile) and S-methyl methanethiosulfonate, all already described for *Brassica* vegetables (Hanschen et al., 2018; Hanschen and Schreiner, 2017; Urooj, 2018). These substances are breakdown products of glucosinolates and are well-investigated for their anti-cancer potential, antioxidant effects, anti-inflammatory properties, and antimicrobial activity (Dufour et al., 2015; Šamec et al., 2017). Allyl isothiocyanate, for example, is effective against *Staphylococcus aureus* and *Candida albicans* (Blažević et al., 2019). Therefore, these volatile compounds are likely involved in the overall wound-healing effect of freshly squeezed cabbage leaves.
Future research will aim to isolate and structurally elucidate these different main components. Furthermore, these pure substances can then be subjected again to *in vitro* assays to determine their role in the effects of the bioactive fractions and investigate potential additive or synergistic effects. Taken together, these findings substantiate the traditional use of fresh cabbage leaves to treat wounds and support the revival of this tradition as an easily accessible, low-cost, and ubiquitously available wound-healing preparation.

Supplementary material

Figure S1 HPLC-DAD comparison of DCM extracts gained from 3 different cabbages at 190 nm. Each cabbage was extracted with prior incubation (lower chromatograms – blue, green, orange) and without incubation (upper chromatograms – pink, purple, red). ***Figure S2*** TLC comparison of the aqueous extract W and its fractions W_H_2O, W_50%, and W_MeOH after derivatization with anisaldehyde/sulfuric acid at daylight (stationary phase: silica 60 F_{254}, mobile phase: DCM-MeOH-1% FA (8+5+1)). ***Figure S3*** TLC comparison of the DCM extract D and its fractions D_H_2O, D_15%, D_40%, D_70%, and D_MeOH after derivatization with anisaldehyde/sulfuric acid at daylight (left) and underivatized at 366 nm (right) (stationary phase: silica 60 F_{254}, mobile phase: DCM-MeOH (9+1)). ***Figure S4*** Screening of dose dependency of Nrf2 pathway activation in HepG2-ARE/Nrf2-luc cells of **a)** the active extract D compared to the positive control Iberin 3 µM, and **b)** the most active fraction D_70% compared to the positive control CDDO-Im at 10 nM. The negative control was DMSO 0.1%. The samples were tested at the mentioned concentrations. Bars represent the mean ± SD of

three independent experiments, n= 3, ****p < 0.0001 (One-way ANOVA with Dunnett's post hoc test vs. vehicle control). ***Figure S5*** Screening of dose dependency of inhibition of TNF-α-stimulated NF-κB transactivation activity in HEK-293/NF-κB-luc cells of **a)** the active extract D compared to the positive control parthenolide 10 µM, and **b)** the most active fractions D_15% and D_40% compared to the positive control parthenolide 10 µM. The negative control was DMSO 0.1%. The samples were tested at the mentioned concentrations. Bars represent the mean ± SD of three independent experiments, n= 3, ****p < 0.0001 (One-way ANOVA with Dunnett's post hoc test vs. vehicle control). ***Figure S6*** HPLC-ELSD chromatograms of fractions D_15% (green), D_40% (red), and D_70% (blue). The main peaks were labeled by numbers and characterized using HPLC-DAD and UHPLC-ESIMS. ***Table S1*** UV spectra and mass spectra (positive mode upper spectrum, negative mode lower spectrum) of main peaks found in the fractions D_15% (Peaks 1-5), D_40% (Peaks 6-12), and D_70% (Peaks 13-15). ***Table S2*** Mass spectra of compounds in the fresh cabbage extract identified by databases (WILEY, NIST) with a similarity score of at least 75%.

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5. Discussion and Conclusion

The VOLKSMED database was screened for herbal applications used for wound healing in Austrian folk medicine. *Hypericum* sp., *Arnica montana*, and *Calendula officinalis* were the most cited hits. Numerous publications and reviews already confirm their wound-healing effects [40,60,61], proving the plausibility of the data incorporated into the database. A literature review of selected lesser-known genera and their wound-healing potential was conducted to find possible candidates for further investigations. Three of these lesser-known plants were followed up due to their status quo of relevant literature: the exudates of *Picea abies* have already been the subject of clinical studies, and extracts of *Brassica oleracea* var. *capitata* f. *alba* have been tested *in vivo* for wound healing in rats. Contrarily, *Sanicula europaea* appeared as an almost forgotten wound-healing plant but was particularly interesting due to the possible confusion with other plants in the Austrian vernacular language (**Publication I**).

First, the phytochemical composition and impact on *in vitro* re-epithelialization of Norway spruce balm, fractions, and pure substances were investigated. This work revealed the balm's complex composition. The main bioactive constituents were various DRAs with different numbers of hydroxylation. Non-hydroxylated DRAs were structurally highly similar and, therefore, posed challenges for chromatographic separation (**Publication II**). So far, the method of choice for separating these DRAs was GC-MS, demanding a time-consuming derivatization step [52,62,63]. Thus, we developed a UHPSFC-MS protocol that provides a simple method for separating and quantifying DRAs quickly without any need for derivatization. This protocol was used to quantitate the content of DRAs in spruce balm samples collected in different locations at different time points. It revealed a highly diverging content, even in samples harvested from the same tree, underlining the importance of well-established chromatographic methods for quality control. Yet more significant differences in the content of DRAs were found for two different commercial products. These products contain different exudates of Norway spruce and different concentrations thereof, which can well explain the divergent values (10% spruce resin vs. 20% spruce balm). Moreover, the balm ointment was based on lard, whereas the resin-based preparation consisted of petrolatum and further additives, giving rise to matrix effects of the semi-solid vehicles that might interfere with the substances of interest [64] (**Publication III**). Further investigations should focus on these possible matrix effects. However, this UHPSFC-MS method and newly developed TLC and HPLC-DAD protocols were applied to compare the phytochemical composition of Norway spruce balm with Norway spruce resin, larch balm, and pine resin. In

addition, their influence on *in vitro* re-epithelialization was investigated. Only larch balm showed significant results in this assay, underlining the importance of current research on this plant [65]. Furthermore, herbal positive controls (birch, St. John's wort, Manuka honey, and pot marigold) varied in their potential to enhance re-epithelialization. On the one hand, these findings underline the relevance of this *in vitro* assay since some of these well-known herbal wound-healing agents revealed a significant reduction of cell-free area. On the other hand, they stress the importance of additional testing systems for wound-healing plants in order to assess the effects on different targets and cell types involved in the healing process. However, the developed chromatographic methods represent an essential prerequisite for the quality control of spruce balm samples and commercial products, maintaining pharmaceutical quality and detecting possible adulterations with other Pinaceae exudates (**Publication IV**). One commercial product in Austria and the respective monograph in the Austrian Pharmacopeia are based on the traditional preparation of Norway spruce balm ointments with lard. A more modern and wider acceptable ointment base could pass the use of spruce balm preparations to a broader audience. Therefore, different semi-solid vehicles were prepared and rheologically evaluated regarding their storage stability at different temperatures. The most stable preparation, consisting of wool wax and castor oil, was subjected to permeation studies in Franz-type diffusion cells. The permeation potential of pinoresinol, 15-hydroxy dehydroabietic acid, dehydroabietic acid, and seven DRAs with the same molecular weight (302 g/mol) was analyzed using HPLC-DAD. The AUCs of respective permeated substances were compared to those from the lard-based commercial product. Pinoresinol and 15-hydroxy dehydroabietic acid showed a higher permeation rate from the lard-based ointment than from the wool wax/castor oil ointment. In contrast, the more apolar substances dehydroabietic acid and the 302 g/mol DRAs revealed similar behavior for both preparations. These differences could have occurred due to the different vehicles or due to the varying spruce balm batches with different contents of respective substances in the two formulations (**Publication V**). Current research focuses on the permeation potential of the compounds from these two preparations containing the same spruce balm batch and further developing the HPLC-DAD method for absolute quantitation of permeated substances via internal standard.

In summary, this work contributed to a better understanding of the traditional application of Norway spruce balm in terms of its phytochemical composition and provided the first evidence for a more modern ointment base. These findings boost the revival of Norway spruce balm ointments as additional wound-healing preparations.

Sanicula europaea was another subject of interest in this doctoral thesis due to the synonymous use of the term "Radix Saniculae" for the underground parts of sanicle and

drooping bittercress in the Austrian dialect. Therefore, chromatographic methods were established to analyze the main components in the roots and rhizomes of both plants. Furthermore, the phytochemical composition of the underground parts of sanicle was compared to the aerial parts, as the latter have already been investigated in the context of wound healing [66]. Both parts of sanicle revealed the same major substance classes: saccharides, hydroxycinnamic acid derivatives, and triterpene saponins. Contrarily, drooping bittercress contained saccharides and glucosinolates (**Manuscript I**). This work demonstrates the potential mix-up of the correct botanical source in the VOLKSMED database. Even though the database might still hold many forgotten plant applications, these results showed that the botanical source of certain herbal drugs should be treated with caution. However, the chromatographic methods developed within this work serve as important tools for quickly detecting mutual adulterations. Future research should isolate the main components from both plants and subject them to *in vitro* wound-healing assays or *in vivo* experiments to evaluate the true wound-healing potential of these two traditionally used plants.

The wound-healing potential of *Brassica oleracea* var. *capitata* extracts has already been investigated in various *in vitro* studies [67,68]. In Austrian folk medicine, the fresh leaves of white cabbage are crushed and used as a compress for wound healing. The active principles behind this tradition have not yet been examined. Therefore, fresh cabbage leaves were extracted according to the traditional way of preparation and tested in different *in vitro* assays concerning their re-epithelialization-enhancing, antioxidant, and anti-inflammatory activity. The extracts were fractionated, and the resulting fractions were re-tested. After the development of various chromatographic methods, the active fractions were characterized. As the prepared extracts of different polarity revealed significant but distinct effects in the performed bioassays, it seems that the wound-healing potential of cabbage compresses is based on a multi-target activity due to various phytochemicals, such as saccharides, fatty acids, and isothiocyanates (**Manuscript II**). This work provides first insights into the wound-healing effects of the traditional application of fresh cabbage leaves and the responsible substance classes. However, isolation and structure elucidation of the main components still need to be done to identify the bioactive substances and potential synergistic or additive effects.

Picea abies, *Sanicula europaea*, and *Brassica oleracea* var. *capitata* f. *alba* belong to different families, namely Pinaceae, Apiaceae, and Brassicaceae. However, besides their traditional use for wound healing, their deeper investigation showed one similarity: their complex composition. Norway spruce balm, methanolic extracts of sanicle, and different

extracts of white cabbage all consisted of numerous substance classes and single compounds of different polarities. Therefore, the choice of extraction solvent and method determines the compounds found in the resulting extract [69]. Moreover, phytochemical analysis is often challenging due to the complex medley of compounds in herbal extracts. Different chromatographic techniques following time-intensive method optimizations are required to detect all substances in multicomponent mixtures simultaneously. However, these separation and analysis methods are pivotal for the quality control of herbal materials [70,71]. As a trade-off for the analytical demands, multicomponent mixtures provide the benefit of multi-target activity, which is immensely favorable in a multifactorial process such as wound healing. Therefore, herbal extracts can promote wound healing through several effects, such as pro-proliferative, promigratory, antioxidant, anti-inflammatory, or antimicrobial activities [8]. Subjecting herbal extracts to only one *in vitro* bioassay, such as the keratinocyte-based wound healing assay, can be proven successful, as in the case of the betulin-enriched extract, where these findings were supported by *ex vivo* and clinical studies [43,44]. Norway spruce resin revealed a phytochemical composition similar to Norway spruce balm, which suggests similar bioactivities. Therefore, the use of only one *in vitro* assay for the bioactivity-guided fractionation of the balm was justified by the amount of data concerning wound-healing effects already published for the resin. Nonetheless, in terms of unraveling the mode of action of Norway spruce balm, further *in vitro* bioassays should be considered. However, if certain extracts exhibit their wound-healing activity by affecting other processes in the wound-healing mechanism, they would be regarded as inactive in this keratinocyte-based assay and could be excluded from further investigations. Therefore, the dichloromethane extract of white cabbage, which showed no activity in the re-epithelialization assay, was subjected to further *in vitro* assays. The promising results emphasize that only the use of various *in vitro* assays covering different bioactivities can capture the full potential of wound-healing plants. For this purpose, the wound-healing potential of herbal extracts and pure substances is often assessed by investigating their effect on keratinocyte and fibroblast migration, proliferation, and protein expression or their antimicrobial activity by disc diffusion assays. Furthermore, anti-inflammatory activities such as inhibiting NF-κB or eicosanoid synthesis or antioxidant effects, e.g., using the 2,2-diphenyl-1-picrylhydrazine (DPPH) assay, should not be overlooked [72,73]. The most promising herbal extracts can be subjected to *in vivo* studies to investigate their effect on wounds. However, it must be remembered that the used animal models may not optimally reflect the human skin structure. The highest similarities to humans can be found in pig skin [74], which has already been used to investigate the wound-healing potential of spruce balm and spruce resin ointments *in vivo*. The 20% spruce balm containing

lard-based ointment, the petrolatum-based formulation with 10% spruce resin, lard, antibiotic blue spray, or nothing were applied on piglet castration wounds. Although the differences in wound size between the groups were not significant, the preparations containing spruce exudates resulted in a higher number of completely closed wounds. Furthermore, wounds treated with Norway spruce balm showed less wound secretion and fungal contamination [75]. The ultimate phase of analyzing the wound-healing effects of herbal extracts involves human cases or even clinical studies, a milestone that has already been achieved with spruce resin [50]. Further investigations of spruce balm, sanicle, and white cabbage will aim to further advance *in vivo* animal and human studies.

Taken together, this dissertation sheds light on some hidden treasures in the VOLKSMED database regarding traditional wound-healing plants. The established chromatographic techniques deliver essential tools for further analysis of these herbal medicines. The successful implementation of the traditional lard-based spruce balm ointment as a monograph in the Austrian Pharmacopeia prompts further investigations, especially of sanicle and white cabbage, to also reintroduce these valuable traditional applications in modern wound management.

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A. Appendix

A.1 List of Peer-Reviewed Articles

- Goels T., **Eichenauer E.**, Langeder J., et al. Norway Spruce Balm: Phytochemical Composition and Ability to Enhance Re-epithelialization *In Vitro*. *Planta Med*. 2020, 86(15), 1080-1088.
- Goels T., **Eichenauer E.**, Tahir A., et al. Exudates of *Picea abies*, *Pinus nigra*, and *Larix decidua*: Chromatographic Comparison and Pro-Migratory Effects on Keratinocytes In Vitro. *Plants* 2022, 11(5), 599.
- Goels T., **Eichenauer E.**, Langeder J., et al. Ultra High-Performance Supercritical Fluid Chromatography for the Quantitation of Diterpene Resin Acids in Norway Spruce Samples. *Front. Pharmacol.* 2022, 13, 906411.
- **Eichenauer E.**, Jozić M., Glasl S., Klang V. Spruce Balm-Based Semisolid Vehicles for Wound Healing: Effect of Excipients on Rheological Properties and Ex Vivo Skin Permeation. *Pharmaceutics* 2023, 15, 1678.
- **Eichenauer E.**, Saukel J., Glasl S. VOLKSMED Database: A Source for Forgotten Wound Healing Plants in Austrian Folk Medicine. *Planta Med.* 2024, 90, 498-511.

A.2 Conference Contributions

The presenting author is underlined.

Poster presentations

- **Eichenauer E.**, Saukel J., Glasl S. Revival of a traditional Austrian wound healing preparation. 72nd International Congress on Natural Product Research in Krakow, Poland (July 13th-17th, **2024**).[1]
- **Eichenauer E.**, Jozić M., Hittmair E., Klang V., Glasl S. Spruce balm ointments - Investigating an Austrian traditional vulnerary drug. Tetranational Congress Phytotherapy 2024 - Herbal Products for Human and Animal Healthcare in Utrecht, Netherlands (May 30th-31st, **2024**).[2]
- **Eichenauer E.**, Jozić M., Klang V., Glasl S. Die komplexe Zusammensetzung des Fichtenbalsams - chromatographische Herausforderungen bei der Qualitätskontrolle. Phytotherapiekongress 2023 - Phytotherapie im 21. Jahrhundert in Bamberg, Germany (June 15th-17th, **2023**).[3]
- **Eichenauer E.**, Goels T., Jozić M., Klang V., Glasl S. Analysis and quantitation of bioactive compounds in Norway spruce balm. 70th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research in Thessaloniki, Greece (August 28th-31st, **2022**).[4]

Oral presentations

- **Eichenauer E.** Phytos für die Haut - traditionelles Wissen und bewährte Arzneimittel. 38. Südtiroler Herbstgespräche in Waidhofen/Ybbs, Austria (September 13th-15th, **2024**).
- **Eichenauer E.**, Glasl S. Die komplexe Zusammensetzung des Fichtenbalsams - chromatographische Herausforderungen bei der Qualitätskontrolle. 6. GPT Young Researcher Workshop in Bamberg, Germany (June 14th-15th, **2023**).
- **Eichenauer E.** Analysis of Wound Healing Pinaceae Balms. 1st International Postgraduate Meeting on Medicinal Plants online (November 25th, **2022**).
- **Eichenauer E.**, Heiss E.H., Glasl S. Phytochemical composition and active principles of traditional wound healing plants. HMPPA Symposium "Next Generation in Pharmacognosy" in St. Urban, Austria (May 19th-20th, **2022**).

[1]See page 139.
[2]See page 140.
[3]See page 141.
[4]See page 142.

Video presentation

- Goels T., **Eichenauer E.**, Tahir A., Prochaska P., Hoeller F., Heiss E.H., Glasl S. Balms of selected species of Pinaceae and their influence on keratinocyte re-epithelialization in vitro. 69th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research online (September 5th-8th, **2021**).

Revival of a traditional Austrian wound healing preparation

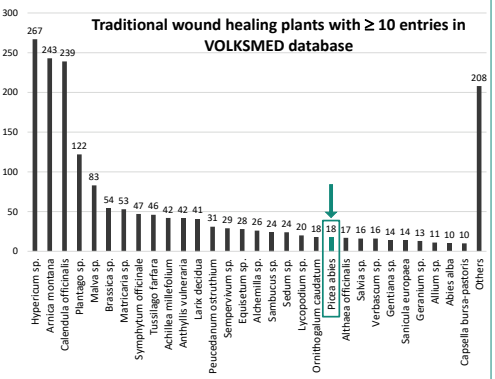
Elisabeth Eichenauer[a,b], Johannes Saukel[a], Sabine Glasl[a]

a Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna, Vienna, Austria;
b Vienna Doctoral School of Pharmaceutical, Nutritional and Sport Sciences, University of Vienna, Vienna, Austria

Introduction

Herbal preparations represent a promising therapy option in the treatment of acute and chronic wounds. However, with the ongoing increase in chronic wounds further therapy alternatives than the already used herbal formulations are highly appreciated [1]. Traditional folk medicine might hold a plethora of old knowledge about already forgotten wound healing plants.

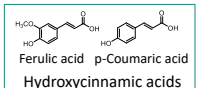
The **VOLKSMED database** was created to compile and preserve Austrian folk medicine and consists of more than 43,000 records about the traditional use of plants and fungi, but also other materials of animal or mineral origin. It can be used to reveal promising folkloric traditions in the field of wound healing. Besides well-known wound healing plants such as *Calendula officinalis*, the database also holds lesser-known and forgotten traditions, such as the **preparation of a wound healing ointment made of lard and Norway spruce balm** (*Picea abies*, Pinaceae) [2,3]. The latter was investigated in depth to scientifically substantiate its revival in modern medicine.



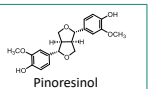
Methods

Results – Phytochemistry and wound healing bioactivities

The main substances were identified using different methods, such as TLC, HPLC, UV-VIS, SFC, LC-MS, and NMR. A keratinocyte-based *in vitro* wound healing assay accompanied the bioactivity-guided fractionation and isolation. Further activities contributing to an overall wound healing effect and representatives of the most important substance classes are depicted below [4,5].

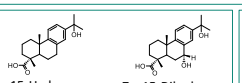


Hydroxycinnamic acids
antioxidant, antimicrobial, anti-inflammatory

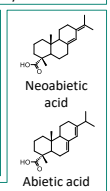


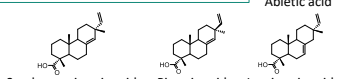
Lignans
antifungal, antioxidant, anti-inflammatory, re-epithelialization boosting

Diterpene resin acids (300 Da)
antimicrobial, anti-inflammatory, re-epithelialization boosting



Hydroxylated diterpene resin acids
antimicrobial, anti-inflammatory, re-epithelialization boosting



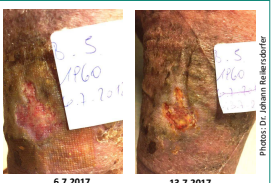


Diterpene resin acids (302 Da)
antimicrobial, anti-inflammatory, antioxidant, re-epithelialization boosting

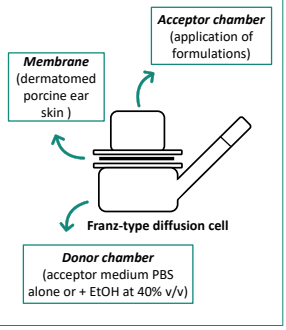
Stationary phase: Silica Gel 60 F_{254}
Mobile Phase: $CHCl_3$:MeOH:TFA (97+3+0.1)
Detection: Vis after derivatization with anisaldehyde/sulphuric acid

Results – *In vivo* testing and formulation development

In vivo experiments with a lard-based spruce balm ointment in castration wounds in piglets revealed the potential of this formulation, such as lower wound secretion and wound contamination with fungi [6]. Furthermore, first case studies in an Austrian nursing home were conducted using the lard-based formulation, resulting in visible wound improvement within one week.



Since lard is not considered an ideal raw material in terms of standardized quality, chemical stability, or patient compliance, other semisolid formulations were developed, and rheologically analyzed. So far, a wool wax/castor oil formulation was found to be a suitable alternative to traditional lard for the production of Norway spruce balm ointments. Moreover, the permeation potential of bioactive compounds in an *ex vivo* porcine ear model using Franz-type diffusion cells was investigated [5]. Latest results suggest a higher permeation rate of substances into the skin from lard than from wool wax/castor oil, which might be negligible in terms of the disrupted skin barrier occurring in wounds.



Status quo

Since 2016, the Austrian Pharmacopeia contains the monograph "**Piceae abietis balsamum**". In 2019, the monograph "**Unguentum Piceae abietis balsami officinale**", an ointment consisting of 80 g of pig lard and 20 g of Norway spruce balm, was added. Furthermore, a **medical device** is available on the Austrian market, which follows this traditional preparation and is also based on lard.

Conclusion

The revival of this folkloric tradition represents an excellent example of the hidden and forgotten treasures that still can be found in Austrian folk medicine.

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[1] Vitale, S., et al. Phytochemistry and Biological Activity of Medicinal Plants in Wound Healing: An Overview of Current Research. DOI: 10.3390/ molecules27113566
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[5] Eichenauer, E., et al. Spruce Balm-Based Semisolid Vehicles for Wound Healing: Effect of Excipients on Rheological Properties and Ex Vivo Skin Permeation. DOI: 10.3390/pharmaceutics15061678
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Acknowledgment:
We thank Dr. Johann Reikersdorfer for the realization of the case studies, as well as all Prof. Johannes Saukel for initiating this project.

Spruce balm ointments – Investigating an Austrian traditional vulnerary drug

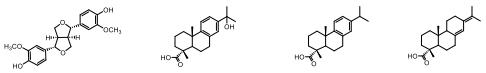
Elisabeth Eichenauer[1,2], Martina Jozić[1], Elisabeth-Sophie Hittmair[1], Victoria Klang[3], Sabine Glasl[1]

1 Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna, Vienna, Austria;
2 Vienna Doctoral School of Pharmaceutical, Nutritional and Sport Sciences, University of Vienna, Vienna, Austria;
3 Division of Pharmaceutical Technology and Biopharmaceutics, Department of Pharmaceutical Sciences, University of Vienna, Vienna, Austria

Introduction

Norway spruce balm is an exudate of *Picea abies* (L.) H.Karst. (Pinaceae), which exhibits a longstanding tradition in the Austrian ethnomedicine as wound healing agent. For this purpose, the balm is traditionally processed with lard to produce an ointment [1]. This traditional lard-based preparation can be found in the monograph "Unguentum Piceae abietis balsami officinale" in the Austrian Pharmacopeia, as well as in a medical device on the Austrian market.

However, nowadays, lard is not considered an ideal vehicle in terms of stability, standardized quality, and patient acceptance. Therefore, alternative formulation strategies were explored and tested for rheological properties, physical stability, and *ex vivo* skin permeation of relevant active compounds (Pinoresinol, 15-Hydroxydehydroabietic acid, Dehydroabietic acid, and 7 different 302 g/mol diterpene resin acids (DRAs) with Neoabietic acid depicted as representative) [2].

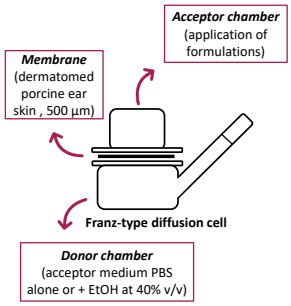


Pinoresinol | 15-Hydroxy-dehydroabietic acid | Dehydroabietic acid | Neoabietic acid

Methods

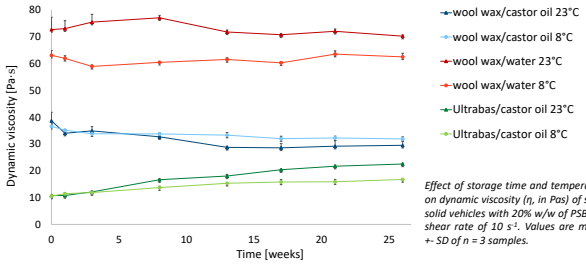
Flow curves were recorded with a modular compact rheometer (MCR 302, Anton Paar, Germany) at room temperature (23°C). For stability monitoring the samples were analyzed in regular intervals during six months of storage at 23°C or 8°C.

Franz-type diffusion cells in a 32°C water bath were used for **skin permeation studies**. The acceptor medium was analyzed after predefined intervals. For further experiments, the porcine skin and the remaining formulation were extracted with acetone. Samples were analyzed via HPLC-DAD and the peak areas of four selected peaks were determined for comparison of the permeation behavior of the respective substances.



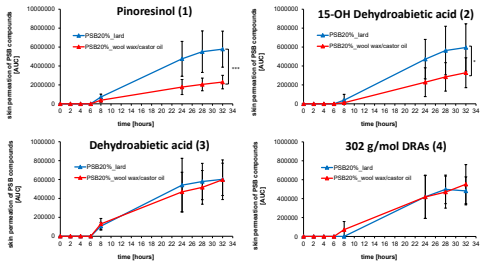
Results – Rheology, skin permeation with two different balm batches

Different formulations were developed and stored at room temperature (23°C) or refrigerated (8°C). After analyzing flow curves during six months of storage, formulations appeared to be stored preferably at a temperature of +8°C.



Effect of storage time and temperature on dynamic viscosity (η, in Pas) of semi-solid vehicles with 20% w/w of PSB at a shear rate of 10 s^{-1}. Values are means +- SD of n = 3 samples.

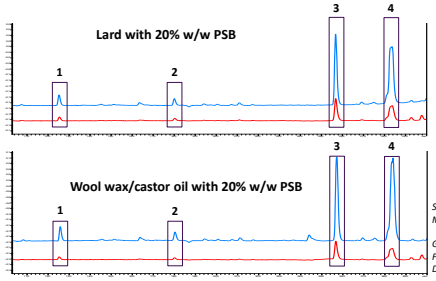
The most rheologically stable and homogenous formulation was 20% w/w balm in wool wax/castor oil. Therefore, this formulation was chosen for permeation studies and compared to a lard-based medical device. The **acceptor medium** (PSB + EtOH 40% v/v) was analyzed after predefined intervals, and selected substances were analyzed. It has to be kept in mind, that two different balm batches were used for the two tested formulations.



*Data represent means of n = 9 cells ± SD. Statistically significant differences between formulations are marked for AUC values after 32 h with * for p < 0.05, ** for p < 0.01 and *** for p < 0.001.*

Results – Skin permeation with the same balm batch

To investigate if the differences in permeation rates occurred due to the different vehicles or different used balm batches, the experiments were repeated with a wool wax/castor oil and a lard-based formulation with 20% w/w of the same balm batch. Furthermore, to analyze the distribution of substances between the three compartments, the **formulation**, the **skin**, and the **acceptor medium** (in this case without EtOH for more physiological conditions) were analyzed via HPLC-DAD after 24 h.



Blue – Residue of formulation
Red – Skin membrane
Both extracted with acetone

Stationary phase: Acclaim® 120 C18 3 µm
Mobile Phase: A – H2O + 0.1% FA, B – ACN + 0.1% FA
Gradient: 15-95% B in 25 min
Flow: 0.3 mL/min
Detection: 190 nm

No substances were detected in the acceptor medium. Comparing the peak areas, a higher amount of substances permeated into the skin from the lard-based device than from wool wax/castor oil, whereas in the wool wax/castor oil formulation, more substances remain in the ointment. Currently, an absolute quantitation of permeated and remaining substances is under investigation. However, these results show, that different balm batches exhibit a highly varying content of bioactive constituents, as well as that the used vehicle has a strong influence on the permeation rate of substances into the skin, potentially due to different solubilizing potential for the involved compounds.

Conclusion

Different spruce balms collected in different areas and at different time points may exhibit highly variable contents, and, therefore, may lead to differences in permeation rates. Using the same spruce balm batch, higher permeation rates into the skin were found for lard compared to wool wax/castor oil. Therefore, the vehicle has an impact on the permeation behavior through intact porcine skin. However, wool wax/castor oil appears to represent a suitable alternative to traditional lard in terms of rheological properties.

Tetranational Congress Phytotherapy 2024
Herbal products for human and animal healthcare
May 30th – 31st, 2024
Utrecht, The Netherlands

References:
[1] Goels, T., Eichenauer, E., Langeder, J., Hoeller, F., Sykora, C., Tahir, A., Urban, E., Heiss, E.H., Saukel, J., Glasl, S., 2020. Norway Spruce Balm: Phytochemical Composition and Ability to Enhance Re-epithelialization In Vitro. Planta Med. 86, 1080-1088.
[2] Eichenauer, E., Jozić, M., Glasl, S., Klang, V., 2023. Spruce Balm-Based Semisolid Vehicles for Wound Healing: Effect of Excipients on Rheological Properties and Ex Vivo Skin Permeation. Pharmaceutics 15, 1678.

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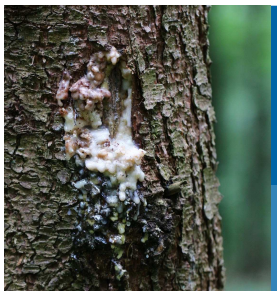


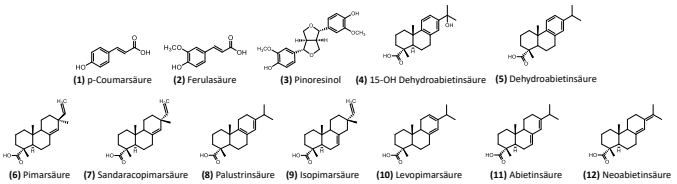
Die komplexe Zusammensetzung des Fichtenbalsams – chromatographische Herausforderungen bei der Qualitätskontrolle

Elisabeth Eichenauer[1,3], Martina Jozić[1], Victoria Klang[2], Sabine Glasl-Tazreiter[1]

1 Universität Wien, Department für pharmazeutische Wissenschaften, Division für Pharmakognosie, Wien, Österreich;
2 Universität Wien, Department für pharmazeutische Wissenschaften, Division für pharm. Technologie, Wien, Österreich;
3 Universität Wien, Doctoral School PhaNuSpo, Wien, Österreich

Einleitung

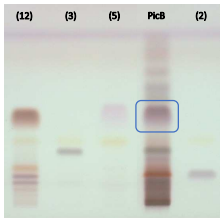
Der Fichtenbalsam ist eine Ausscheidung der Gemeinen Fichte (*Picea abies* (L.) H. Karst., Pinaceae) und wird in der österreichischen Volksmedizin zur Wundheilung verwendet. Traditionell wird dafür eine Salbe aus Schweineschmalz und Fichtenbalsam hergestellt. Seit 2019 gibt es im Österreichischen Arzneibuch die Monographie „Unguentum balsami Piceae Abietis officinale“, eine Salbe aus 20% Fichtenbalsam in Schweineschmalz. Der Balsam enthält verschiedenste Substanzklassen unterschiedlicher Polaritäten wie Hydroxyzimtsäuren, Lignane und Diterpen-Harzsäuren. Die Erstellung einer Methode zur Erfassung aller Substanzklassen wird durch die sehr komplexe Zusammensetzung erschwert, ist aber essentiell für die Qualitätskontrolle [1-3].



Ergebnisse

1. Dünnschichtchromatographie

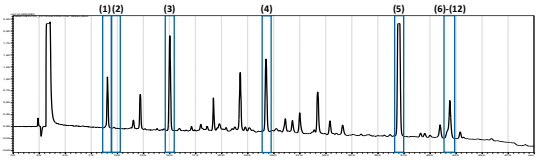
Nach dem Besprühen mit Anisaldehyd/ Schwefelsäure-Reagenz weist der Fichtenbalsams **(PicB)** eine Hauptbande bei R_f 0.5 auf. Diese besteht aus Dehydroabietinsäure **(5)** und sieben Harzsäuren, die alle ein Molekulargewicht von 302 g/mol haben **(6-12)** und sich lediglich in der Position der Doppelbindungen oder in der Stereochemie der Seitenkette unterscheiden. Im Dünnschichtchromatogramm sind exemplarisch die Verbindungen **(5)** und **(12)** dargestellt.



Stationäre Phase: Silica Gel 60 F_{254}
Mobile Phase: $CHCl_3$:MeOH:TFA (97+3+0.1)
Detektion: Vis nach Derivatisierung

2. High performance liquid chromatography

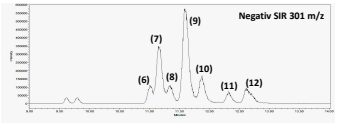
Unter Verwendung einer RP18e-Säule und Detektion bei 190 nm wurde eine HPLC-Methode entwickelt, um alle unterschiedlichen Substanzklassen gleichzeitig zu detektieren. Im Vergleich zur DC ist mit dieser Technik eine Trennung der Dehydroabietinsäure **(5)** von den sieben Harzsäuren der Größe 302 g/mol **(6-12)** möglich. Letztere eluieren allerdings bei 42 min zusammen als ein Peak [2].



Stationäre Phase: RP18e
Mobile Phase: 15-95% ACN + 0.1% FA in 45 min (1 mL/min)
Detektion: 190 nm

3. Supercritical fluid chromatography

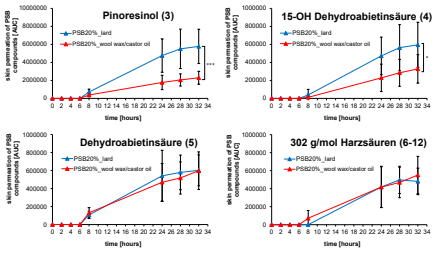
Um die sieben Harzsäuren der Größe 302 g/mol **(6-12)** aufzutrennen, wurde die SFC als weitere Trennmethode gewählt. Mit einer 2-Picolylamin-Säule und einer mobile Phase bestehend aus CO_2 und Ethanol war es schlussendlich möglich, dieses Ziel zu erreichen [3].



Stationäre Phase: Torus 2-Picolylamin
Mobile Phase: 0-5.5% Ethanol in 15 min (1 mL/min)
Detektion: QDa Mass detector (150-700 Da), SIR

Permeationsversuche mit Franz-Diffusionszellen

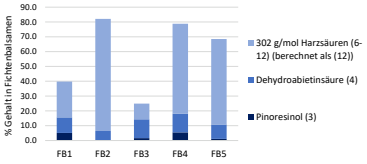
Die entwickelte HPLC-Methode wurde verwendet, um das Permeationsverhalten bestimmter Inhaltsstoffe des Balsams zu analysieren. Dafür wurden zwei verschiedene Salbengrundlagen mit jeweils 20% Fichtenbalsam (Schweineschmalz vs. Wollwachs/Rizinusöl) in Franz-Diffusionszellen auf eine Membran aus porziner Ohrenhaut aufgetragen und der Gehalt an **(3)**, **(4)**, **(5)** und **(6-12)** im Akzeptormedium (Ethanol/PBS 4+6) nach definierten Zeitpunkten gemessen. Die Ergebnisse zeigten für die zwei stärker polaren Substanzen **(3)** und **(4)** eine signifikant höhere Permeationsrate aus Schweineschmalz als aus Wollwachs/Rizinusöl.



Daten repräsentieren Mittelwerte aus n = 9 Zellen ± SD. Statistisch signifikante Unterschiede zwischen Formulierungen sind markiert für die AUC Werte nach 32h ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)*

Heterogenität verschiedener Fichtenbalsame

Um herauszufinden, ob die Unterschiede der Permeationsversuche an den unterschiedlichen Vehikeln oder an verwendeten Fichtenbalsamen liegen, wurde die HPLC-Methode zur Quantifizierung mittels internem Standard (Isoferulasäure) der Substanzen **(3)**, **(5)** und **(6-12)** verwendet. Es wurden fünf verschiedene Fichtenbalsame analysiert, die zu unterschiedlichen Zeitpunkten an unterschiedlichen Stellen in Österreich gesammelt wurden. Die Ergebnisse zeigen eine enorme Variabilität im Gehalt der jeweiligen Substanzen.



Conclusio

Für die Analyse der komplexen Zusammensetzung des Fichtenbalsams sind mehrere chromatographische Techniken nötig, die die Variabilität im Gehalt verschiedener Fichtenbalsame deutlich machten. Der Einsatz all dieser Methoden ermöglicht die Entwicklung neuer Formulierungen sowie die Qualitätskontrolle und –sicherung.

Quellen
[1] Goels T, Eichenauer E, Langeder J, et al. Norway Spruce Balm: Phytochemical Composition and Ability to Enhance Re-epithelialization In Vitro. *Planta Med* 2020; 86: 1080–1088.
[2] Goels T, Eichenauer E, Tahir A, et al. Exudates of *Picea abies*, *Pinus nigra*, and *Larix decidua*: Chromatographic Comparison and Pro-Migratory Effects on Keratinocytes In Vitro. *Plants* 2022; 11: 599.
[3] Goels T, Eichenauer E, Langeder J, et al. Ultra High-Performance Supercritical Fluid Chromatography for the Quantitation of Diterpene Resin Acids in Norway Spruce Samples. **Front. Pharmacol**. 2022, 13, 906411.

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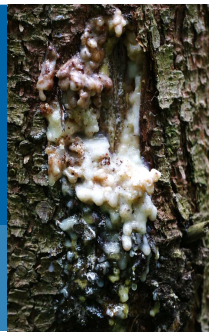
Division of Pharmaceutical Technology

Analysis and quantitation of bioactive compounds in Norway spruce balm

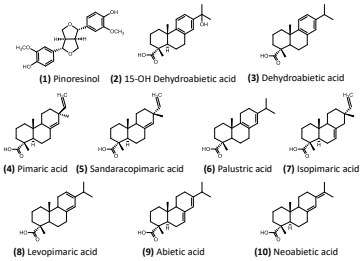


Elisabeth Eichenauer[1,2], Thomas Göls[1,2], Martina Jozić[1], Victoria Klang[1], Sabine Glasl-Tazreiter[1]

1 Department of Pharmaceutical Sciences, Faculty of Life Sciences, University of Vienna;
2 Vienna Doctoral School of Pharmaceutical, Nutritional and Sport Sciences, University of Vienna

Introduction

The resinous excretion of *Picea abies* (L.) H. Karst. (Pinaceae), also known as Norway spruce balm, has been used in European folk medicine since centuries to treat acute, chronic and infected wounds. Traditionally, the balm is processed with lard or another animal fat to an ointment. Lignans like pinoresinol and different diterpene resin acids (DRAs) are known to be part of the wound healing mechanism [1]. Different chromatographic methods for the analysis and quantitation of the bioactive compounds in Norway spruce balm have to be provided in order to guarantee consistent pharmaceutical quality of the balm and commercial products thereof [2].



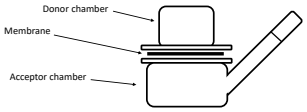
Methods

Chromatographic techniques

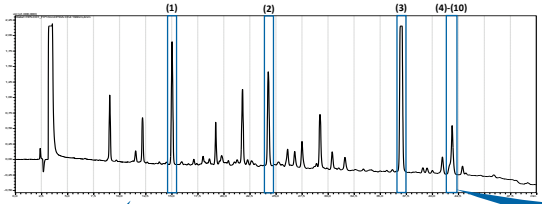
A HPLC-UV/DAD method was established, using a RP-18 column and gradient elution with water/acetonitrile + 0.1% formic acid. Furthermore, an ultra-high performance supercritical fluid-chromatography (UHPSFC) instrumentation was used, hyphenated to a quadrupole mass detector. The stationary phase was a Torus 2-Picolylamin column, as mobile phase CO_2 and ethanol were utilized, again using gradient elution.

Skin permeation

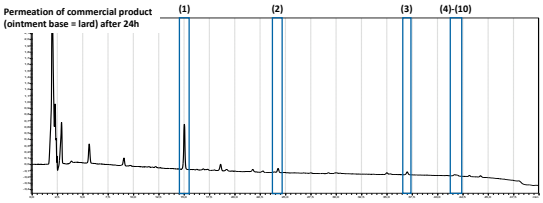
Skin permeation can be imitated using Franz diffusion cells. An ointment is applied in the donor chamber, substances diffuse through a membrane into the acceptor chamber and can be analyzed in the acceptor medium. As membrane porcine ear skin cut to a thickness of 400 µm was used, since this material shows the highest similarity to human skin. As acceptor medium ethanol 96% + PBS (4+6) was chosen.



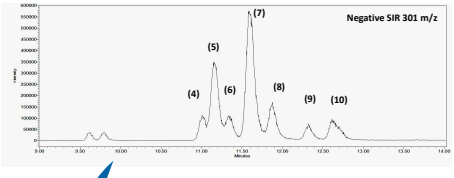
Results



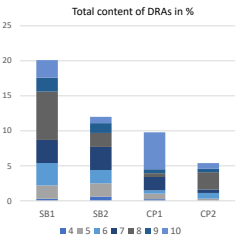
Franz diffusion cells were used in order to determine, if bioactive compounds permeate through a membrane made of pig skin. Samples of the acceptor medium were drawn after 8h, 24h, 28h and 32h, and afterwards analyzed by HPLC-UV/DAD. It was shown, that the most important bioactive compounds are able to permeate through the skin. The next step will be the quantitation of permeating substances.



HPLC-UV/DAD at 190 nm represents a good technique to analyze the bioactive compounds in raw spruce balm, like (1), (2) and (3). However, seven DRAs ((4)-(10)) show high structural similarity and only differ in the position of a double bond or stereochemistry. These DRAs cannot be separated sufficiently by HPLC-UV/DAD. Therefore, an UHPSFC-MS method was established, with which the seven DRAs were separated.



This method was used for the quantitation of these seven DRAs. The composition of raw Norway spruce balm samples (SB1 & 2) and commercial products (CP1 & 2) were approximatively matching, but a difference in the content was detected. This may be due to matrix effects, e.g. the influence of components of the ointment base on the ionization efficiency. These effects still have to be investigated.



Conclusion

The established HPLC-UV/DAD and UHPSFC-MS methods represent an important and essential tool for quality control of raw Norway spruce balm samples and presently available ointments thereof, as well as for the development of new Norway spruce balm products.

Society for Medicinal Plant and Natural Product Research GA

70th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA) August 28-31, 2022 in Thessaloniki, Greece

References

[1] Goels T, Eichenauer E, Langeder J, et al. Norway Spruce Balm: Phytochemical Composition and Ability to Enhance Re-epithelialization In Vitro. *Planta Med* 2020; 86: 1080–1088. doi:10.1055/a-1141-0921

[2] Goels T, Eichenauer E, Tahir A, et al. Exudates of *Picea abies*, *Pinus nigra*, and *Larix decidua*: Chromatographic Comparison and Pro-Migratory Effects on Keratinocytes In Vitro. *Plants* 2022; 11: 599. doi:10.3390/plants11050599

Acknowledgement

The authors thank Johannes Saukel for the collection and identification of the *Picea abies* balm samples as well as the Vienna Anniversary Foundation for Higher Education for funding. This work was supported by the Austrian Federal Forest by supplying the crude plant material of *Picea abies*.

A.3 Supplementary Materials

Supplementary material

Supporting Information

VOLKSMED Database: A Source for Forgotten Wound Healing Plants in Austrian Folk Medicine

Elisabeth Eichenauer[1, 2], Johannes Saukel[1], Sabine Glasl[1]

Affiliations

[1]Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna, Vienna, Austria

[2]Vienna Doctoral School of Pharmaceutical, Nutritional and Sport Sciences, University of Vienna, Vienna, Austria

Correspondence

Sabine Glasl

Division of Pharmacognosy

Department of Pharmaceutical Sciences

University of Vienna

Josef-Holaubek-Platz 2

1090 Vienna

Austria

Phone: +43 14277 55207

sabine.glasl@univie.ac.at

Supplementary material

Table 1S Information about diploma theses used for the establishment of the monographs in the VOLKSMED database.

Diploma thesis	Region	Nr. of interviewed persons	Year
1	Waldviertel/Lower Austria	50	1985
3	Southern Styria	65	1992
4	Vorarlberg	50	1992
5	Salzkammergut	41	1990
6	Lungau/Salzburg	62	1992
7	Alpenvorland/Upper Austrian	52	1993
8	Hochschwab, Ötscherland/Lower Austria	84	1989
9	East Tyrol	50	1988
10	Lower Mühlviertel/Upper Austria	59	1989
11	Upper Innviertel/Upper Austria	42	1989
12	Lower Carinthia	40	1987
13	Upper Mühlviertel/Upper Austria	33	1990
14	Hügelland/West Styria	29	1990
15	Inntal/Tyrol	50	1992
16	Bucklige Welt/Styria	55	1990
17	Mühlviertel/Upper Austria	42	1990
18	Innviertel/Upper Austria	38	1987
19	Mölltal/Carinthia	28	1992
20	Mur, Mürztal/Styria	38	1985
21	Traisental/Lower Austria	30	1990
23	Southern Styria	55	1992
24	Southern Styria	55	1992
25	Montafon/Vorarlberg	27	1991
26	Pinzgau/Salzburg	41	1991
27	Central Styria	52	1989
28	Dunkelsteiner Wald/Lower Austria	25	1991
29	Rax, Schneeberg/Lower Austria	12	1989
30	Flachgau/Salzburg	39	1991
31	Upper Ennstal/Salzburg	49	1989
32	Mur, Mürztal/Styria	50	1990
33	Eastern Styria	33	1983
34	South Tyrol	30	1994
35	Nockgebiet/Carinthia	32	1990
36	Tyrol	53	1994
37	Bregenzerwald/Vorarlberg	25	1987
38	Rosental/Carinthia	29	1986
39	Southern and Eastern Carinthia	34	1987
40	Burgenland	60	1988
50	Lesachtal/East Tyrol	38	1995
52	Innviertel/Upper Austria	42	1995
54	East Tyrol	55	1994
55	South Tyrol	83	1995
56	Phyrn-Eisenwurzen/Upper Austria	47	1998
57	Ötztal/Tyrol	31	2004
58	Southeastern Bavarian Forest/Germany and Austria	80	1995
59	Lower Austria/Upper Austria/Styria/Burgenland	99	1983

Information about the authors of the diploma theses is available from the authors of this publication.

Supplementary material

Table 2S Traditional wound healing plants with less than 10 entries in the VOLKSMED database. The table contains currently accepted species. If the database contains synonyms, they are marked by superscripts and explained in the footnote. The application form indicates the usage of dry (d), fresh (f) plant material, or both (df), or the information is not available (-).

Genus	Species	Family	Part used	Application form	Entries
Agrimonia	*eupatoria* L. *repens*[1] L.	Rosaceae	Herb Leaves	Tea (for rinsing, as a compress) (d), herb directly on wounds (as a compress) (-), ointment (d) Leaves directly on wounds (f)	7
Aristolochia	*clematitis* L.	Aristolochiaceae	Herb Leaves	Tea (as a bath or compress) (df), herb directly on wounds (-) Leaves directly on wounds (f)	7
Solidago	*virgaurea* L.	Asteraceae	Herb	Tea (as a bath or compress) (-)	7
Echinacea	*angustifolia* DC. *purpurea* (L.) Moench	Asteraceae	Leaves Root Flower	Leaves directly on wounds (-), ointment (-) Ointment (d), tea (for rinsing, as a compress) (d) Alcoholic extract (-)	6
Linum	*usitatissimum* L.	Linaceae	Seeds	Boiled in water or milk and applied on wounds (as a compress) (d)	6
Populus	sp. *balsamifera* L.	Salicaceae	Buds	Alcoholic extract (f), buds directly on wounds (as a compress) (f)	6
Rumex	sp. *acetosa* L.	Polygonaceae	Leaves Herb	Leaves directly on wounds (f), tea (d) Herb directly on wounds (-)	6
Tropaeolum	*majus* L.	Tropaeolaceae	Leaves	Leaves directly on wounds (f)	6
Chelidonium	*majus* L.	Papaveraceae	Herb	Herb directly on wounds (as a compress) (f)	5
Levisticum	*officinale* W.D.J.Koch	Apiaceae	Herb (d) Leaves (-) Root (-)	Ointment (d), tea (as a bath) (d) Tea (as a bath) (-) Tea (as a compress) (-)	5
Quercus	sp. *petraea* (Matt.) Liebl. *robur* L. *pubescens* Willd.	Fagaceae	Bark Root	Tea (for rinsing) (d), boiled in milk and applied on wounds (-), ointment (-) Tea (as a bath) (-)	5

Supplementary material

Drimia	*maritima*[2] (L.) Stearn	Asparagaceae	Leaves	Leaves directly on wounds (f)	5
Viola	*odorata* L. *tricolor* L.	Violaceae	Herb	Tea (df), mixed with honey applied on wounds (-), ointment (d)	5
Aloe	sp.	Asphodelaceae	Leaves	Leaves directly on wounds (df), tea from powdered leaves (for rinsing) (d)	4
Betula	*pendula* Roth *pubescens* Ehrh.	Betulaceae	Leaves	Tea (for rinsing, as a bath) (-), leaves directly on wounds (f)	4
Centaurium	sp. *erythraea* Rafn	Gentianaceae	Herb Root	Tea (for rinsing, as a compress) (d) Ointment (d), tea (for rinsing, as a compress) (d)	4
Dryopteris	sp. *filix-mas* (L.) Schott	Polypodiaceae	Herb Root	Alcoholic extract (-), tea (for rinsing, as a compress) (d) Extracted with vinegar and applied on wounds (as a compress) (f)	4
Galium	*aparine* L. *verum* L.	Rubiaceae	Herb	Tea (for rinsing, as a compress) (df), juice from herb on wounds (f)	4
Glechoma	*hederacea* L.	Lamiaceae	Herb	Ointment (-), tea (for rinsing, as a compress) (-)	4
Hieracium	*intybaceum* All.	Asteraceae	Flower	Alcoholic extract (-), flower directly on wounds (-), in oil (-)	4
Juglans	*regia* L.	Juglandaceae	Leaves	Tea (for rinsing, as a bath) (d), leaves directly on wounds (f)	4
Lilium	*candidum* L.	Liliaceae	Flower	In oil (d)	4
Prunus	*avium* (L.) L.	Rosaceae	Resin	Resin directly on wounds (-)	4
	cerasus L. *domestica* L.	Rosaceae	Fruits	Alcoholic extract (f)	
Solanum	*lycopersicum* L.	Solanaceae	Fruits	Fruits directly on wounds (f)	4
	tuberosum L.	Solanaceae	Tuber	Tuber directly on wounds (f)	
Alnus	sp. *glutinosa* (L.) Gaertn. *incana* (L.) Moench	Betulaceae	Leaves	Leaves directly on wounds (f)	3
Arctium	*lappa* L. *minus* (Hill) Bernh. *tomentosum* Mill.	Asteraceae	Root Leaves	Tea (as a bath or compress) (df) Leaves directly on wounds (f)	3
Armoracia	*rusticana* G.Gaertn., B.Mey. & Scherb.	Brassicaceae	Leaves	Leaves directly on wounds (-)	3

Supplementary material

Daucus	*carota* L.	Apiaceae	Root	Root browned in fat or ground root directly on wounds (f)	3
Melissa	*officinalis* L.	Lamiaceae	Leaves	In oil (d), leaves directly on wounds (f), tea (df)	3
Pinus	*cembra* L.	Pinaceae	Cones Resin	Ointment (d) Resin directly on wounds (f)	3
Tanacetum	*balsamita*[3] L.	Asteraceae	Leaves	Leaves directly on wounds (f), tea (d)	3
Thymus	sp. *vulgaris* L.	Lamiaceae	Herb Leaves	Herb directly on wounds (df), in oil (-) Tea (as a bath) (-)	3
Verbena	*officinalis* L.	Verbenaceae	Herb	Juice from herb on wounds (f), tea (-)	3
Elymus	*repens*[4] (L.) Gould	Poaceae	Herb Root	Tea (for rinsing, as a compress) (df) Tea (for rinsing, as a compress) (df)	2
Anthemis	*arvensis* L.	Asteraceae	Herb	Tea (as a bath) (d)	2
Bellis	*perennis* L.	Asteraceae	Flower	Alcoholic extract (d), juice from flower directly on wounds (f)	2
Camelina	*sativa* (L.) Crantz	Brassicaceae	Seeds	Oil gained by pressing seeds applied on wounds (-)	2
Cyclamen	*purpurascens* Mill.	Primulaceae	Flower Leaves	Boiled in milk with honey and applied on wounds (-) Leaves with lard directly on wounds (df)	2
Cardamine	*enneaphyllos*[5] (L.) Crantz	Brassicaceae	Root	Ointment (-)	2
	Graminis flos	Poaceae	Flower	Tea (as a bath) (d)	2
Pimpinella	*major* (L.) Huds.	Apiaceae	Root	Ointment (d), tea (d)	2
Polygonatum	sp. *multiflorum* (L.) All. *verticillatum* (L.) All.	Asparagaceae	Root	Ointment (d), tea (-)	2
Polygonum	*aviculare* L.	Polygonaceae	Herb Leaves	Powdered herb on wounds (df) Leaves directly on wounds (f)	2
Tilia	*cordata* Mill. *platyphyllos* Scop.	Malvaceae	Bark	Ash or bark directly on wounds (-)	2
Urtica	*dioica* L. *urens* L.	Urticaceae	Leaves	Alcoholic extract (-), juice from leaves on wounds (f)	2
Veronica	sp.	Plantaginaceae	Herb	Juice from herb on wounds (f), ointment (d)	2

Supplementary material

	persica Poir.				
Acorus	*calamus* L.	Acoraceae	Rhizome	Alcoholic extract (-)	1
Aesculus	*hippocastanum* L.	Sapindaceae	Seeds	Tea (d)	1
Angelica	*archangelica* L.	Apiaceae	Root	Alcoholic extract (-)	1
Artemisia	*splendens*[6] Willd.	Asteraceae	Herb	Tea (for rinsing) (-)	1
Beta	*vulgaris* subsp. *vulgaris*[7]	Amaranthaceae	Leaves	Leaves directly on wounds (-)	1
Blitum	*bonus-henricus*[8] (L.) Rchb.	Amaranthaceae	Leaves	Leaves directly on wounds (f)	1
Crocus	*sativus* L.	Iridaceae	Stylus	Ointment (-)	1
Cucurbita	*pepo* L.	Cucurbitaceae	Fruits	Fruits directly on wounds (f)	1
Eriophorum	*angustifolium* Honck.	Cyperaceae	Herb	Herb directly on wounds (-)	1
Fagopyrum	*esculentum* Moench	Polygonaceae	Seeds	Grounded seeds with honey on wounds (-)	1
Filipendula	*ulmaria* (L.) Maxim.	Rosaceae	Flower	Tea (for rinsing, as a compress) (-)	1
Fragaria	sp.	Rosaceae	Leaves	Leaves directly on wounds (as a compress) (f)	1
Hedera	*helix* L.	Araliaceae	Leaves	Leaves directly on wounds (f)	1
Hepatica	*nobilis* Schreb.	Ranunculaceae	Herb	Tea (as a bath) (-)	1
Humulus	*lupulus* L.	Cannabaceae	Cones	Tea (as a compress) (-)	1
Hyoscyamus	*niger* L.	Solanaceae	Herb	Tea (for rinsing) (-)	1
Iris	× *germanica* L.	Iridaceae	Root	Ointment (-)	1
Juniperus	*communis* L.	Cupressaceae	Fruits	Fruits directly on wounds (as a compress) (f)	1
Lithospermum	*officinale* L.	Boraginaceae	Seeds	Fumigation (df)	1
Lysimachia	*vulgaris* L.	Primulaceae	Herb	Tea (as a bath) (-)	1
Malus	sp.	Rosaceae	Fruits	Fruits directly on wounds (f)	1
Ocimum	*basilicum* L.	Lamiaceae	Herb	Tea (as a compress) (d)	1
Paris	*quadrifolia* L.	Melanthiaceae	Leaves	Leaves directly on wounds (f)	1

Supplementary material

Potentilla	*erecta* (L.) Raeusch.	Rosaceae	Root	Root directly on wounds (f)	1
Pulmonaria	*officinalis* L.	Boraginaceae	Herb	Ash applied on wounds (-)	1
Ranunculus	*acris* L.	Ranunculaceae	Herb	Herb directly on wounds (f)	1
Rhododendron	*tomentosum*[9] Harmaja	Ericaceae	Herb	Cold maceration in water (for rinsing) (-)	1
Secale	*cereale* L.	Poaceae	Fruits	Rye bread crumbled, mixed with honey applied on wounds (-)	1
Senecio	*nemorensis* L.	Asteraceae	Herb	Tea (as a bath) (d)	1
Taraxacum	sect. *Taraxacum*[10] F.H.Wigg.	Asteraceae	Leaves	Leaves directly on wounds (-)	1
Taxus	*baccata* L.	Taxaceae	Fruits	Fruits directly on wounds (df)	1
Tragopogon	*pratensis* L.	Asteraceae	Flower	Alcoholic extract (df)	1
Trifolium	sp.	Fabaceae	Herb	Tea (as a compress) (d)	1
Trigonella	*foenum-graecum* L.	Fabaceae	Seeds	Boiled in water and applied on wounds (df)	1
Vitis	*vinifera* L.	Vitaceae	Leaves	Leaves directly on wounds (f)	1

[1]*Agrimonia odorata* Mill.
[2]*Urginea maritima* (L.) Baker
[3]*Chrysanthemum balsamita* (L.) Baill.
[4]*Agropyron repens* (L.) P.Beauv.
[5]*Dentaria enneaphyllos* L.
[6]*Artemisia mutellina* S.G.Gmel.
[7]*Beta vulgaris* var. *cicla* L.
[8]*Chenopodium bonus-henricus* L.
[9]*Ledum palustre* L.
[10]*Taraxacum officinale* F.H.Wigg.

Supporting Information

Norway Spruce Balm: Phytochemical Composition and Ability to Enhance Re-epithelialization *in vitro*

Thomas Goels[1], Elisabeth Eichenauer[1], Julia Langeder[1], Franziska Hoeller[1], Christina Sykora[1], Ammar Tahir[1], Ernst Urban[2], Elke H. Heiss[1], Johannes Saukel[1], Sabine Glasl[1]

Affiliation

[1] Department of Pharmacognosy, Faculty of Life Sciences, University of Vienna, Vienna, Austria

[2] Department of Pharmaceutical Chemistry, Faculty of Life Sciences, University of Vienna, Vienna, Austria

Correspondence

ao. Univ.-Prof. Mag. Dr. Sabine Glasl

Department of Pharmacognosy

Faculty of Life Sciences

University of Vienna

Althanstrasse 14

Vienna

Austria

Phone: +43-1-4277-55207

Fax: +43-1-4277-855207

sabine.glasl@univie.ac.at

*Dedicated to emer. Univ.-Prof. Dr. Wolfgang Kubelka on behalf of his 85th birthday

Data of compounds **1** - **9**:

p-Coumaric acid (**1**): yellowish white powder, RT 6.75 min, (HPLC: LiChrospher RP-18e; mobile phase: water + 0.1% FA/acetonitrile + 0.1% FA; gradient: 20–95% acetonitrile + 0.1% FA in 37.5 min); UV_{max} 310 nm (acetonitrile/water); HR-ESI-MS (pos. ion mode) m/z 165.0549 $[M+H]^+$ (calculated for $C_9H_9O_3$ 165.05517).

Caffeic acid (**2**): brown powder, RT 5.26 min, (HPLC: LiChrospher RP-18e; mobile phase: water + 0.1% FA/acetonitrile + 0.1% FA; gradient: 20–95% acetonitrile + 0.1% FA in 37.5 min), UV_{max} 320 nm (acetonitrile/water); ESI-MS (pos. ion mode) m/z 163.0 $[M - H_2O + H]^+$; 181.0 $[M+H]^+$; ESI-MS (neg. ion mode) m/z 179.0 [M-H].

Ferulic acid (**3**): yellowish white powder, RT 7.28 min (HPLC: LiChrospher RP-18e; mobile phase: water + 0.1% FA/acetonitrile + 0.1% FA; gradient: 20–95% acetonitrile + 0.1% FA in 37.5 min), UV_{max} 323 nm (acetonitrile/water); HR ESIMS (pos. ion mode) m/z 195.0656 $[M+H]^+$ (calculated for $C_{10}H_{11}O_4$ 195.06573).

(+)-Pinoresinol (**4**): white powder, RT 13.10 min (HPLC: LiChrospher RP-18e; mobile phase: water + 0.1% FA/acetonitrile + 0.1% FA; gradient: 20–95% acetonitrile + 0.1% FA in 37.5 min), $[\alpha]_D^{20}$ = + 33.6 (c = 0,1429 g/100 mL, MeOH), UV_{max} 230 nm, 280 nm (acetonitrile/water); ^1H NMR (CD_3OD, 500 MHz) δ 6.95 (2H, d, H-2, H-2'), δ 6.82 (2H, dd, H-6, H-6'), δ 6.77 (2H, d, H-5, H-5'), δ 4.71 (2H, d, H-7, H-7'), δ 4.24 (2H, dd, H-9, H-9'), δ 3.85 (4H, m, H-9, H-9', OCH_3, OCH_3'), δ 3.16 (1H, m, H-8, H-8'); ^{13}C NMR (CD_3OD, 125 MHz) δ 149.1 (C, C-3, C-3'), δ 147.3 (C, C-4, C-4'), δ 133.8 (C, C-1, C-1'), δ 120.0 (CH, C-6, C-6'), δ 116.1 (CH, C-5, C-5'), δ 110.9 (CH, C-2, C-2'), δ 87.5 (CH, C-7, C-7'), δ 72.6 (CH_2, C-9, C-9'), δ 55.4 (CH, C-8, C-8'), δ 53.4 (CH_3, OCH_3, OCH_3'); HR-ESI-MS (pos. ion mode) m/z 359.1506 $[M+H]^+$, 717,2934 $[2M+H]^+$ (calculated for $C_{20}H_{23}O_6$ 359.14947).

7*α*,15-Dihydroxy-DHAA (**5**): yellowish resin, RT 15.24 min (HPLC: LiChrospher RP-18e; mobile phase: water + 0.1% FA/acetonitrile + 0.1% FA; gradient: 20–95% acetonitrile + 0.1% FA in 37.5 min), $[\alpha]_D^{20}$ = −7.7 (c = 0.27 g/100 mL, $CHCl_3$), UV_{max} 220 nm, 266 nm (acetonitrile/water); ^{1}H NMR (CD_3OD, 500 MHz) *δ* 7.41 (1H, d, H-14), *δ* 7.33 (1H, dd, H-12), *δ* 7.23 (1H, d, H-11), *δ* 4.71 (1H, d, H-7), *δ* 2.52 (1H, dd, H-5), *δ* 2.35 (1H, d, H-1), *δ* 2.10 (1H, td, H-6), *δ* 1.93 (1H, m, H-3), *δ* 1.84 (1H, d, H-2), *δ* 1.72 (1H, d, H-2), *δ* 1.63 (2H, m, H-3, H-6), *δ* 1.49 (6H, d, H-16, H-17), *δ* 1.45 (1H, dd, H-1), *δ* 1.24 (3H, s, H-19), *δ* 1.13 (3H, dd, H-20); ^{13}C NMR (CD_3OD, 125 MHz) *δ* 182.1 (C, C-18), *δ* 148.8 (C, C-9), *δ* 148.2 (C, C-13), *δ* 136.8 (C, C-8), *δ* 127.6 (CH, C-14), *δ* 125.6 (CH, C-12), *δ* 124.8 (CH, C-11), *δ* 72.8 (C, C-15), *δ* 68.5 (CH, C-7), *δ* 48.3 (C, C-4), *δ* 40.9 (CH, C-5), *δ* 39.0 (CH_2, C-1), *δ* 38.5 (C, C-10), *δ* 37.6 (CH_2, C-3), *δ* 32.4 (CH_2, C-6), *δ* 31.8 (CH_3, C-16, C-17), *δ* 24.7 (CH_3, C-20), *δ* 19.7 (CH_2, C-2), *δ* 17.1 (CH_3, C-19); ESI-MS (pos. ion mode) m/z 297.2 $[M - 2H_2O + H]^+$, 687.4 $[2M + Na]^+$; ESI-MS (neg. ion mode) m/z 331.1 $[M - H]^-$, 663.2 $[2M - H]^-$.

7*β*,15-Dihydroxy-DHAA (**6**): yellowish resin, RT 14.36 min (HPLC: LiChrospher RP-18e, mobile phase: water + 0.1% FA/acetonitrile + 0.1% FA; gradient: 20–95% acetonitrile + 0.1% FA in 37.5 min), $[\alpha]_D^{20}$ = +20.3 (c = 0,5714 g/100 mL, EtOH), UV_{max} 220 nm, 258 nm (acetonitrile/water); ^{1}H NMR ($CDCl_3$, 500 MHz) *δ* 7.66 (1H, d, H-14), *δ* 7.31 (1H, d, H-12), *δ* 7.19 (1H, d, H-11), *δ* 4.90 (1H, dd, H-7), *δ* 2.30 (1H, t, H-1), *δ* 2.28 (1H, t, H-5), *δ* 1.92 (1H, m, H-6), *δ* 1.85 (1H, m, H-6), *δ* 1.82 (1H, m, H-2), *δ* 1.81 (1H, m, H-3), *δ* 1.75 (1H, m, H-2), *δ* 1.73 (1H, m, H-3) *δ* 1.56 (6H, s, H-16, H-17), *δ* 1.45 (2H, t, H-1), *δ* 1.29 (3H, d, H-19), *δ* 1.28 (3H, d, H-20); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ* 182.9 (C, C-18), *δ* 147.7 (C, C-9), *δ* 146.9 (C, C-13), *δ* 137.5 (C, C-8), *δ* 124.4 (CH, C-11), *δ* 124.2 (CH, C-12), *δ* 123.3 (CH, C-14), *δ* 72.8 (C, C-15), *δ* 70.8 (CH, C-7), *δ* 47.2 (C, C-4), *δ* 43.5 (CH, C-5), *δ* 38.0 (CH_2, C-1), *δ* 37.7 (C, C-10), *δ* 36.3 (CH_2, C-3), *δ* 32.7 (CH_2, C-6), *δ* 31.8 (CH_3, C-16, C-17), *δ* 25.5 (CH_3, C-20), *δ* 18.5 (CH_2, C-2), *δ* 16.4 (CH_3, C-19); ESI-MS (pos. ion mode) m/z 297.2 $[M - 2H_2O +$

$H]^+$, 315.2 $[M - H_2O + H]^+$, 687.4 $[2M + Na]^+$; ESI-MS (neg. ion mode) m/z 331.1 $[M - H]^-$, 663.2 [2M − H].

15-Hydroxy-DHAA (**7**): colorless resin, RT 20.63 min (HPLC: LiChrospher RP-18e; mobile phase: water + 0.1% FA/acetonitrile + 0.1% FA; gradient: 35–60 % acetonitrile + 0.1% FA in 45 min), $[\alpha]_D^{20}$ = +52.9 (c = 0.2740 g/100 mL, $CHCl_3$), UV_{max} 220 nm, 267 nm (acetonitrile/water); ^1H NMR (CD_3OD, 500 MHz) *δ* 7.17 (2H, s, H-11, H-12), *δ* 7.09 (1H, s, H-14), *δ* 2.85 (2H, d, J = 6.0 Hz, H-7), *δ* 2.33 (1H, d, J = 12.9 Hz, H-1), *δ* 2.14 (1H, d, J = 12.4 Hz, H-5), *δ* 1.83 (1H, s, H-6), *δ* 1.80 (1H, s, H-2), *δ* 1.78 (1H, s, H-3), *δ* 1.68 (1H, d, J = 10.3 Hz, H-2), *δ* 1.63 (1H, d, J = 9.9 Hz, H-3), *δ* 1.50 (1H, s, H-6), *δ* 1.46 (6H, s, H-16, H-17), *δ* 1.40 (1H, s, H-1), *δ* 1.24 (3H, s, H-19), *δ* 1.18 (3H, s, H-20); ^{13}C NMR (CD_3OD, 125 MHz) *δ* 182.6 (C, C-18), *δ* 148.8 (C, C-9), *δ* 147.6 (C, C-13), *δ* 135.4 (C, C-8), *δ* 126.0 (CH, C-14), *δ* 125.0 (CH, C-11), *δ* 123.2 (CH, C-12), *δ* 72.8 (C, C-15), *δ* 48.4 (C, C-4), *δ* 46.5 (CH, C-5), *δ* 39.4 (CH_2, C-1), *δ* 38.0 (CH_2, C-3), *δ* 38.0 (C, C-10), *δ* 31.9 (CH_3, C-17), *δ* 31.8 (CH_3, C-16), *δ* 31.4 (CH_2, C-7), *δ* 25.5 (CH_3, C-20), *δ* 22.9 (CH_2, C-6), *δ* 19.7 (CH_2, C-2), *δ* 17.0 (CH_3, C-19); ESI-MS (pos. ion mode) m/z 299.1 $[M - H_2O + H]^+$, 654.7 $[2M + Na + H]^+$; ESI-MS (neg. ion mode) m/z 315.0 $[M - H]^-$, 653.0 [2M − 2H + Na].

7-Hydroxy-DHAA (**8**): colourless resin, RT 13.11 min (HPLC: LiChrospher RP-18e; mobile phase: water + 0.1% FA/acetonitrile + 0.1% FA; gradient: 50 % acetonitrile + 0.1% FA isocratic in 30 min), $[\alpha]_D^{20}$ = +71.3 (c = 0,1810 g/100 mL, MeOH), UV_{max} 220 nm, 275 nm (acetonitrile/water); ^1H NMR (CD_3OD, 500 MHz) *δ* 7.33 (1H, s, H-14), *δ* 7.13 (1H, d, *J* = 8.3 Hz, H-11), *δ* 7.03 (1H, d, *J* = 7.3 Hz, H-12), *δ* 4.70 (1H, s, H-7), *δ* 2.82 (1H, s, H-15), *δ* 2.30 (2H, d, *J* = 12.0 Hz, H-1), *δ* 2.17 (1H, d, *J* = 12.0 Hz, H-5), *δ* 1.78 (4H, s, H-2, H-3, H-6), *δ* 1.66 (2H, s, H-2, H-3), *δ* 1.26 (6H, s, H-19, H-20), *δ* 1.20 (3H, s, H-17), *δ* 1.19 (3H, s H-16); ^{13}C NMR (CD_3OD, 125 MHz) *δ* 182.2 (C, C-18), *δ* 148.1 (C, C-9), *δ* 147.3 (C, C-13), *δ* 139.1 (C, C-8), *δ* 126.6 (CH, C-12), *δ* 126.4 (CH, C-14), *δ* 125.1 (CH, C-11), *δ* 71.3 (CH, C-7), *δ*

48.2 (C, C-4), *δ* 45.0 (CH, C-5), *δ* 39.4 (CH_2, C-1), *δ* 38.7 (C, C-10), *δ* 37.8 (CH_2, C-3), *δ* 35.1 (CH, C-15), *δ* 33.4 (CH_2, C-6), *δ* 25.8 (CH_3, C-20), *δ* 24.5 (CH_3, C-16, C-17), *δ* 19.5 (CH_2, C-2), *δ* 17.0 (CH_3, C-19); ESI-MS (pos. ion mode) m/z 299.2 $[M - H_2O + H]^+$, 597.3 $[2M - 2H_2O + H]^+$; ESI-MS (neg. ion mode) m/z 315.0 $[M - H]^-$, 630.6 $[2M - H]^-$.

DHAA (**9**): white crystals, RT 31.67 min (HPLC: LiChrospher RP-18e; mobile phase: water + 0.1% FA/acetonitrile + 0.1% FA; gradient: 20–95% acetonitrile + 0.1% FA in 37.5 min), $[\alpha]_D^{20}$ = +57.5 (c = 2.000 g/100 mL, MeOH), UV_{max} 220 nm, 270 nm (acetonitrile/water); ^1H NMR ($CDCl_3$, 500 MHz) *δ* 7.17 (1H, d, J = 8.0 Hz, H-11), *δ* 7.00 (1H, d, J = 7.9 Hz, H-12), *δ* 6.89 (1H, a, H-14), *δ* 2.93 (1H, m, H-7), *δ* 2.89 (1H, m, H-7), *δ* 2.82 (1H, septet, H-15), *δ* 2.31 (1H, t, J = 12.3 Hz, H-1), *δ* 2.25 (1H, d, J = 12.4 Hz, H-5), *δ* 1,85 (1H, m, H-6), *δ* 1,80 (1H, m, H-2), *δ* 1,79 (1H, m, H-3), *δ* 1,73 (1H, m, H-3) *δ* 1,72 (1H, m, H-2), *δ* 1,53 (1H, m, H-6), *δ* 1,50 (1H, m, H-1), *δ* 1.28 (3H, s, H-19), *δ* 1.23 (6H, d, J = 3.8 Hz, H-16, H-17), *δ* 1.21 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ* 184.6 (C, C-18), *δ* 146.7 (C, C-9), *δ* 145.7 (C, C-13), *δ* 134.7 (C, C-8), *δ* 126.9 (CH, C-14), *δ* 124.1 (CH, C-11), *δ* 123.9 (CH, C-12), *δ* 47.4 (C, C-4), *δ* 44.6 (CH, C-5), *δ* 37.9 (CH_2, C-1), *δ* 36.8 (CH_2, C-10), *δ* 36.7 (CH_2, C-3), *δ* 33.4 (CH, C-15), *δ* 30.0 (CH_2, C-7), *δ* 25.1 (CH_3, C-20), *δ* 24.0 (CH_3, C-16, C-17), *δ* 21.7 (CH_2, C-6), *δ* 18.5 (CH_2, C-2), *δ* 16.2 (CH_3, C-19); ESI-MS (pos. ion mode) m/z 301.3 $[M + H]^+$; ESI-MS (neg. ion mode) m/z 299.4 $[M - H]^-$, 345.1 $[M + FA - H]^-$.

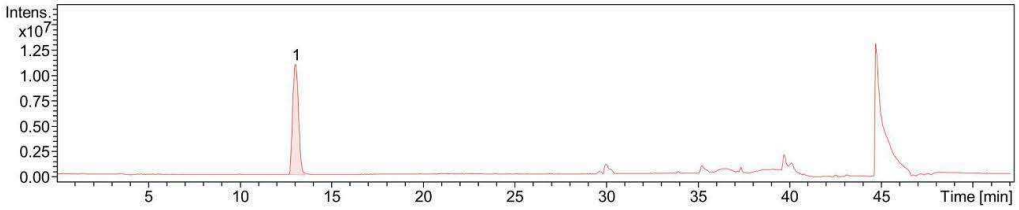
Compound Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data_TS_data\Lisi\20170619\SGtg17_FC01_8_pos_1007_RA5_01_2580.d				
Method	lcms_20_to_95_in_37min_posms1.m			Operator	JW_DD
Sample Name	SGtg17_FC01_8_pos_1007			Instrument	maXis HD 1820881.21300
Comment					

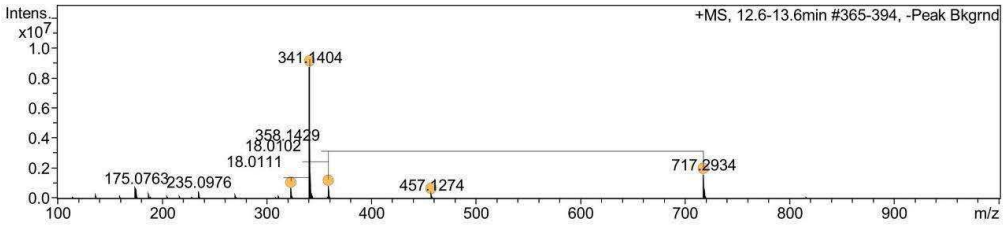
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.8 Bar
Focus	Active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	7.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Source
		Set Corona	0 nA	Set APCI Heater	0 °C



#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
1	13.1	238893584	Manual	11048584	532.3	BPC +All MS	341.1404	

Cmpd 1, 13.1 min



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule
323.1293	1	C20H19O4	323.1278	-4.7	10.2	1	63.02	11.5	even	ok
	2	C21H15N4	323.1291	-0.6	23.4	2	100.00	16.5	even	ok
	1	C20H19O4	323.1278	-4.7	10.2	1	63.02	11.5	even	ok
	2	C21H15N4	323.1291	-0.6	23.4	2	100.00	16.5	even	ok
341.1404	1	C20H21O5	341.1384	-6.0	18.4	1	57.07	10.5	even	ok
	2	C21H17N4O	341.1397	-2.1	31.4	2	100.00	15.5	even	ok
	1	C20H21O5	341.1384	-6.0	18.4	1	57.07	10.5	even	ok
	2	C21H17N4O	341.1397	-2.1	31.4	2	100.00	15.5	even	ok
359.1506	1	C20H23O6	359.1489	-4.6	9.1	1	61.26	9.5	even	ok
	2	C17H15N10	359.1476	-8.3	10.5	2	19.58	15.5	even	ok
	3	C21H19N4O2	359.1503	-0.8	22.4	3	100.00	14.5	even	ok
	1	C20H23O6	359.1489	-4.6	9.1	1	61.26	9.5	even	ok
457.1274	1	C16H21N6O10	457.1314	8.7	6.1	1	12.07	9.5	even	ok
	2	C14H9N20	457.1314	8.6	13.0	3	10.76	20.5	even	ok
717.2934	1	C40H45O12	717.2906	-4.0	17.2	10	20.72	18.5	even	ok

Fig. 1S: MS spectrum and analysis settings of **4.**

Generic Display Report

Analysis Info

Analysis Name	D:\Data_TS_data_Lisi\FC09-9_BA6_01_4658.d		
Method	acn_20_95_lcms_uv_ams1_170410_4658.m	Operator	User
Sample Name	FC09-9	Instrument	HCT
Comment			

Mode

Mass Range Mode	Std/Enhanced
Ion Polarity	Positive
Ion Source Type	ESI
Alternating Ion Polarity	on
Current Alternating Ion Pol	Positive
Divert Valve	to Source

Tune SPS

Target Mass	600 m/z
Compound Stability	100 %
Trap Drive Level	100 %
Optimize	Wide
Smart Parameter Setting	active

Tune Source

Trap Drive	62.1
Octopole RF Amplitude	200.0 Vpp
Lens 2	-60.0 Volt
Capillary Exit	127.0 Volt
Dry Temp (Set)	340 °C
Nebulizer (Set)	26.00 psi
Dry Gas (Set)	9.00 l/min
HV Capillary	-3700 V
HV End Plate Offset	-500 V

Trap

Rolling	off
Scan Begin	80 m/z
Scan End	2500 m/z
Averages	5 Spectra
Max. Accu Time	10000 µs
(Smart) ICC Target	200000
ICC	on

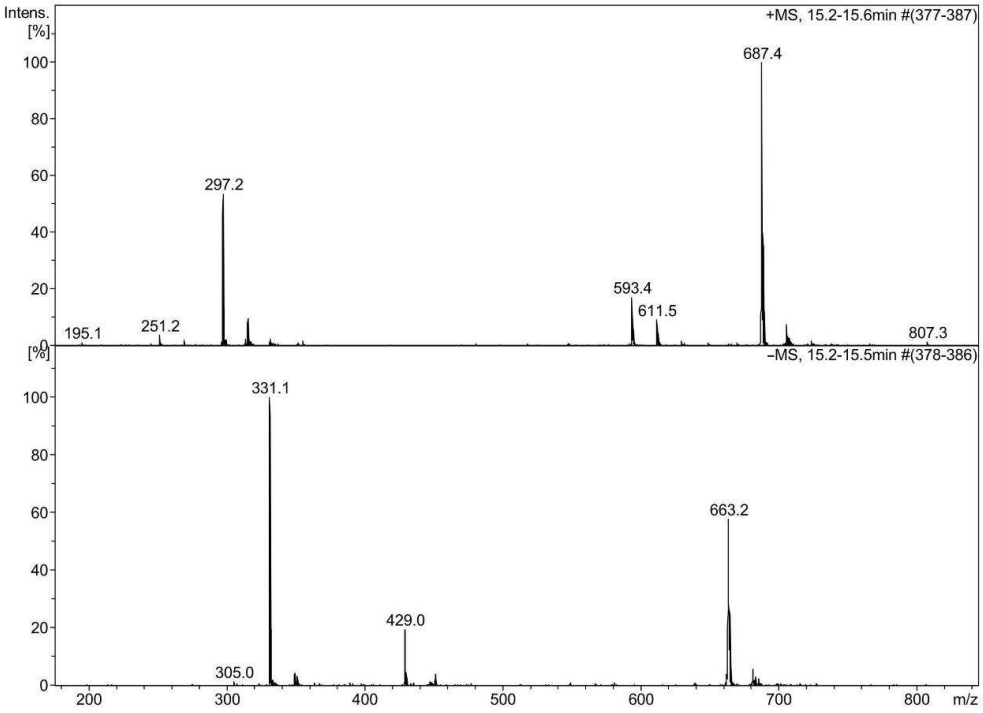


Fig. 2S. MS spectrum and analysis settings of **5.**

Generic Display Report

Analysis Info

Analysis Name	D:\Data_TS_data_Lisi\FC09-9_BA6_01_4658.d		
Method	acn_20_95_lcms_uv_ams1_170410_4658.m	Operator	User
Sample Name	FC09-9	Instrument	HCT
Comment			

Mode

Mass Range Mode	Std/Enhanced
Ion Polarity	Positive
Ion Source Type	ESI
Alternating Ion Polarity	on
Current Alternating Ion Pol	Positive
Divert Valve	to Source

Tune SPS

Target Mass	600 m/z
Compound Stability	100 %
Trap Drive Level	100 %
Optimize	Wide
Smart Parameter Setting	active

Tune Source

Trap Drive	62.1
Octopole RF Amplitude	200.0 Vpp
Lens 2	-60.0 Volt
Capillary Exit	127.0 Volt
Dry Temp (Set)	340 °C
Nebulizer (Set)	26.00 psi
Dry Gas (Set)	9.00 l/min
HV Capillary	-3700 V
HV End Plate Offset	-500 V

Trap

Rolling	off
Scan Begin	80 m/z
Scan End	2500 m/z
Averages	5 Spectra
Max. Accu Time	10000 µs
(Smart) ICC Target	200000
ICC	on

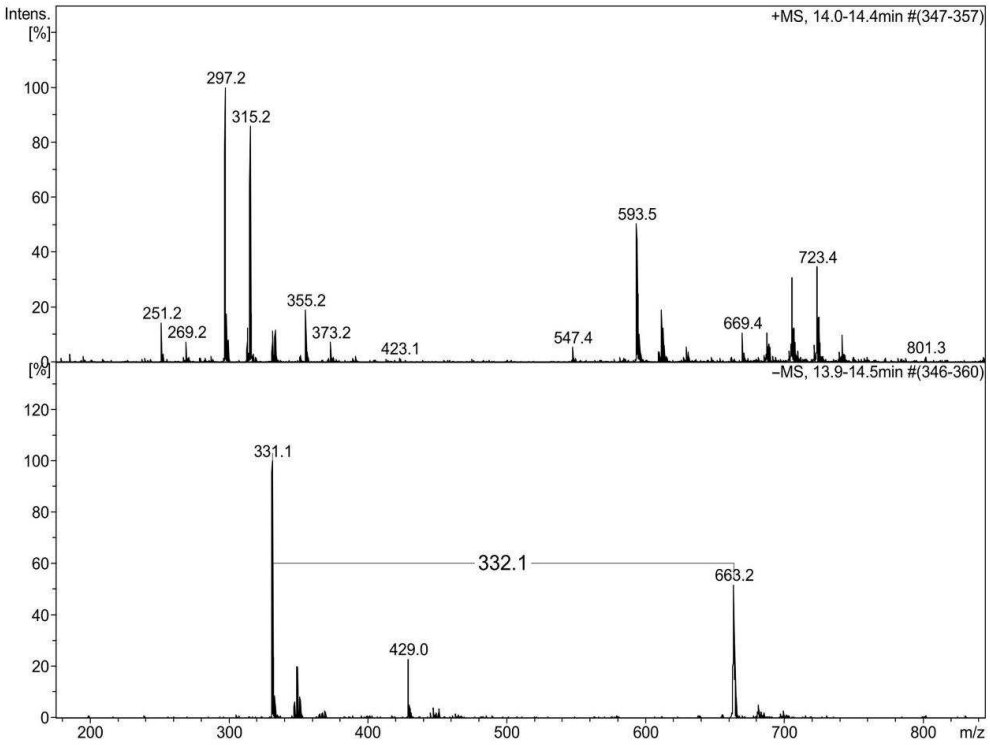


Fig. 3S. MS spectrum and analysis settings of **6**

Generic Display Report

Analysis Info

Analysis Name	D:\Data_TS_data_Thomas\FC07_9_U_RB1_01_4375.d		
Method	fc07_9u_lcms_ams1_161110_4375.m	Operator	User
Sample Name	FC07_9_U	Instrument	HCT
Comment			

Mode

Mass Range Mode	Std/Enhanced
Ion Polarity	Positive
Ion Source Type	ESI
Alternating Ion Polarity	on
Current Alternating Ion Pol	Positive
Divert Valve	to Source

Tune SPS

Target Mass	600 m/z
Compound Stability	100 %
Trap Drive Level	100 %
Optimize	Wide
Smart Parameter Setting	active

Tune Source

Trap Drive	62.1
Octopole RF Amplitude	200.0 Vpp
Lens 2	-60.0 Volt
Capillary Exit	127.0 Volt
Dry Temp (Set)	340 °C
Nebulizer (Set)	26.00 psi
Dry Gas (Set)	9.00 l/min
HV Capillary	-3700 V
HV End Plate Offset	-500 V

Trap

Rolling	off
Scan Begin	80 m/z
Scan End	2500 m/z
Averages	5 Spectra
Max. Accu Time	10000 µs
(Smart) ICC Target	80000
ICC	on

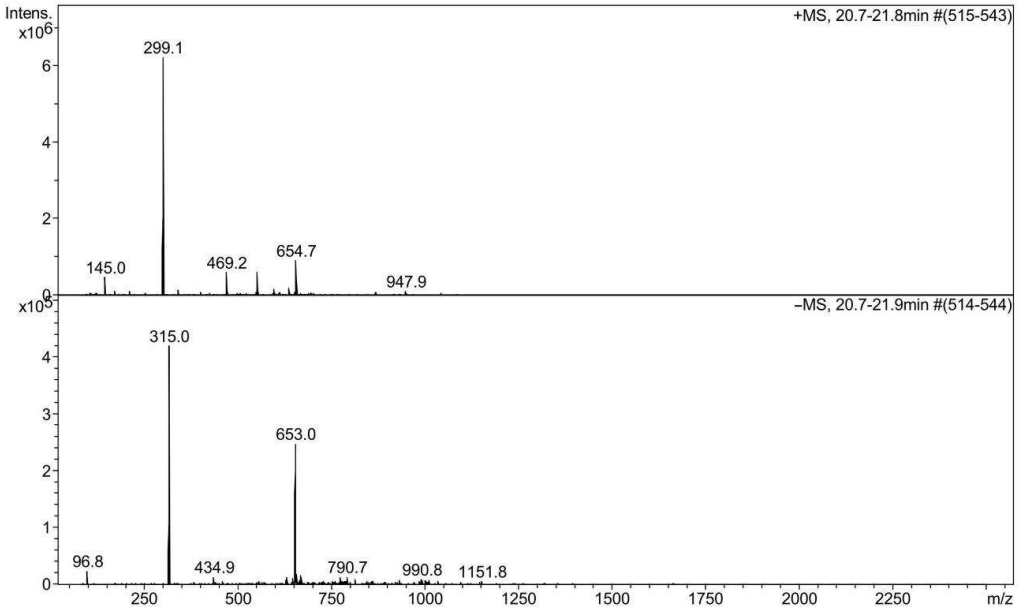


Fig. 4S. MS spectrum and analysis settings of **7.**

Generic Display Report

Analysis Info

Analysis Name	D:\Data_TS_data_Thomas\F11_RB3_01_4377.d		
Method	f11_lcms_ams1_161110_4377.m	Operator	User
Sample Name	F11	Instrument	HCT
Comment			

Mode

Mass Range Mode	Std/Enhanced
Ion Polarity	Negative
Ion Source Type	ESI
Alternating Ion Polarity	on
Current Alternating Ion Pol	Negative
Divert Valve	to Source

Tune SPS

Target Mass	600 m/z
Compound Stability	100 %
Trap Drive Level	100 %
Optimize	Wide
Smart Parameter Setting	active

Tune Source

Trap Drive	72.1
Octopole RF Amplitude	200.0 Vpp
Lens 2	60.0 Volt
Capillary Exit	-127.0 Volt
Dry Temp (Set)	340 °C
Nebulizer (Set)	26.00 psi
Dry Gas (Set)	9.00 l/min
HV Capillary	3500 V
HV End Plate Offset	-500 V

Trap

Rolling	off
Scan Begin	80 m/z
Scan End	2500 m/z
Averages	5 Spectra
Max. Accu Time	10000 µs
(Smart) ICC Target	30000
ICC	on

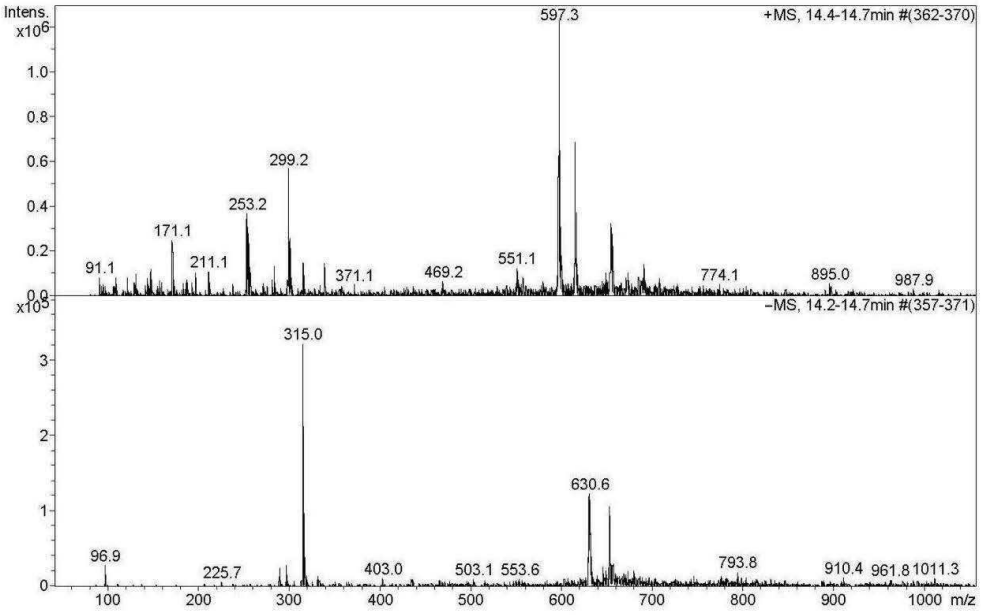


Fig. 5S. MS spectrum and analysis settings of **8.**

Generic Display Report

Analysis Info

Analysis Name	D:\Data_TS_data_Thomas\SFC02_03_RB6_01_4380.d		
Method	sfc02_03_lcms_ams1_161110_4380.m	Operator	User
Sample Name	SFC02_03	Instrument	HCT
Comment			

Mode

Mass Range Mode	Std/Enhanced
Ion Polarity	Positive
Ion Source Type	ESI
Alternating Ion Polarity	on
Current Alternating Ion Pol	Positive
Divert Valve	to Source

Tune SPS

Target Mass	600 m/z
Compound Stability	100 %
Trap Drive Level	100 %
Optimize	Wide
Smart Parameter Setting	active

Tune Source

Trap Drive	62.1
Octopole RF Amplitude	200.0 Vpp
Lens 2	−60.0 Volt
Capillary Exit	127.0 Volt
Dry Temp (Set)	340 °C
Nebulizer (Set)	26.00 psi
Dry Gas (Set)	9.00 l/min
HV Capillary	−3700 V
HV End Plate Offset	−500 V

Trap

Rolling	off
Scan Begin	80 m/z
Scan End	2500 m/z
Averages	5 Spectra
Max. Accu Time	10000 µs
(Smart) ICC Target	80000
ICC	on

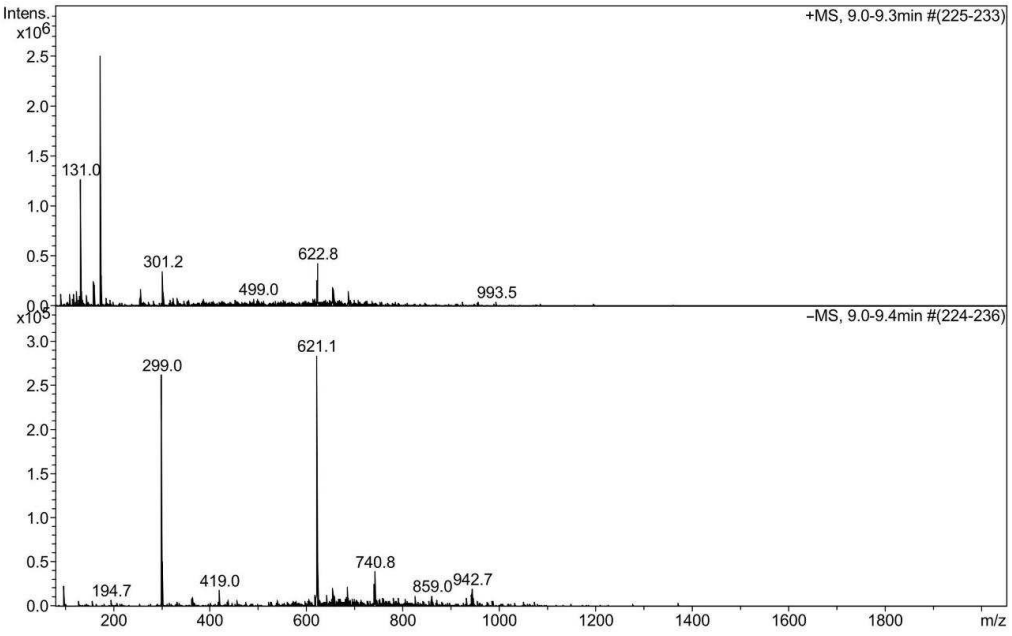


Fig. 6S. MS spectrum and analysis settings of **9.**

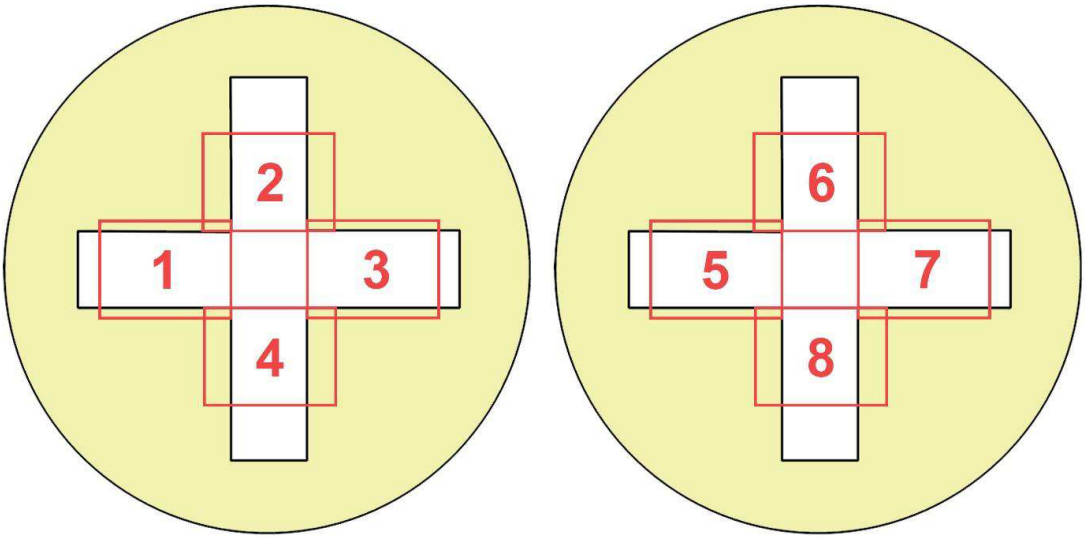


Fig. 7S. Cross shaped cell-free area and image sections corresponding to the horizontal (1, 3, 5, 7) and vertical (2, 4, 6, 8) pictures taken in the landscape format.

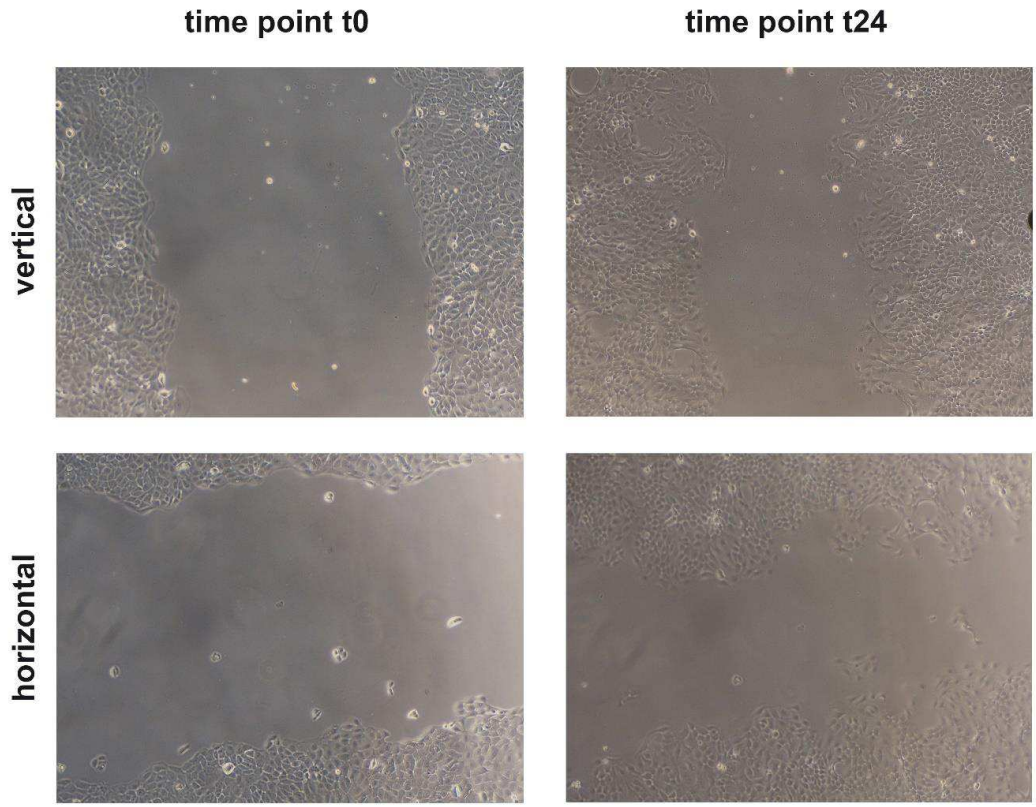


Fig. 8S. Sample images of the bioassay for the vertical and horizontal vehicle controls.

frontiers

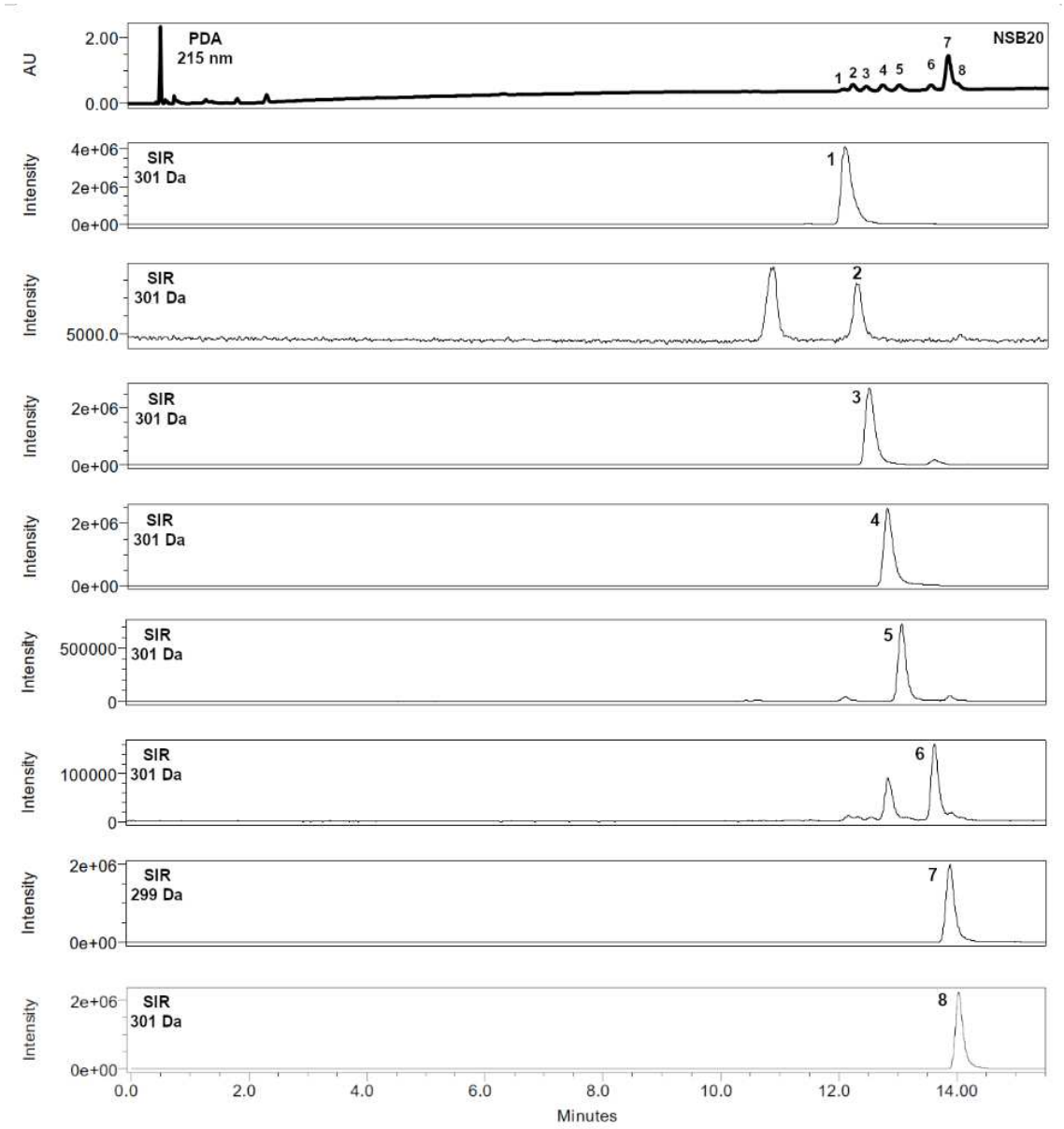
Supplementary Material

1. Norway spruce balm samples

Batch #	Date of harvest	Harvest location	Commercial product	Batch #	Expiration date	Storage conditions
NSB01 - NSB08	09.07.2016	Promau Kitzhütte, Hollenstein, Lower Austria	NSB-CP1	552026	06/21	stored at room temperature
NSB09 - NSB12	03.07.2016	Gaflenz Heiligenstein, Upper Austria	NSB-CP2	552014	01/19	stored at room temperature
NSB13	2015	Lungau, Salzburg	NSB-CP3	552014	01/19	stored at 2 – 8°C
NSB14	15.05.2016	Zillingtaler Wald, Burgenland	NSB-CP4	160301	03/20	stored at room temperature
NSB15	15.05.2016		NSB-CP5	160301	03/20	stored at 2 – 8°C
NSB16	Spring 2016	Pinsdorf, Upper Austria				
NSB17	10.07.2015	unknown				
NSB18	24.08.2016	Lungau, Salzburg				
NSB19	Harvest 2010-2016	Austria				
NSB20	Harvest 2010-2016	Austria				
NSB21	Harvest 2010-2016	Austria				
NSB22	23.08.2017	Lungau, Salzburg				

Supplementary Table S1. Details for Norway Spruce Balm Samples NSB01-22 and Commercial Products Samples

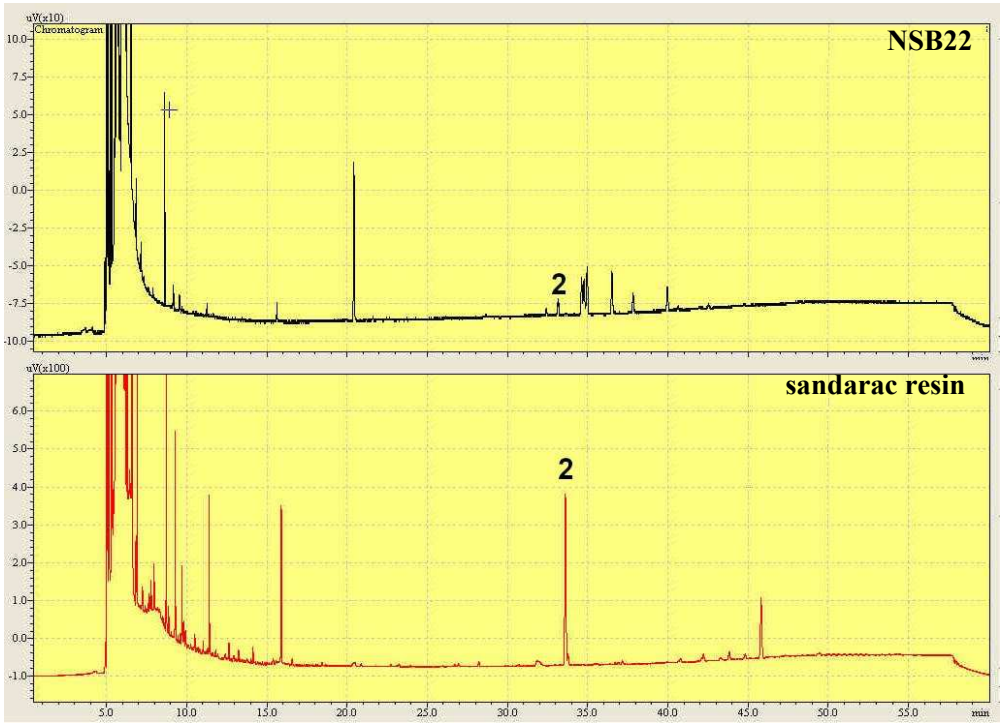
2. Sample chromatogram



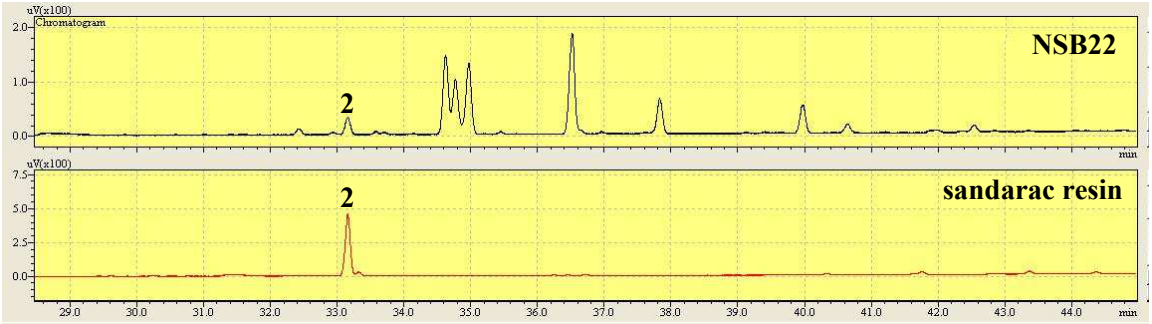
Supplementary Figure S1. PDA chromatogram of spruce balm sample NSB20 (at 215 nm) and UHPSFC-ESI-MS Extracted Ion Chromatograms (negative mode, 299 and 301 Da) of diterpene resin acids. Compound **1**: Pimaric acid, **2**: Sandaracopimaric acid (sample: sandarac resin), **3**: Palustric acid, **4**: Isopimaric acid, **5**: Levopimaric acid, **6**: Abietic acid, **8**: Neoabietic acid

3. Validation

3.1.Sandarac resin



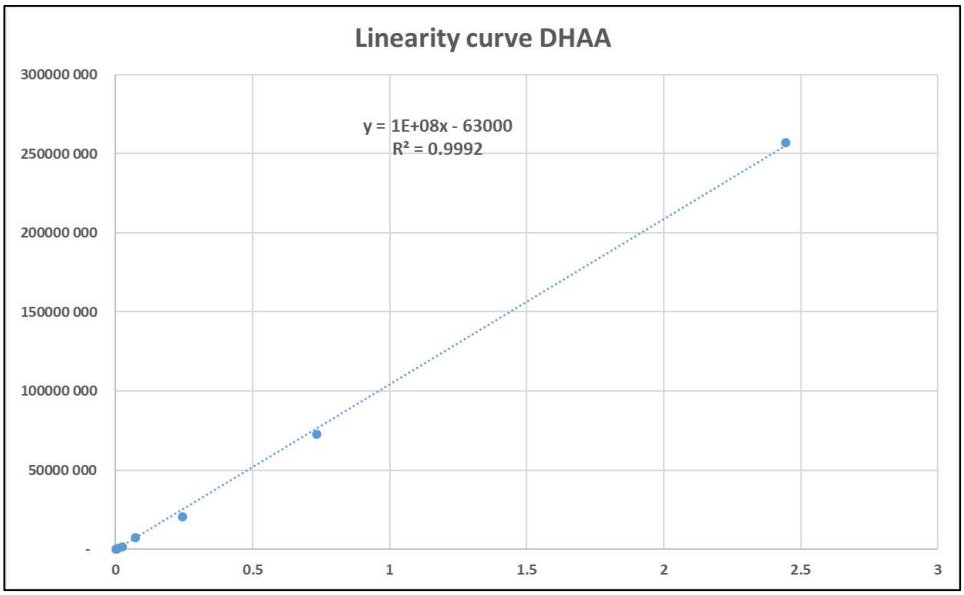
Supplementary Figure S2. GC-FID chromatogram of spruce balm sample NSB22 and sandarac resin. Compound **2**: sandaracopimaric acid. GC-System Shimadzu GC-2010: stationary phase ZB-5 Zebron, 60 m, 0.25 mm, 0.25 µm; mobile phase: He (5.0), flow: 50 ml/min; temperature program: 5 min 100°C, 100-200°C rate 15°C/min, 200-250°C rate 2°C/min; injection volume: 1 µl, split ratio 1:100; detection: synthetic air (5.0), H_2 (5.0)



Supplementary Figure S3. Detailed section (RT 28.5-45 min) of GC-FID chromatogram of spruce balm sample NSB22 and sandarac resin. Compound **2**: sandaracopimaric acid. GC-System Shimadzu GC-2010: stationary phase ZB-5 Zebron, 60 m, 0.25 mm, 0.25 µm; mobile phase: He (5.0), flow: 50

ml/min; temperature program: 5 min 100°C, 100-200°C rate 15°C/min, 200-250°C rate 2°C/min; injection volume: 1 µl, split ratio 1:100; detection: synthetic air (5.0), H_2 (5.0)

3.2. Linearity

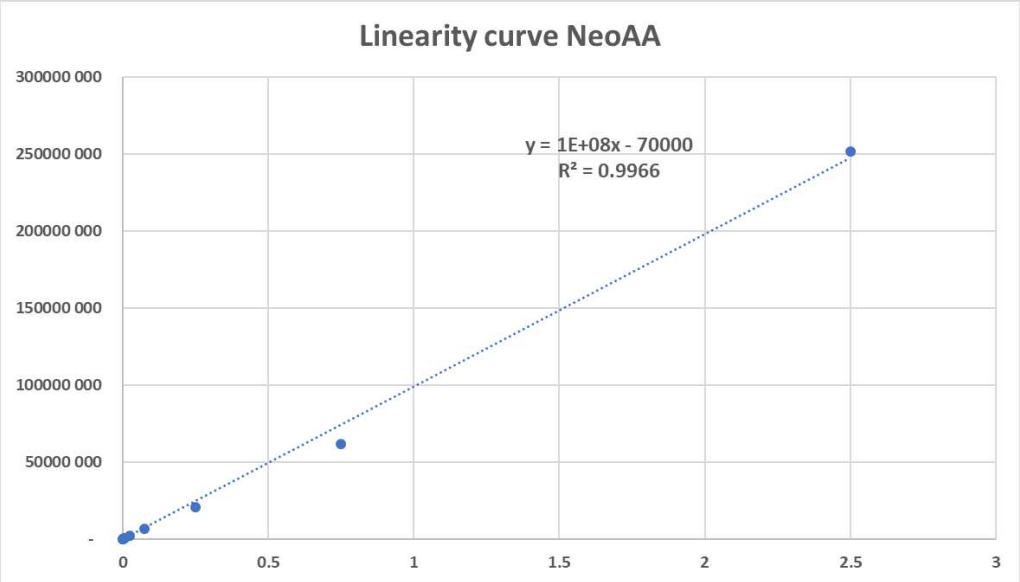


Supplementary Figure S4. Linearity curve of dehydroabietic acid

c [mg/ml]	Run	RT [min]	Area	Mean
7.335	1	13.725	475 897 268	466 371 025
	2	13.738	419 734 916	
	3	13.726	503 480 890	
2.445	1	13.766	255 190 971	257 088 347
	2	13.760	257 112 675	
	3	13.766	258 961 397	
0.7335	1	13.812	73 152 487	72 593 616
	2	13.823	72 625 126	
	3	13.808	72 003 234	
0.2445	1	13.870	19 252 645	20 360 947
	2	13.884	20 679 791	
	3	13.874	21 150 404	
0.07335	1	13.901	7 228 218	7 234 994
	2	13.914	7 313 775	
	3	13.904	7 162 989	

0.02445	1	13.927	1 602 098	1 641 123
	2	13.929	1 603 410	
	3	13.928	1 717 862	
0.007335	1	13.956	715 245	697 620
	2	13.959	708 727	
	3	13.932	668 887	
0.002445	1	13.934	199 290	192 139
	2	13.938	200 749	
	3	13.959	176 379	
0.000734	1	13.938	71 607	81 348
	2	13.956	85 217	
	3	13.966	87 219	

Supplementary Table S2. Experimental data for the calculation of the linearity curve of dehydroabietic acid



Supplementary Figure S5. Linearity curve of neoabietic acid

c [mg/ml]	Run	RT [min]	Area	Mean
7.5	1	13.917	499795141	498 286 885
	2	13.916	498704548	
	3	13.914	496360967	
2.5	1	13.929	252165844	251 661 129
	2	13.931	251317481	
	3	13.931	251500063	

0.75	1	13.987	62355659	61 879 886
	2	13.990	61778821	
	3	13.996	61505179	
0.25	1	14.042	20672692	20 945 889
	2	14.027	21217309	
	3	14.027	20947667	
0.075	1	14.075	6912575	6 827 319
	2	14.055	6805296	
	3	14.076	6764087	
0.025	1	14.085	2173278	2 145 625
	2	14.087	2162849	
	3	14.091	2100747	
0.0075	1	14.090	731793	729 842
	2	14.109	709099	
	3	14.112	748635	
0.0025	1	14.094	378984	368 469
	2	14.106	368446	
	3	14.092	357978	
0.00075	1	14.107	72901	74 350
	2	14.098	75606	
	3	14.127	74542	

Supplementary Table S3. Experimental data for the calculation of the linearity curve of neoabietic acid

3.3. Specificity

Reference substances		**Extr. Ion**	**Extract**		**Deviation**
Resin acid	**RT [min]**	**[Da]**	**Peak number**	**RT [min]**	**[min]**
Pimaric acid	12.072	301^-	1	12.160	-0.088
Sandaracopimaric acid*	12.387	301^-	2	12.313	0.074
Palustric acid	12.477	301^-	3	12.508	-0.031
Isopimaric acid	12.792	301^-	4	12.801	-0.009
Levopimaric acid	13.064	301^-	5	12.963	0.101
Abietic acid	13.586	301^-	6	13.575	0.011
Dehydroabietic acid	13.873	299^-	7	13.885	-0.012
Neoabietic acid	14.024	301^-	8	13.975	0.049

*sandarac extract was tested

Supplementary Table S4. References of the resin acids were compared to the respective constituent of *Picea abies* balm extract NSB22. The deviation of the reference substance to the resin acid of the extract was calculated.

3.4. Accuracy

		DHAA Area	Recovery rate
1	NSB22 2mg/ml	20 352 108	
	DHAA 0.183 mg/ml	10 933 543	
	DHAA 0.244 mg/ml	15 036 184	
	DHAA 0.305 mg/ml	18 396 690	
	NSB22 2 mg/ml+DHAA 0.183 mg/ml	32 220 874	**97.1**
	NSB22 2 mg/ml+DHAA 0.244 mg/ml	38 094 500	**92.9**
	NSB22 2 mg/ml+DHAA 0.305 mg/ml	43 683 516	**88.7**
2	NSB22 2mg/ml	19 722 408	
	DHAA 0.183 mg/ml	10 802 031	
	DHAA 0.244 mg/ml	15 237 185	
	DHAA 0.305 mg/ml	17 770 642	
	NSB22 2 mg/ml+DHAA 0.183 mg/ml	31 473 598	**97.0**
	NSB22 2 mg/ml+DHAA 0.244 mg/ml	37 361 626	**93.6**
	NSB22 2 mg/ml+DHAA 0.305 mg/ml	43 739 038	**85.7**
3	NSB22 2mg/ml	19 531 898	
	DHAA 0.183 mg/ml	10 768 794	
	DHAA 0.244 mg/ml	14 706 166	
	DHAA 0.305 mg/ml	17 849 075	
	NSB22 2 mg/ml+DHAA 0.183 mg/ml	31 312 698	**96.8**
	NSB22 2 mg/ml+DHAA 0.244 mg/ml	36 838 298	**92.9**
	NSB22 2 mg/ml+DHAA 0.305 mg/ml	43 734 973	**85.5**
Mean	NSB22 2mg/ml	19 868 805	
	DHAA 0.183 mg/ml	10 834 789	
	DHAA 0.244 mg/ml	14 993 178	
	DHAA 0.305 mg/ml	18 005 469	
	NSB22 2 mg/ml+DHAA 0.183 mg/ml	31 669 057	**97.0**
	NSB22 2 mg/ml+DHAA 0.244 mg/ml	37 431 475	**93.1**
	NSB22 2 mg/ml+DHAA 0.305 mg/ml	43 719 176	**86.6**

Supplementary Table S5. The spruce balm sample NSB22 was spiked with three different concentrations of dehydroabietic acid (DHAA) and analyzed in triplicates. The recovery rate of dehydroabietic acid in the spiked samples was calculated.

		NeoAA Area	Recovery rate
1	NSB22 2 mg/ml	43 792	
	NeoAA_0.0013 mg/ml	66 768	
	NeoAA_0.00174 mg/ml	93 922	
	NeoAA_0.0022 mg/ml	124 029	
	NSB22 2 mg/ml+NeoAA_0.0013 mg/ml	113 330	**102.5**
	NSB22 2 mg/ml+NeoAA_0.00174 mg/ml	161 836	**117.5**
	NSB22 2 mg/ml+NeoAA_0.0022 mg/ml	193 022	**115.0**
2	NSB22 2 mg/ml	41 530	
	NeoAA_0.0013 mg/ml	67 831	
	NeoAA_0.00174 mg/ml	111 900	
	NeoAA_0.0022 mg/ml	148 402	
	NSB22 2 mg/ml+NeoAA_0.0013 mg/ml	121 101	**110.7**
	NSB22 2 mg/ml+NeoAA_0.00174 mg/ml	157 672	**102.8**
	NSB22 2 mg/ml+NeoAA_0.0022 mg/ml	202 629	**106.7**
3	NSB22 2 mg/ml	48 613	
	NeoAA_0.0013 mg/ml	72 781	
	NeoAA_0.00174 mg/ml	102 771	
	NeoAA_0.0022 mg/ml	140 906	
	NSB22 2 mg/ml+NeoAA_0.0013 mg/ml	120 861	**99.6**
	NSB22 2 mg/ml+NeoAA_0.00174 mg/ml	147 631	**97.5**
	NSB22 2 mg/ml+NeoAA_0.0022 mg/ml	194 101	**102.4**
Mean	NSB22 2 mg/ml	44 645	
	NeoAA_0.0013 mg/ml	69 127	
	NeoAA_0.00174 mg/ml	102 864	
	NeoAA_0.0022 mg/ml	137 779	
	NSB22 2 mg/ml+NeoAA_0.0013 mg/ml	118 431	**104.1**
	NSB22 2 mg/ml+NeoAA_0.00174 mg/ml	155 713	**105.6**
	NSB22 2 mg/ml+NeoAA_0.0022 mg/ml	196 584	**107.8**

Supplementary Table S6. The spruce balm sample NSB22 was spiked with three different concentrations of neoabietic acid (NeoAA) and analyzed in triplicates. The recovery rate of neoabietic acid in the spiked samples was calculated.

3.5. Precision – Repeatability

0,625 mg/ml	Run 1		Run 2		Run 3		Mean	SD	RSD
	RT	Area	RT	Area	RT	Area			
1	11.947	100 734	12.000	99 370	12.032	96 899	99 001	1 587	1.6
2	12.122	358 872	12.180	373 379	12.166	384 815	372 355	10 616	2.9
3	12.199	119 338	12.261	105 639	12.256	123 405	116 127	7 600	6.5
4	12.637	552 477	12.676	549 646	12.690	564 260	555 461	6 328	1.1
5	12.719	204 037	12.786	195 530	12.789	198 150	199 239	3 557	1.8
6	13.487	41 983	13.475	38 784	13.522	44 494	41 754	2 337	5.6
7	13.733	4 729 646	13.776	4 777 050	13.768	4 809 858	4 772 185	32 927	0.7
8	13.799	34 033	13.856	30 066	13.836	34 935	33 011	2 115	6.4

1.250 mg/ml	Run 1		Run 2		Run 3		Mean	SD	RSD
	RT	Area	RT	Area	RT	Area			
1	11.743	215 858	11.932	195 089	11.950	211 049	207 332	8 877	4.3
2	11.894	760 452	12.125	804 084	12.132	741 675	768 737	26 143	3.4
3	11.962	277 328	12.199	227 231	12.196	271 967	258 842	22 459	8.7
4	12.420	1 202 828	12.622	1 122 395	12.646	1 191 860	1 172 361	35 614	3.0
5	12.507	378 278	12.726	430 982	12.743	429 799	413 020	24 571	5.9
6	13.213	65 718	13.462	63 592	13.416	71 167	66 826	3 190	4.8
7	13.517	9 894 656	13.749	9 827 699	13.751	10 048 112	9 923 489	92 264	0.9
8	13.603	65 470	13.810	62 235	13.820	75 362	67 689	5 584	8.2

1.875 mg/ml	Run 1		Run 2		Run 3		Mean	SD	RSD
	RT	Area	RT	Area	RT	Area			
1	11.926	313 752	11.927	339 717	11.921	316 352	323 274	11 676	3.6
2	12.099	1 120 618	12.100	1 112 769	12.108	1 128 706	1 120 698	6 506	0.6
3	12.156	473 630	12.159	429 758	12.169	397 591	433 660	31 165	7.2
4	12.652	1 933 482	12.616	1 777 968	12.626	1 824 312	1 845 254	65 192	3.5
5	12.719	517 400	12.706	574 492	12.716	554 283	548 725	23 637	4.3
6	13.391	108 108	13.420	101 959	13.417	124 673	111 580	9 592	8.6
7	13.722	15 403 853	13.708	15 179 091	13.716	14 747 474	15 110 139	272 365	1.8
8	13.793	99 771	13.783	102 051	13.779	108 963	103 595	3 908	3.8

Supplementary Table S7. Three different concentrations of sample NSB22 were analyzed in triplicates to assess the repeatability. The relative standard deviation was calculated for each resin acid at each concentration (pimaric acid (**1**), sandaracopimaric acid (**2**), palustric acid (**3**), isopimaric acid (**4**), levopimaric acid (**5**), abietic acid (**6**), dehydroabietic acid (**7**), and neoabietic acid (**8**))

3.5. Precision - Intermediate Precision

1,875 mg/ml	1 - Day 1		1 - Day 2		1 - Day 3		Mean	SD	RSD
	RT	Area	RT	Area	RT	Area			
1	12.233	351 699	12.221	339 092	12.169	307 323	332 705	18 671	5.6
2	12.399	1 550 033	12.390	1 462 874	12.329	1 197 898	1 403 602	149 743	10.7
3	12.530	255 956	12.586	252 296	12.479	221 569	243 274	15 420	6.3
4	12.876	3 151 372	12.881	2 994 062	12.803	2 469 172	2 871 535	291 672	10.2
5	13.080	147 524	13.076	269 641	12.956	255 158	224 108	54 475	24.3
6	13.648	206 573	13.669	215 831	13.573	151 821	191 408	28 246	14.8
7	13.961	16 786 248	13.956	15 400 355	13.881	13 284 812	15 157 138	1 439 764	9.5
8	14.026	96 778	14.026	91 851	13.947	71 104	86 578	11 125	12.8

1,875 mg/ml	2 - Day 1		2 - Day 2		2 - Day 3		Mean	SD	RSD
	RT	Area	RT	Area	RT	Area			
1	12.275	360 760	12.213	354 763	12.161	262 696	326 073	44 881	13.8
2	12.439	1 477 809	12.378	1 407 038	12.337	1 184 938	1 356 595	124 771	9.2
3	12.574	254 729	12.516	258 742	12.463	250 286	254 586	3 454	1.4
4	12.936	3 186 087	12.883	2 923 543	12.845	2 457 941	2 855 857	301 093	10.5
5	13.120	170 620	13.056	247 813	13.026	225 742	214 725	32 462	15.1
6	13.738	203 439	13.647	174 961	13.598	169 427	182 609	14 901	8.2
7	14.018	16 141 384	13.963	15 426 084	13.929	12 893 322	14 820 263	1 393 495	9.4
8	14.093	94 815	14.020	94 711	13.979	78 992	89 506	7 435	8.3

1,875 mg/ml	3 - Day 1		3 - Day 2		3 - Day 3		Mean	SD	RSD
	RT	Area	RT	Area	RT	Area			
1	12.261	338 056	12.211	308 585	12.183	289 265	311 969	20 062	6.4
2	12.429	1 265 966	12.370	1 397 354	12.367	1 107 744	1 257 021	118 402	9.4
3	12.569	247 782	12.501	269 735	12.487	216 667	244 728	21 772	8.9
4	12.943	2 735 245	12.873	2 895 296	12.863	2 277 077	2 635 873	261 986	9.9
5	13.143	143 091	13.042	243 962	13.027	245 517	210 857	47 922	22.7
6	13.715	190 935	13.651	206 293	13.630	169 931	189 053	14 904	7.9
7	14.092	14 212 017	13.954	15 477 498	13.936	11 927 762	13 872 426	1 468 933	10.6
8	14.092	75 108	14.023	89 201	13.990	75 734	80 014	6 501	8.1

Supplementary Table S8. Spruce balm sample NSB22 was analyzed at a concentration of 1,875 mg/ml in triplicates over three days to assess the intermediate precision (pimaric acid (**1**), sandaracopimaric acid (**2**), palustric acid (**3**), isopimaric acid (**4**), levopimaric acid (**5**), abietic acid (**6**), dehydroabietic acid (**7**), and neoabietic acid (**8**)). Table S10 summarizes the mean values, SD and RSD of all intermediate precision – experiments

All experiments	Mean	SD	RSD
1	323 582	31 568	9.8
2	1 339 073	145 159	10.8
3	247 529	16 325	6.6
4	2 787 755	305 009	10.9
5	216 563	46 226	21.3
6	187 690	20 685	11.0
7	14 616 609	1 534 054	10.5
8	85 366	9 461	11.1

Supplementary Table S9. The results of the intermediate precision experiments are summarized. The mean value, SD and RSD are calculated for each resin acid (pimaric acid (**1**), sandaracopimaric acid (**2**), palustric acid (**3**), isopimaric acid (**4**), levopimaric acid (**5**), abietic acid (**6**), dehydroabietic acid (**7**), and neoabietic acid (**8**))**.**

3.6. Detection limit (LOD) and Quantitation limit (LOQ)

#	RT [min]	Height	#	RT [min]	Height	#	RT [min]	Height
1	1.007	1965	6	6.045	1149	11	11.050	1404
2	2.010	1196	7	7.003	1479	12	12.005	1061
3	2.996	2100	8	7.971	1063	13	13.072	2042
4	4.006	1479	9	9.011	1576	14	15.126	1296
5	5.042	1106	10	10.067	1238	15	15.927	1641
							Mean:	**1453**

Supplementary Table S10. Noise value at 10 different time points of a solvent blank for the calculation of the signal/noise ratio of dehydroabietic acid

	c [mg/ml]	height	S/N factor	Mean
LOD	**0.00075**	8661	6.0	**5.7**
		8376	5.8	
		7807	5.4	
LOQ	**0.0025**	18403	12.7	**13.4**
		19554	13.5	
		20432	14.1	

Supplementary Table S11. Limit of detection (LOD) and limit of quantitation (LOQ) of dehydroabietic acid

#	RT [min]	Height	#	RT [min]	Height	#	RT [min]	Height
1	1.036	1073	6	6.011	1726	11	10.979	1124
2	1.976	1109	7	7.010	1577	12	11.979	1276
3	2.893	1127	8	7.943	865	13	13.000	1572
4	3.979	1317	9	9.009	1440	14	15.004	1085
5	5.068	1707	10	10.031	1154	15	15.996	1280
							Mean:	**1295**

Supplementary Table S12. Noise value at 10 different time points of a solvent blank for the calculation of the signal/noise ratio of neoabietic acid

	c [mg/ml]	height	S/N factor	Mean
LOD	0.001	6269	4.8	**5.0**
		5553	4.3	
		7690	5.9	
LOQ	0.002	11294	8.7	**9.3**
		12484	9.6	
		12475	9.6	

Supplementary Table S13. Limit of detection (LOD) and limit of quantitation (LOQ) of neoabietic acid

4. Quantitation

	1	2	3	4	5	6	7	8	Total
Ret.-time ([min])	12.150±0.03	12.316±0.02	12.518±0.03	12.812±0.05	13.065±0.05	13.629±0.04	13.931±0.04	14.058±0.05	
NSB01	0.3 (±0.005)	1.3 (±0.038)	3.8 (±0.026)	3.4 (±0.046)	4.6 (±0.089)	2.4 (±0.072)	7.1 (±0.051)	2.3 (±0.049)	25.1 (±0.181)
NSB02	0.3 (±0.006)	1.4 (±0.011)	3.1 (±0.042)	2.5 (±0.023)	4.9 (±0.02)	2.0 (±0.015)	7.7 (±0.054)	1.8 (±0.045)	23.7 (±0.122)
NSB03	0.3 (±0.011)	1.5 (±0.012)	0.2 (±0.016)	2.2 (±0.004)	0.02 (±0.021)	0.5 (±0.008)	10.5 (±0.062)	0.0 (±0.004)	15.3 (±0.076)
NSB04	0.3 (±0.012)	1.1 (±0.010)	2.7 (±0.019)	3.2 (±0.031)	3.5 (±0.003)	1.8 (±0.024)	10.2 (±0.133)	1.0 (±0.034)	23.8 (±0.187)
NSB05	0.4 (±0.018)	1.6 (±0.038)	4.5 (±0.118)	3.7 (±0.043)	7.8 (±0.060)	2.6 (±0.032)	5.7 (±0.046)	3.1 (±0.037)	29.3 (±0.270)
NSB06	0.4 (±0.006)	1.6 (±0.03)	4.7 (±0.007)	3.7 (±0.044)	10.8 (±0.108)	2.0 (±0.158)	3.8 (±0.023)	3.9 (±0.135)	31.0 (±0.415)
NSB07	0.2 (±0.007)	1.5 (±0.028)	3.2 (±0.056)	2.7 (±0.042)	6.9 (±0.021)	2.8 (±0.004)	5.3 (±0.062)	3.6 (±0.025)	26.3 (±0.062)
NSB08	0.3 (±0.020)	1.9 (±0.031)	3.2 (±0.041)	3.3 (±0.020)	6.9 (±0.032)	2.0 (±0.020)	9.1 (±0.044)	2.5 (±0.019)	29.1 (±0.077)
NSB09	0.4 (±0.008)	1.3 (±0.031)	0.5 (±0.030)	2.0 (±0.020)	0.4 (±0.018)	0.4 (±0.009)	10.3 (±0.085)	0.1 (±0.008)	15.4 (±0.125)
NSB10	0.6 (±0.016)	2.0 (±0.032)	4.1 (±0.032)	3.5 (±0.013)	10.4 (±0.047)	2.4 (±0.040)	5.6 (±0.002)	3.7 (±0.053)	32.2 (±0.059)
NSB11	0.5 (±0.003)	2.0 (±0.047)	4.0 (±0.006)	2.5 (±0.053)	9.2 (±0.076)	1.6 (±0.194)	5.2 (±0.019)	2.4 (±0.188)	27.4 (±0.556)
NSB12	0.4 (±0.011)	1.5 (±0.022)	5.5 (±0.013)	2.0 (±0.049)	30.8 (±0.138)	1.9 (±0.059)	1.7 (±0.017)	3.1 (±0.085)	46.9 (±0.275)
NSB13	0.1 (±0.034)	0.6 (±0.132)	0.2 (±0.077)	1.4 (±0.315)	0.1 (±0.017)	0.3 (±0.093)	5.6 (±1.217)	0.0 (±0.002)	8.4 (±1.879)
NSB14	0.2 (±0.004)	0.8 (±0.014)	1.1 (±0.004)	3.8 (±0.027)	0.5 (±0.043)	1.4 (±0.007)	6.8 (±0.052)	0.4 (±0.006)	15.0 (±0.094)
NSB15	0.2 (±0.016)	1.3 (±0.028)	0.3 (±0.018)	1.7 (±0.027)	0.1 (±0.022)	0.2 (±0.009)	9.0 (±0.075)	0.0 (±0.004)	12.8 (±0.078)
NSB16	0.2 (±0.022)	1.2 (±0.018)	2.2 (±0.025)	2.1 (±0.045)	5.8 (±0.068)	1.6 (±0.133)	5.3 (±0.034)	1.6 (±0.118)	20.0 (±0.360)
NSB17	0.4 (±0.016)	1.3 (±0.024)	2.2 (±0.034)	2.3 (±0.030)	3.9 (±0.121)	1.1 (±0.067)	6.7 (±0.034)	1.2 (±0.025)	19.1 (±0.324)
NSB18	0.4 (±0.013)	1.6 (±0.016)	2.8 (±0.035)	2.8 (±0.017)	0.6 (±0.004)	3.3 (±0.011)	8.0 (±0.019)	0.8 (±0.4)	20.2 (±0.348)
NSB19	0.7 (±0.008)	1.6 (±0.013)	0.2 (±0.016)	1.7 (±0.062)	0.1 (±0.021)	0.2 (±0.006)	9.3 (±0.032)	0.0 (±0.002)	13.8 (±0.133)
NSB20	0.6 (±0.01)	1.9 (±0.041)	1.9 (±0.016)	3.3 (±0.046)	2.0 (±0.071)	1.4 (±0.038)	8.8 (±0.032)	0.9 (±0.016)	20.9 (±0.203)
NSB21	0.5 (±0.005)	2.3 (±0.025)	2.6 (±0.064)	3.6 (±0.025)	3.2 (±0.001)	2.3 (±0.022)	7.8 (±0.012)	1.8 (±0.017)	24.0 (±0.070)
NSB22	0.2 (±0.009)	0.8 (±0.01)	0.3 (±0.01)	1.9 (±0.01)	0.1 (±0.033)	0.2 (±0.007)	9.2 (±0.112)	0.0 (±0.001)	12.7 (±0.132)
Mean	0.4 (±0.150)	1.5 (±0.398)	2.5 (±1.612)	2.7 (±0.743)	5.2 (±6.601)	1.6 (±0.908)	7.2 (±2.215)	1.6 (±1.316)	22.7 (±8.404)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	Total
Ret.-time ([min])	12.150±0.03	12.316±0.02	12.518±0.03	12.812±0.05	13.065±0.05	13.629±0.04	13.931±0.04	14.058±0.05	
NSB-CP1	n.d.	n.d.	0.02 (±0.001)	0.07 (±0.001)	0.11 (±0.002)	0.15 (±0.005)	0.28 (±0.008)	0.05 (±0.007)	0.64 (±0.014)
NSB-CP 2	n.d.	n.d.	0.02 (±0.001)	0.08 (±0.002)	0.11 (±0.004)	0.17 (±0.003)	0.26 (±0.005)	0.06 (±0.002)	0.70 (±0.006)
NSB-CP 3	n.d.	n.d.	0.03 (±0.001)	0.08 (±0.001)	0.12 (±0.004)	0.17 (±0.004)	0.26 (±0.004)	0.07 (±0.002)	0.73 (±0.009)
NSB-CP4	0.05 (±0.004)	0.24 (±0.009)	0.79 (±0.016)	0.48 (±0.007)	1.9 (±0.012)	0.53 (±0.009)	0.57 (±0.005)	0.71 (±0.021)	5.27 (±0.052)
NSB-CP5	0.06 (±0.003)	0.26 (±0.011)	0.81 (±0.008)	0.50 (±0.014)	2.46 (±0.026)	0.48 (±0.013)	0.5 (±0.005)	0.82 (±0.026)	5.88 (±0.049)

Supplementary Table S14. Retention times (min ±SD), contents and total contents (% ±SD) of the eight diterpene acids (pimaric acid (**1**), sandaracopimaric acid (**2**), palustric acid (**3**), isopimaric acid (**4**), levopimaric acid (**5**), abietic acid (**6**), dehydroabietic acid (**7**), and neoabietic acid (**8**)) in Norway Spruce Balm (samples NSB01-22) and commercial products (NSB-CP1-5). Contents of compounds **1**-**6** were calculated as neoabietic acid (**8**).

Exudates of *Picea abies*, *Pinus nigra* and *Larix decidua*: Chromatographic comparison and pro-migratory effects on keratinocytes in vitro

Thomas Goels, Elisabeth Eichenauer, Ammar Tahir, Paul Prochaska, Franziska Hoeller, Elke H. Heiß, Sabine Glasl*

Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna, Althanstraße 14, Vienna, Austria
* Correspondence: sabine.glasl@univie.ac.at; Tel.: +43 1 4277 55207

Table S1. Summary of the qualitative chromatographic comparison.

Technique	Stationary phase	Mobile phase	Detection	Results
TLC (see Figure 2)	Silica 60 F_{254} aluminium sheets (9.5-11.5 µm)	chloroform – methanol - trifluoroacetic acid (97 + 3 + 0.1)	derivatization with anisaldehyde/sulphuric acid solution	➢ Pinoresinol (**4**, R_f 0.3) ➢ Neoabietic acid (**10-7**), dehydroabietic acid (**9**) and other diterpene resin acids (**10-1** – **10-6**): no separation by TLC (overlap at R_f 0.5) ➢ Ferulic acid (**3**, R_f 0.2) ➢ High similarity of *Picea abies* resin and balm with slight differences in color and intensity at R_f 0.3 (band corresponds to pinoresinol **4**) ➢ Differentiation of *Larix decidua* by two additional prominent bands at R_f 0.7 and 0.8; less intensive bands at R_f 0.2-0.3 than in *Picea* and *Pinus* ➢ Differentiation of *Pinus nigra* by one additional band at R_f 0.7 and a missing band at R_f 0.3 (corresponds to pinoresinol **4**)
HPLC-DAD (see Figure 3)	LiCrospher 100 RP18e, 4x250 mm, 5 µm	acetonitrile – water (both containing 0.1% formic acid) start: 15%, gradient: 1.77%/min (0-45 min), flow: 1 mL/min	Shimadzu Diode Array Detector SPD-M20A, detection wave length: 190 nm	➢ Diterpene resin acids with m/z 302 (**10-1** – **10-7**) are not separated and elute between 41.5-42.5 min ➢ Dehydroabietic acid (**9**, m/z 300) is well separated from the other resin acids (R_t 37.2 min) ➢ Hydroxylated derivatives of dehydroabietic acid (**7**, **8**) elute more than 10 min earlier than **9** (25.2 and 27.5 min) ➢ Dihydroxylated derivatives of dehydroabietic acid (**5**, **6**) elute more than 18 min earlier than **9** (17.8 and 19.7 min) ➢ Pinoresinol (**4**) elutes at 15.0 min; detectable in the balm of *Picea* and in the resin of *Pinus* (confirmation of the results from TLC) ➢ Hydroxycinnamic acids (**1**-**3**) elute within the first 10 minutes but are of minor importance for the composition of the exudates; only p-coumaric acid (**2**, R_t 9.4 min) is detectable in *Picea* balm ➢ *Larix* balm: characterized by two additional peaks with unknown identity (38.0 and 46.0 min, m/z values see below) ➢ *Picea* balm and resin: similar qualitative composition; *Picea* balm: additional peaks with unknown identity at 12.5 min and 22.0 min
UHPLC-ESI-MS	Kinetex RP18 Phenomenex, 2.1×150 mm, 2.6 µm, 100 Å	acetonitrile/ methanol (80+20) – water (both containing 0.02% formic acid) start: 5% isocratic (0-2 min), gradient: 2.9%/min (2-30 min), flow: 350 µL/min	AB Sciex ESI X500 QTOF peak detection: negative mode	➢ Confirmation of the HPLC-DAD results (see Figure 3) by m/z values and sum formulas ➢ MS-data of unknown compounds: 29.5 min: m/z 318 ($C_{20}H_{30}O_3$ suggestion: hydroxylated derivative of abietic acid) 30.3 min: m/z 318 ($C_{20}H_{30}O_3$ suggestion: hydroxylated derivative of abietic acid) 31.0 min: m/z 334 ($C_{20}H_{30}O_4$ suggestion: dihydroxylated derivative of abietic acid) 38.0 min: m/z 312 ($C_{21}H_{28}O_2$) 46.0 min: m/z 388 ($C_{24}H_{36}O_4$)
UHSFC-MS (see Figure 4)	Torus 2-Picolylamin, 3.0x100.0 mm, 1.7 µm	supercritical CO_2 – ethanol ethanol gradient: 0-3% (0-8 min), hold for 2 min, 3-5.5% (10-15 min)	Waters Acquity QDa peak detection: negative mode	➢ Separation of the diterpene resin acids with m/z 302 (**10-1** – **10-7**) ➢ *Picea* balm and *Pinus* resin: contain all seven diterpene resin acids ➢ *Picea* resin: no levopimaric acid (**10-5**) ➢ *Larix* balm: no levopimaric acid (**10-5**) and no pimaric acid (**10-1**)

Supplementary Materials:

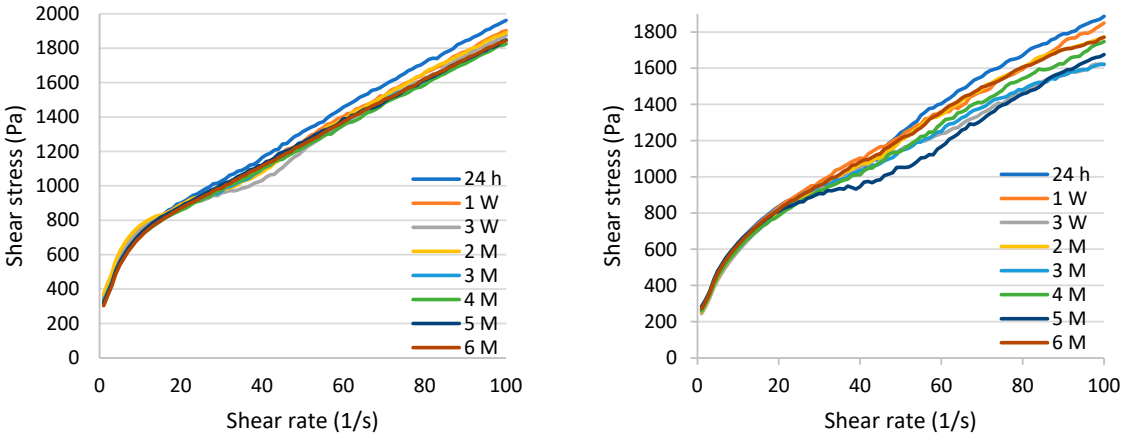


Figure S1. Flow curves of (**a**) wool wax/water stored at room temperature (23°C) and (**b**) wool wax/water stored in the refrigerator (8°C). The colored curves are means of n = 3 formulations analyzed at room temperature (23°C) at a shear rate of 1-100 s^{-1}. SDs are omitted for better visualization. The refrigerated samples were left to acclimate for six hours at 23°C before each measurement. Abbreviations: h = hour/s, W = week/s, and M = month/s.

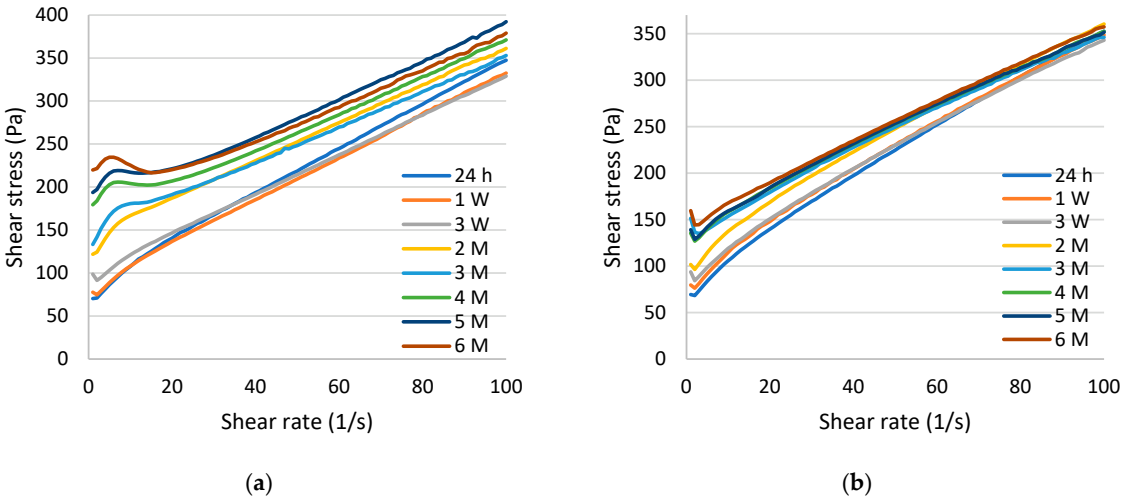


Figure S2. Flow curves of (**a**) Ultrabas/castor oil stored at room temperature (23°C) and (**b**) Ultrabas/castor oil stored in the refrigerator (8°C). The colored curves are means of n = 3 formulations analyzed at room temperature (23°C) at a shear rate of 1-100 s^{-1}. SDs are omitted for better visualization. The refrigerated samples were left to acclimate for six hours at 23°C before each measurement. Abbreviations: h = hour/s, W = week/s, and M = month/s.

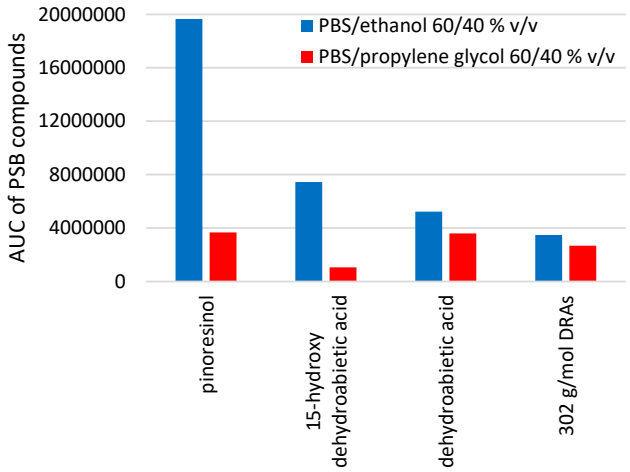


Figure S3. Comparison of the relative solubility of PSB compounds in different acceptor media. AUCs were obtained after dissolving an identical surplus amount of purified spruce balm (PSB) in either phosphate-buffered saline/ethanol at 60:40% *v/v* (blue bars) or phosphate-buffered saline/propylene glycol at 60:40% *v/v* (red bars).

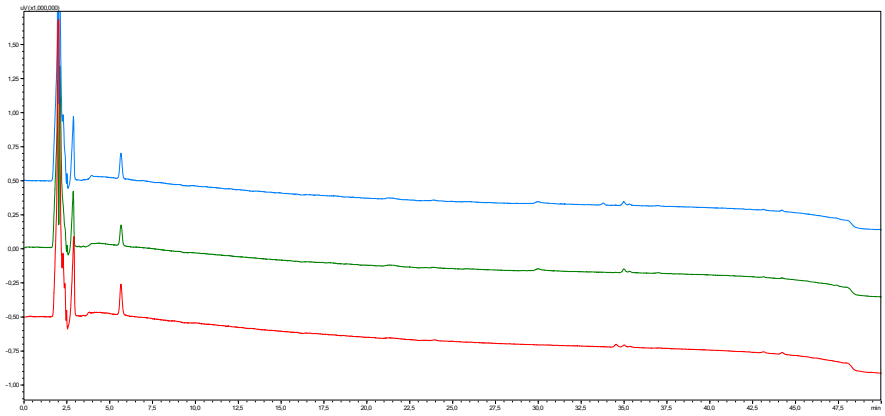


Figure S4. HPLC-DAD chromatograms of samples drawn from permeation experiments after 32 h using no ointment (red), pig fat without PSB (green), and wool wax/castor oil without PSB (blue) at 190 nm.

Table S1. Batch numbers (PSB), internal assignment (FP), date of harvest, and harvest location of analyzed PSB batches.

PSB batch	Date of harvest	Harvest location
PSB 1 (FP6)	07/2016	Promau Kitzhütte, Hollenstein, Lower Austria
PSB 2 (FP12)	07/2016	Gaflenz Heiligenstein, Upper Austria
PSB 3 (FP13)	2015	Lungau, Salzburg
PSB 4 (FP21)	2010-2016	Austria
PSB 5 (FP23)	07/2021	Schellgaden, Tamsweg, Lungau, Salzburg

Supplementary Materials:

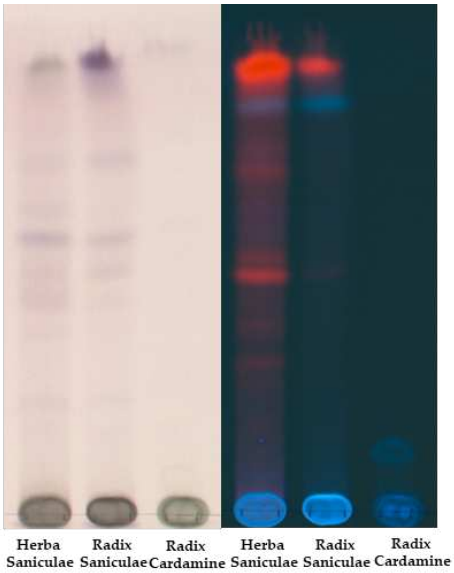


Figure S1. TLC comparison of the extracts in system A after derivatization with anisaldehyde (left) at visible light, and without derivatization at 365 nm (right).

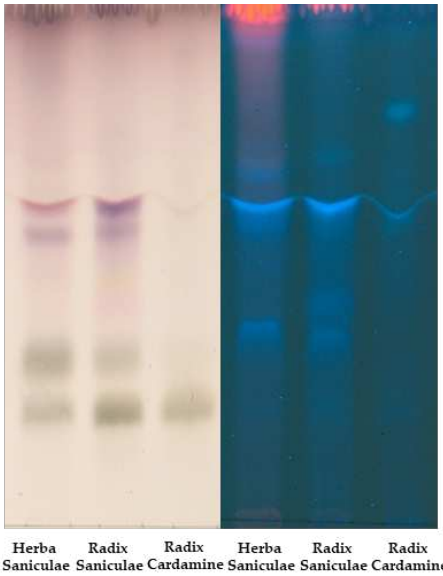


Figure S2. TLC comparison of the extracts in system B after derivatization with anisaldehyde (left) at visible light, and without derivatization at 365 nm (right).

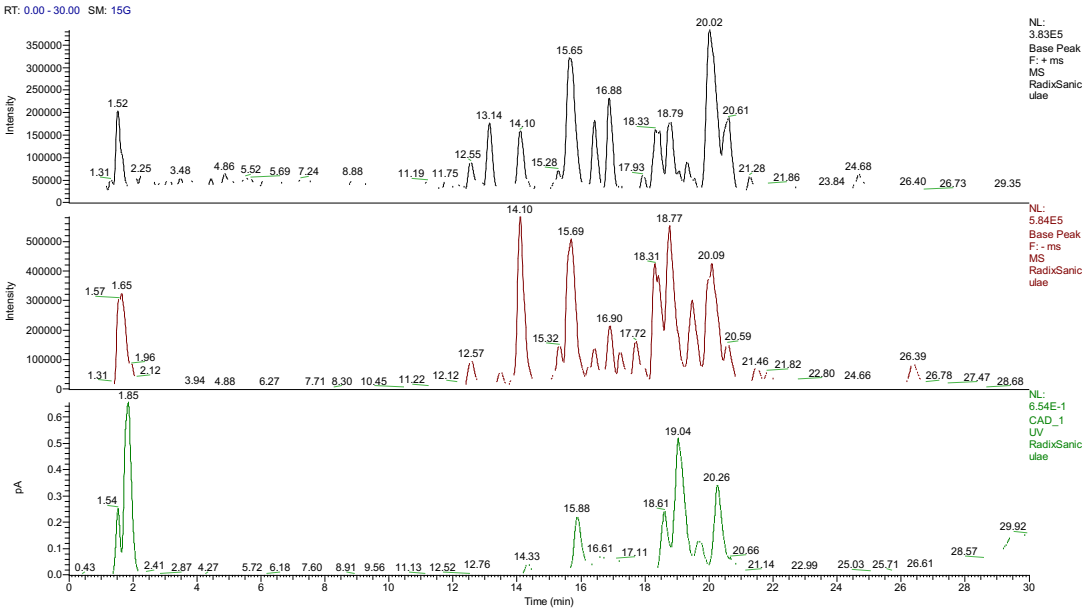


Figure S3. UHPLC-ESIMS base peak chromatogram in positive mode (upper), in negative mode (middle) and CAD chromatogram (lower) of Radix Saniculae.

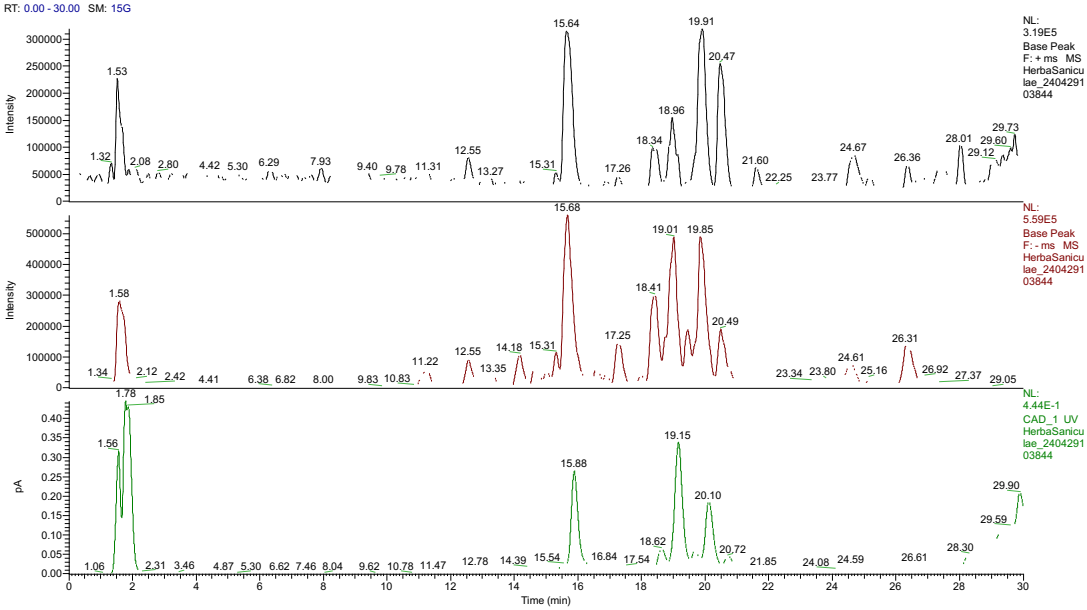


Figure S4. UHPLC-ESIMS base peak chromatogram in positive mode (upper), in negative mode (middle) and CAD chromatogram (lower) of Herba Saniculae.

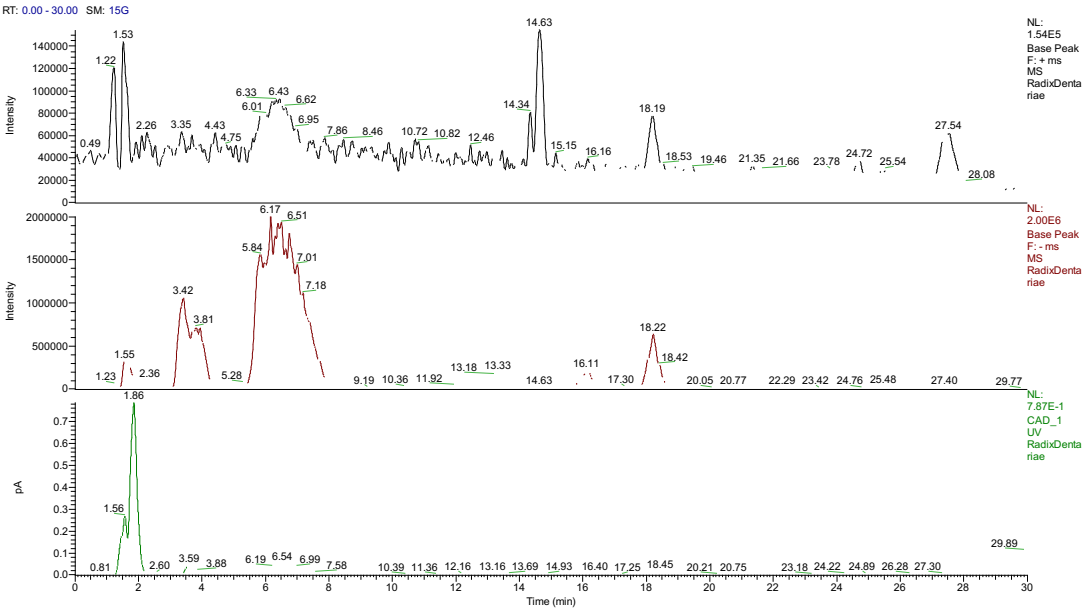
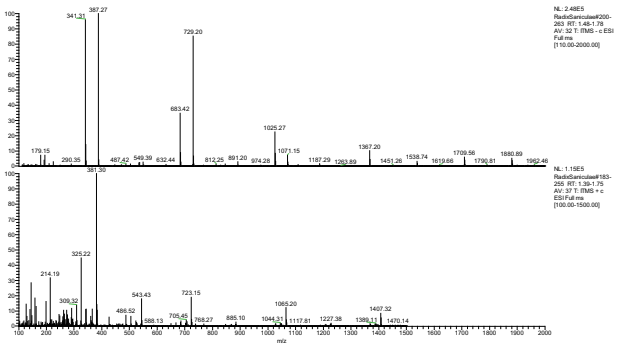
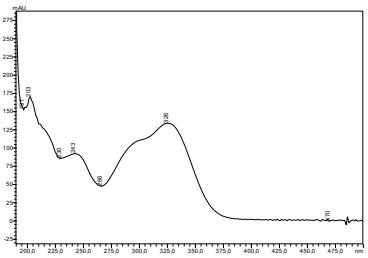
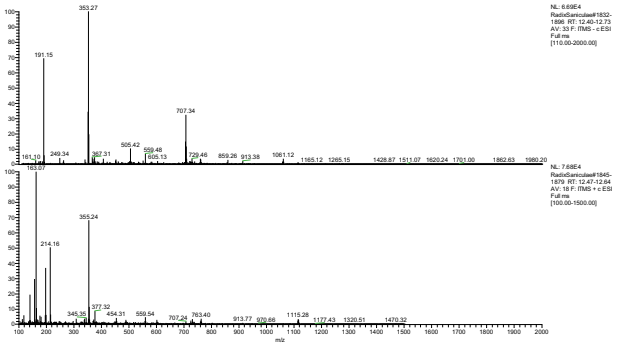


Figure S5. UHPLC-ESIMS base peak chromatogram in positive mode (upper), in negative mode (middle) and CAD chromatogram (lower) of Radix Cardamines enneaphyllos.

Table S1. UV spectra and mass spectra (negative mode upper spectrum, positive mode lower spectrum) of main peaks found in the extracts of Radix Saniculae.

Peak Nr.	UV spectrum	ESIMS
1	nd	
2		

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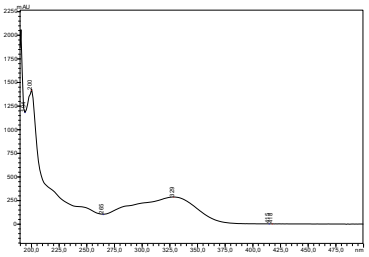

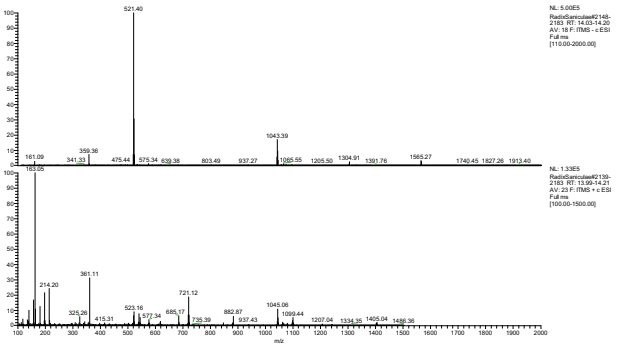

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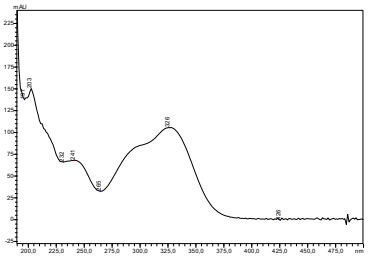

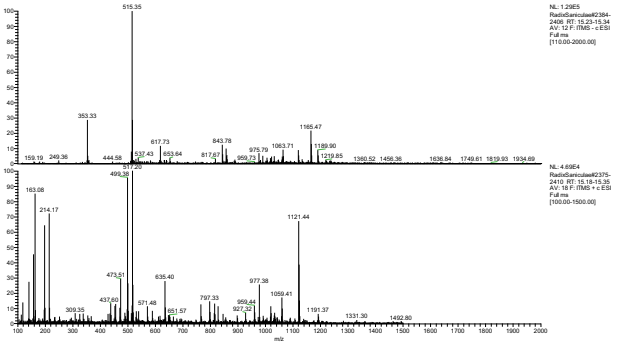

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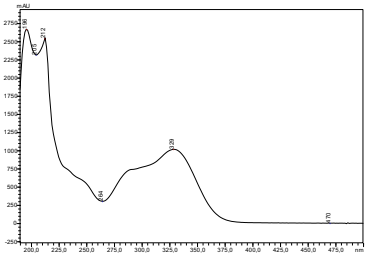

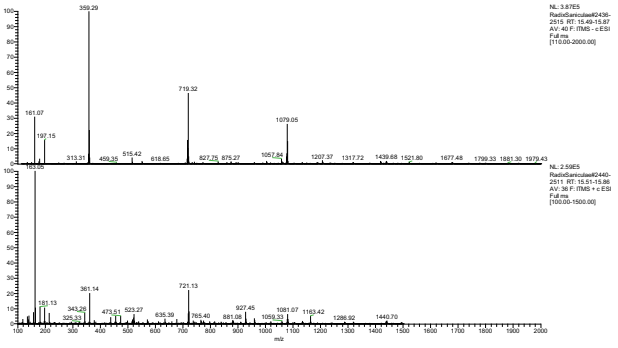

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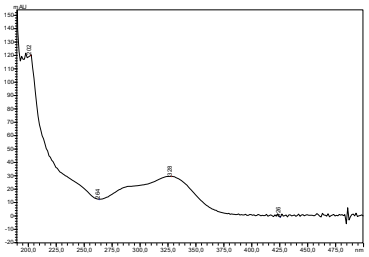

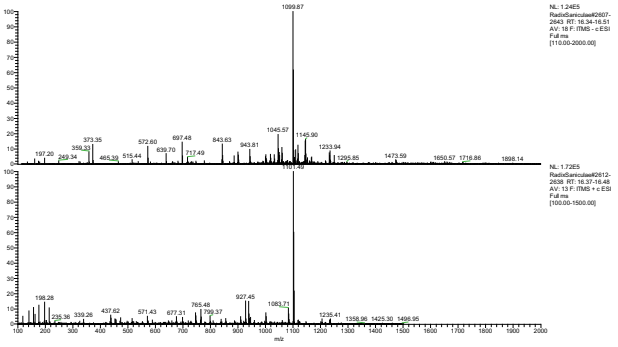

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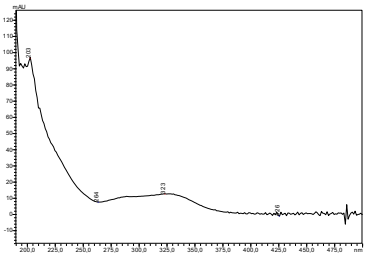

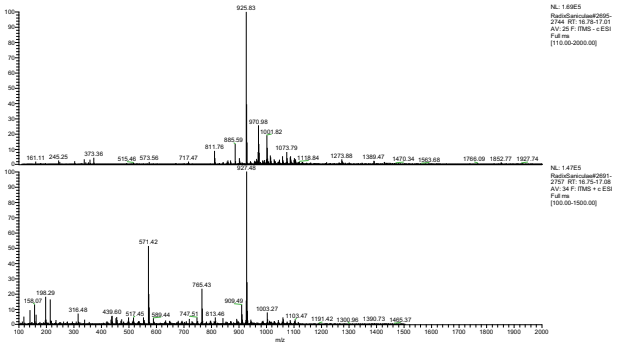

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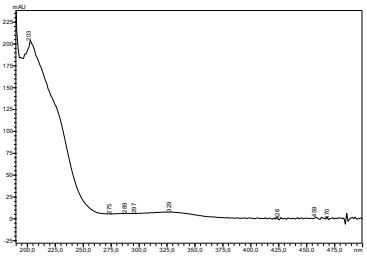

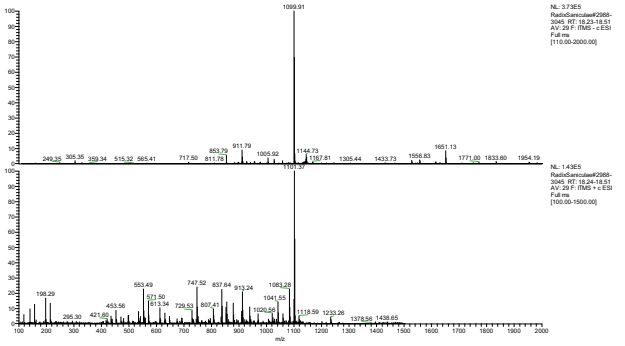

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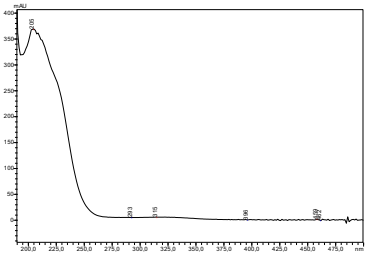

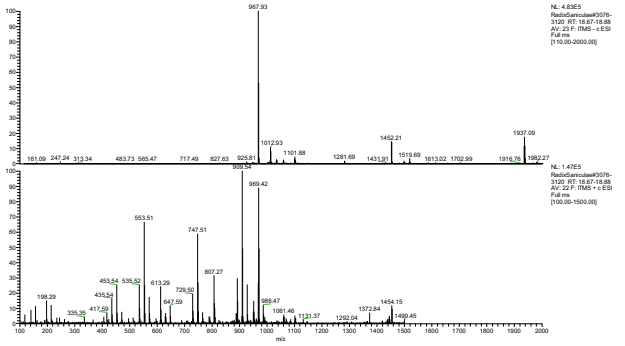

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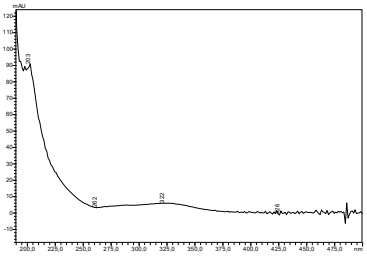
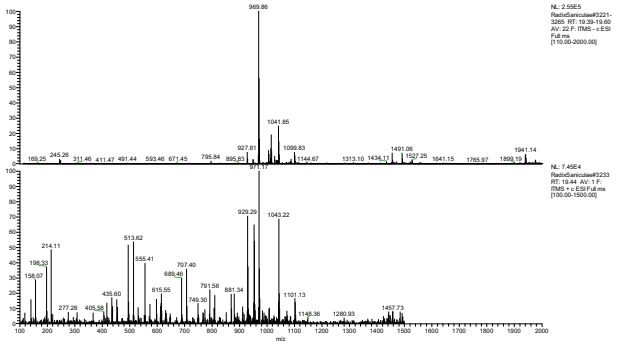




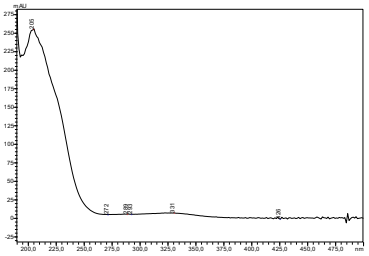
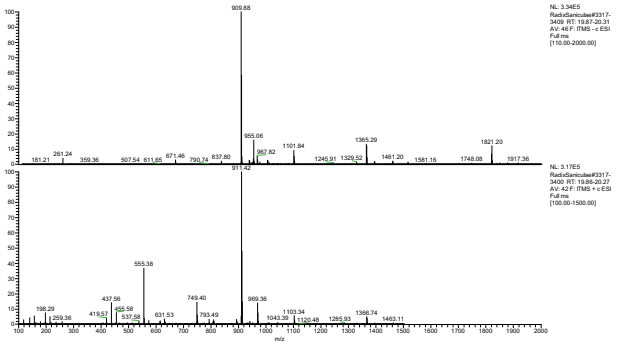
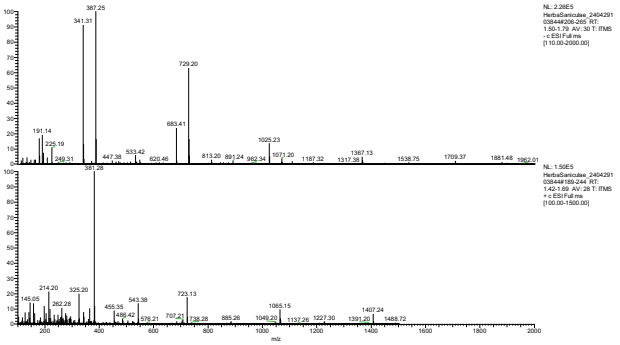
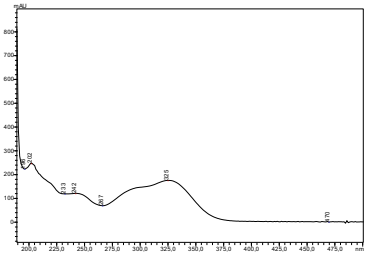
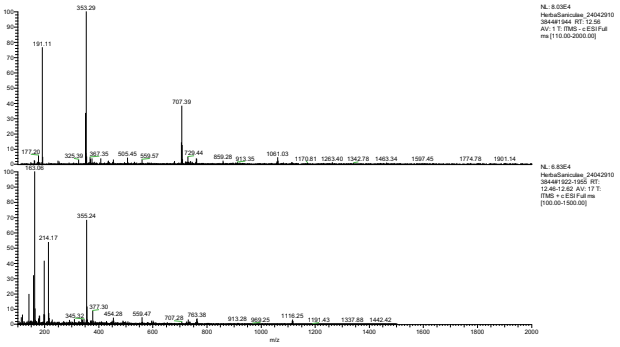
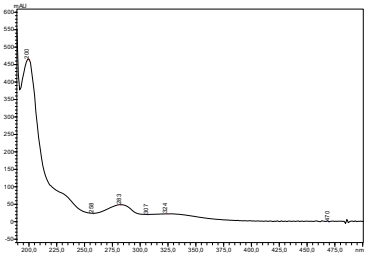
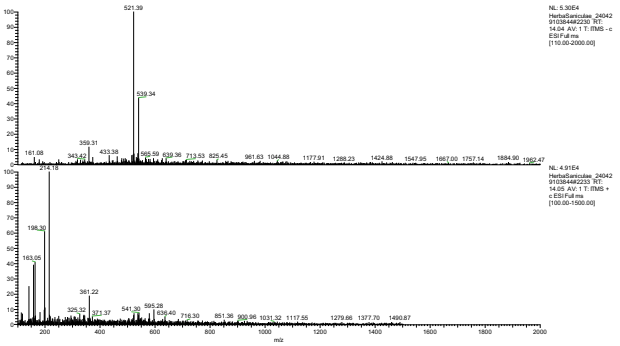
11		

Table S2. UV spectra and mass spectra (negative mode upper spectrum, positive mode lower spectrum) of main peaks found in the extracts of Herba Saniculae.

Peak Nr.	UV spectrum	ESIMS
1	nd	
2		
3		

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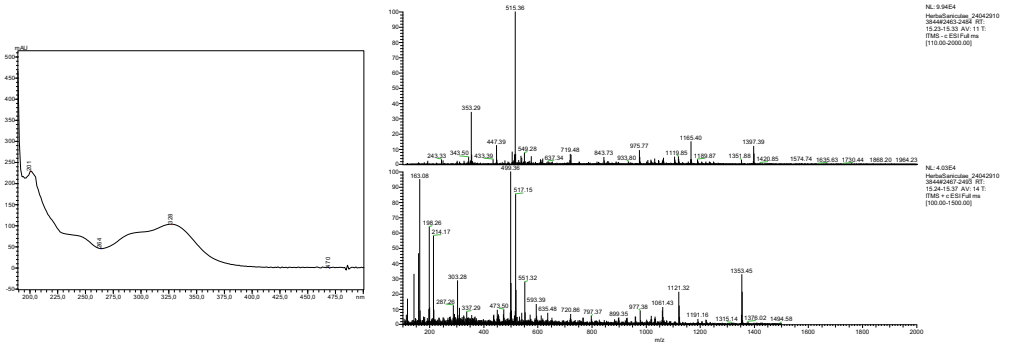

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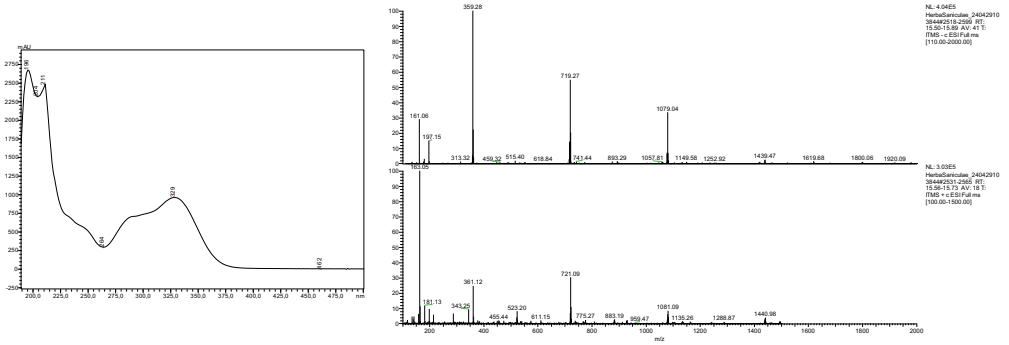

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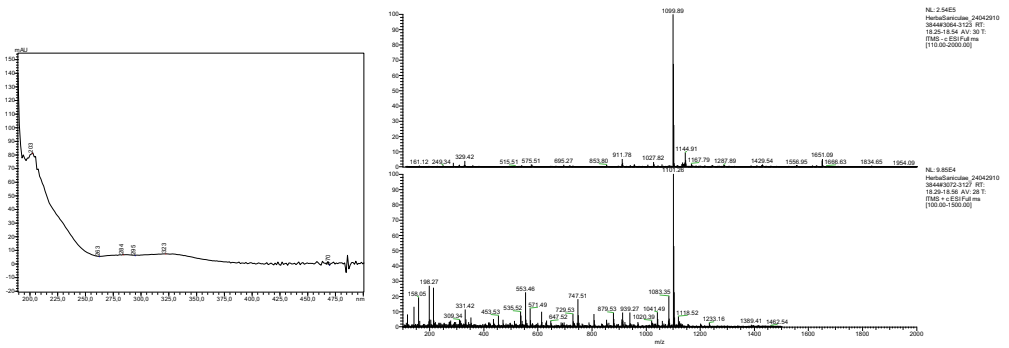

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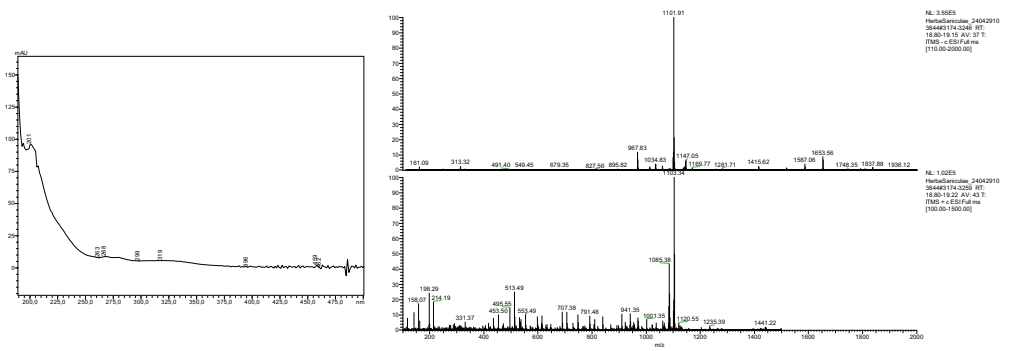


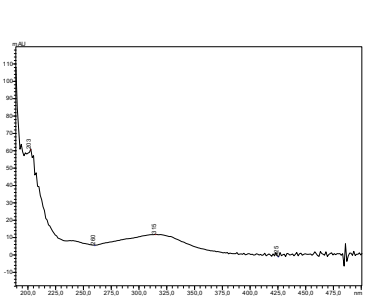
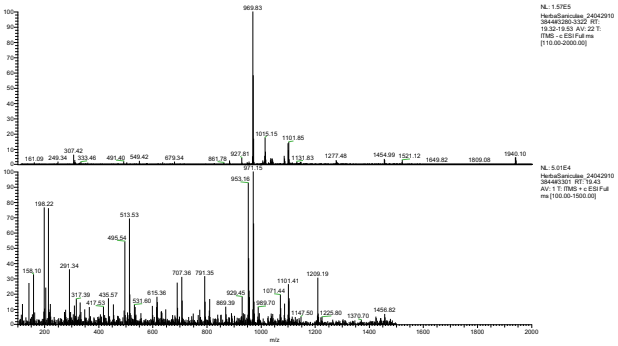
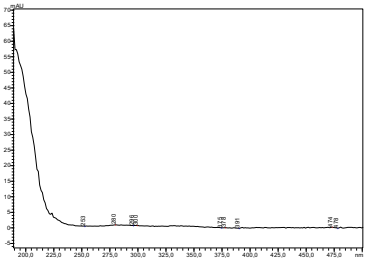
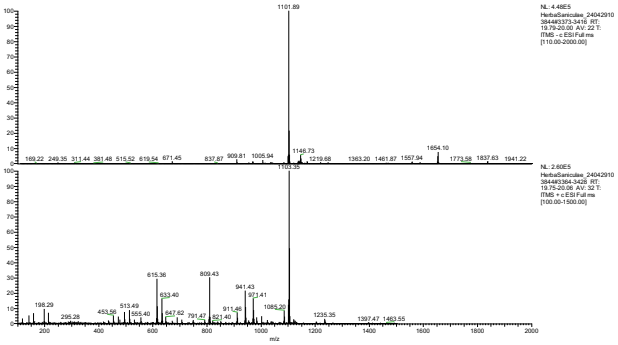
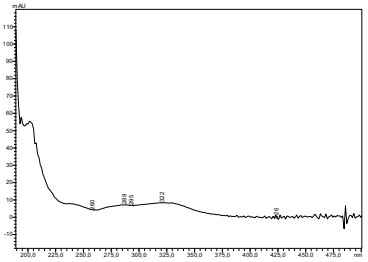
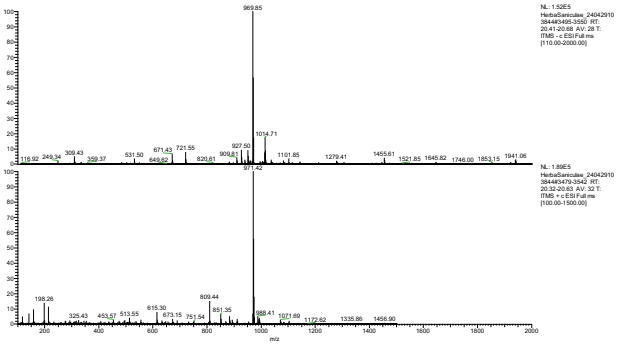
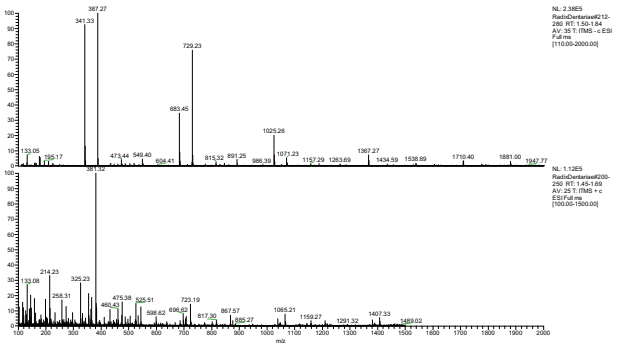
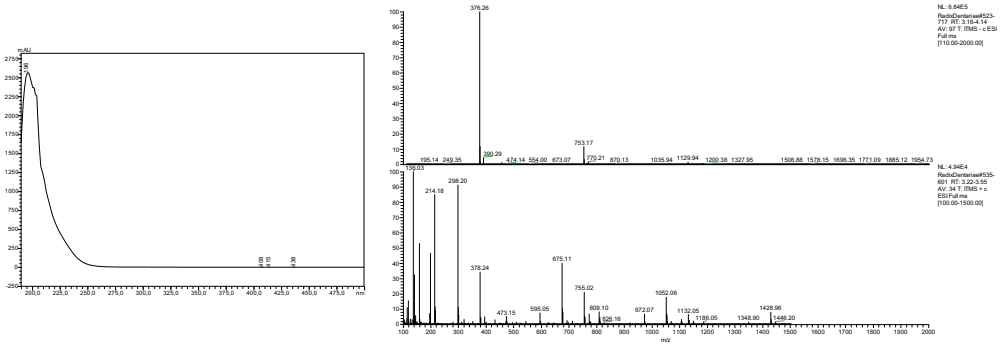
Peak Nr.	UV spectrum	ESIMS
8		
9		
10		
1	nd	

Table S3. UV spectra and mass spectra (negative mode upper spectrum, positive mode lower spectrum) of main peaks found in the extracts of Radix Cardamines enneaphyllos.

2



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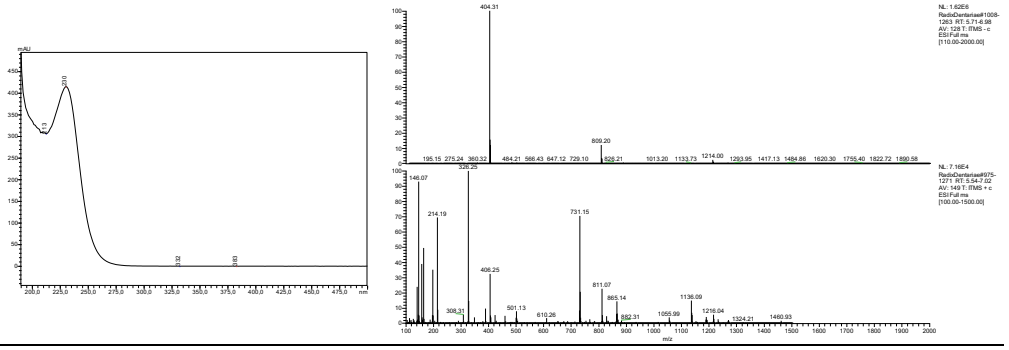


White Cabbage as Wound-Healing Agent: Phytochemical Analysis and Bioactivity Screening

Elisabeth Eichenauer[a,b], Barbara Braunböck[a], Karin Ortmayr[a], Elke H. Heiss[a], Sabine Glasl[a,*]

[a] Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna, Josef-Holaubek-Platz 2, 1090 Vienna, Austria
[b] Vienna Doctoral School of Pharmaceutical, Nutritional and Sport Sciences, University of Vienna, Josef-Holaubek-Platz 2, 1090 Vienna, Austria
* Correspondence: sabine.glasl@univie.ac.at; Phone: +43-1-4277-55207

Supplementary material

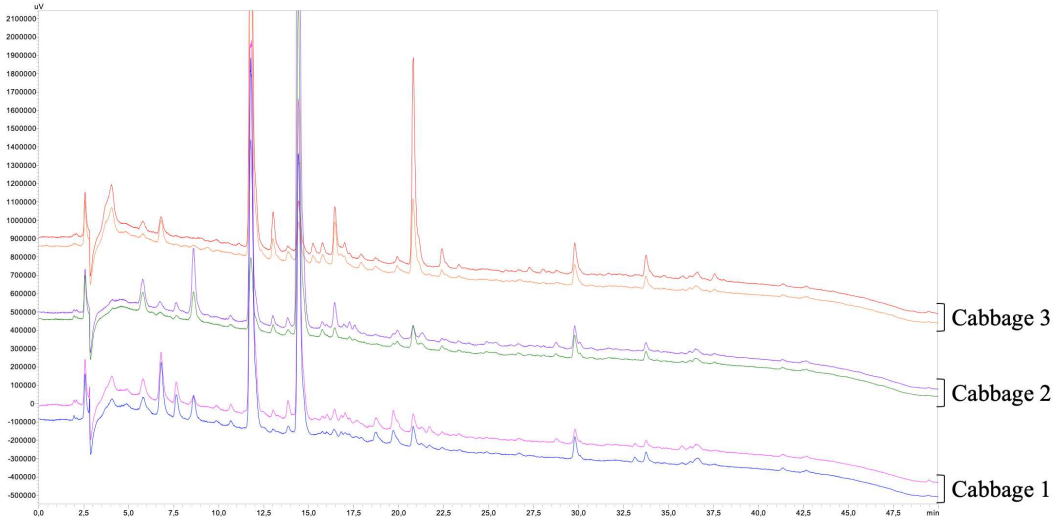


Figure S1 HPLC-DAD comparison of DCM extracts gained from 3 different cabbages at 190 nm. Each cabbage was extracted with prior incubation (lower chromatograms – blue, green, orange) and without incubation (upper chromatograms – pink, purple, red).

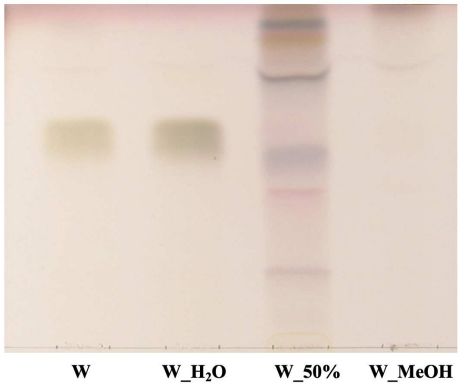


Figure S2 TLC comparison of the aqueous extract W and its fractions W_H_2O, W_50%, and W_MeOH after derivatization with anisaldehyde/sulfuric acid at daylight (stationary phase: silica 60 F_{254}, mobile phase: DCM-MeOH-1% FA (8+5+1)).

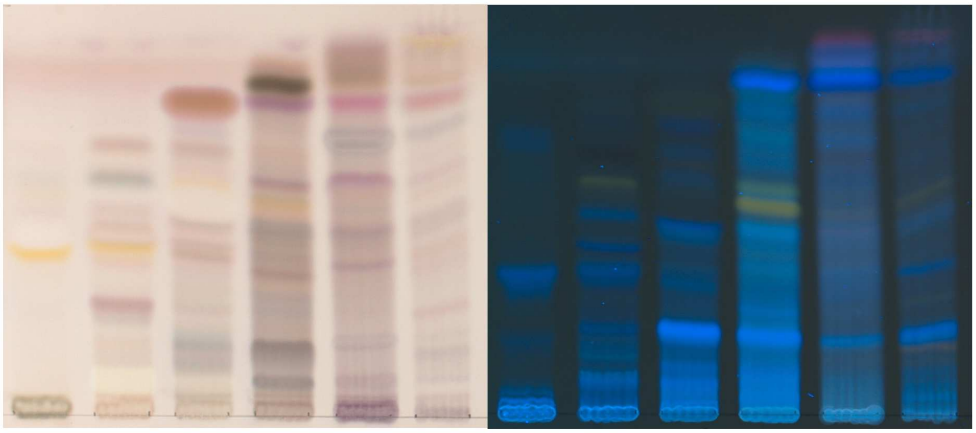
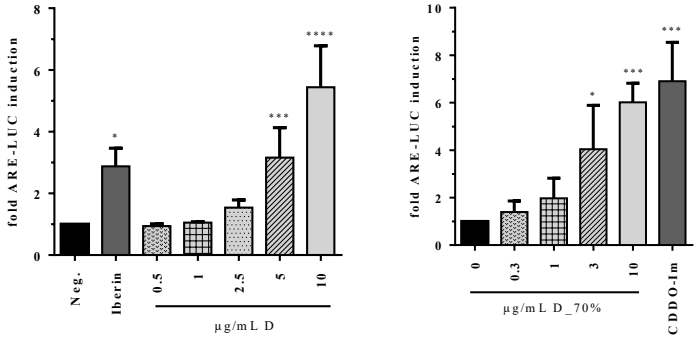
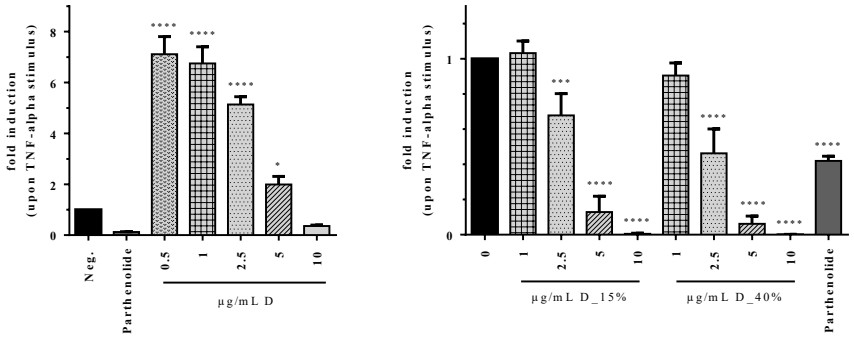


Figure S3 TLC comparison of the DCM extract D and its fractions D_H_2O, D_15%, D_40%, D_70%, and D_MeOH after derivatization with anisaldehyde/sulfuric acid at daylight (left) and underivatized at 366 nm (right) (stationary phase: silica 60 F_{254}, mobile phase: DCM-MeOH (9+1)).



a) **b)**

Figure S4 Screening of dose dependency of Nrf2 pathway activation in HepG2-ARE/Nrf2-luc cells of **a)** the active extract D compared to the positive control Iberin 3 µM, and **b)** the most active fraction D_70% compared to the positive control CDDO-Im at 10 nM. The negative control was DMSO 0.1%. The samples were tested at the mentioned concentrations. Bars represent the mean ± SD of three independent experiments, n= 3, ****p < 0.0001 (One-way ANOVA with Dunnett's post hoc test vs. vehicle control).



a) **b)**

Figure S5 Screening of dose dependency of inhibition of TNF-α-stimulated NF-κB transactivation activity in HEK-293/NF-κB-luc cells of **a)** the active extract D compared to the positive control parthenolide 10 µM, and **b)** the most active fractions D_15% and D_40% compared to the positive control parthenolide 10 µM. The negative control was DMSO 0.1%. The samples were tested at the mentioned concentrations. Bars represent the mean ± SD of three independent experiments, n= 3, ****p < 0.0001 (One-way ANOVA with Dunnett's post hoc test vs. vehicle control).

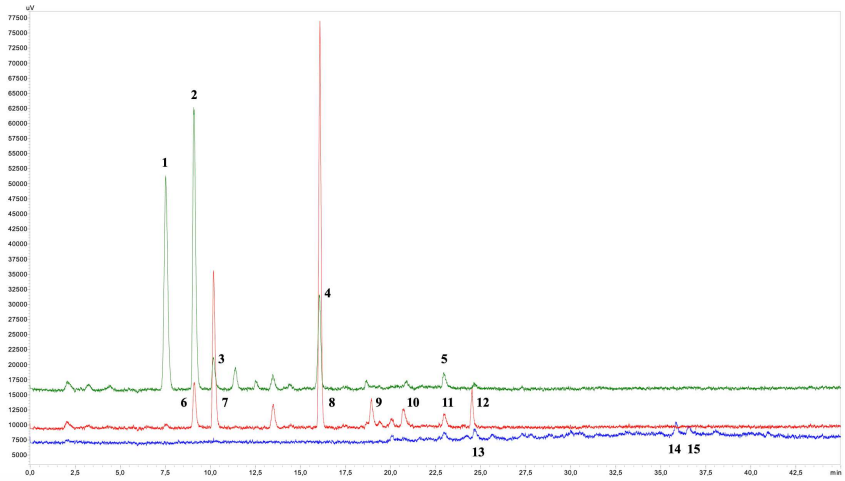
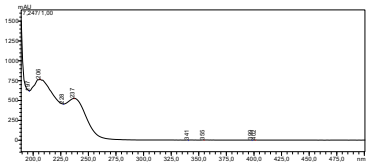
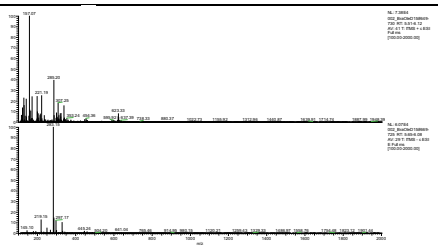
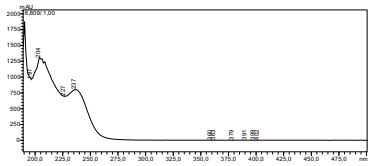
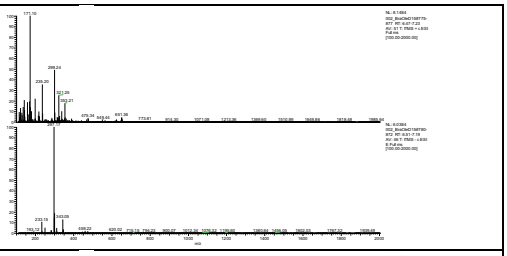
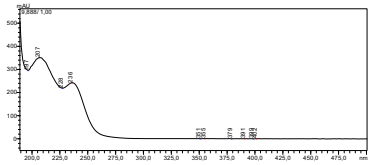
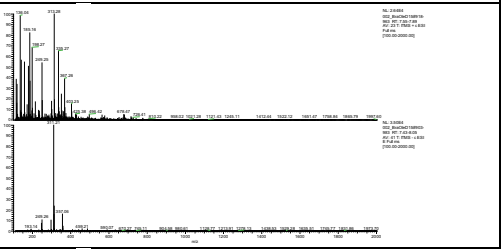
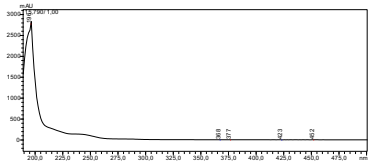
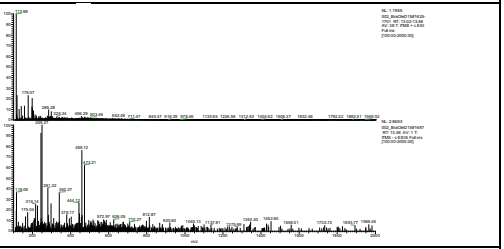
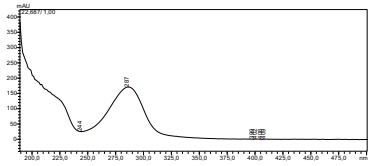
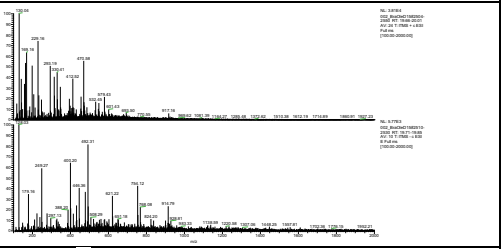
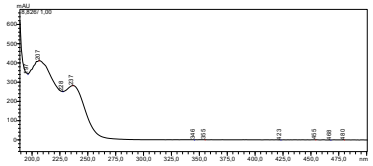
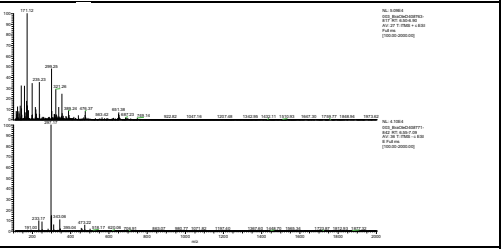
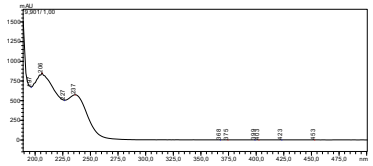
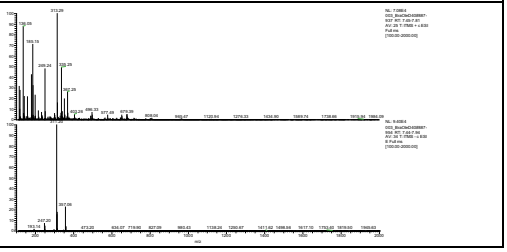
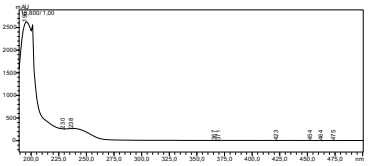
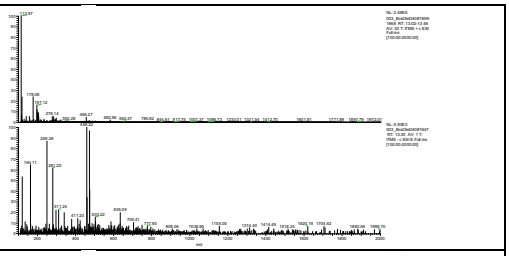
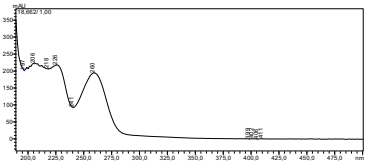
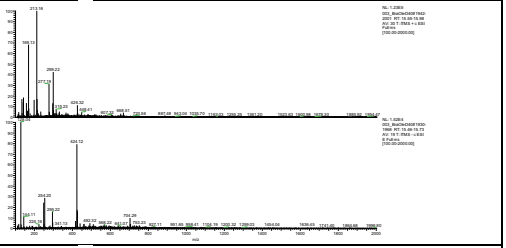
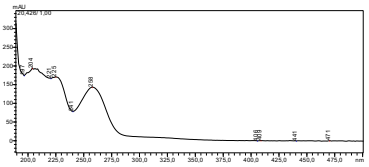
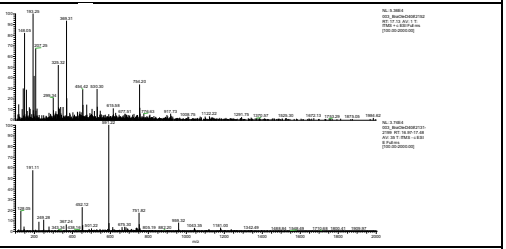
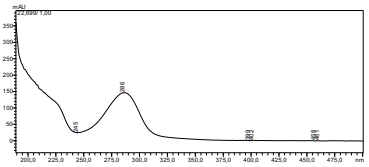
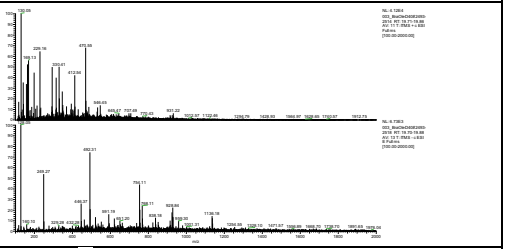
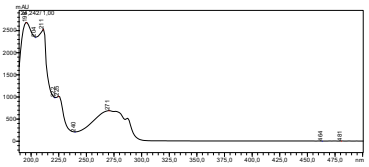
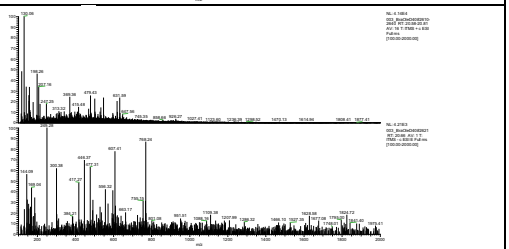
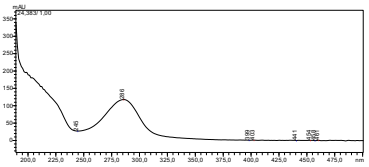
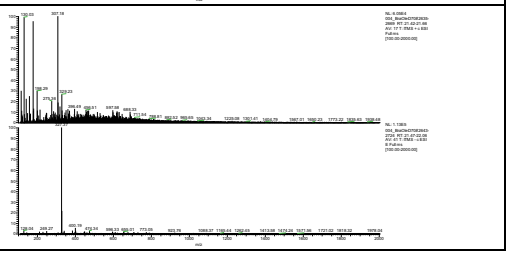


Figure S6 HPLC-ELSD chromatograms of fractions D_15% (green), D_40% (red), and D_70% (blue). The main peaks were labeled by numbers and characterized using HPLC-DAD and UHPLC-ESIMS.

Table S1 UV spectra and mass spectra (positive mode upper spectrum, negative mode lower spectrum) of main peaks found in the fractions D_15% (Peaks 1-5), D_40% (Peaks 6-12), and D_70% (Peaks 13-15).

Peak Nr.	UV spectrum	ESIMS
1		

2		
3		
4		
5		
6		
7		

8		
9		
10		
11		
12		
13		

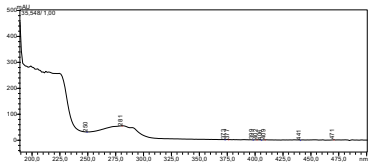
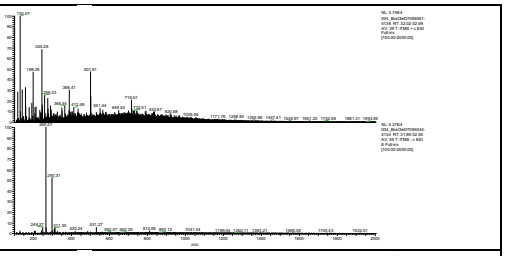
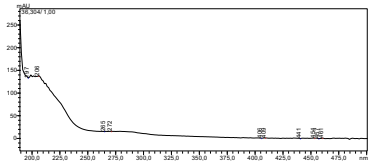
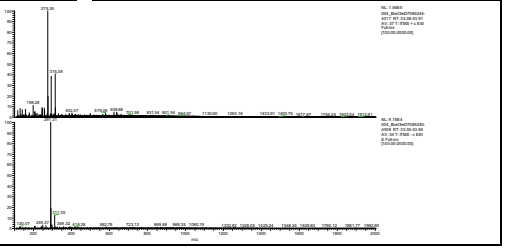
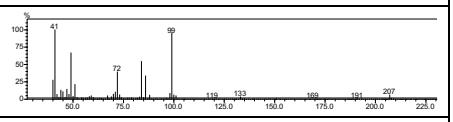
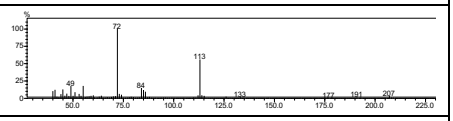
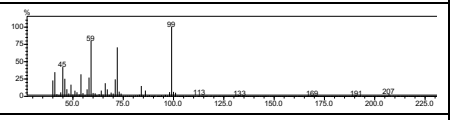
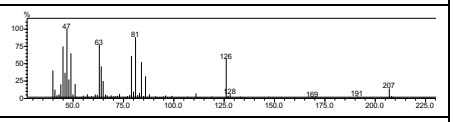
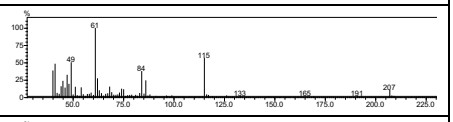
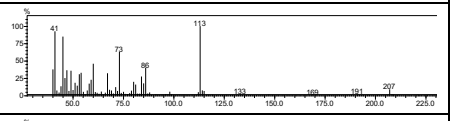
14		
15		

Table S2 Mass spectra of compounds in the fresh cabbage extract identified by databases (WILEY, NIST) with a similarity score of at least 75%.

Peak Nr.	Compound name (Abbreviation)	Molecular weight [g/mol]	Mass spectrum
1	Allyl isothiocyanate (AITC)	99	
2	3-Butenyl isothiocyanate (3-BITC)	113	
3	Cyclopropyl isothiocyanate (CPITC)	99	
4	S-Methyl methanethiosulfonate (MMTS)	126	
5	4-(Methylthio)butylnitrile (4-MTBN)	115	
6	Thiiranepropanenitrile (TPN)	113	
7	5-(Methylthio)pentanenitrile (5-MTPN)	129