WARSZAWSKI UNIWERSYTET MEDYCZNY

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WYDZIAŁ FARMACEUTYCZNY

Warszawski Uniwersytet Medyczny

Wydział Farmaceutyczny

Katedra i Zakład Biologii Farmaceutycznej

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu w dyscyplinie nauki farmaceutyczne

Charakterystyka fitochemiczna i aktywność biologiczna wyciągu z liści dzikiego czarnego bzu – wskazanie składników o potencjale przeciwzapalnym

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Obrona rozprawy doktorskiej przed Radą Dyscypliny Nauk Farmaceutycznych Warszawskiego Uniwersytetu Medycznego

Warszawa, 2024

Spis treści

1. Wykaz publikacji będących podstawą pracy doktorskiej

Podstawę ubiegania się o stopień doktora nauk medycznych i nauk o zdrowiu w dyscyplinie nauki farmaceutyczne stanowi cykl trzech artykułów naukowych opublikowanych w latach 2022-2024 w czasopismach naukowych o łącznym wskaźniku cytowań (Impact Factor) = 16,2 i punktacji Ministerstwa Edukacji i Nauki = 420.

Publikacja nr 1 (IF = 5,4; MEiN = 140) – artykuł oryginalny

Skowrońska W., Granica S., Czerwińska M. E., Osińska E., Bazylko A.; *Sambucus nigra* L. leaves inhibit TNF-α secretion by LPS-stimulated human neutrophils and strongly scavenge reactive oxygen species; *Journal of Ethnopharmacology*, 290 (2022): 115116; DOI: 10.1016/j.jep.2022.115116

Publikacja nr 2 (IF = 5,4; MEiN = 140) – artykuł oryginalny

Skowrońska W., Granica S., Piwowarski J. P., Jakupović L., Zovko-Končić M., Bazylko A.; Wound healing potential of extract from *Sambucus nigra* L. leaves and its fractions; *Journal of Ethnopharmacology*, 320 (2024): 117423; DOI: 10.1016/j.jep.2023.117423

Publikacja nr 3 (IF = 5,4; MEiN = 140) – praca przeglądowa

Skowrońska W., Bazylko A.; The potential of medicinal plants and natural products in the treatment of burns and sunburn – a review; *Pharmaceutics*, 15.2 (2023): 633; DOI: 10.3390/pharmaceutics15020633

Pełne teksty manuskryptów oraz oświadczenia współautorów publikacji zamieszczono w Załącznikach (str. 65).

2. Wykaz stosowanych skrótów

ABTS – sól diamoniowa kwasu 2,2'-azyno-bis(3-etylobenzotiazolino-6-sulfonowego), *(diammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate));* **API** – substancja czynna *(active pharmaceutical ingredient);* **AZS** – atopowe zapalenie skóry *(atopic dermatitis);* **CCR** – receptor C-C chemokin *(C-C chemokine receptor);* **CD** – antygen różnicowania komórkowego *(cluster of differentiation);* **CLA** – antygen związany z limfocytami skóry *(cutaneous lymphocyte-associated antigen);* **CRTH2** – cząsteczka homologiczna z receptorem chemoatraktantu ulegająca ekspresji na komórkach Th2 *(chemoattractant receptor-homologous molecule expressed on Th2 cells);* **CTGF** – czynnik wzrostu tkanki łącznej *(connective tissue growth factor);* **CXCL8 (IL-8)** – chemokina 8 / interleukina 8 *(chemokine (C-X-C motif) ligand 8);* **DC** – komórka dendrytyczna *(dendritic cell);* **DPPH** – 2,2-difenylo-1-pikrylohydrazyl *(2,2-diphenyl-1-picrylhydrazyl);* **ECM** – macierz pozakomórkowa *(extracellular matrix);* **EGF** – nabłonkowy czynnik wzrostu *(epidermal growth factor);* **Eo** – eozynofil *(eosinophil);* **EX** – wyciąg *(extract);* **FGF** – czynnik wzrostu fibroblastów *(fibroblast growth factor);* **f-MLP** – *N*-formylo-metionylo-leucylo-fenyloalanina *(N-formyl-methionyl-leucyl-phenylalanine);* **FR** – frakcja *(fraction);* **FRAP** – metoda oznaczania zdolności redukowania jonów Fe(II) *(ferric ion reducing antioxidant parameter);* **GMCSF** – czynnik stymulujący tworzenie kolonii granulocytów i makrofagów *(granulocyte-macrophage colony stimulating factor);* **GF** – czynnik wzrostu *(growth factor);* **HaCaT** – nienowotworogenna, unieśmiertelniona linia komórkowa keratynocytów skóry ludzkiej osoby dorosłej *(spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin);* **HGF** – czynnik wzrostu hepatocytów *(hepatocyte growth factor);* **HPLC-DAD** – wysokosprawna chromatografia cieczowa z detekcją diodową *(High-performance liquid chromatography, diode array detection);* **IC_{50}** – połowa maksymalnego stężenia hamującego *(half-maximal inhibitory concentration);* **IDEC** – zapalna dendrytyczna komórka naskórka *(inflammatory dendritic epidermal cell);* **IDO** – 2,3-dioksygenaza indoloaminowa *(indoleamine 2,3-dioxygenase);* **IFN-γ** – interferon γ *(interferon γ);* **IGF-1** – insulinopodobny czynnik wzrostu 1 *(insulin-like growth factor 1);* **IL** – interleukina *(interleukin);* **ILC** – wrodzona komórka limfoidalna *(innate lymphoid cell);* **JAK** – kinazy janusowe *(Janus-activated kinases);* **KGF** – czynnik

wzrostu keratynocytów *(keratinocyte growth factor);* **KLK** – peptydaza związana z kalikreiną *(kallikrein-related peptidase);* **LC** – komórka limfoidalna *(lymphoid cell);* **LOX** – lipooksygenaza *(lipoxygenase);* **LPS** – lipopolisacharyd *(lipopolysaccharide);* **LTA** – kwas lipotejchojowy *(lipoteichoic acid);* **MC** – komórka tuczna *(mast cell);* **MMP** – metaloproteinazy macierzy pozakomórkowej *(matrix metalloproteinases);* **NAP-2** – peptyd aktywujący neutrofile 2 *(neutrophil activating peptide 2);* **NF-κB** – jądrowy czynnik kappa B *(nuclear factor kappa-light-chain-enhancer of activated B cells);* **NHDF** – prawidłowe fibroblasty skóry ludzkiej *(normal human dermal fibroblasts);* **NMF** – naturalny czynnik nawilżający *(natural moisturising factor);* **NMR** – spektroskopia magnetycznego rezonansu jądrowego *(nuclear magnetic resonance);* **$O_2^{-\bullet}$** – anionorodnik ponadtlenkowy *(superoxide anion radical);* **PAR2** – receptor aktywowany proteazą 2 *(protease-activated receptor 2);* **PDGF** – płytko-pochodny czynnik wzrostu *(platelet-derived growth factor);* **PF-4** – płytkowy czynnik 4 *(platelet factor 4);* **PLE** – ciśnieniowa/przyspieszona ekstrakcja za pomocą cieczy *(pressurized liquid extraction);* **ROS** – reaktywne formy tlenu *(reactive oxygen species);* **SDF-1** – czynnik 1 z komórek zrębowych *(stromal-cell-derived factor 1);* **STAT** – przekaźnik sygnału i aktywator transkrypcji *(signal transducer and activator of transcription);* **TARC** – chemokina CCL17 *(CC chemokine ligand 17; thymus- and activation-regulated chemokine);* **TGF-β1** – transformujący czynnik wzrostu β1 *(transforming growth factor* β1*);* **Teff** – efektorowe limfocyty T *(effector T cells);* **Tem** – limfocyty T pamięci efektorowej *(effector memory T cells);* **Th** – pomocnicze limfocyty T *(helper T cells);* **TIMP1** – tkankowy inhibitor metaloproteinaz 1 *(tissue inhibitor of metalloproteinases 1);* **TLR** – receptory *toll*-podobne *(toll-like receptors);* **Tnaive** – naiwne limfocyty T (*naive T cells*)*;* **TNF-α** – czynnik martwicy nowotworów α *(tumor necrosis factor α);* **Treg** – limfocyty T regulatorowe *(regulatory T cells);* **Trm** – limfocyty T pamięci rezydujące w tkance *(tissue-resident memory T cells);* **TSLP (IL-2)** – interleukina 2, limfopoetyna zrębowa grasicy *(thymic stromal lymphopoietin);* **UHPLC-DAD-MS^n** – ultra-wysokosprawna chromatografia cieczowa z detekcją diodową i spektrometrią mas (*Ultra high-performance liquid chromatography, diode array detection, mass spectrometry);* **UV** – promieniowanie ultrafioletowe *(ultraviolet);* **VEGF** – czynnik wzrostu śródbłonka naczyniowego *(vascular endothelial growth factor)*

3. Słowa kluczowe

3.1. Słowa kluczowe w języku polskim

Czarny bez, aktywność przeciwzapalna, aktywność przeciwutleniająca, gojenie ran, neutrofile, keratynocyty, fibroblasty, oparzenia, oparzenia słoneczne, fitochemia, flawonoidy, kwasy fenolowe, lignany, glikozydy cyjanogenne

3.2. Słowa kluczowe w języku angielskim *(Keywords)*

Elderberry, anti-inflammatory activity, antioxidant activity, wound healing, neutrophils, keratinocytes, fibroblasts, burns, sunburn, phytochemistry, flavonoids, phenolic acids, lignans, cyanogenic glycosides

4. Streszczenie

4.1. Streszczenie w języku polskim

Sambucus nigra L., znany jako czarny bez, jest gatunkiem z rodziny kalinowatych (Viburnaceae) występującym powszechnie w Europie i Azji Zachodniej (Waswa et al., 2022). W medycynie wykorzystywane są głównie właściwości lecznicze jego kwiatów i owoców. Działają one napotnie i przeciwgorączkowo, w związku z czym są stosowane w łagodzeniu objawów przeziębienia (Młynarczyk et al., 2018). Natomiast w medycynie ludowej wykorzystywane były również liście czarnego bzu, głównie zewnętrznie, w leczeniu ran, oparzeń i schorzeń skóry o podłożu zapalnym (Menendez-Baceta et al., 2014). Jednakże dotychczas ich działanie, jak i skład chemiczny nie były dokładnie przebadane.

Głównym celem pracy była analiza fitochemiczna i zbadanie aktywności biologicznej wyciągów z liści czarnego bzu w odniesieniu do ich tradycyjnego zastosowania w schorzeniach skóry.

W pierwszej części pracy wykonano cztery ekstrakty z liści *S. nigra* – wodne i 70% (*v/v*) etanolowe w temperaturze pokojowej i w temperaturze wrzenia rozpuszczalnika. Poddano je jakościowej i ilościowej analizie fitochemicznej, wykorzystując odpowiednio metody UHPLC-DAD-MS^n i HPLC-DAD. Następnie oceniono ich właściwości przeciwutleniające i przeciwzapalne, badając wpływ na wymiatanie rodników w układach *in vitro* (DPPH, H_2O_2, NO, $O_2^{-}\cdot$), hamowanie aktywności lipooksygenazy, a także obniżanie wydzielania reaktywnych form tlenu (ROS) i mediatorów stanu zapalnego (tj. TNF-α, IL-1β i IL-8) przez ludzkie neutrofile *ex vivo*. Analiza fitochemiczna wykazała, że w wyciągach dominowały związki z grup kwasów fenolowych i flawonoidów. Zawartość głównych substancji chemicznych – izomerów kwasu kawoilo-treoninowego, kwasu chlorogenowego, pochodnych kwercetyny i kemferolu była najwyższa w 70% (*v/v*) etanolowym wyciągu sporządzonym w temperaturze pokojowej. Wyniki badań *in vitro* wskazywały na silne działanie przeciwutleniające badanych ekstraktów, przede wszystkim wobec tlenku azotu. W badaniach *ex vivo* wykazano, że wyciągi silnie i

zależnie od stężenia hamują uwalnianie ROS i TNF-α przez izolowane z krwi neutrofile stymulowane odpowiednio f-MLP i LPS. (Skowrońska et al., 2022).

Na podstawie wyników uzyskanych w pierwszym etapie do dalszych badań wybrano wyciąg 70% (*v/v*) etanolowy sporządzony w temperaturze pokojowej. Charakteryzował się on najwyższą zawartością głównych składników, działał silnie przeciwutleniająco w układach *in vitro*, a także wykazywał istotną aktywność przeciwzapalną *ex vivo* przy najniższym negatywnym wpływie na żywotność komórek. Wytypowany ekstrakt sporządzono w większej skali, a następnie poddano frakcjonowaniu za pomocą rozpuszczalników o wzrastającej polarności – dichlorometanu, eteru dietylowego, octanu etylu i n-butanolu. Skład ekstraktu, poszczególnych frakcji i pozostałości wodnej oceniono jakościowo metodą UHPLC-DAD-MS^n. Następnie, wykorzystując metody chromatografii kolumnowej i preparatywnej chromatografii cieczowej, wykonano izolację związków chemicznych. Otrzymano 11 substancji, których strukturę ustalono na podstawie analizy widm ^{1}H NMR. Zidentyfikowano związki z grupy flawonoidów, kwasów fenolowych, lignanów i glikozydów cyjanogennych.

W celu weryfikacji tradycyjnego zastosowania liści czarnego bzu w leczeniu ran i schorzeń skóry o podłożu zapalnym wykonano badania podstawowe wyciągu i otrzymanych z niego frakcji. Analizowano ich wpływ na odpowiedź zapalną komórek obecnych w skórze, migrację keratynocytów do miejsca zranienia, a także na aktywność enzymów zaangażowanych w procesy naprawcze zachodzące w skórze. Wykazano, że ekstrakt i część frakcji istotnie przyspiesza proces zamykania rany przez komórki naskórka oraz obniża wydzielanie przez nie chemotaktycznej interleukiny 8. Ponadto, wyciąg i frakcje działały przeciwzapalnie na fibroblasty skóry ludzkiej, obniżając uwalnianie interleukin 6 i 8. Aktywność enzymów hamowana była w różnym stopniu, substancje działały najsilniej wobec kolagenazy i lipooksygenazy (Skowrońska et al., 2024).

Podsumowując, przeprowadzenie zaprezentowanych badań umożliwiło częściowe uzasadnienie stosowania liści czarnego bzu w medycynie ludowej w leczeniu ran, oparzeń i schorzeń dermatologicznych o podłożu zapalnym. W ramach pracy opracowano trzy modele badania odpowiedzi zapalnej na komórkach skóry, które są

aktualnie wykorzystywane rutynowo w pracy naukowej Katedry i Zakładu Biologii Farmaceutycznej.

Dodatkowo, wykonano przegląd literatury dotyczący stosowania produktów pochodzenia naturalnego w leczeniu oparzeń i oparzeń słonecznych. Przygotowano zestawienie i ocenę opublikowanych w latach 2010-2022 wyników badań klinicznych i badań na zwierzętach, w których analizowano wpływ na gojenie zarówno pojedynczych substancji roślinnych, jak i ich mieszanin (Skowrońska and Bazylko, 2023).

4.3. Streszczenie w języku angielskim (*Abstract*)

Sambucus nigra L., known as the black elderberry, is a species of the Viburnaceae family native to Europe and West Asia (Waswa et al., 2022). In medicine, the healing properties of its flowers and fruits are mainly used. They have a diaphoretic and antipyretic effect and are used to relieve the symptoms of common cold (Młynarczyk et al., 2018). However, in folk medicine elderberry leaves have also been used, mainly externally, for the treatment of wounds, burns and inflammatory skin conditions (Menendez-Baceta et al., 2014). So far, their activity and chemical composition have not been studied in detail.

The main aim of this study was to perform a phytochemical analysis and to investigate the biological activity of elderberry leaves extracts in relation to their traditional use in skin diseases.

In the first part of the work, four extracts of *S. nigra* leaves were prepared - aqueous and 70% (*v/v*) ethanolic at room temperature and at the boiling point of the solvent. They were subjected to qualitative and quantitative phytochemical analysis using UHPLC-DAD-MS^n and HPLC-DAD methods, respectively. Subsequently, their antioxidant and anti-inflammatory properties were evaluated by studying the effect on radicals scavenging in *in vitro* systems (DPPH, H_2O_2, NO, $O_2^{-}\cdot$), inhibition of lipoxygenase activity, and reduction of secretion of reactive oxygen species (ROS) and inflammatory mediators (TNF-α, IL-1β and IL-8) by human neutrophils *ex vivo*. Phytochemical analysis showed that the extracts were dominated by phenolic acids and flavonoids. The 70% (*v/v*) ethanolic extract prepared at room temperature had the highest content of the major chemical compounds - isomers of caffeoyl-threonic acid, chlorogenic acid, quercetin and kaempferol derivatives. The results of *in vitro* tests indicated a strong antioxidant effect of the tested extracts, mainly against nitric oxide. *Ex vivo* studies showed that the extracts exert a potent and concentration-dependent inhibitory effect on the release of ROS and TNF-α by neutrophils isolated from blood stimulated with f-MLP and LPS, respectively (Skowrońska et al., 2022).

Based on the results obtained in the first step, the 70% (*v/v*) ethanolic extract prepared at room temperature was selected for further investigation. This extract had the highest content of main ingredients, exhibited a strong antioxidant effect *in vitro*, and demonstrated significant anti-inflammatory activity *ex vivo*, while having the lowest negative impact on cell viability. The extract was prepared on a larger scale and then fractionated using solvents of increasing polarity: dichloromethane, diethyl ether, ethyl acetate, and n-butanol. The composition of the extract, individual fractions, and aqueous residue was qualitatively assessed using the UHPLC-DAD-MS^n method. Chemical compounds were isolated using column chromatography and preparative liquid chromatography methods. Eleven substances were obtained and identified using ^{1}H NMR spectra. These compounds belong to the groups of flavonoids, phenolic acids, lignans, and cyanogenic glycosides.

To verify the traditional use of elderberry leaves in treating wounds and inflammatory skin diseases, a basic research was carried out on the extract and its fractions. Their impact on the inflammatory response of cells present in the skin, the migration of keratinocytes to the site of injury, and the activity of enzymes involved in the skin repair processes were analyzed. Research has demonstrated that the extract and certain fractions can expedite wound closure by epidermal cells and decrease their secretion of chemotactic interleukin 8. Additionally, the extract and fractions exhibit anti-inflammatory properties on human skin fibroblasts, reducing the release of interleukins 6 and 8. The substances were found to inhibit enzyme activity, with the strongest effect observed on collagenase and lipoxygenase (Skowrońska et al., 2024).

In summary, the research presented provides partial justification for the use of elderberry leaves in folk medicine for treating wounds, burns, and inflammatory dermatological diseases. The work also involved developing three models for testing the inflammatory response on skin cells, which are now routinely used in the scientific work of the Department of Pharmaceutical Biology.

Additionally, a literature review was conducted on the use of natural products in treating burns and sunburn. Clinical trials and animal studies published between 2010

and 2022 on the effects of individual botanicals and their mixtures on the healing of burns and sunburn were compiled and evaluated (Skowrońska and Bazylko, 2023).

5. Dotychczasowa aktywność naukowa doktorantki

5.1. Sylwetka naukowa

Doktorantka rozpoczęła jednolite studia magisterskie na kierunku farmacja Warszawskiego Uniwersytetu Medycznego w październiku 2012 roku. W trakcie studiów otrzymywała Stypendium Rektora dla najlepszych studentów. Od 2014 roku była członkiem Studenckiego Koła Naukowego „Herbarium" działającego przy Katedrze Farmakognozji i Molekularnych Podstaw Fitoterapii. Na prowadzenie badań naukowych otrzymała mini-grant studencki pt. „Badania porównawcze składu i aktywności ekstraktów z części nadziemnych i korzeni gatunków *Arctium lappa* i *Arctium tomentosum*" przyznany przez Warszawski Uniwersytet Medyczny. W czerwcu 2017 roku doktorantka obroniła pracę magisterską pt. „Zbadanie i porównanie aktywności przeciwutleniającej i przeciwzapalnej oraz wstępna analiza składu chemicznego ekstraktów z części nadziemnych i korzeni *Arctium tomentosum* Mill. z różnych stanowisk naturalnych rejonu Podkarpacia" realizowaną pod opieką dr hab. Agnieszki Bazylko. Po ukończeniu praktyki zawodowej w aptece ogólnodostępnej w marcu 2018 roku otrzymała prawo wykonywania zawodu farmaceuty.

W październiku 2018 roku doktorantka rozpoczęła studia doktoranckie, kontynuując działalność naukową w Katedrze Farmakognozji i Molekularnych Podstaw Fitoterapii na Wydziale Farmaceutycznym Warszawskiego Uniwersytetu Medycznego. W trakcie studiów otrzymywała Stypendium Rektora dla najlepszych doktorantów. Promotorem jej pracy pt. „Charakterystyka fitochemiczna i aktywność biologiczna wyciągu z liści dzikiego czarnego bzu – wskazanie składników o potencjale przeciwzapalnym" jest dr hab. Agnieszka Bazylko. Na przełomie 2021 i 2022 roku doktorantka odbyła 6-cio miesięczny staż naukowy w Chorwacji w ramach programu Erasmus+ (Zavod za farmakognoziju, Farmaceutsko-biokemijski fakultet, Sveučilište u Zagrebu) pod opieką profesor Marijany Zovko Končić. Podczas praktyk wykonała oznaczenia wpływu ekstraktu z liści czarnego bzu i frakcji z niego otrzymanych na aktywność enzymów zaangażowanych w procesy naprawcze skóry.

Oprócz prowadzenia badań w ramach pracy doktorskiej, doktorantka jest zaangażowana w realizację innych projektów naukowych, realizowanych nie tylko w Katedrze. W 2021 roku została kierownikiem grantu naukowo-wdrożeniowego Inkubator Innowacyjności 4.0 pt. „Ekstrakt z liści brzozy o wyjątkowej zawartości składników aktywnych" o akronimie BetulaEBM przyznanego przez Ministerstwo Edukacji i Nauki. W projekcie była odpowiedzialna za badania aktywności hamującej adhezję uropatogennego szczepu *Escherichia coli* do komórek nabłonka pęcherza, a także za prowadzenie badań *in vivo* aktywności diuretycznej na modelu zwierzęcym – szczurach Sprague-Dawley. Dodatkowo, była zaangażowana w analizę chemiczną moczu i ocenę zawartych w nim metabolitów powstających z podawanego innowacyjnego ekstraktu z liści brzozy. W związku z prowadzonymi badaniami uczestniczyła w szkoleniu dotyczącym pracy ze zwierzętami laboratoryjnymi (dla osób odpowiedzialnych za planowanie procedur i doświadczeń oraz za ich przeprowadzanie, wykonujących procedury, uśmiercających zwierzęta wykorzystywane w procedurach oraz uczestniczących w wykonywaniu procedur). Doktorantka była również zaangażowana w analizę rynku dla innowacyjnego produktu leczniczego do stosowania w nawracających zakażeniach układu moczowego u kobiet, łączącego aktywność przeciwzapalną, antyadhezyjną, diuretyczną i quorum quenching (tj. hamowania quorum sensing, czyli zdolności wykrywania i odpowiedzi na komórkową gęstość populacji poprzez regulację genową). Ponadto, doktorantka jest współtwórcą zgłoszenia patentowego (P.441246) w Urzędzie Patentowym Rzeczpospolitej Polskiej, które powstało na podstawie wyników uzyskanych podczas realizacji projektu. Dotyczy ono technologii otrzymywania ekstraktu z liści brzozy o wysokiej zawartości 3-*O*-β-D-glukopiranozydu 3-hydroksy-1-(4-hydroksyfenyl)propan-1-onu, charakteryzującego się wyjątkową aktywnością biologiczną.

W 2022 roku doktorantka została kierownikiem grantu Młody Badacz przyznawanego przez Warszawski Uniwersytet Medyczny pt. „Wpływ wyciągów roślinnych na skład mikrobioty sromu i pochwy oraz produkowane z zawartych w nich substancji metabolity postbiotyczne". W ramach projektu badała *ex vivo* wzajemne interakcje pomiędzy mikrobiotą sromu i pochwy, a ekstraktami roślinnymi bogatymi w garbniki. Analizowała

powstawanie metabolitów postbiotycznych z wyciągów roślinnych, a także badała zmiany jakościowe i ilościowe składu pobranej od ochotniczek mikrobioty. W 2023 roku, w nawiązaniu do prowadzonych badań, odbyła staż w Belgii (Departement Bioingenieurswetenschappen, Universiteit Antwerpen) w ramach wymiany Erasmus+ dla nauczycieli akademickich, gdzie pod opieką prof. Sarah Lebeer miała możliwość przyjrzenia się technikom badawczym stosowanym w stworzonym przez nią Lab of Applied Microbiology and Biotechnology. Dodatkowo, doktorantka poznała projekt naukowy ISALA, którego pomysłodawcą jest prof. Lebeer, skupiający się na analizach mikrobiomu pochwy, a także na badaniach naukowych związanych ze zdrowiem kobiet.

W 2023 roku doktorantka została wykonawcą w projekcie Preludium pt. „Wspomaganie terapii trudno gojących się ran z zastosowaniem kory oczaru wirginijskiego", którego kierownikiem jest dr inż. Karolina Pawłowska. Celem projektu jest uzasadnienie tradycyjnego stosowania miejscowego *Hamamelidis cortex* w leczeniu infekcji i stanów zapalnych skóry oraz gojeniu ran. Rolą doktorantki było przeprowadzenie badań aktywności wyciągu i wyizolowanych z niego głównych związków chemicznych na modelach komórkowych skóry *in vitro* – keratynocytach i fibroblastach. Badała odpowiedź zapalną komórek na opracowanych i wdrożonych w ramach doktoratu modelach. Dodatkowo, była odpowiedzialna za analizę powstających w wyniku ich inkubacji *ex vivo* z mikrobiotą skóry metabolitów postbiotycznych, a także na przeprowadzeniu badań aktywności głównego powstającego związku.

Od kwietnia 2024 roku doktorantka jest wykonawcą w grancie Lider XIV pt. „Biorafineria HERB do przetwarzania konopi siewnych". Projekt polega na opracowaniu ciągu technologicznego Biorafinerii HERB (ang. Hemp Extraction, Refining and Biogasification) do pozyskiwania związków chemicznych o wysokiej wartości rynkowej z konopi siewnych, a także zaprojektowanie, skonstruowanie i uruchomienie prototypu biorafinerii w celu doświadczalnej weryfikacji jej parametrów technicznych i ekonomicznych. Kierownikiem projektu jest dr inż. Jan Krzysztoforski z Zakładu Inżynierii i Dynamiki Reaktorów Chemicznych na Wydziale Inżynierii Chemicznej i Procesowej Politechniki Warszawskiej. Doktorantka odpowiada za przestrzeganie

aspektów prawnych uprawy i przetwarzania konopi siewnych, a także za szczegółowe analizy fitochemiczne produktów otrzymywanych na każdym etapie realizacji projektu.

Od 2019 roku doktorantka pracuje w Katedrze i Zakładzie Biologii Farmaceutycznej (wcześniej Katedra Farmakognozji i Molekularnych Podstaw Fitoterapii) na stanowisku asystenta naukowo-badawczego. W ramach pensum dydaktycznego prowadzi ćwiczenia laboratoryjne i seminaria dla studentów III, IV i V roku kierunku farmacja z przedmiotów Farmakognozja i Lek Pochodzenia Naturalnego, a także zajęcia dla Fakultatywnego Bloku Programowego Farmacja Analityczna. Od 2022 roku jest opiekunem naukowym prac magisterskich realizowanych w Katedrze, a także studentów zagranicznych odbywających praktyki w ramach programu Erasmus+. Ponadto, w październiku 2023 roku doktorantka została opiekunem Studenckiego Koła Naukowego „Herbarium", działającego przy Katedrze i Zakładzie Biologii Farmaceutycznej.

5.2. Udział w projektach badawczych i naukowo-wdrożeniowych

- Wykonawca w projekcie naukowo-wdrożeniowym **Lider XIV** (0161/L-14/2023); Narodowe Centrum Badań i Rozwoju
- Wykonawca w projekcie naukowym **Preludium** (2021/41/N/NZ7/00602); Narodowe Centrum Nauki
- Kierownik w projekcie naukowym **Młody Badacz** (F/MB/05/22); Warszawski Uniwersytet Medyczny
- Kierownik w projekcie badawczo-wdrożeniowym **Inkubator Innowacyjności 4.0** (FW25/FS249/ZW/JSt/WS/22); Ministerstwo Edukacji i Nauki
- Kierownik w projekcie naukowym **Mini-grant** studencki (FW25/NM2/17); Warszawski Uniwersytet Medyczny

5.3. Praktyki, staże naukowe i szkolenia

- **21-28.08.2023** – Zagraniczny staż naukowy w Belgii – Departement Bio-ingenieurswetenschappen, Universiteit Antwerpen – Opiekun naukowy: prof. Sarah Lebeer

- **02.06.2023** – warsztat „Test ELISA – kompendium, dobre praktyki, najczęstsze błędy"; Fundacja na rzecz promocji nauki i rozwoju TYGIEL
- **06-28.02.2023** – Szkolenie z pracy ze zwierzętami laboratoryjnymi dla osób odpowiedzialnych za planowanie procedur i doświadczeń oraz ich przeprowadzanie, wykonujących procedury, uśmiercających zwierzęta wykorzystywane w procedurach oraz uczestniczących w wykonywaniu procedur; Warszawski Uniwersytet Medyczny
- **01.10.2021 – 16.01.2022** – Zagraniczny staż naukowy w Chorwacji – Zavod za farmakognoziju, Farmaceutsko-biokemijski fakultet, Sveučilište u Zagrebu – Opiekun naukowy: prof. Marijana Zovko Končić.
- **01.10.2017 – 31.03.2018** – Praktyki zawodowe w aptece ogólnodostępnej – Apteka Zachodnia, Aleja Wojska Polskiego 50A, 05-800 Pruszków. Opiekun stażu – mgr farm. Marta Bednarska
- **01-31.08.2016** – Praktyki zawodowe – Narodowy Instytut Leków, Zakład Leków Pochodzenia Naturalnego i Suplementów Diety, ul. Chełmska 30/34, 00-725 Warszawa
- **01-31.07.2016** – Praktyki zawodowe w aptece szpitalnej – Apteka Szpitalna Centralnego Szpitala Klinicznego Warszawskiego Uniwersytetu Medycznego, ul. Nielubowicza 5, 02-097 Warszawa

5.4. Lista publikacji, które nie weszły w cykl rozprawy doktorskiej

- Dos Santos Szewczyk K., Skowrońska W., Kruk A., Makuch-Kocka A., Bogucka-Kocka A., Miazga-Karska M., Grzywa-Celińska A., Granica S.; Chemical composition of extracts from leaves, stems and roots of wasabi (*Eutrema japonicum*) and their anti-cancer, anti-inflammatory and anti-microbial activities; ***Scientific Reports***, 13.1 (2023): 9142. DOI: 10.1038/s41598-023-36402-y; IF = 4,6; MEiN = 140
- Korczak M., Roszkowski P., Skowrońska W., Żołdak K., Popowski D., Granica S., Piwowarski J.; Urolithin A conjugation with NSAIDs inhibits its glucuronidation and maintains improvement of Caco-2 monolayers' barrier function;

Biomedicine & Pharmacotherapy, 169 (2023): 115932. DOI: 10.1016/j.biopha.2023.115932; IF = 7,5; MEiN = 140

- Melnyk N., Vlasova I., Skowrońska W., Bazylko A., Piwowarski J., Granica S.; Current knowledge on interactions of plant materials traditionally used in skin diseases in Poland and Ukraine with human skin microbiota; ***International Journal of Molecular Sciences***, 23.17 (2022): 9644. DOI: 10.3390/ijms23179644; IF = 5,6; MEiN = 140
- Pawłowska K., Baracz T., Skowrońska W., Piwowarski J., Majdan M., Malarz J., Stojakowska A., Zidorn C., Granica S.; The contribution of phenolics to the anti-inflammatory potential of the extract from Bolivian coriander (*Porophyllum ruderale* subsp. *ruderale*); ***Food Chemistry***, 371 (2022): 131116. DOI: 10.1016/j.foodchem.2021.131116; IF = 8,8; MEiN = 200
- Laskowska A., Wilczak A., Skowrońska W., Michel P., Melzig M., Czerwińska M.; Fruits of *Hippophaë rhamnoides* in human leukocytes and Caco-2 cell monolayer models—A question about their preventive role in lipopolysaccharide leakage and cytokine secretion in endotoxemia; ***Frontiers in Pharmacology***, 13 (2022): 981874. DOI: 10.3389/fphar.2022.981874; IF = 5,6; MEiN = 100
- Skowrońska W., Granica S., Dziedzic M., Kurkowiak J., Ziaja M., Bazylko A.; *Arctium lappa* and *Arctium tomentosum*, sources of *Arctii radix*: comparison of anti-lipoxygenase and antioxidant activity as well as the chemical composition of extracts from aerial parts and from roots; ***Plants***, 10.1 (2021): 78. DOI: 10.3390/plants10010078; IF = 4,658; MEiN = 70
- Ilina T., Skowrońska W., Kashpur N., Granica S., Bazylko A., Kovalyova A., Goryacha O., Koshovyi O.; Immunomodulatory activity and phytochemical profile of infusions from Cleavers herb; ***Molecules***, 25.16 (2020): 3721. DOI: 10.3390/molecules25163721; IF = 4,412; MEiN = 140

5.5. Udział w konferencjach naukowych[1]

5.5.1. Prezentacje ustne

- Skowrońska W., Pawłowska K., Piwowarski J., Granica S.; Changes in the biological activity of *Hamamelidis cortex* extract after incubation with skin microbiota; Natural Cosmetics International Meeting; 20-22.09.2023; Rzeszów (Kielnarowa), Polska. **Nagroda Phytochemical Society of Europe za najlepszą prezentację ustną.**
- Skowrońska W., Naruszewicz M., Sacharczuk M., Piwowarski J., Granica S.; Urolithin A – a postbiotic metabolite of natural ellagitannins as an innovative molecule for skin inflammations; Natural Cosmetics International Meeting; 20-22.09.2023; Rzeszów (Kielnarowa), Polska.
- Skowrońska W., Nowoszewska J., Granica S., Bazylko A.; Anti-inflammatory activity of *Serpylli herba* on human skin keratinocytes; 22nd International Congress of International Society for Ethnopharmacology & 10th International Congress of Society of Ethnopharmacology; 24-26.02.2023; Imphal, Manipur, Indie.
- Skowrońska W., Czerwińska M., Osińska E., Bazylko A.; Właściwości przeciwutleniające i przeciwzapalne liści czarnego bzu; Carpre Herbarium – Ogólnopolska Studencka Konferencja Naukowa; 24-26.05.2019; Karpacz, Polska.
- Wilczak A., Skowrońska W., Czerwińska M.; Wyciągi i związki izolowane z owoców *Hippophaë rhamnoides* oraz ich wpływ na uwalnianie cytokin przez ludzkie neutrofile; Carpre Herbarium – Ogólnopolska Studencka Konferencja Naukowa; 24-26.05.2019; Karpacz, Polska.
- Skowrońska W., Dziedzic M., Ziaja M., Bazylko A.; Zbadanie i porównanie aktywności przeciwutleniającej i przeciwzapalnej oraz wstępna analiza składu chemicznego wyciągów z korzeni i części nadziemnych *Arctium lappa* L. i *Arctium tomentosum* Mill. z różnych stanowisk naturalnych rejonu Podkarpacia; IX Mini-Sympozjum Młodych Naukowców; 19.02.2019; Warszawa, Polska.

[1] Podkreśleniem zaznaczono autora prezentującego wyniki.

5.5.2. Plakaty

- Skowrońska W., Michalak A., Nowoszewska J., Granica S., Bazylko A.; Anti-inflammatory activity of *Serpylli herba* on human skin cells; Natural Cosmetics International Meeting; 20-22.09.2023; Rzeszów (Kielnarowa), Polska.
- Skowrońska W., Pawłowska K., Piwowarski J., Granica S.; Modifications of chemical composition and biological activity of *Hamamelidis cortex* extract after incubation *ex vivo* with skin microbiota; 12th Probiotics, Prebiotics & New foods: Nutraceuticals, Botanicals & Phytochemicals for Nutrition & Human, Animal and Microbiota Health, 3rd Science & Business Symposium; 16-19.09.2023; Rzym, Włochy.
- Skowrońska W., Piwowarski J., Popowski D., Kawka M., Granica S.; The interplay of vaginal microbiota and oak bark; 12th Probiotics, Prebiotics & New foods: Nutraceuticals, Botanicals & Phytochemicals for Nutrition & Human, Animal and Microbiota Health, 3rd Science & Business Symposium; 16-19.09.2023; Rzym, Włochy.
- Skowrońska W., Pawłowska K., Piwowarski J., Granica S.; *Hamamelidis cortex* reduces the inflammatory response of cells involved in wound healing; American Society of Pharmacognosy Annual Meeting : Innovation Through Interaction; 22-26.07.2023; Waszyngton (Rockville), Maryland, Stany Zjednoczone.
- Skowrońska W., Granica S., Bazylko A.; Wound healing potential of extracts and fractions of elderberry (*Sambucus nigra* L.) leaves; 70th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA); 28-31.08.2022; Thessaloniki, Grecja. Abstrakt opublikowany w ***Planta medica*** 2022, 88(15), DOI: 10.1055/s-0042-1759157
- Piwowarski J., Sacharczuk M., Skowrońska W., Granica S.; Application of urolithin A – a postbiotic metabolite produced by human gut microbiota, in topical treatment of atopic dermatitis; 70th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA); 28-31.08.2022; Thessaloniki, Grecja. Abstrakt opublikowany w ***Planta medica*** 2022, 88(15), DOI: 10.1055/s-0042-1759005

- Popowski D., Skowrońska W., Korczak M., Kruk A., Pawłowska K., Piwowarski J., Granica S.; Main non-flavonoid constituent of birch leaves water extract – 3-hydroxy-1-(4- hydroxyphenyl)-propan-1-one 3-O-β-D-glucoside: isolation, permeability, and antiadhesive activity; 70th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA); 28-31.08.2022; Thessaloniki, Grecja. Abstrakt opublikowany w ***Planta medica*** 2022, 88(15), DOI: 10.1055/s-0042-1759084
- Skowrońska W., Czerwińska M., Bazylko A.; Preliminary analysis of the chemical composition and antioxidant as well anti-inflammatory activity of extracts from elderberry leaves; 4th International Conference on Natural Products Utilization: From Plants to Pharmacy Shelf; 29.05-01.06.2019; Albena Resort, Bułgaria.
- Skowrońska W., Dziedzic M., Rogowska M., Ziaja M., Bazylko A.; *Arctium tomentosum* and *A. lappa* from Sub Carpathian Region of Poland. Comparison of antioxidant and enzyme inhibition activities, as well chemical composition; Joint meeting: GA2018 (The 66th Annual Meeting of the Society for Medicinal Plant and Natural Product Research) and S-TCM 2018 (The 11th Shanghai International Conference on Traditional Chinese Medicine and Natural Medicine); 26-29.08.2018; Shanghai, Chiny.
- Skowrońska W., Ziaja M., Bazylko A.; Qualitative and quantitative composition comparison between aqueous and ethanolic extracts of *Stellaria media* and *S. nemorum*; 2nd International Young Scientists Symposium "Plants in Pharmacy & Nutrition"; 15-17.09.2016; Wrocław, Polska.
- Skowrońska W., Grochowski D., Bazylko A., Granica S., Tomczyk M.; Comparison of antioxidant and enzyme inhibition activities as well chemical composition of different extracts and fractions of *Rubus caesius* leaves; 9th Joint Natural Products Conference, Copenhagen, Dania. Abstrakt opublikowany w ***Planta medica*** 2016, 82(S 01), DOI: 10.1055/s-0036-1596916

5.6. Dorobek naukowy w liczbach (na dzień 04.04.2024)

ORCID: 0000-0002-6960-2152

Łączna liczba publikacji: 10

(4 publikacje – pierwszy autor, 2 publikacje – autor korespondencyjny)

- 8 publikacji – artykuły oryginalne
- 2 publikacje – prace przeglądowe

Sumaryczny Impact Factor: 57,37.

Łączna punktacja MEiN: 1350.

Sumaryczna liczba cytowań (wg bazy Scopus): 65.

Index Hirscha (wg bazy Scopus, bez autocytowań): 6.

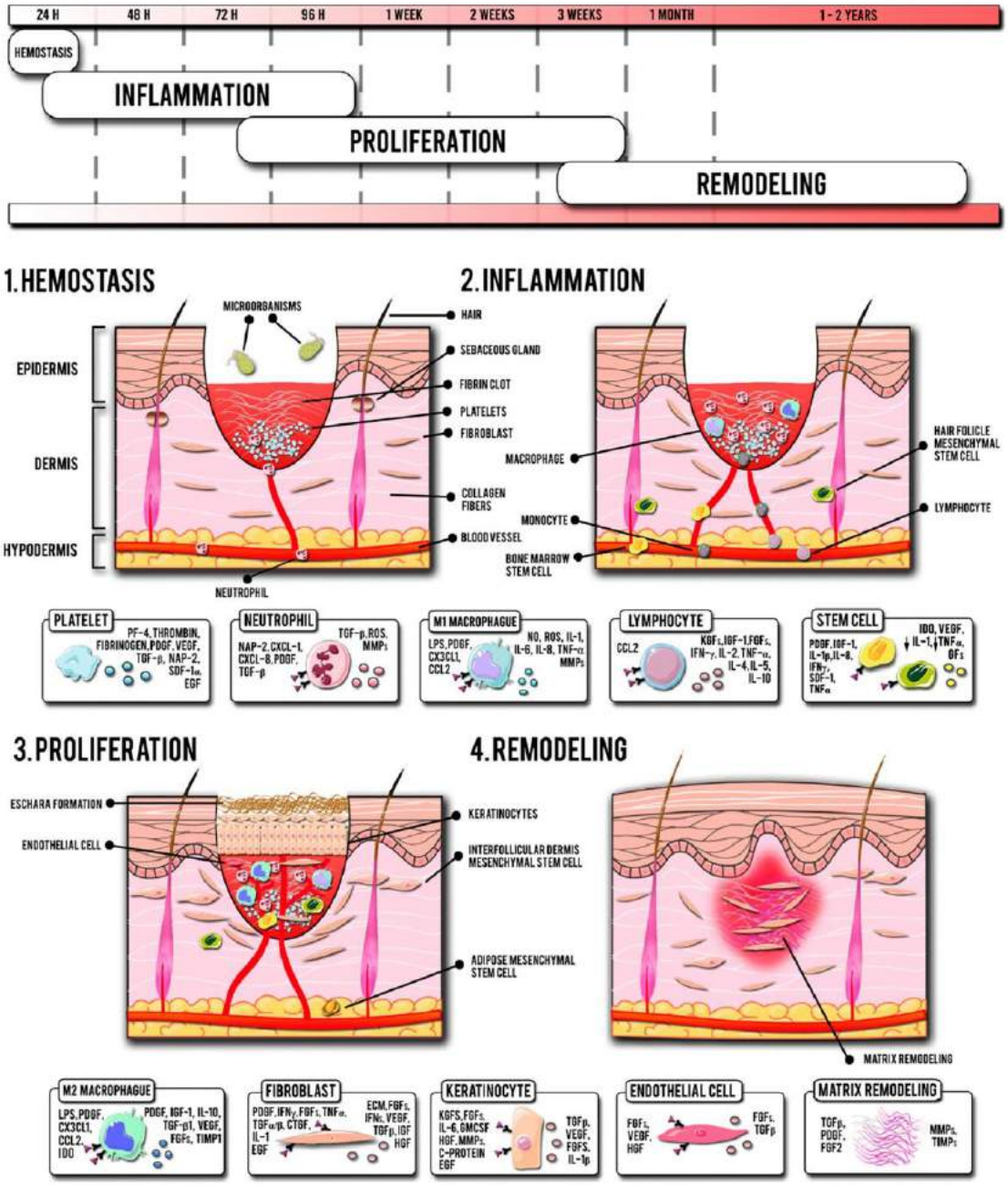
6. Wprowadzenie z uzasadnieniem tematyki badań

Skóra stanowi zewnętrzną powłokę ciała człowieka. Zbudowana jest ona z trzech podstawowych warstw – naskórka, skóry właściwej i tkanki podskórnej. W organizmie pełni wiele funkcji fizjologicznych, z których najważniejsze to termoregulacja, odbieranie bodźców i ochrona przed czynnikami zewnętrznymi (Proksch et al., 2006). Choroby skóry lub jej uszkodzenie prowadzą do upośledzenia jej funkcji, a także wiążą się z negatywnym wpływem na komfort psychiczny i jakość życia człowieka.

6.1. Gojenie ran

Rany powstają w wyniku naruszenia integralności skóry lub błony śluzowej. W celu przywrócenia funkcji fizjologicznych i spójności warstwy ochronnej uruchamiana jest dobrze zorganizowana kaskada zdarzeń, nazywana gojeniem ran. Fizjologicznie, proces ten jest podzielony na cztery główne etapy obejmujące hemostazę, zapalenie, proliferację i przebudowę (**Rycina 1.**) (Singh et al., 2017).

Faza hemostazy rozpoczyna się niezwłocznie po wystąpieniu urazu, aby jak najszybciej przywrócić funkcje ochronne skóry. Dochodzi do zwężenia naczyń krwionośnych, agregacji płytek krwi i ostatecznie do wytworzenia skrzepu, który stanowi swoiste rusztowanie dla napływających do miejsca urazu komórek skóry i układu odpornościowego (Las Heras et al., 2020). W wyniku aktywacji między innymi płytek krwi, w ranie gromadzi się wiele białek i cząsteczek, takich jak trombina, fibrynogen, czynnik płytkowy 4, czynniki pro- i antyangiogenne, np. czynnik wzrostu śródbłonka naczyń (VEGF), czynniki wzrostu, np. naskórkowy czynnik wzrostu (EGF), płytko-pochodny czynnik wzrostu (PDGF) i transformujący czynnik wzrostu (TGF-β), a także cytokiny i chemokiny, np. peptyd aktywujący neutrofile 2 (NAP-2). Wydzielane substancje indukują aktywację i migrację do miejsca urazu neutrofili, makrofagów, fibroblastów, komórek śródbłonka i innych (Rodrigues et al., 2019).



Rycina 1. Etapy prawidłowego gojenia ran: hemostaza (1), zapalenie (2), proliferacja (3) i przebudowa (4). W okienkach z poszczególnymi komórkami przedstawiono cytokiny, czynniki wzrostu itd., które pobudzają komórki (lewa część okienka), a także czynniki wydzielane przez te komórki (prawa strona okienka). Rycina i opis zaadaptowane z: Las Heras, K., Igartua, M., Santos-Vizcaino, E. & Hernandez, R. M. Chronic wounds: Current status, available strategies and emerging therapeutic solutions. *Journal of Controlled Release* vol. 328 532–550; doi.org/10.1016/j.jconrel.2020.09.039 (2020) (Las Heras et al., 2020)

Następnie rozpoczyna się faza zapalna gojenia, którą, oprócz obecnych cytokin, chemokin i mediatorów płytkowych, napędzają również produkty pochodzenia bakteryjnego. W wyniku wzrostu przepuszczalności naczyń, do miejsca urazu niemal natychmiast infiltrują neutrofile, a następnie makrofagi, które zabijają bakterie i usuwają resztki uszkodzonych białek macierzy. Pojawiające się neutrofile uwalniają metaloproteinazy macierzy pozakomórkowej (MMP), reaktywne formy tlenu (ROS), peptydy przeciwdrobnoustrojowe, czynniki wzrostu oraz szereg cytokin i chemokin prozapalnych, np. czynnik martwicy nowotworów (TNF-α) i interleukiny: 6 (IL-6), 8 (CXCL-8, IL-8), czy 1 (IL-1). Następnie, w miejscu uszkodzenia zjawiają się monocyty, ulegające transformacji do makrofagów typu M1 (prozapalnych), które przyczyniają się do wzrostu poziomu specyficznych mediatorów stanu zapalnego w tkance. Kolejnymi komórkami docierającymi do rany są limfocyty. Komórki typu αβ+T (CD4+, CD8+ oraz Treg) odpowiadają przede wszystkim za zwalczanie patogenów, natomiast typu γδ+T wydzielają czynniki wzrostu, takie jak insulinopodobny czynnik wzrostu 1 (IGF-1), a także czynniki wzrostu fibroblastów (FGFs) i keratynocytów (KGFs) (Shah and Amini-Nik, 2017; Shukla et al., 2019).

Pod koniec fazy zapalnej w miejscu urazu przeważają makrofagi typu M2 (przeciwzapalne / wspierające gojenie ran), które, poprzez uwalnianie specyficznych czynników, takich jak VEGF, PDGF, IGF-1, FGF, TGF-β1 i IL-10, sprzyjają angiogenezie i granulacji tkanki. Powoduje to płynne przejście w trzecią fazę gojenia, czyli proliferację. Najważniejszą rolę odgrywają w niej keratynocyty i fibroblasty, które aktywowane cytokinami i czynnikami wzrostu zaczynają migrować i proliferować, odbudowując barierę naskórkową. Ponadto, następuje intensywna odbudowa naczyń krwionośnych zarówno w procesie waskulogenezy, czyli tworzenia nowych struktur *de novo*, jak i angiogenezy – rozwoju struktur z migrujących i proliferujących komórek śródbłonka naczyń (Las Heras et al., 2020).

Przebudowa jest ostatnią, a zarazem najdłuższą fazą gojenia. W zależności od ciężkości urazu może trwać rok lub nawet dłużej. Główną rolę odgrywają w niej fibroblasty, produkujące składniki macierzy pozakomórkowej, przede wszystkim kolagen typu I. Jednocześnie, wydzielone MMP (np. kolagenazy i elastazy) rozkładają

niepotrzebne białka macierzy oraz kolagen zdezorganizowany, głównie typu III, przejściowo budowany w ranie. Zaburzenia równowagi pomiędzy MMP i ich inhibitorami mogą prowadzić do nieprawidłowego zakończenia procesu, np. w przypadku nadmiernej degradacji macierzy pozakomórkowej mogą powstawać rany przewlekłe, a w razie jej nieproporcjonalnej akumulacji – blizny przerostowe (Pastar et al., 2014; Reinke and Sorg, 2012).

6.2. Trudno gojące się rany

Gojenie rany jest niezwykle skomplikowanym procesem, w którym wiele zdarzeń, zachodzących w sposób sekwencyjny, musi być ściśle kontrolowanych. Trudno gojące się rany, nazywane również przewlekłymi, stanowią coraz większy problem kliniczny. Mimo, iż brak jednoznacznej definicji, tym mianem określa się rany, które nie goją się w zakładanym czasie (zwykle przyjmuje się brak redukcji powierzchni rany o co najmniej 40% w przeciągu miesiąca) (Falanga et al., 2022).

W ranie przewlekłej obserwujemy zaburzenia równowagi w poziomie cytokin, chemokin, czynników wzrostu, czy aktywności proteaz. Prowadzi to do utrzymywania się stanu zapalnego, nieprawidłowej angiogenezy i utrudnionej proliferacji (Shukla et al., 2019). Na poziomie komórkowym obserwujemy zmiany fenotypowe neutrofili, które prowadzą do ich dłuższego przebywania w miejscu urazu i nadmiarowego wydzielania mediatorów stanu zapalnego. Ponadto, dochodzi do zaburzenia równowagi stosunku makrofagów typu M1 i M2, na korzyść komórek prozapalnych, wydzielających kolejne czynniki wzmacniające odpowiedź immunologiczną. Uwalniane mediatory prowadzą do nieprawidłowości w funkcjach keratynocytów i fibroblastów. Keratynocyty charakteryzują się nadmierną proliferacją lecz upośledzoną zdolnością do migracji w kierunku zranienia i brakiem ekspresji markerów różnicowania, co uniemożliwia zamknięcie rany. Natomiast proliferacja fibroblastów zostaje zahamowana, pojawiają się trudności w formowaniu macierzy pozakomórkowej i tkanki ziarninowej (Las Heras et al., 2020). Dlatego też, zachowanie równowagi stanu zapalnego podczas procesu gojenia wydaje się najlepszą strategią, by zapobiegać przekształceniu rany w ranę przewlekłą (Shukla et al., 2019).

Dotychczas zidentyfikowano wiele czynników, które mogą wpływać negatywnie na naprawę uszkodzenia. Do najważniejszych z nich należą choroby współistniejące (np. cukrzyca, otyłość, niedożywienie), stosowane leki (np. steroidy, niesteroidowe leki przeciwzapalne), interwencje onkologiczne (np. radio- i chemioterapia), a także nawyki związane ze stylem życia (np. spożywanie alkoholu, palenie tytoniu) (Anderson and Hamm, 2012).

Rany przewlekłe możemy zaklasyfikować do jednej z trzech kategorii: owrzodzenia żylne i tętnicze, owrzodzenia cukrzycowe oraz odleżyny. Owrzodzenia naczyniowe, stanowiące ponad 70% wszystkich ran przewlekłych, występują na kończynach dolnych i zwykle dotyczą osób, które są w podeszłym wieku, zmagają się z otyłością lub są po przebytych urazach bądź zakrzepicy żył głębokich. Owrzodzenia cukrzycowe najczęściej zaczynają się od niewielkiego otarcia lub zranienia na skórze stóp pacjentów chorych na cukrzycę. Z powodu rozwijającej się podczas choroby neuropatii, rana często pozostaje niezauważona i jest podatna na infekcje. Osłabiony układ odpornościowy nie jest w stanie ich zwalczyć, co uniemożliwia dalsze gojenie. Odleżyny mogą pojawić się na skórze pacjentów z niedotlenieniem tkanek spowodowanym unieruchomieniem ciała. W wyniku długotrwałego ucisku i zaburzeń krążenia pojawia się ognisko martwicy, podatne na zakażenia (Irfan-Maqsood, 2018).

W związku z rosnącą częstością występowania i wysokimi kosztami leczenia, rany przewlekłe istotnie obciążają system opieki zdrowotnej (Sen, 2023). Ponadto, wpływają negatywnie na jakość życia pacjentów, zarówno w obszarze fizycznym, jak i emocjonalnym oraz społecznym (Falanga et al., 2022).

6.3. Oparzenia i oparzenia słoneczne

Oparzenie może być definiowane jako uszkodzenie skóry wywołane wysoką temperaturą, elektrycznością, odczynnikiem chemicznym lub promieniowaniem. Ze względu na głębokość urazu, możemy je sklasyfikować w czterostopniowej skali. Oparzenia I stopnia najczęściej nie wymagają interwencji lekarza – obejmują wyłącznie naskórek i charakteryzują się zaczerwienieniem i dyskomfortem. Oparzenia II stopnia możemy podzielić na powierzchniowe i głębokie. Oparzenia powierzchniowe,

obejmujące naskórek i część skóry właściwej, są bolesne i charakteryzują się wystąpieniem pęcherzy wypełnionych płynem surowiczym. Oparzenia głębokie, obejmujące naskórek i skórę właściwą, niekiedy wymagają interwencji chirurgicznej i powodują powstanie blizn. Ze względu na uszkodzenie receptorów, mogą wydawać się mniej bolesne. Oparzenia III stopnia obejmują naskórek, skórę właściwą i tkankę podskórną. Powodują martwicę tkanek, uszkodzenie nerwów i wymagają oczyszczenia chirurgicznego. Gojenie jest długotrwałe i pozostawia blizny, a najczęściej niezbędny jest przeszczep skóry. W przypadku oparzeń IV stopnia martwica obejmuje również mięśnie i ścięgna, czasem kości. Charakterystyczne jest zwęglenie, a konsekwencją jest amputacja dotkniętej urazem kończyny lub śmierć pacjenta (Jeschke et al., 2020; Markiewicz-Gospodarek et al., 2022).

Rany oparzeniowe są niezwykle podatne na infekcje, które stanowią najczęstszą przyczynę zgonów pacjentów. Zakażenia wywołane przez *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* lub *Staphylococcus aureus* są niezależnymi czynnikami predykcyjnymi śmiertelności (Wang et al., 2018). W związku z powyższym stosowanie leków przeciwdrobnoustrojowych, najczęściej miejscowe, wciąż stanowi podstawę leczenia oparzeń w wielu krajach (Atiyeh et al., 2007).

Oparzenie słoneczne jest ostrą reakcją zapalną skóry na nadmierną ekspozycję na promieniowanie UV. Za powstanie rumienia spowodowanego światłem naturalnym odpowiedzialne jest głównie promieniowanie UVB w zakresie 290-320 nm (Abeck et al., 2003). Przebieg kliniczny oparzenia zależy przede wszystkim od dawki promieniowania przyjętej przez pacjenta, jak i jego typu skóry. Tradycyjnie, rodzaje skóry klasyfikujemy według 6-stopniowej skali Fitzpatricka:

Skóra typu I – zawsze ulega oparzeniom, nigdy się nie opala, jest nadwrażliwa na promieniowanie UV

Skóra typu II – często ulega oparzeniom, minimalnie się opala

Skóra typu III – niekiedy ulega oparzeniom, zwykle opala się równomiernie

Skóra typu IV – rzadko ulega oparzeniom, zawsze dobrze się opala

Skóra typu V – bardzo rzadko ulega oparzeniom, opala się bardzo łatwo

Skóra typu VI – nie ulega oparzeniom, jest najmniej wrażliwa na promieniowanie UV (Goon et al., 2021).

Zaprezentowana skala może być wykorzystywana w celu określenia ryzyka wystąpienia oparzenia słonecznego, jednak, zgodnie z najnowszymi doniesieniami, nie powinna być stosowana w szacowaniu ryzyka zachorowania na raka skóry w wyniku ekspozycji na promieniowanie UV. Niezależnie od fenotypu skóry, powinno się stosować filtry ochronne, by zminimalizować ryzyko wystąpienia nowotworów, uszkodzeń i fotostarzenia skóry (Goon et al., 2021).

6.4. Najważniejsze choroby skóry o podłożu zapalnym

6.4.1. Atopowe zapalenie skóry

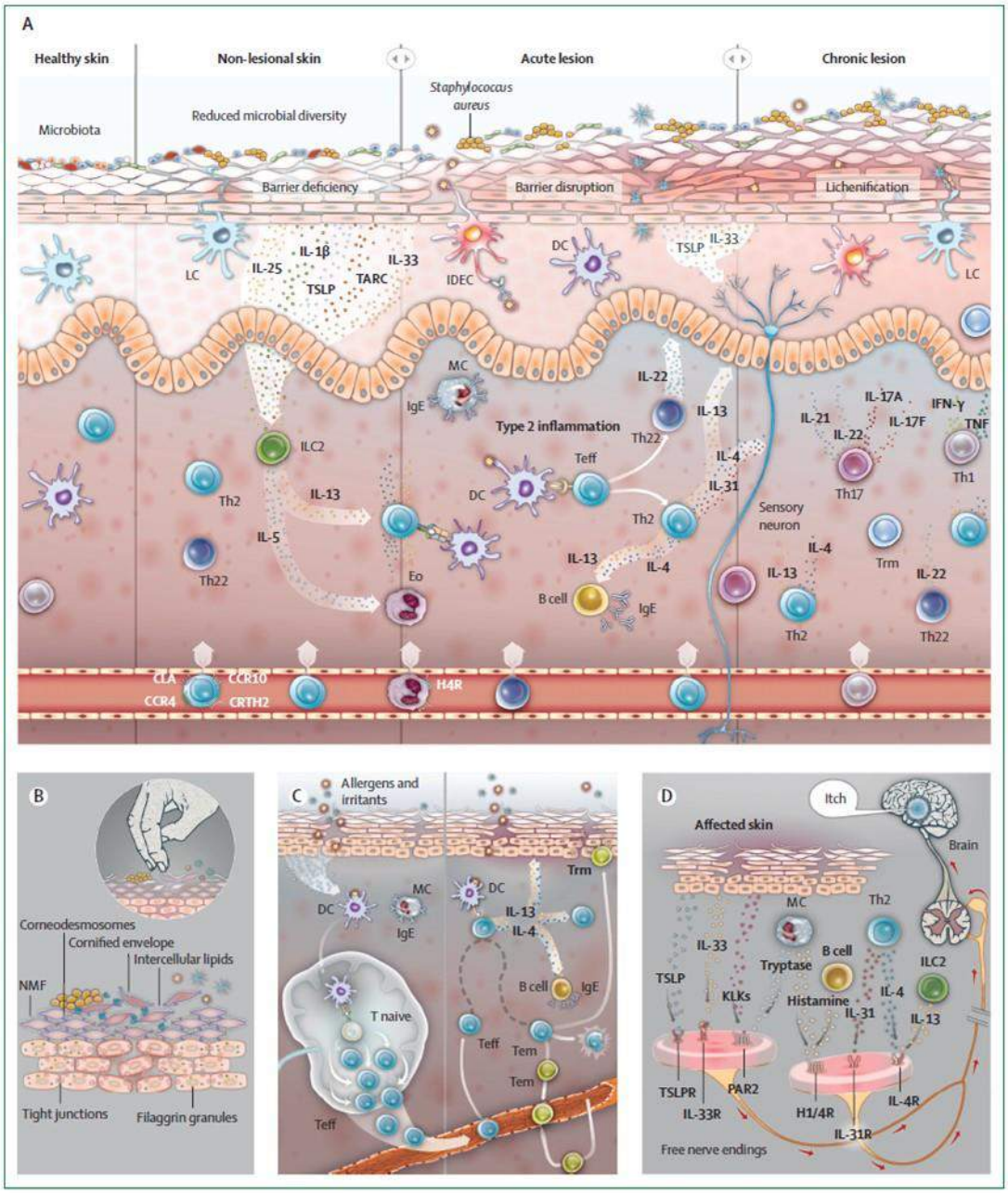
Atopowe zapalenie skóry (AZS) jest przewlekłą chorobą skóry, charakteryzującą się intensywnym swędzeniem i nawracającymi zmianami wypryskowymi. Pierwsze zmiany skórne najczęściej pojawiają się zwykle w 3-6 miesiącu życia dziecka, choć choroba może wystąpić w każdym wieku. W krajach rozwijających się dotyka niemal 20% dzieci i 10% dorosłych. Globalnie obserwujemy wzrost częstości zachorowań, choć w krajach o wysokich dochodach utrzymuje się ona na stałym poziomie (Langan et al., 2020).

Atopowe zapalenie skóry charakteryzuje duża różnorodność objawów klinicznych i ich nasilenia. Najistotniejszymi cechami są wspomniane już zmiany wypryskowe, intensywny świąd i nawracający lub przewlekły przebieg choroby. U niemowląt zmiany obejmują zwykle twarz, policzki i tułów. Cechują się niewielkim zaczerwienieniem i obrzękiem, a niekiedy drobnymi pęcherzykami wypełnionymi płynem surowiczym. U dzieci powyżej 2 roku życia zmiany zaczynają charakteryzować się suchością i zgrubieniem, a najczęściej obejmują powierzchnie zginaczy. Wyprysk u nastolatków i dorosłych jest zwykle rozlany, choć niekiedy nasilone zmiany obejmują powieki, dłonie i zgięcia (Fortson et al., 2017).

Przyczyny AZS są niezwykle złożone i heterogeniczne. Obejmują złożoną interakcję między dysfunkcjami bariery nabłonkowej, nieprawidłowościami w składzie mikrobioty

skóry oraz zaburzoną regulacją immunologiczną. Spośród czynników genetycznych choroby jako najistotniejszą wymienia się mutację genu kodującego główne białko strukturalne bariery skórnej – filagrynę. Skóra, osłabiona w wyniku niedoboru filagryny, a także przez fizyczne uszkodzenie spowodowane drapaniem, charakteryzuje się m.in. zwiększoną przepuszczalnością, utratą integralności, nadmierną przeznaskórkową utratą wody, podwyższonym pH, zmniejszoną retencją wody, a także zmienionym składem lipidowym (Fortson et al., 2017). Mikrobiota skóry osób cierpiących na AZS cechuje się zmniejszoną różnorodnością, a dominującym kolonizatorem jest *Staphylococcus aureus*. Jego obecność nasila lokalną odpowiedź zapalną, wywołaną głównie przez komórki Th1. Wydzielane czynniki mobilizują pozostałe komórki do reakcji immunologicznej, a także działają bezpośrednio na wolne zakończenia nerwowe wzmacniając uczucie świądu (Iwamoto et al., 2019; Kim et al., 2019) (**Rycina 2.**).

Leczenie atopowego zapalenia skóry sprowadza się do łagodzenia objawów i opracowania długoterminowej kontroli choroby. Podstawą jest stosowanie preparatów nawilżających, a w okresach nasilenia choroby stosowanie miejscowych leków przeciwzapalnych. W przypadku ciężkiego przebiegu choroby można stosować fototerapię (głównie UVB), ogólnoustrojową terapię konwencjonalną (podawanie cyklosporyny, metotreksatu, azatiopryny lub mykofenolanu mofetylu) lub immunomodulacyjną (specyficzne przeciwciała monoklonalne lub inhibitory kinaz JAK) (Langan et al., 2020).



Rycina 2. Patogeneza, główne mechanizmy i patofizjologia atopowego zapalenia skóry. Rycina przedstawia patogenezę w oparciu o etapy i główne mechanizmy atopowego zapalenia skóry (A). Klinicznie nienaruszona skóra ma dysfunkcję bariery naskórkowej i zmniejszoną różnorodność mikrobiomu powierzchniowego. W skórze ze zmianami komórki Langerhansa, zapalne komórki dendrytyczne naskórka niosące specyficzną IgE związaną z receptorem o wysokim powinowactwie dla IgE oraz komórki dendrytyczne skóry wchłaniają alergeny i antygeny. Cytokiny typu 2: IL-4, IL-13 i IL-31 bezpośrednio aktywują nerwy czuciowe, co sprzyja świądowi. Wraz ze wzrostem przewlekłości następuje postępujący wzrost cytokin pochodzących z keratynocytów i komórek Th. Swędzenie jest wywoływane przez różne czynniki wywołujące świąd (np. antygeny i mediatory molekularne, takie jak histamina i inne substancje), a pośredniczą w nim skórne pierwotne nerwy czuciowe, które przekazują sygnał świądowy poprzez włókna doprowadzające do rdzenia kręgowego (B–D). Rycina i opis zaadaptowane z: Langan, S. M., Irvine, A. D. & Weidinger, S. Atopic dermatitis. *The Lancet* vol. 396 345–360; doi.org/10.1016/S0140-6736(20)31286-1 (2020) (Langan et al., 2020)

6.4.2. Łuszczyca

Łuszczyca jest przewlekłą zapalną chorobą autoimmunologiczną dotykającą około 2-3% światowej populacji (Elkhawaga et al., 2023). Wywołujące ją czynniki genetyczne i epigenetyczne mogą być wyzwalane pod wpływem uwarunkowań środowiskowych. Obraz kliniczny nie jest jednorodny i możemy wyróżnić kilka form choroby. Najpopularniejszą, obejmującą 85-90% przypadków, jest łuszczyca zwyczajna (Deng et al., 2016). Charakterystycznymi objawami są czerwone, łuszczące się i swędzące zmiany na skórze wywołane hiperproliferacją keratynocytów, rozszerzonymi naczyniami krwionośnymi i zapalnym naciekiem leukocytów do skóry właściwej. Oprócz skóry, przewlekły stan zapalny obejmuje przede wszystkim stawy i paznokcie, ale również inne narządy (Rendon and Schäkel, 2019).

Odpowiedź immunologiczna dotyka wielu komórek i pośredniczy w niej wiele cytokin, a najważniejszymi są IFN-γ, TNF-α, IL-23, IL-17, IL-22, czy IL-9 (Deng et al., 2016). IFN-γ i TNF-α wpływają przede wszystkim na aktywację i proliferację keratynocytów oraz komórek śródbłonka naczyń, poprzez aktywację szlaków sygnalizacyjnych, odpowiednio JAK/STAT i NF-κB. Blokowanie sygnalizacji TNF-α stanowi podstawę terapii celowanej łuszczycy, wykorzystując przeciwciała monoklonalne, m.in. infliksymab, golimumab oraz adalimumab (Lowes et al., 2007). We krwi i w skórze pacjentów chorujących na łuszczycę odnotowuje się wysoki poziom IL-17, będącej bezpośrednim czynnikiem wzmacniającym proliferację keratynocytów. Dodatkowo, za sprawą dodatniego sprzężenia zwrotnego, keratynocyty promują rekrutację komórek Th17 i wydzielanie przez nie IL-17. Wzrost stężenia IL-17 i ilości komórek Th17 jest ściśle powiązany z ilością cytokiny IL-23, która również przyczynia się do hiperproliferacji keratynocytów. Zależność pomiędzy cytokinami IL-17 i IL-23, tak zwana oś IL-17/IL-23, stanowi ważny szlak nowoczesnych terapii celowanych chorób o podłożu zapalnym z wykorzystaniem przeciwciał monoklonalnych (Liu et al., 2020). Natomiast cytokiny IL-22 i IL-9, których podwyższony poziom także obserwujemy w przebiegu łuszczycy, przyczyniają się m.in. do wzrostu wydzielania odpowiednio IL-23 i IL-17, również wzmagając stan zapalny i proliferację keratynocytów (Lowes et al., 2007).

Leczenie łuszczycy zwyczajnej najczęściej polega na miejscowym stosowaniu środków złuszczających oraz hamujących nadmierną proliferację komórek naskórka. W celu usunięcia łusek można stosować np. maści mocznikowe i salicylowe, a następnie, aby zredukować wzrost keratynocytów, nanosić ditranol, dziegcie, czy kortykosteroidy. Terapia ogólna, wdrażana w przypadku nieskutecznej terapii miejscowej lub ciężkiego przebiegu choroby, może opierać się na podawaniu leków immunosupresyjnych, takich jak metotreksat, albo przeciwciał monoklonalnych skierowanym przeciwko specyficznym cytokinom (Rendon and Schäkel, 2019).

6.4.3. Trądzik pospolity

Trądzik pospolity dotyka niemal 80% osób w wieku od 11 do 30 lat (Mahto, 2017). Jest to choroba przewlekła, która może się utrzymywać również w wieku dojrzałym. Najważniejsze procesy wpływające na przebieg i stopień zaawansowania obejmują uwalnianie mediatorów stanu zapalnego do skóry, modyfikację keratynizacji prowadzącą do powstawania zmian trądzikowych, zwiększoną produkcję sebum pod wpływem androgenów, a także kolonizację mieszków włosowych przez *Cutibacterium acnes* (dawniej *Propionibacterium acnes*) (Williams et al., 2012). Procesy te zachodzą niemal równocześnie i wzajemnie na siebie oddziałują, prowadząc do rozwoju choroby. Bakterie rozwijające się w beztlenowym, bogatym w lipidy środowisku mieszków włosowych, wywołują odpowiedź zapalną komórek poprzez pobudzanie receptorów TLR na powierzchni komórek naskórka, a także prowadzą do hiperkeratynizacji. Reakcja może być wzmacniana przez utlenianie lipidów obecnych w sebum, prowadzące do aktywacji szlaku NF-κB i wzmożonej odpowiedzi immunologicznej. Czynnikiem, który może również wpływać na ciężkość przebiegu choroby jest równoczesne zakażenie gatunkiem *Staphylococcus epidermidis*. Namnażające się bakterie *C. acnes* i *S. epidermidis* mogą powodować powstawanie biofilmów blokujących pory i mieszki włosowe. Tworzą w ten sposób optymalne środowisko beztlenowe pod skórą, prowadząc do zaostrzenia objawów i utrudniając leczenie (Bharti and Vadlamudi, 2021; Williams et al., 2012).

Łagodna i umiarkowana postać trądziku może być skutecznie leczona preparatami miejscowymi – nadtlenkiem benzoilu, kwasem azelainowym, antybiotykami i retinoidami lub kombinacją powyższych. W ciężkich przypadkach trądziku włącza się doustną terapię hormonalną, podaje się antybiotyki lub izotretynoinę (Mohsin et al., 2022).

6.5. Znaczenie produktów pochodzenia naturalnego w dermatologii

Produkty pochodzenia roślinnego od lat cieszą się rosnącym zainteresowaniem. Szacuje się, że wielkość światowego rynku produktów do pielęgnacji skóry opartych o składniki naturalne wycenianego w 2021 roku na 6,4 mld dolarów, do końca 2030 roku osiągnie 12,27 mld dolarów (Grand View Research, 2022). Jest to związane z wieloma czynnikami, m.in. wzrostem nacisku społeczeństwa na estetyczny wygląd, a także skłonności konsumentów do wybierania produktów wolnych od substancji chemicznych, a zawierających bezpieczne i lecznicze substancje roślinne. Trend ten obejmuje również dermokosmetyki i preparaty lecznicze, w składzie których zaczynają dominować produkty pochodzenia naturalnego. Wiąże się to z nasileniem rozwoju badań dotyczących skuteczności i bezpieczeństwa stosowania takich substancji (Yazarlu et al., 2021; Zhang et al., 2012).

Do najbardziej znanych leczniczych substancji roślinnych stosowanych w schorzeniach skóry należą m.in. kwiat nagietka i rumianku, ziele wąkroty azjatyckiej, jeżówki purpurowej, dziurawca i krwawnika, a także kora dębu i oczaru. Wszystkie z wymienionych posiadają monografie Europejskiej Agencji Leków jako tradycyjne produkty lecznicze roślinne wskazane do stosowania w leczeniu chorób skóry i niewielkich ran (European Medicines Agency, 2024). Najczęściej wykazują one silne właściwości przeciwzapalne i przeciwutleniające, ponieważ odpowiedź zapalna towarzyszy zarówno uszkodzeniom skóry, jak i jej schorzeniom.

Korzystne działanie substancji roślinnych potwierdzono w wielu badaniach klinicznych. W jednym z nich testowano skuteczność działania kremu zawierającego ekstrakt z ziela dziurawca (*Hypericum perforatum*), standaryzowany na zawartość

hiperforyny (1,5%), w leczeniu atopowego zapalenia skóry (Schempp et al., 2003). Po czterech tygodniach stosowania kremu zaobserwowano, że w porównaniu do nośnika, krem istotnie obniżał nasilenie zmian wypryskowych, a także zmniejszał kolonizację mikrobioty skóry przez *S. aureus*. W innym badaniu klinicznym potwierdzono korzystne działanie ekstraktu z kwiatów nagietka (*Calendula officinalis*) w zapobieganiu wystąpienia zapalenia skóry (Pommier et al., 2004). Pacjentkami były kobiety operowane z powodu raka piersi, a następnie poddane napromieniowaniu pooperacyjnemu. Wykazano, że w porównaniu do trolaminy (niesteroidowy preparat przeciwzapalny), maść zawierająca ekstrakt z kwiatów nagietka istotnie obniżała odczucie bólu wywołanego radioterapią oraz zmniejszała ryzyko wystąpienia ostrego zapalenia skóry. Natomiast w leczeniu oparzeń kilka badań klinicznych potwierdziło skuteczność stosowania żelu otrzymanego z wewnętrznej części liści aloesu (*Aloe vera*). Wykazano, że istotnie skracał czas gojenia rany oparzeniowej (Khorasani et al., 2009), jak również uśmierzał ból (Shahzad and Ahmed, 2010) skuteczniej niż stosowany w grupie kontrolnej 1% krem sulfatiazolu srebra.

Pomimo niezaprzeczalnie korzystnych efektów działania substancji pochodzenia naturalnego w dermatologii, należy mieć świadomość iż ich stosowanie niesie również ryzyko wystąpienia działań niepożądanych. Najczęstszymi są fitodermatozy, które możemy podzielić na nieimmunologiczne i immunologiczne (Reuter et al., 2010). Zapalenia skóry o podłożu nieimmunologicznym dzielimy na toksyczne, wywoływane przez np. forbol z grupy związków diterpenowych występujący m.in. w oleju krotonowym czy wilczomleczu obrotnym (*Euphorbia helioscopia*), a także fototoksyczne, jak wywoływane przez kumaryny popularnie występujące w roślinach z rodziny selerowatych (Apiaceae). Do fitodermatoz o podłożu immunologicznym zaliczamy przede wszystkim alergiczne i fotoalergiczne kontaktowe zapalenie skóry. Alergiczne zapalenie skóry występuje bardzo często. Wywołują je głównie rośliny z rodziny astrowatych (Asteraceae), w których są obecne laktony seskwiterpenowe zwierające grupę funkcyjną α-metyleno-γ-laktonu. Fotoalergiczne kontaktowe zapalenie skóry występuje stosunkowo rzadko. Może być wywołane przez niektóre związki chemiczne z grupy laktonów seskwiterpenowych obecne m.in. w zielu wrotyczu

(*Tanacetum vulgare*) lub roślinach z rodzaju złocień (*Chrysantemum*) (Novak-Bilić et al., 2018; Reuter et al., 2010; Rozas-Muñoz et al., 2012; Sheehan, 2020).

6.6. *Sambucus nigra* L.

6.6.1. Opis botaniczny

Sambucus nigra L. (**Rycina 3.**), powszechnie znany jako bez czarny, jest gatunkiem krzewu lub małego drzewa z rodziny kalinowatych (Viburnaceae). Dorasta zwykle do 3-6 metrów wysokości. Posiada szeroką i rozłożystą koronę oraz brązowe, gładkie pędy pokryte licznymi, jasnymi kropkami. Liście są złożone, nieparzystopierzaste i zawierają 5-7 listków piłkowanych na brzegach o długości 5-12 cm. Wierzchnia strona listków jest ciemnozielona, a spodnia jest jaśniejsza. Kwiaty są małe, białe lub kremowe, zebrane w duże, płaskie baldachogrona o średnicy do 20 cm. Kwitnie od maja do lipca. Owoce to małe, okrągłe jagody, które dojrzewają do ciemnopurpurowej lub niemal czarnej barwy.

Rycina 3. *Sambucus nigra* L. Źródło: Franz Eugen Köhler, *Köhler's Medizinal-Pflanzen (https://pl.wikipedia.org/wiki/Bez_czarny* dostęp: 28.02.2024)

6.6.2. Systematyka

Królestwo: Rośliny (Plantae)

Podkrólestwo: Rośliny naczyniowe (Tracheobionta)

Nadgromada: Nasienne (Spermatophyta)

Gromada: Okrytonasienne (Magnoliophyta)

Klasa: Dwuliścienne (Magnoliopsida)

Rząd: Szczeciowce (Dipsacales)

Rodzina: Kalinowate (Viburnaceae)

Rodzaj: Bez (*Sambucus*)

Gatunek: Dziki bez czarny (*Sambucus nigra*) (ITIS, 2024)

6.6.3. Rozmieszczenie geograficzne

Sambucus nigra jest gatunkiem rodzimym dla niemal całego obszaru Europy. Występuje od Skandynawii i Wysp Brytyjskich na północy, po Półwysep Iberyjski na południu. Rośnie powszechnie w Europie Środkowej i Wschodniej. W Polsce występuje pospolicie na terenie całego kraju. Gatunek występuje rodzimie również na terenie Azji Zachodniej – Turcji i części regionu Kaukazu, oraz w Afryce Północnej – Maroko i Tunezji. Bez czarny został introdukowany w Ameryce Północnej, Australii i Nowej Zelandii, a także w niektórych rejonach Ameryki Południowej i Południowej Afryki (Atkinson and Atkinson, 2002).

6.6.4. Zastosowanie w lecznictwie

W lecznictwie znalazły zastosowanie kwiaty i owoce *Sambucus nigra*. Kwiaty czarnego bzu posiadają monografię Europejskiej Agencji Leków jako tradycyjny produkt leczniczy roślinny stosowany w leczeniu wczesnych symptomów przeziębienia ze względu na działanie napotne i przeciwgorączkowe. Zaleca się doustne podanie naparu sporządzonego z wysuszonej substancji roślinnej *Sambuci flos* (European Medicines Agency, 2007).

Owoce czarnego bzu, podobnie jak kwiaty, są stosowane w lecznictwie w łagodzeniu objawów przeziębienia i grypy, choć nie posiadają monografii EMA. Oprócz działania napotnego i przeciwgorączkowego, wykazują również właściwości moczopędne i przeczyszczające (European Medicines Agency, 2013).

Obie substancje roślinne były przedmiotem wielu badań *in vitro* i *in vivo*. Wykazano w nich m.in. ich aktywność przeciwutleniającą, przeciwzapalną, przeciwgrypową, przeciwbakteryjną, przeciwcukrzycową i neuroprotekcyjną. Dodatkowo, potwierdzono ochronne działanie owoców czarnego bzu na układ krążenia (Liu et al., 2022; Młynarczyk et al., 2018; Ulbricht et al., 2014). W ostatnich latach, ze względu na pandemię choroby COVID-19 wywołanej koronawirusem SARS-CoV-2, wzrosło zainteresowanie preparatami naturalnymi wykazującymi działanie przeciwwirusowe, jako alternatywnymi środkami zapobiegania i leczenia. Aktualne wyniki wskazują, że owoce czarnego bzu mogą zapobiegać wystąpieniu chorób wirusowych i wspomagać ich początkowe leczenie poprzez stymulację układu odpornościowego. Jednakże, głównie ze względu na ryzyko wystąpienia burzy cytokin po podaniu, brak wystarczających dowodów, by rozważać stosowanie *Sambuci fructus* w przypadku COVID-19 (Asgary and Pouramini, 2022). Interesujące jednak są interakcje lektyn roślinnych, występujących w czarnym bzie, z N- i O-glikanami obecnymi w białku S tworzącym homotrimeryczne kolce patogennych beta-koronawirusów, które mogłyby uniemożliwić zakażenie wirusowe m.in. SARS-CoV-2 (Simplicien et al., 2023).

6.6.5. Zastosowanie liści *S. nigra* w medycynie tradycyjnej

Liście czarnego bzu (*Sambuci folium*) były stosowane w medycynie ludowej w leczeniu ran i łagodzeniu chorób dermatologicznych, głównie na Półwyspie Bałkańskim i terenach należących obecnie do Turcji. Świeże, rozdrobnione liście aplikowano bezpośrednio na rany i zadrapania, by przyspieszyć ich gojenie (Ecevit Genç and Özhatay, 2006; Pieroni and Gray, 2008; Savo et al., 2011) lub wyleczyć obejmującą je infekcję (Guarrera, 2005). W przypadku wrzodów, ropni i trądziku stosowano miejscowo okłady ze świeżo zmiażdżonych liści, które zmniejszały obrzęk i zaczerwienienie zmian skórnych (Cornara et al., 2009). Liście wykorzystywano również do łagodzenia objawów

ukąszeń owadów, oparzeń i oparzeń słonecznych (Guarrera, 2005; Menendez-Baceta et al., 2014; Pieroni and Gray, 2008). Napary i odwary sporządzone z liści wykorzystywano do przemywań hemoroidów, wyprysków, a także oczu, w przypadku wydzieliny ropnej, bólu i stanu zapalnego (Calvo and Cavero, 2016; Menendez-Baceta et al., 2014). Stosowano je również na błonę śluzową jamy ustnej w postaci płukanek do łagodzenia zapalenia dziąseł i bólu zębów (Ballero et al., 2001; Cornara et al., 2009). Maści z dodatkiem zmielonych liści aplikowano na suchą i zrogowaciałą skórę (Cornara et al., 2009). Istnieją również doniesienia o stosowaniu liści *S. nigra* w weterynarii, w leczeniu ran lub w przypadku parchu u bydła, owiec i koni (Menale and Muoio, 2014).

Napary stosowane wewnętrznie działały przeczyszczająco (Tuzlacı and Sadıkoğlu, 2007), choć istnieją informacje o ich doustnym podawaniu w łagodzeniu objawów zapalenia gruczołu krokowego i pęcherzycy (Yesilada, 1997). Natomiast w postaci inhalacji łagodziły bóle głowy i obniżały gorączkę (Menendez-Baceta et al., 2014).

6.6.6. Skład chemiczny liści *S. nigra*

Dotychczas przeprowadzone badania składu chemicznego liści *Sambucus nigra* wykazały obecność głównie związków fenolowych, glikozydów cyjanogennych i terpenoidów. Z grupy kwasów fenolowych zidentyfikowano obecność kwasu kawowego i jego pochodnych, kwasu p-kumaroilochinowego i kwasów kawoilo- i dikawoilochinowych (Kiprovski et al., 2021). Flawonoidy są reprezentowane przez pochodne kwercetyny, kemferolu i izoramnetyny (Dawidowicz et al., 2006; Kiprovski et al., 2021; Senica et al., 2019), antocyjany przez pochodne cyjanidyny (Kiprovski et al., 2021), a glikozydy cyjanogenne przez prunazynę, sambunigrynę, holokalinę, 6-acetyloholokalinę i zierynę (DellaGreca et al., 2000). Ponadto, w ekstraktach z liści czarnego bzu wykryto obecność związków lipofilowych, jak kwas palmitynowy i linoleinian metylu (Szymański et al., 2020), jak również terpenoidów, jak kwas ursolowy i oleanowy (Inoue and Sato, 1975).

6.6.7. Aktywność biologiczna liści *S. nigra*

W przeprowadzonych do tej pory badaniach potwierdzono aktywność przeciwutleniającą (Azari et al., 2015; Dawidowicz et al., 2006; Tundis et al., 2019), przeciwzapalną (Yeşilada et al., 1997), przeciwdrgawkową (Ataee et al., 2016), przeciwdepresyjną (Mahmoudi et al., 2014), przeciwbakteryjną (Cybulska et al., 2011) i przeciwko toksoplazmie (Daryani et al., 2015). W żadnych z przytoczonych badań nie analizowano aktywności wyciągów z liści *Sambucus nigra* w kierunku leczenia schorzeń dermatologicznych. Ponadto, dotyczyły one ekstraktów, które nie były sporządzone metodami tradycyjnymi zgodnie z opisami zastosowań w medycynie ludowej.

W badaniach Azari et al. (Azari et al., 2015) analizowano aktywność przeciwutleniającą ekstraktów metanolowych z liści czarnego bzu sporządzonych trzema metodami – ekstrakcji wspomaganej ultradźwiękami, perkolacji i ekstrakcji w aparacie Soxhleta. Zaobserwowano, że wyciągi uzyskane za pomocą ultradźwięków najsilniej spośród analizowanych wymiatały rodnik DPPH (IC_{50}=21,6±1,1 µg/mL), tlenek azotu (IC_{50}=13,2±0,9 µg/mL), nadtlenek wodoru (IC_{50}=228,3±10,9 µg/mL) i posiadały najwyższą zdolność chelatowania jonów Fe^{2+} (IC_{50}=860,8±11,4 µg/mL). W innych badaniach aktywności przeciwutleniającej (Tundis et al., 2019) zaobserwowano, że metanolowe i etanolowe maceraty z liści *S. nigra* wymiatają rodniki DPPH (IC_{50} odpowiednio 41,3±2,6 i 42,1±3,1 µg/mL) i ABTS (IC_{50} odpowiednio 66,0±4,0 i 80,3±3,3 µg/mL), a także posiadają zdolność do redukcji żelaza w teście FRAP (IC_{50} odpowiednio 84,4±3,4 i 102,7±5,9 µM Fe(II)/g). Natomiast 80% (*v/v*) etanolowe ekstrakty sporządzone metodą PLE (Pressurized Liquid Extraction) w temperaturze 20 i 100°C wymiatały rodnik DPPH odpowiednio o 16,76±0,32 i 48,52±0,34%, a także zmniejszały stratę oksydacyjną β-karotenu o odpowiednio 4,25±0,14 i 19,13±0,29% (Dawidowicz et al., 2006).

Aktywność przeciwutleniającą wyciągów wodnego i metanolowego analizowano poprzez ich wpływ na poziom cytokin (IL-1α, IL-1β i TNF-α) w krwi pobranej od zdrowych dawców *ex vivo* po narażeniu na obecność lipopolisacharydu. Zaobserwowano, że ekstrakty nie mają istotnego wpływu na wydzielanie IL-1α i IL-1β, natomiast hamują uwalnianie TNF-α. Silniejszą aktywność wykazywał wyciąg metanolowy (Yeşilada et al., 1997).

W innym badaniu (Ataee et al., 2016) oceniano aktywność przeciwdrgawkową metanolowego ekstraktu zastosowanego u myszy poddanych elektrowstrząsom. Wykazano, że wyciąg w stężeniu 250 i 500 mg/kg masy ciała istotnie opóźnia wystąpienie drgawek i skraca czas ich trwania.

Aktywność przeciwdepresyjną metanolowych wyciągów sporządzonych metodą perkolacji badano na myszach w behawioralnych testach wymuszonego pływania i zawieszania za ogon (Mahmoudi et al., 2014). Zaobserwowano, że podanie ekstraktu w dawce 1200 mg/kg działało porównywalnie z dawką 10 mg/kg imipraminy, stosowanej jako kontrola, skracając czas unieruchomienia zwierzęcia.

Aktywność przeciwbakteryjną 55% (*v/v*) etanolowego wyciągu z liści czarnego bzu badano *in vitro* wobec bakterii *Neisseria gonorrhoeae* (Cybulska et al., 2011). Ustalono, że minimalne stężenie hamujące wynosi 512 µg/mL. W badaniu aktywności *in vitro* metanolowych wyciągów z liści *S. nigra* (5-50 mg/mL) wobec *Toxoplasma gondii* wykazano istotny wpływ na śmiertelność pierwotniaków względem kontroli negatywnej, lecz aktywność nie była istotna względem kontroli pozytywnej – pirymetaminy w dawce 100 mg/mL.

6.7. Uzasadnienie wyboru materiału roślinnego do badań

Liście *Sambucus nigra*, choć obecnie nie są popularną leczniczą substancją roślinną, to w medycynie ludowej były wykorzystywane zewnętrznie w łagodzeniu schorzeń skóry, gojeniu ran i oparzeń. Jednakże do tej pory nie zweryfikowano ich aktywności biologicznej w tym kierunku. Na podstawie badań wstępnych przeprowadzonych w Katedrze i Zakładzie Biologii Farmaceutycznej (Mainka et al., 2021) zaobserwowano, że liście czarnego bzu są interesującym materiałem roślinnym ze względu na swoje właściwości przeciwutleniające i przeciwzapalne. Ponadto, w dostępnej literaturze istnieją doniesienia o korzystnym działaniu ekstraktu z liści *Sambucus ebulus*, gatunku występującego częściej w Południowej Europie, Południowowschodniej Azji i Północnej Afryce, na gojenie ran (Süntar et al., 2010).

7. Cel pracy

Głównym celem pracy było zbadanie składu chemicznego i aktywności biologicznej liści *Sambucus nigra* w odniesieniu do ich tradycyjnego zastosowania w medycynie ludowej w leczeniu ran i schorzeń dermatologicznych o podłożu zapalnym.

W ramach projektu zrealizowano następujące zadania:

- Wykonanie czterech różnych wyciągów z liści *Sambucus nigra* L. – wodnych i 70% (*v/v*) etanolowych w temperaturze pokojowej i w temperaturze wrzenia rozpuszczalnika.
- Analiza fitochemiczna otrzymanych ekstraktów – oznaczenie całkowitej zawartości związków fenolowych, analiza jakościowa metodą UHPLC-DAD-MS^n oraz analiza ilościowa metodą HPLC-DAD.
- Zbadanie aktywności przeciwzapalnej i przeciwutleniającej wyciągów na modelach bezkomórkowych – hamowania aktywności lipooksygenazy (LOX), a także wymiatania anionorodnika ponadtlenkowego ($O_2^{-}\cdot$), nadtlenku wodoru (H_2O_2), tlenku azotu (NO) i rodnika 2,2,-difenylo-1-pikrylohydrazylu (DPPH).
- Zbadanie wpływu ekstraktów *ex vivo* na żywotność ludzkich neutrofili, izolowanych z krwi zdrowych dawców z wykorzystaniem jodku propidyny (cytometria przepływowa).
- Zbadanie zdolności ekstraktów do hamowania produkcji reaktywnych form tlenu (ROS) przez neutrofile stymulowane f-MLP.
- Analiza wpływu wyciągów na wydzielanie cytokin i chemokin przez neutrofile stymulowane LPS z *E. coli*– TNF-α, IL-1β i IL-8.
- Porównanie otrzymanych wyników i wytypowanie ekstraktu o najwyższej aktywności przeciwzapalnej i przeciwutleniającej.
- Sporządzenie wybranego wyciągu w większej skali i jego frakcjonowanie ciecz-ciecz z zastosowaniem rozpuszczalników o wzrastającej polarności – dichlorometanu, eteru dietylowego, octanu etylu i n-butanolu.
- Jakościowa analiza fitochemiczna ekstraktu i jego frakcji metodą UHPLC-DAD-MS^n.

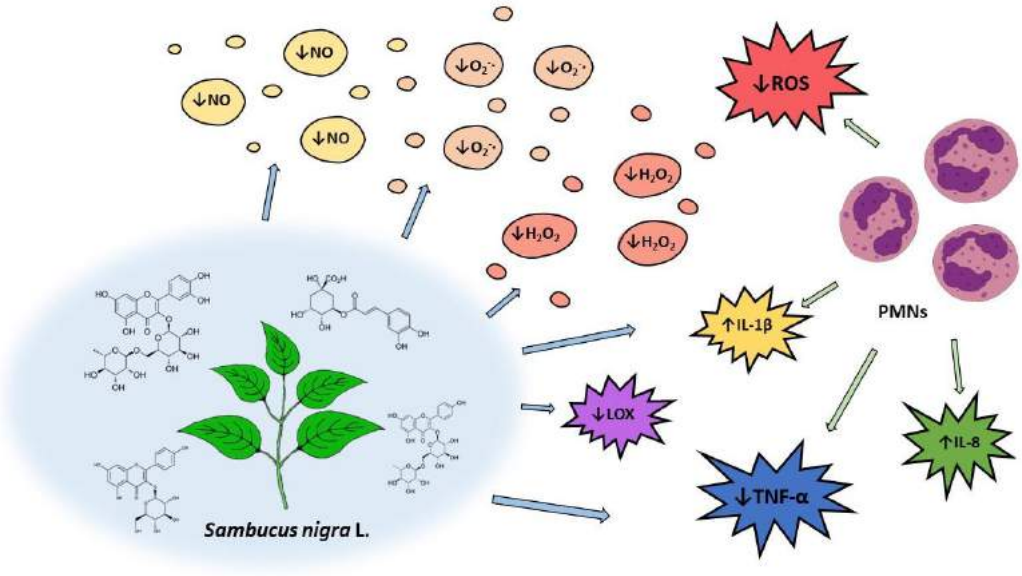
- Izolacja 11 związków chemicznych z frakcji octanu etylu i n-butanolu i ich identyfikacja metodą NMR.
- Zbadanie wpływu ekstraktu i jego frakcji na żywotność komórek zaangażowanych bezpośrednio w gojenie ran – neutrofili, keratynocytów i fibroblastów.
- Analiza wpływu wyciągu i frakcji na odpowiedź zapalną komórek:
    - Produkcję ROS przez neutrofile *ex vivo* stymulowane f-MLP
    - Wydzielania TNF-α, IL-1β i IL-8 przez neutrofile stymulowane LPS z *E. coli*
    - Uwalniania IL-6 i IL-8 przez:
        - Keratynocyty narażone na promieniowanie UVB
        - Keratynocyty stymulowane mieszaniną TNF-α i IFN-γ
        - Fibroblasty stymulowane LTA z *S. aureus*
- Ocena działania ekstraktu i frakcji na migrację keratynocytów do miejsca urazu w teście gojenia ran *in vitro*.
- Zbadanie wpływu wyciągu i frakcji na aktywność enzymów biorących udział w procesach naprawczych i zapalnych skóry, a także inwazji drobnoustrojów – elastazy, kolagenazy, lipooksygenazy, hialuronidazy i tyrozynazy.
- Dodatkowo, wykonano przegląd literatury dotyczący substancji roślinnych, które wykazują potencjał w leczeniu oparzeń i oparzeń słonecznych. Zebrano i podsumowano przeprowadzone w latach 2010-2022 badania kliniczne i na modelach zwierzęcych dla aktywności zarówno pojedynczych substancji roślinnych, jak i ich mieszanin.

9. Komentarz do publikacji

9.1. Publikacja nr 1

Skowrońska W., Granica S., Czerwińska M. E., Osińska E., Bazylko A.; *Sambucus nigra* L. leaves inhibit TNF-α secretion by LPS-stimulated human neutrophils and strongly scavenge reactive oxygen species; *Journal of Ethnopharmacology*, 290 (2022): 115116; DOI: 10.1016/j.jep.2022.115116

Celem niniejszej pracy było zbadanie aktywności przeciwzapalnej i przeciwutleniającej różnych wyciągów z liści czarnego bzu i ich wstępna analiza fitochemiczna (**Rycina 4.**).



Rycina 4. Abstrakt graficzny Publikacji 1.

Pierwszym etapem było sporządzenie ekstraktów z liści czarnego bzu, dostarczonych przez prof. Ewę Osińską z pola uprawnego Szkoły Głównej Gospodarstwa Wiejskiego w Warszawie. Wyciągi sporządzono w temperaturze pokojowej i w temperaturze wrzenia rozpuszczalnika z użyciem najczęściej stosowanych w medycynie tradycyjnej rozpuszczalników – wody i 70% (*v/v*) etanolu. Otrzymane ekstrakty wodne zagęszczono i zliofilizowano. Natomiast z wyciągów alkoholowych odparowano pod zmniejszonym ciśnieniem rozpuszczalnik organiczny, a pozostałość wodną poddano ekstrakcji ciecz-

ciecz chloroformem w celu oczyszczenia ekstraktu z chlorofilu. Następnie oczyszczoną wodną warstwę również zagęszczono i liofilizowano. Suche ekstrakty przechowywano w lodówce.

Prawidłowa analiza fitochemiczna wyciągów roślinnych stosowanych w badaniach biologicznych jest kluczowym elementem w zapewnieniu odpowiedniej jakości otrzymywanych danych. Na skład chemiczny ekstraktów roślinnych wpływa wiele czynników na poszczególnych etapach – m.in. wzrostu roślin (np. środowisko, gleba, dostęp do wody, nasłonecznienie, temperatura, wysokość nad poziomem morza), zbioru i stabilizacji materiału roślinnego (np. okres wegetacji, pora dnia, metoda suszenia), przygotowania wyciągów (np. dobór rozpuszczalników, temperatura, ciśnienie, czas, stosunek masowy materiału roślinnego do rozpuszczalnika). Standaryzacja uzyskanych ekstraktów umożliwia sformułowanie odpowiednich wniosków dotyczących bezpieczeństwa klinicznego i aktywności biologicznej badanych substancji roślinnych. Badania fitochemiczne przygotowanych wyciągów obejmowały kolorymetryczne oznaczenie całkowitej zawartości polifenoli z odczynnikiem Folina-Ciocâlteu, jakościową analizę składu z zastosowaniem UHPLC-DAD-MS^n, a także analizę ilościową najważniejszych składników ekstraktów metodą HPLC-DAD. Głównymi składnikami otrzymanych wyciągów były kwasy fenolowe i flawonoidy, pochodne kwercetyny i kemferolu. Analiza ilościowa wykazała, że wyższe stężenia tych związków są obecne w ekstraktach etanolowych niż wodnych, a także w sporządzonych w temperaturze wrzenia, w porównaniu do sporządzonych w temperaturze pokojowej. Po raz pierwszy w ekstraktach z liści czarnego bzu stwierdzono obecność izomerów kwasu kawoilo-treoninowego.

Badania biologiczne rozpoczęto od wstępnych analiz aktywności przeciwutleniającej w układach bezkomórkowych. Oceniano wpływ wyciągów na wymiatanie anionorodnika ponadtlenkowego ($O_2^{-}\cdot$), nadtlenku wodoru (H_2O_2), tlenku azotu (NO) i rodnika 2,2,-difenylo-1-pikrylohydrazylowego (DPPH). Zaobserwowano, że ekstrakty silnie wymiatają badane substancje w sposób zależny od stężenia, a ich aktywność jest skorelowana z zawartością związków fenolowych. Następnie, zbadano zdolność ekstraktów do hamowania aktywności 5-lipooksygenazy biorącej udział w przemianach

kwasu arachidonowego do leukotrienów. Wykazano, że inhibicja działania enzymu była nieznaczna, osiągając wartości IC_{50} rzędu 376 – 433 µg/mL.

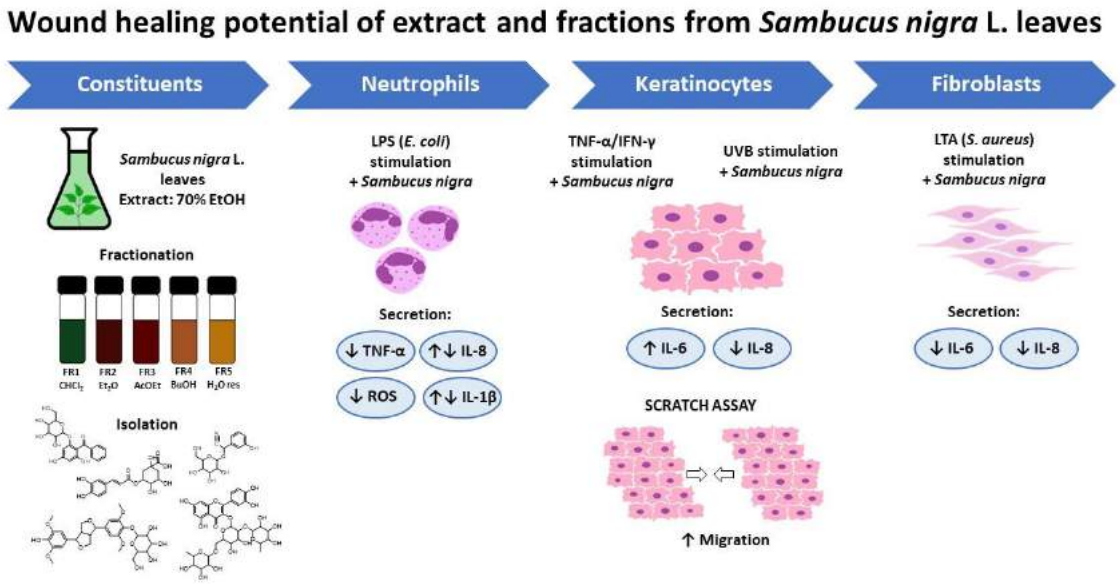
Kolejnym etapem badań biologicznych była analiza wpływu wyciągów na wydzielanie reaktywnych form tlenu (ROS), cytokin (TNF-α, IL-1β) i chemokin (IL-8) przez ludzkie neutrofile *ex vivo*. Są one pierwszymi komórkami pojawiającymi się w miejscu zranienia, które wydzielając czynniki prozapalne zwalczają patogeny i mobilizują pozostałe komórki do naprawy uszkodzenia. Jednak ich dłuższa obecność w ranie nasila stan zapalny mogąc prowadzić do powstania zmiany przewlekłej. Dlatego też, zdecydowano się przeanalizować wpływ ekstraktów na wydzielane przez nie najważniejsze mediatory stanu zapalnego. Komórki izolowano z kożuszków płytkowo-leukocytarnych, zakupionych w Regionalnym Centrum Krwiodawstwa i Krwiolecznictwa w Warszawie, metodą sedymentacji na dekstranie i odwirowania w gradiencie na medium do separacji komórek (Pancoll human) (Böyum, 1968). Aktywność ekstraktów hamującą wydzielanie ROS zbadano metodą chemiluminescencji zależnej od luminolu po stymulacji komórek chemotaktycznym peptydem N-formylo-metionylo-leucylo-fenyloalaniną (f-MLP). Zaobserwowano, że wyciągi silnie hamują wydzielanie ROS. W najniższym stężeniu, 5 µg/mL, obniżały ich sekrecję o ponad 50%, a w wyższych stężeniach zmniejszały ją o 80-95%. Wpływ ekstraktów na wydzielanie cytokin i chemokin przez neutrofile zbadano po stymulacji komórek lipopolisacharydem z błony komórkowej *Escherichia coli*. Stężenie mediatorów po 24 godzinach inkubacji zmierzono w supernatantach poprzez użycie komercyjnych zestawów testów immunoenzymatycznych ELISA (enzyme-linked immunosorbent assay). Zaobserwowano, że wszystkie badane wyciągi hamują wydzielanie TNF-α zależnie od stężenia, a silniej działały wyciągi etanolowe. Uwalnianie IL-8 było istotnie stymulowane przez ekstrakt wodny sporządzony w temperaturze pokojowej w stężeniach 50-100 µg/mL, natomiast pozostałe wyciągi nie miały istotnego wpływu na jej wydzielanie. W przypadku IL-1β zaobserwowano, że wszystkie wyciągi w stężeniu 100 µg/mL istotnie zwiększają jej uwalnianie przez neutrofile. Silniej stymulowały to działanie ekstrakty wodne, w szczególności wyciąg sporządzony w temperaturze pokojowej.

Przeprowadzone badania wskazują, że ekstrakty z liści *S. nigra* są dobrym źródłem związków o działaniu przeciwutleniającym, głównie kwasu chlorogenowego i flawonoidów, pochodnych kwercetyny i kemferolu. Ich korzystne działanie wynika przede wszystkim z wymiatania reaktywnych form tlenu, zarówno w układach bezkomórkowych, jak i na modelu ludzkich neutrofili. Dodatkowo, zaobserwowano, że wyciągi z liści czarnego bzu istotnie obniżały wydzielanie TNF-α, który pełni kluczową rolę w odpowiedzi zapalnej, głównie poprzez indukcję czynnika transkrypcyjnego NF-κB. Występujący w wyciągu kwas chlorogenowy może być jednym ze związków odpowiedzialnych za korzystne działanie liści czarnego bzu w leczeniu ran i schorzeń skóry. W badaniach *in vitro* i *in vivo* jego aktywności biologicznej wykazano, że posiada on zdolność hamowania odpowiedzi zapalnej komórek skóry, wzmaga polaryzację makrofagów do formy M2, wspomaga angiogenezę oraz migrację keratynocytów i fibroblastów do miejsca urazu (Bagdas et al., 2014; Huang et al., 2023; Moghadam et al., 2017).

9.2. Publikacja nr 2

Skowrońska W., Granica S., Piwowarski J. P., Jakupović L., Zovko-Koncić M., Bazylko A.; Wound healing potential of extract from *Sambucus nigra* L. leaves and its fractions; *Journal of Ethnopharmacology*, 320 (2024): 117423.

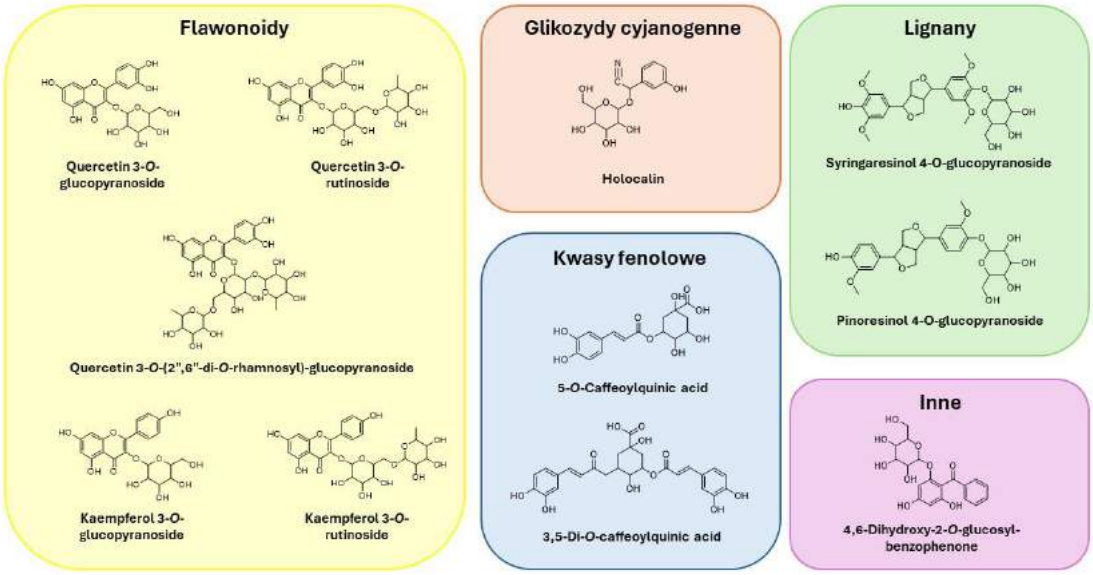
Celem badań zawartych w publikacji była analiza wpływu ekstraktu z liści czarnego bzu na procesy biorące udział w gojeniu ran (**Rycina 5.**).



Rycina 5. Abstrakt graficzny Publikacji 2.

Na podstawie wcześniejszych badań do dalszych analiz wybrano 70% (*v/v*) etanolowy wyciąg sporządzony w temperaturze pokojowej. Charakteryzował się on najwyższą aktywnością przeciwutleniającą i najsilniejszym hamowaniem wydzielania TNF-α przez ludzkie neutrofile stymulowane LPS, jednocześnie nie wpływając negatywnie na ich żywotność. Wyselekcjonowany ekstrakt sporządzono w większej skali, a następnie frakcjonowano rozpuszczalnikami o wzrastającej polarności. Następnie zbadano wpływ otrzymanych próbek na odpowiedź zapalną komórek bezpośrednio zaangażowanych w gojenie ran (neutrofili, keratynocytów i fibroblastów), migrację keratynocytów do miejsca zranienia, a także na aktywność enzymów biorących udział w procesach naprawczych i stanie zapalnym skóry. Dodatkowo, wykonano analizę jakościową składu

chemicznego badanego wyciągu i frakcji z niego otrzymanych, a także wyizolowano 11 czystych związków chemicznych, które zidentyfikowano na postawie widm NMR (**Rycina 6.**).



Rycina 6. Wzory związków chemicznych wyizolowanych z 70% (*v/v*) etanolowego wyciągu z liści *Sambucus nigra*.

Wyciąg sporządzono z 2 kg suchych, rozdrobnionych liści *Sambucus nigra*, zebranych z pola uprawnego Szkoły Głównej Gospodarstwa Wiejskiego w Warszawie. Materiał poddano trzykrotnej, 24-godzinnej maceracji z 70% (*v/v*) etanolem, za każdym razem stosując 15 L rozpuszczalnika na 1 kg substancji roślinnej. Po przefiltrowaniu, odparowaniu etanolu i liofilizacji otrzymano ok. 0,6 kg suchego ekstraktu. Część wyciągu (ok. 5 g) pozostawiono do dalszych badań, a pozostałą zawieszono w wodzie i poddano frakcjonowaniu ciecz-ciesz stosując kolejno dichlorometan, eter dietylowy, octan etylu i n-butanol. Z powstałych frakcji odparowano rozpuszczalniki pod zmniejszonym ciśnieniem, a pozostałość wodną zliofilizowano. Otrzymane próbki oznaczono jako: EX (wyciąg), FR1 (frakcja dichlorometanu), FR2 (frakcja eteru dietylowego), FR3 (frakcja octanu etylu), FR4 (frakcja n-butanolu), FR5 (pozostałość wodna). Ich skład chemiczny został zbadany jakościowo za pomocą UHPLC-DAD-MS^n. W ekstrakcie i frakcjach wykryto łącznie 41 związków chemicznych. Obecność części z nich po raz pierwszy zidentyfikowano w ekstraktach z liści *Sambucus nigra*. Dodatkowo, frakcje FR3 i FR4

poddano dalszym rozdziałom z zastosowaniem chromatografii kolumnowej i preparatywnej chromatografii cieczowej. W rezultacie wyizolowano 11 związków chemicznych z grupy flawonoidów, kwasów fenolowych, lignanów i glikozydów cyjanogennych, których identyfikację przeprowadzono na podstawie analizy widm ^{1}H NMR (**Rycina 6.**).

Badania *in vitro* wpływu ekstraktu i frakcji na aktywność enzymów przeprowadzono podczas zagranicznego stażu naukowego na Uniwersytecie w Zagrzebiu pod nadzorem prof. Marijany Zovko Končić. Zbadano wpływ na aktywność elastazy, kolagenazy, lipoksygenazy, hialuronidazy i tyrozynazy. Kolagen i elastyna to białka włókniste, które są obecne w tkance łącznej. Stanowią istotny element budulcowy ścięgien, wiązadeł i naczyń krwionośnych. Ich obecność w skórze zapewnia jej odpowiednią integralność, sprężystość i elastyczność. Z wiekiem produkcja tych białek przez fibroblasty maleje, co objawia się utratą jędrności i pogorszeniem właściwości mechanicznych skóry. Enzymami odpowiedzialnymi za ich rozpad jest elastaza i kolagenaza, należące do metaloproteinaz macierzy pozakomórkowej. Kolejnym enzymem wpływającym negatywnie na kondycję skóry w przypadku nadmiernego pobudzania jego aktywności jest hialuronidaza, rozkładająca kwas hialuronowy. Zbyt intensywna aktywność wspomnianych enzymów, pobudzana między innymi przez nadmierny stres oksydacyjny wzmacniany przez promieniowanie UV, stres czy palenie tytoniu, prowadzi m.in. do zmniejszenia jędrności i pogorszenia wyglądu skóry. Może także powodować trudności w przebiegu procesów naprawczych – gojenia ran, tworzenia tkanki ziarninowanej, migracji komórek i angiogenezy. Natomiast tyrozynaza jest enzymem, który w skórze bierze udział w produkcji melaniny z tyrozyny. Preparaty hamujące jej aktywność znajdują zastosowanie w rozjaśnianiu istniejących przebarwień skóry lub zapobieganiu powstawaniu nowych zmian. Ekstrakt z liści *S. nigra* i jego frakcje hamowały badane enzymy w różnym stopniu. Aktywność elastazy była najsilniej hamowana przez frakcję butanolową, kolagenazy przez frakcje dichlorometanu i eteru etylowego, hialuronidazy przez frakcję dichlorometanu i ekstrakt, a lipooksygenazy przez frakcję eteru dietylowego. Wyciąg i frakcje nie wpływały istotnie na aktywność tyrozynazy.

Badania na modelach komórkowych przeprowadzono na izolowanych z ludzkiej krwi neutrofilach i dwóch liniach komórkowych skóry ludzkiej – unieśmiertelnionych keratynocytach (HaCaT) i prawidłowych fibroblastach (NHDF). Na modelu neutrofili zbadano, podobnie jak w Publikacji nr 1, wpływ na produkcję ROS po stymulacji f-MLP, a także uwalnianie TNF-α, IL-1β i IL-8 po stymulacji LPS. Zarówno wyciąg, jak i frakcje silnie hamowały produkcję ROS. Dodatkowo niemal wszystkie badane próbki, oprócz FR1, istotnie obniżały wydzielanie TNF-α. Natomiast uwalnianie IL-1β było pobudzane, za wyjątkiem FR2, która je hamowała zależnie od stężenia. Ekstrakt i frakcje FR3-5 nie wpływały na sekrecję IL-8, podczas gdy frakcje FR1-2 znacznie ją pobudzały.

Na keratynocytach linii HaCaT przeprowadzono badania wpływu ekstraktu i frakcji na migrację komórek do miejsca zadrapania, a także na wydzielanie IL-6 i IL-8 po naświetlaniu promieniowaniem UVB oraz po stymulacji mieszaniną TNF-α i IFN-γ. Nadmierna ekspozycja na promieniowanie UVB powoduje wystąpienie rumienia lub oparzenia słonecznego. W łagodzeniu objawów znalazło zastosowanie wiele substancji roślinnych o potwierdzonym działaniu antyoksydacyjnym i przeciwzapalnym, m.in. liście aloesu lub kwiaty nagietka czy rumianku. Natomiast model pobudzania odpowiedzi zapalnej keratynocytów przez mieszaninę TNF-α i IFN-γ ma odzwierciedlać przewlekły proces zapalny, charakterystyczny np. dla atopowego zapalenia skóry lub łuszczycy, w którym infiltrujące do naskórka limfocyty Th1 produkują powyższe cytokiny w sposób niekontrolowany. Prowadzi to do zaostrzania stanu zapalnego i upośledzenia funkcji pozostałych komórek naskórka i skóry. Badania odpowiedzi zapalnej rozpoczęto od optymalizacji modeli, które opisywane były w literaturze naukowej. Dobrano odpowiednią dawkę promieniowania, która wywołuję reakcję immunologiczną nie wpływając negatywnie na żywotność komórek, a także dostosowano stężenie TNF-α i IFN-γ. Następnie dobrano odpowiednie rozcieńczenia supernatantów pobieranych znad komórek w celu oznaczenia zawartości poszczególnych cytokin testami ELISA. Zaobserwowano, że ekstrakt i frakcje FR1, FR2 i FR4 istotnie pobudzają migrację komórek, przyspieszając zamknięcie rany. Dodatkowo, modulują odpowiedź zapalną, pobudzając wydzielanie IL-6, a hamując uwalnianie IL-8.

Mikrobiota skóry stanowi zespół mikroorganizmów, żyjących w ścisłej korelacji z komórkami naskórka. Prawidłowe środowisko mikrobioty skóry powinno składać się z wielu gatunków drobnoustrojów. Niestety u ponad 20% zdrowej populacji i niemal 90% pacjentów z AZS dominującym gatunkiem jest gronkowiec złocisty (*Staphylococcus aureus*). W wyniku uszkodzenia bariery naskórkowej bakterie mogą wnikać w głąb skóry wywołując ostry stan zapalny i infekcje. Jest to szczególnie istotne w przypadku pacjentów z AZS, których sucha i podrażniona skóra jest podatna na urazy. Aby odzwierciedlić powyższą sytuację na modelu komórkowym, opracowano nowy model wywoływania odpowiedzi zapalnej w komórkach fibroblastów wykorzystując kwas lipotejchojowy (LTA) obecny w ścianie komórkowej *S. aureus*. Dobrano jego odpowiednie stężenie i opracowano sposób rozcieńczania supernatantów w celu wykrycia poszczególnych cytokin w supernatantach znad hodowli. Zaobserwowano, że po narażeniu na obecność LTA wzrasta ilość IL-6 i IL-8 wydzielanych przez fibroblasty, natomiast zarówno ekstrakt, jak i wszystkie frakcje istotnie obniżały ich sekrecję zmniejszając odpowiedź zapalną.

Przeprowadzone badania świadczą o potencjalnym korzystnym działaniu 70% (*v/v*) etanolowego wyciągu z liści czarnego bzu na gojenie ran i łagodzenie stanów zapalnych skóry. Jednak ze względu na niejednoznaczne wyniki uzyskane dla poszczególnych frakcji, nie było możliwe wytypowanie grupy związków odpowiedzialnych za takie działanie.

9.3. Publikacja nr 3

Skowrońska W., Bazylko A.; The potential of medicinal plants and natural products in the treatment of burns and sunburn – a review; *Pharmaceutics*, 15.2 (2023): 633.

Praca przeglądowa powstała w celu zestawienia badań z udziałem ludzi i zwierząt opublikowanych w latach 2010-2022, dotyczących zastosowania produktów pochodzenia naturalnego w leczeniu oparzeń i oparzeń słonecznych. Analizę wykonano zarówno dla preparatów zawierających jedną substancję roślinną, jak i złożonych z kilku produktów naturalnych a następnie dokonano krytycznej oceny przedstawionych badań.

Leczenie oparzeń stanowi wyzwanie szczególnie w krajach o niskim dochodzie i rozwijających się, w których system ochrony zdrowia nie jest w stanie zapewnić pacjentom dostępu do nowoczesnych terapii. W wielu krajach standardem leczenia jest stosowanie preparatów przeciwdrobnoustrojowych, zawierających głównie sól srebrową sulfatiazolu, pomimo iż w wielu badaniach wykazano, że mogą one wydłużać proces gojenia, powodować wzrost ryzyka komplikacji i prowadzić do zwiększenia oporności na antybiotyki. Ze względu na zróżnicowany mechanizm działania, obejmujący aktywność przeciwzapalną, przeciwdrobnoustrojową i wspomagającą gojenie, a także bezpieczeństwo i stosunkowo niskie koszty pozyskiwania, produkty naturalne mogą konkurować z lekami syntetycznymi.

W zaprezentowanych badaniach klinicznych preparaty zawierające ekstrakty z kory *Albizia julibrissin*, korzeni *Arnebia euchroma*, liści *Centella asiatica*, owoców *Hippophaë rhamnoides*, a także żelu wewnętrznego liści *Aloe vera* wpływały korzystniej na gojenie oparzeń od zastosowanego w grupie kontrolnej 1% kremu z sulfatiazolem srebra. Również preparaty z ekstraktem z kory *Betula pendula* i *Betula pubescens* oraz z owoców *Juglans regia* działały lepiej niż stosowane w grupie kontrolnej terapie, odpowiednio lek Octenilin® i autoprzeszczep skóry. Dlatego też, kontynuacja badań nad poszukiwaniem nowych substancji pochodzenia naturalnego, które znajdą zastosowanie w leczeniu oparzeń wydaje się być uzasadniona. Natomiast należy zwrócić szczególną uwagę na

przygotowanie preparatów, ponieważ materiał roślinny charakteryzuje się dużą zmiennością. Konieczne jest precyzyjne określenie części rośliny wykorzystanej do sporządzenia preparatu, zastosowane rozpuszczalniki, użyte stężenie i inne informacje, umożliwiające odtworzenie preparatu. Jak zaobserwowano podczas przygotowania zestawienia w pracy przeglądowej, nie zawsze wszystkie wymagane dane zostały udostępnione. Dodatkowo, w niektórych badaniach zaobserwowano brak odpowiedniej analizy statystycznej otrzymanych wyników. Takie niedopatrzenia prowadzą do utraty wiarygodności i zaufania wśród innych naukowców i lekarzy, którzy mogliby rekomendować wspomaganie terapii stosowanych standardowo dostępnymi preparatami naturalnymi.

9.4. Podsumowanie i wnioski

Po raz pierwszy kompleksowo przebadano potencjał wyciągu z liści *Sambucus nigra* i frakcji z niego otrzymanych w leczeniu ran i stanów zapalnych skóry, wykorzystując badania aktywności przeciwutleniającej i przeciwzapalnej, wpływ na aktywność enzymów, a także działanie przeciwzapalne na komórkach zaangażowanych w proces gojenia i zdolność regeneracji uszkodzeń *in vitro*. Umożliwiło to częściowe uzasadnienie tradycyjnego zewnętrznego zastosowania liści czarnego bzu w terapiach schorzeń skóry. Dodatkowo, po raz pierwszy wykonano dokładną analizę fitochemiczną, jakościową i ilościową, ekstraktu i frakcji.

W pracy przeglądowej zaprezentowano badania z lat 2010-2022 dotyczące wpływu preparatów zawierających substancje pochodzenia naturalnego w leczeniu oparzeń i oparzeń słonecznych. Dokonano krytycznej oceny zaprezentowanych wyników, jak i sposobu przedstawiania danych i ich analizy.

W ramach prowadzonych prac opracowano i wdrożono trzy nowe modele badań na liniach komórkowych. Dwa z nich dotyczyły stymulacji komórek linii HaCaT promieniowaniem UVB i mieszaniną TNF-α/IFN-γ. Na podstawie istniejących doniesień literaturowych opracowano odpowiednie dawki i stężenia, a także rozcieńczenia supernatantów do oznaczeń ilościowych cytokin. Trzeci model, stymulacji fibroblastów linii NHDF, został opracowany od podstaw i wprowadzony jako jeden z modeli stosowanych w Katedrze. Do wywoływania odpowiedzi immunologicznej wybrano kwas lipotejchojowy obecny w ścianie komórkowej bakterii *Staphylococcus aureus*. Dobrano stężenia i opracowano protokół postępowania.

Na podstawie opracowanych w ramach doktoratu modeli wykonano wiele badań w Katedrze i Zakładzie Biologii Farmaceutycznej Warszawskiego Uniwersytetu Medycznego, m.in.:

- Badania wstępne do projektu badawczo-wdrożeniowego Lider XI „Synteza API, opracowanie formulacji oraz przeprowadzenie badań *in vivo* dla kremu zawierającego postbiotyczny metabolit mikrobioty jelitowej człowieka –

U228 do stosowania miejscowego w terapii atopowych stanów zapalnych skóry" prof. Jakuba Piwowarskiego

- Badania do projektu naukowego Preludium BIS „Interakcje mikrobioty skóry ludzkiej z ekstraktami roślinnymi tradycyjnie stosowanymi w leczeniu schorzeń skóry" prof. Sebastiana Granicy
- Badania do projektu naukowego Preludium „Wspomaganie terapii trudno gojących się ran z zastosowaniem kory oczaru wirginijskiego" dr inż. Karoliny Pawłowskiej
- Badania w pracach magisterskich realizowanych w Katedrze:
    - „Badania, *in vitro* na komórkach skóry ludzkiej, aktywności przeciwzapalnej oraz badania składu chemicznego ziela *Thymus serpyllum*" mgr Julii Nowoszewskiej
    - „Badanie wpływu wyciągów i frakcji z ziela macierzanki na gojenie ran i aktywność fibroblastów skóry ludzkiej" mgr. Aleksandry Michalak
    - „Badanie aktywności przeciwzapalnej wyciągu wodno-etanolowego z kwiatu rumianku z użyciem modelu komórkowego skóry ludzkiej" mgr Aleksandry Nyczki
    - "Evaluation of cytotoxicity and anti-inflammatory effects of *Matricaria chamomilla* flower extracts on HaCaT cells" M. Pharm. Laura Lopez Rodero
    - "Evaluation of the cytotoxicity and anti-inflammatory activity of *Melittis melissophyllum* on HaCaT cell line" M. Pharm. Barbara Belmar Sanchez

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11. Załączniki

Journal of Ethnopharmacology 290 (2022) 115116

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Sambucus nigra L. leaves inhibit TNF-α secretion by LPS-stimulated human neutrophils and strongly scavenge reactive oxygen species

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ARTICLE INFO

Keywords:
Sambucus nigra L.
Elderberry
TNF-α
ROS
Nitric oxide

ABSTRACT

Ethnopharmacological relevance: Sambucus nigra (elderberry) leaves were used in folk medicine to treat skin inflammations, ulcers, burns or boils, as well as to treat wounds, including infected and chronic ones. For centuries, elderberry leaves have been used mainly in eastern and southern Europe, as well as in western Asia.

Aim of the study: The study aimed to investigate the anti-inflammatory and antioxidant activity of four different extracts, such as aqueous and ethanolic prepared at room temperature and the solvent's boiling point, from the leaves of elderberry.

Materials and methods: The effect of extracts both on the secretion of cytokines (TNF-α, IL-1β, and IL-8) and reactive oxygen species (ROS) by neutrophils stimulated with bacteria-derived products was investigated. The cytotoxicity of extracts was analyzed by staining with propidium iodide measured by flow cytometry. The anti-inflammatory activity of extracts was also investigated through their influence on lipoxygenase activity. The antioxidant properties, including scavenging superoxide anion, hydrogen peroxide, nitric oxide, and 2,2-diphenyl-1-picrylhydrazyl radical were investigated in cell-free systems. The total content of phenolic compounds was tested using the Folin-Ciocalteu reagent. The qualitative and quantitative determination of the content of individual phenolic acids and flavonoids was performed by HPLC-DAD-MS^n and HPLC-DAD method, respectively.

Results: Elderberry leaves extracts turned out to affect the inflammatory response of neutrophils by inhibiting the secretion of TNF-α and ROS. The ethanolic and aqueous extracts at a concentration of 50 $\mu g \times mL^{-1}$ reduce the secretion of TNF-α by approximately 40% and 10%, respectively. ROS secretion was decreased by around 50% for all extracts at concentration of 5 $\mu g \times mL^{-1}$. All the extracts were able to inhibit the activity of lipoxygenase. The ethanolic extracts were characterized by a higher content of phenolic compounds and a higher antioxidant activity, especially against nitric oxide, compared to the aqueous extracts.

Conclusions: Our research has confirmed that elderberry leaves are a plant material with anti-inflammatory activity, especially against reactive oxygen species, and a potentially rich source of antioxidants. Preliminary analyses performed in this study could be the first step in confirming the traditional use of elderberry leaves in relieving inflammation.

1. Introduction

Sambucus nigra L. belongs to the Adoxaceae family. It is commonly named elder, European black elder, elderberry, black elderberry, European elderberry, European black elderberry, and common elderberry. The species *Sambucus nigra* includes three subspecies: *Sambucus nigra* ssp. *canadensis* (L.) R. Bolli, *Sambucus nigra* ssp. *cerulea* (Raf.) R. Bolli and *Sambucus nigra* ssp. *nigra* L. (ITIS, 2021). It widely occurs in Europe and North America, as well as it is introduced in North Africa,

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https://doi.org/10.1016/j.jep.2022.115116
Received 14 December 2021; Received in revised form 11 February 2022; Accepted 14 February 2022
Available online 17 February 2022

Abbreviations

AA	ascorbic acid
DAF-2	4,5-diaminofluorescein
DEX	dexamethasone
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FBS	fetal bovine serum
f-MLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid
HPLC-DAD-MS^n	high-performance liquid chromatography with diode array detection coupled with mass spectrometry
HRP	horseradish peroxidase
IC_{50}	concentration required to inhibit 50% activity of enzyme
IL-1β	interleukin 1β
IL-8	interleukin 8
LOX	lipoxygenase
LPS	lipopolysaccharide
NBT	nitrotetrazolium blue chloride
PBS	phosphate-buffered saline
PI	propidium iodide
Q	quercetin
ROS	reactive oxygen species
SC_{50}	concentration required to scavenge 50% of the radical
SNP	sodium nitroprusside
TNF-α	tumor necrosis factor α
VEGF	vascular epithelial growth factor
VEGFR	vascular epithelial growth factor receptor

West Asia, and South America. In Poland, this shrub grows popular throughout the country, often in sunny places. It is found near the edges of forests, on balks and wastelands, close to human settlements, and in parks and gardens. Elderberry leaves are large, dark green, arranged in opposite pairs, and pinnate with serrated leaflets. It blooms from May to June. Small, cream-white flowers on long stalks are gathered in cymose corymb inflorescences. Black berries of *S. nigra* ripen from August to September (Anioł-Kwiatkowska et al., 1993; Rutkowski, 1998).

Elderberry flower (*Sambuci flos*) is well-known plant material described in the monograph of the European Medicines Agency (EMA) as a herbal medicinal product traditionally used to treat early symptoms of cold and flu (EMA, 2016). It is known in folk medicine for its diaphoretic, antipyretic, and diuretic properties (Ożarowski and Jaroniewski, 1987). The preparations of elderberries (*Sambuci fructus*), such as juices, jams, and other preserves, are traditionally used to treat mild constipation and colds (Committee on Herbal Medicinal Products, 2013), and due to their detoxifying properties, for example, in the treatment of gout and rheumatism (Kültür, 2007).

Elderberry leaves (*Sambuci folium*) are a much less known medicinal part of the plant, currently very rarely used, but with a fascinating history of use in traditional medicine. In ancient folk medicine, mainly in the Balkan Peninsula and the territory currently belonging to Turkey, elderberry leaves have been used externally to treat wounds, dermatological diseases such as pemphigus and folliculitis or rheumatic disorders (Cappelletti et al., 1982; Cavero et al., 2011; Yeşilada et al., 1995). Fresh, crushed leaves were applied to the wounds of both human and animals, which allowed and accelerated their healing (Ecevit Genç and Özhatay, 2006). They could also be used when infection had spread to the wound (Guarrera, 2005). In case of ulcers, abscesses, and pimples, compresses of freshly crushed leaves were topically applied as resolvent and decongestant, which allowed the lesion to mature, reduced swelling and redness, and provided relief. Infusions had a soothing and regenerating effect on burned skin (Guarrera, 2005). Decoctions and infusions of leaves were used to wash haemorrhoids, as well as the eyes, in case of rheum, pain, or inflammation (Calvo and Cavero, 2016; Menendez-Baceta et al., 2014). The ointments with the addition of ground leaves were applied to dry, affected, and calloused places (Cornara et al., 2009). Elderberry leaf infusions have also been used internally in inflammation of the prostate gland and pemphigus (Yeşilada et al., 1997).

In studies on an animal model (adult Wistar rats), the influence of elderberry leaf tincture on skin regeneration after third-degree burns were investigated (Mogoşanu et al., 2013). Cold-cream containing 10% herbal extract had been shown to be a local modulator of cellular response, supporting healing, epithelization, and scarring. It also stimulated the process of neoangiogenesis. An identical experimental model showed that the flavonoids and tannins occurring in the elderberry leaf extract were responsible for the astringent, antiseptic, and scarring effect (Mogoşanu et al., 2014).

The skin, the largest organ of the human body, is the body's protective barrier against external factors. As a result of its damage, a series of repair processes take place to restore the tissue and create a scar. The first cells to appear at the wound site are neutrophils, followed by macrophages. By secreting inflammatory mediators, such as IL-1, IL-6, TNF-α, nitric oxide, reactive oxygen species (ROS), or metalloproteinases, they start an inflammatory phase of the healing process (Phillipson and Kubes, 2019). The inflammatory step is necessary to combat pathogens entering the body and remove dead tissue from the injury site. However, an excessive inflammatory response can transform the lesion into a chronic wound. Achieving balance during the inflammatory phase can accelerate the healing process and improve damage repair (Shukla et al., 2019). Every year the number of cases of dermatological diseases increases, which is associated with an increase in treatment costs and a reduction in the quality of life of patients (Flohr and Hay, 2021; Lim et al., 2017). In recent years, there has been a growing interest in evidence-based herbal medicine among patients and doctors in cosmetology and dermatology. The extracts and compounds isolated from them, which affect the inflammatory response, are taken into consideration (Shedoeva et al., 2019; Shukla et al., 2019).

In elderberry leaves, compounds with potential anti-inflammatory activity, such as phenolic acids, phenolic glycosides, flavonoids, lignans, and neolignans, have been identified. In addition, cyanogenic glycosides and their derivatives and cyanohydrins have also been detected (D'Abrosca et al., 2001; DellaGreca et al., 2000a, 2000b; Senica et al., 2019).

Therefore, the main aim of this research was to investigate the anti-inflammatory and antioxidant properties of traditionally used elderberry leaf extracts. In addition, the content of individual phytochemicals was quantified, and a comparative analysis of extracts prepared at different temperatures with the use of traditionally used solvents – water and ethanol was performed. The anti-inflammatory activity was assessed on lipopolysaccharide (LPS) or chemotactic peptide (f-MLP) stimulated human neutrophil model. The secretion of pro-inflammatory cytokines (IL-1β and TNF-α), chemokines (IL-8), and ROS was determined. Moreover, the ability of the extracts to inhibit the activity of lipoxygenase (LOX) *in vitro* was investigated. The antioxidant activity was evaluated in an *in vitro* model by examining the effect of the extracts on scavenging reactive oxygen species such as superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), nitric oxide (NO), and the 2,2-diphenyl-1-picrylhydrazyl (DPPH).

2. Materials and methods

2.1. Chemicals

Ascorbic acid, allopurinol, chlorogenic acid, dexamethasone (DEX), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 4,5-diaminofluorescein (DAF-2), dextran from *Leuconostoc mesenteroides*, formic acid, f-MLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine), hydrogen peroxide, kaempferol 3-*O*-rutinoside, linoleic acid, lipoxygenase from Glycine Max, luminol, nitrotetrazolium blue chloride (NBT), horseradish peroxidase (HRP), propidium iodide (PI), quercetin, quercetin 3-*O*-rutinoside, sodium carbonate, sodium nitroprusside (SNP), xanthine, xanthine oxidase from bovine milk were purchased from Sigma-Aldrich (St. Louis, MO, USA). Indomethacin was purchased from Cayman chemical (Ann Arbor, MI, USA). Chloroform, ethanol, Folin-Ciocalteu reagent, and methanol were purchased from POCh (Gliwice, Poland). Chromatographic grade acetonitrile and lipopolysaccharide from *Escherichia coli* (LPS) were purchased from Merck Millipore (Billerica, MA, USA). Fetal bovine serum (FBS), Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}, penicillin-streptomycin solution, RPMI 1640 w/L-glutamine w/ 25 mM HEPES were purchased from Biowest (Nuaillé, France). Pancoll human (1.077 g $\times$ mL^{-1}) was purchased from PAN-Biotech (Aidenbach, Germany). Sets of enzyme-linked immunosorbent assay (ELISA) for TNF-α, IL-8, IL-1β were purchased from BD Biosciences (Franklin Lakes, NY, USA). Citric acid, sodium citrate, glucose, sodium chloride, and potassium chloride were purchased from Chempur (Piekary Śląskie, Poland). Ultra-pure water was provided by a Merck Millipore Simplicity UV System (Molsheim, France). The equipment of assays, such as 96-well plates and 96-DeepWell plates, were purchased from Nunc (Roskilde, Sjælland, Denmark).

2.2. Plant material and extracts preparation

Sambucus nigra L. leaves were collected after a flowering period in July 2018 from a cultivated field of the Warsaw University of Life Sciences (Poland, 52°16'04.0"N; 21°10'37.1"E). A specimen of the raw material (SNL_201807) is available in the Department of Pharmacognosy and Molecular Basis of Phytotherapy, Medical University of Warsaw (Poland). The raw material was dried in the Leśniczanka type dryer (Hamech, Hajnówka, Poland) with constant airflow at a temperature of 35–40 °C. Plant material was ground with an electric grinder (IKA-WERKE, Staufen im Breisgau, Germany), and then four different extracts were prepared, each with 10.0 g of the raw material.

Extract No. 1 (E1) and extract No. 2 (E2) were prepared by macerating the raw material three times for 24 h at room temperature, each time using 200 mL of distilled water or 70% (*v/v*) ethanol, respectively. Extract No. 3 (E3) and extract No. 4 (E4) were prepared under reflux at the boiling point of the solvent three times for 1 h, each time using 200 mL of distilled water or 70% (*v/v*) ethanol, respectively. The obtained extracts were filtered through cotton and a paper filter grade 41 (Whatman, Marlborough, MA, USA). Ethanol from extracts E2 and E4 was evaporated under vacuum (Laboranta 4000 WB, Heidolph, Schwabach, Germany) at 45 °C. To remove a significant amount of chlorophylls from the ethanolic extracts, three-time liquid/liquid extraction with 200 mL of chloroform was performed on the aqueous residue after ethanol evaporation. The chloroform residue was evaporated from the aqueous layer under a vacuum at 40 °C. The concentrated extracts were frozen and then lyophilized using a laboratory freeze-dryer Cryodos (Telstar, Terrassa, Spain). Yields of 2.52 g, 2.86 g, 2.16 g, and 2.41 g were obtained for E1, E2, E3, and E4, respectively. They were stored in sealed vials at 2–8 °C.

2.3. Isolation of human neutrophils

The buffy coats were purchased at the Warsaw Blood Donation Centre (Poland). They were prepared from peripheral venous blood collected from healthy male donors (18–35 years old). All donors declared that they did not take any medications. They were clinically confirmed to be healthy, and routine laboratory tests showed values within a normal range. Neutrophils were isolated using Böyum's method (Böyum, 1968) by dextran sedimentation and centrifugation in Ficoll Hypaque (Pancoll human) gradient. After isolation, cells were suspended in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin solution or in HBSS.

2.4. Evaluation of ROS production by human neutrophils

The ROS production by f-MLP-stimulated human neutrophils was determined using luminol-dependent chemiluminescence in 96-well white plates. Following isolation, 70 μL of cell suspension in HBSS ($3 \times 10^5 \times mL^{-1}$) was incubated with 50 μL of samples in proper concentration (5, 50 and 100 μg $\times$ mL^{-1}) in HBSS and 50 μL of luminol suspended in HBSS (0.4 mg $\times$ mL^{-1}). The chemiluminescence measurement was performed after adding 30 μL of a solution of f-MLP (1.5 μg $\times$ mL^{-1}) in HBSS. Changes in chemiluminescence were measured over 40 min in a microplate reader (Synergy 4, Biotek, Winooski, VT, USA). The chemiluminescence curve was registered by plotting the luminescence value versus the time (2 min intervals). The percent of inhibition was calculated compared to the control without tested extracts based on the values registered in the maximum of chemiluminescence. Quercetin at the concentration of 5, 10, and 20 μM in HBSS was used as a positive control.

2.5. Evaluation of TNF-α, IL-8, and IL-1β production by human neutrophils

Cell suspension in RPMI 1640 medium ($2 \times 10^6 \times mL^{-1}$) was incubated in 96-well plates with 50 μL of the tested extracts at the concentration of 5, 50 and 100 μg $\times$ mL^{-1} and presence or absence of LPS solution (100 ng $\times$ mL^{-1}) in PBS for 24 h at 37 °C with 5% CO_2. After 24 h, plates were centrifuged (2000 RPM, 10 min, 4 °C), and supernatants were collected. The released cytokines' concentration by stimulated human neutrophils was measured by ELISA tests following the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). Dexamethasone at the concentration of 0.01, 0.1, and 1 μM in PBS was used as a control known to inhibit the secretion of TNF-α, IL-1β and IL-8 (Zielińska et al., 2020).

2.6. Evaluation of neutrophils' viability

The cytotoxicity of the extracts was determined by flow cytometry measurement using PI staining (Granica et al., 2015). After collecting supernatants, neutrophils were washed twice with 500 μL of PBS. After 15 min of incubation with 500 μL propidium iodide solution (0.5 μg $\times$ mL^{-1} in PBS) at the room temperature in the absence of the light, cells were analyzed in BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) by recording 10,000 events per sample. A 0.1% (*v/v*) solution of Triton X-100 was used as a control substrate affecting cell membrane permeability for PI.

2.7. In vitro anti-inflammatory and antioxidant activity

2.7.1. Inhibition of lipoxygenase (LOX) activity

Lipoxygenase inhibition capacity was performed using the previously described method (Skowrońska et al., 2021) with some modifications, based on SIGMA Enzymatic Assay of Lipoxygenase (EC 1.13.11.12). Twenty five microliters of lipoxygenase solution (7695.56 U $\times$ mL^{-1}) was added to 100 μL of the sample in appropriate concentration (62.5, 125, 250 and 500 μg $\times$ mL^{-1}). After 5 min, 75 μL of linoleic acid solution (1.21 mM) was added. All solutions were made in PBS (100 mM; pH 8). The absorbance was measured at 234 nm over a period of 60 min (with an interval of 1 min). For the calculations, the

measurement, for which the maximum absorbance was recorded, was selected. As a positive control indomethacin in the concentration range 15.6–250 μg × mL^{-1} was used.

2.7.2. Scavenging of superoxide anion ($O_2^{-}\cdot$)

The ability to scavenge the superoxide radical was tested with the previously described method (Choi et al., 2002) in the xanthine-xanthine oxidase system with NBT (nitro-blue tetrazolium chloride) reduction, modified for 96-well plates (Kiss et al., 2010). Fifty microliters of the extract dissolved in PBS at the appropriate concentration (5, 10, 25, 75, and 125 μg × mL^{-1}) was mixed with 100 μL of a mixture (1:1, *v/v*) of xanthine (0.4 mM in PBS) with NBT (0.24 mM in PBS) and 50 μL of xanthine oxidase (3.66 mU in PBS, *ex tempore* prepared). The absorbance was measured at 560 nm in a microplate reader over 40 min (with an interval of 10 min) in the dark at 37 °C. Measurement 3 was used for the calculations (after 20 min), for which the maximum absorbance was recorded. The superoxide anion scavenging was calculated related to the control without extracts tested. Ascorbic acid in the concentration range 1–12.5 μg × mL^{-1} was used as a positive control.

At the same time, a measurement of xanthine oxidase inhibition was performed by monitoring uric acid formation to verify whether the impact of the extracts was due to an inhibition of enzyme activity (Schepetkin et al., 2009). Fifty microliters of extract, 100 μL of xanthine, and 50 μL of xanthine oxidase were mixed, and after 20 min of incubation of the plate in the dark at 37 °C, the absorbance was measured at 285 nm. The inhibition was calculated related to the control without extracts. Allopurinol was used as a positive control in the concentration range of 0.25–5 μg × mL^{-1}.

2.7.3. Scavenging of hydrogen peroxide (H_2O_2)

Hydrogen peroxide scavenging was determined by the chemiluminescence method (O'Dowd et al., 2004) modified for 96-well plates (Kiss et al., 2010). Fifty microliters of extracts at a concentration of 5, 15, 50, 75, and 125 μg × mL^{-1} were added to 50 μL of horseradish peroxidase solution (98.9 mU × mL^{-1}), 50 μL of H_2O_2 solution (0.0075%), and 50 μL of luminol solution (0.005 μg × mL^{-1}). All solutions were prepared in PBS. The chemiluminescence was measured 5 min after adding the luminol solution at room temperature in the dark. The reader was set to read luminescence sensitivity 75. The percent of inhibition of the HRP/H_2O_2 system was calculated compared to the control without extracts. Ascorbic acid in the concentration range of 0.5–5 μg × mL^{-1} was used as a positive control.

2.7.4. Scavenging of nitrogen oxide (NO)

Scavenging of nitric oxide was determined by fluorescence method (Chung et al., 2001) modified for 96-well plates (Czerwińska et al., 2012). Fifty microliters of extracts solution in PBS at a concentration of 2.5, 5, 10, 25, and 75 μg × mL^{-1} was mixed with 50 μL of DAF-2 (5 mM) solution in PBS, 50 μL of sodium nitroprusside solution (3 μg × mL^{-1}) in PBS and 50 μL of PBS. Measurements were made after 15 min of incubation at 37 °C in the dark on a black 96-well plate at excitation and emission wavelengths of 495 and 515 nm, respectively. The scavenging of nitrogen oxide was calculated related to controls without tested extracts. Ascorbic acid in the concentration range 1–12 μg × mL^{-1} was used as a positive control.

2.7.5. Scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH)

The DPPH scavenging assay was performed using the previously described method (Choi et al., 2002), which was modified into 96-well plates. One hundred microliters of extracts in 50% (*v/v*) ethanol at a concentration of 20, 50, 75, 125, and 200 μg × mL^{-1} were mixed with 100 μL of ethanolic solution of DPPH (0.02 mM). After 30 min of incubation at room temperature in the absence of the light, absorbance at 518 nm was measured. The scavenging rate of DPPH was calculated related to controls without tested extracts. Ascorbic acid in the concentration range 1–12.5 μg × mL^{-1} was used as a positive control.

2.8. Phytochemical analysis

2.8.1. The total content of phenolic compounds

The total content of phenolic compounds in the extracts was determined by the colorimetric method using the Folin-Ciocalteu reagent on a 96-well plate (Skowrońska et al., 2021). Forty microliters of extract dissolved in 50% (*v/v*) methanol, 105 μL of 10% Folin-Ciocalteu reagent in deionized water were mixed with 85 μL of 1 M sodium carbonate solution in deionized water. After 15 min of incubation at 45 °C, the absorbance was measured at 765 nm. The plate was incubated in a microplate shaker (DTS-2, Elmi, Newbury Park, CA, USA) that allowed the samples to be mixed simultaneously (at 420 RPM). The content of phenolic compounds was calculated to gallic acid based on a calibration curve according to the equation $y = 0.1711x + 0.2706$ ($R^2 = 0.999$).

2.8.2. Qualitative analysis of the composition of extracts

The HPLC-DAD-MS^n analysis was performed using an UltiMate HPLC 3000 system (Dionex, Germany) with DAD detection and splitless connection with an AmaZon SL ion trap mass spectrometer with an ESI interface (Bruker Daltonik GmbH, Germany). Separation was performed on a Kinetex XB-C_{18} (150 × 2.1 mm × 1.7 μm) column (Phenomenex, Torrance, CA, USA). The column temperature was set at 25 °C. The mobile phase (A) was water/formic acid (100:0.1, *v/v*), and the mobile phase (B) was acetonitrile/formic acid (100:0.1, *v/v*). The gradient system with the flow rate 0.2 mL × min^{-1} was 0–60 min, 0–26% B. The extracts at a concentration of 10 mg × mL^{-1} were injected (5 μL). The column was equilibrated between injections for 10 min. The UV–Vis spectra were recorded in the range of 200–450 nm. The chromatograms at 254, 280, 325, and 350 nm were retained. The nebulizer pressure was 40 psi, the dry gas flow was 9 L × min^{-1}, the dry temperature was 300 °C, and the capillary voltage was 4.5 kV. The MS spectra were registered by scanning from *m/z* 70 to 2200. Compounds were analyzed in a negative ion mode. The MS^2 fragmentation was obtained for two of the most abundant ions at the time. The identification was based on analytical standards and comparison with literature data (Kim et al., 2019).

2.8.3. Quantitative analysis of the composition of extracts

Quantitative HPLC-DAD analysis was performed on an apparatus equipped with a dual low-pressure gradient pump LC-10AT, a sampler SIL-20A, a CTO-10AS column oven, and a diode-array detector SPD-M20A (all, Shimadzu, Kyoto, Japan) (Czerwińska et al., 2020). Separation was carried out on a reversed-phase Kinetex XB-C18 (150 × 2.1 mm, 2.6 μm) column (Phenomenex, Torrance, CA, USA). The mobile phase (A) was 0.1% formic acid in water (*v/v*), and the mobile phase (B) was 0.1% formic acid in acetonitrile (*v/v*). A multistep gradient solvent system, 0–17 min, 10–19% B, 17–37 min, 19–21% B, and 37–60 min, 21–40% B was used. The column oven was set at 25 °C and equilibrated using 10% B for 10 min between injections. A sample at a concentration of 10 mg × mL^{-1} was injected in the volume of 2 μL. The flow rate was 0.3 mL × min^{-1}. UV–Vis spectra were recorded over a range of 200–450 nm, and chromatograms were acquired at 325 nm (phenolic acids) and 350 nm (flavonoids). The content of compounds in the tested extracts was calculated using the regression parameters of the calibration curves prepared for the chemical standards, which were chlorogenic acid ($y = 8.5275x + 1.4997$; $R^2 = 0.999$) and quercitrin ($y = 9.1373x - 0.291$; $R^2 = 1$).

3. Statistical analysis

The obtained data were analyzed using the Statistica program (data analysis software system), version 13 (TIBCO Software Inc., 2017; Palo Alto, CA, USA). The Shapiro-Wilk test was used to check the normal distribution. The homogeneity of variance was checked by the Brown-

Forsythe test. Statistical significance was determined using one-way ANOVA, with Dunnett's test and Tuckey's *post-hoc* test, or the Kruskal-Wallis test. Three independent experiments were performed in triplicate for each method. Data were expressed as mean ± SD. Statistical significance was determined at the significance level of $p<0.05$ and $p<0.001$. SC_{50} and IC_{50} values were calculated based on concentration-inhibition curves.

4. Results

4.1. Effect on ROS production by neutrophils

All tested extracts strongly inhibited the production of reactive oxygen species by human neutrophils stimulated with the bacteria-derived peptide (f-MLP) in a dose-dependent manner (Fig. 1). Even at the concentration of 5 μg × mL^{-1}, all extracts inhibited ROS production by more than 60% ($p<0.001$) compared to the stimulated control. The most substantial inhibitory effect on ROS production had aqueous and ethanolic extracts prepared at the solvent's boiling point (E3 and E4) (Fig. 1). The calculated IC_{50} value for quercetin was 13.65 ± 5.06 μM.

4.2. Effect on TNF-α, IL-8, and IL-1β production by neutrophils

The effect of the tested extracts on the secretion of cytokines by LPS-stimulated human neutrophils was investigated using ELISA tests. The secretion of TNF-α was significantly ($p<0.001$) reduced by each tested extract at a concentration of 100 μg x mL^{-1} (Fig. 2). The most potent inhibitory effect on TNF-α release by neutrophils had the ethanolic extracts – E2 and E4, which even at a concentration of 5 μg x mL^{-1} reduced secretion by 31 and 25% ($p<0.001$), respectively. Extracts E2, E3, and E4 had no impact on IL-8 secretion by LPS-stimulated human neutrophils (Fig. 3). The E1 extract at the concentration of 50 and 100 μg × mL^{-1} stimulated the secretion of IL-8 by 22 and 30% ($p<0.001$), respectively, compared to the stimulated control. The secretion of IL-1β by LPS-stimulated neutrophils incubated with the tested extracts was increased (Fig. 4). The aqueous extracts, E1 and E3, at the concentrations of 50 and 100 μg x mL^{-1} strongly stimulated the secretion of IL-1β by over 100% ($p<0.001$) compared to the stimulated control. Ethanolic extracts, E2 and E4, also stimulated IL-1β secretion at a concentration of 100 μg x mL^{-1} by about 80% ($p<0.001$) compared to the stimulated control.

4.3. Cells viability

The effect of the extracts and dexamethasone on the viability of LPS-stimulated human neutrophils is shown in Fig. 5. After 24 h of incubation, cell viability in the non-treated controls was 96.27 ± 0.53 and 97.13 ± 0.21% for the non-stimulated (LPS (−)) and stimulated (LPS (+)) controls, respectively. The viability of cells treated with Triton X-100 was 4.11 ± 0.46%. Cells incubated with extracts E1, E3, and E4 at a concentration of 100 μg × mL^{-1} showed 86.79 ± 1.68, 93.13 ± 0.99, and 92.81 ± 0.41% viability, respectively. It was significantly lower

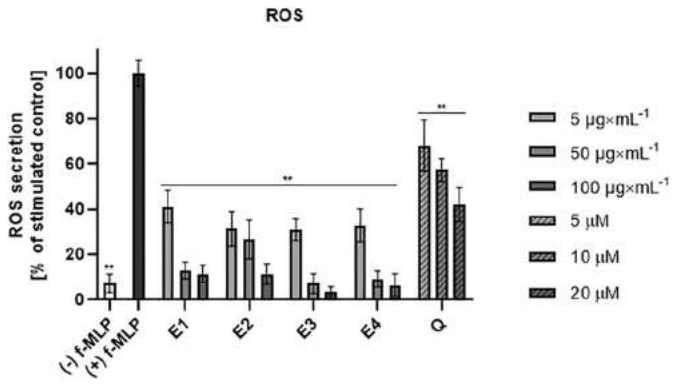

Fig. 1. Effect of extracts from *Sambucus nigra* L. leaves at the concentrations of 5, 50, and 100 μg × mL^{-1} and quercetin (Q) at the concentrations of 5, 10, and 20 μM on ROS production by f-MLP stimulated neutrophils. Statistical significance (**$p<0.001$) is marked in comparison to the stimulated control ((+) f-MLP).

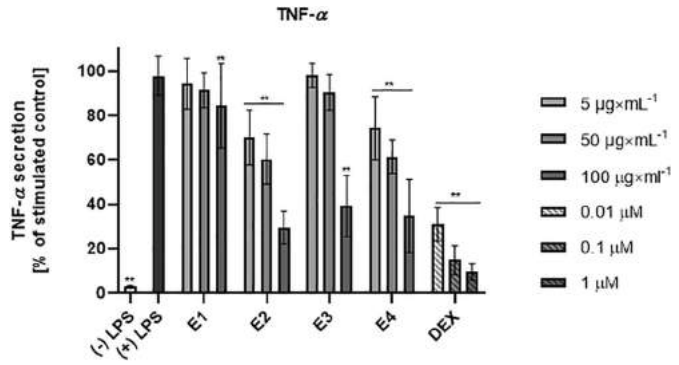

Fig. 2. Effect of extracts from *Sambucus nigra* L. leaves at concentration of 5, 50 and 100 μg × mL^{-1} and dexamethasone (DEX) at concentration of 0.01, 0.1, and 1 μM on TNF-α secretion by LPS-stimulated human neutrophils. Statistical significance (**$p<0.001$) is marked in comparison to the stimulated control ((+) LPS).

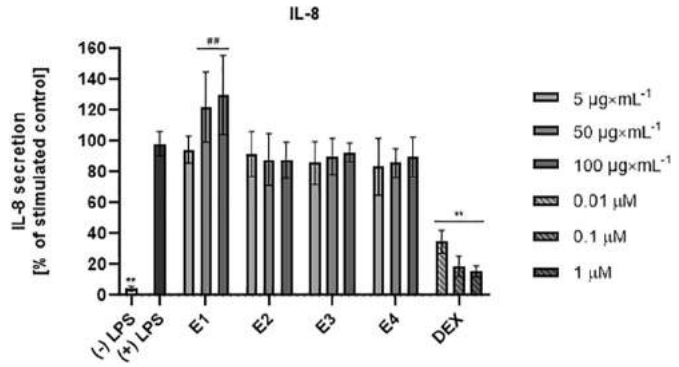

Fig. 3. Effect of extracts from *Sambucus nigra* L. leaves at the concentrations of 5, 50, and 100 μg × mL^{-1} and dexamethasone (DEX) at the concentrations of 0.01, 0.1, and 1 μM on IL-8 secretion by LPS-stimulated human neutrophils. Statistical significance (**$p<0.001$; ##$p<0.001$) is marked in comparison to the stimulated control ((+) LPS).

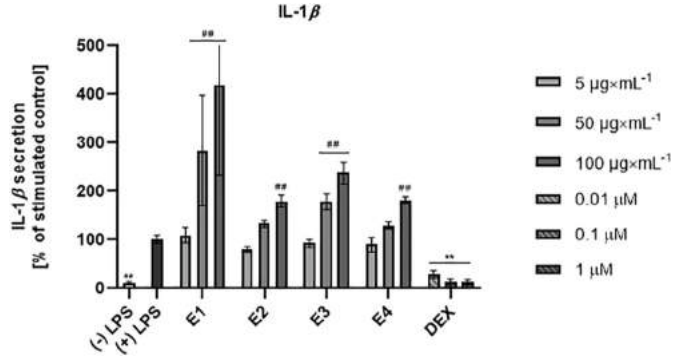

Fig. 4. Effect of extracts from *Sambucus nigra* L. leaves at the concentrations of 5, 50, and 100 μg × mL^{-1} and dexamethasone (DEX) at the concentration of 0.01, 0.1, and 1 μM on IL-1β secretion by LPS-stimulated human neutrophils. Statistical significance (**$p<0.001$; ##$p<0.001$) is marked in comparison to the stimulated control ((+) LPS).

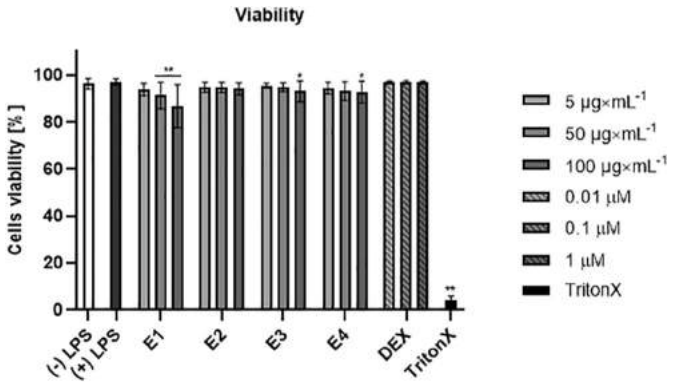

Fig. 5. Neutrophils viability (%) upon 24 h treatment with tested extracts from *Sambucus nigra* L. leaves at the concentrations of 5, 50, and 100 µg × mL^{-1}, dexamethasone (DEX) at the concentrations of 0.01, 0.1, and 1 µM and Triton X-100 at the concentration of 0.1%. Statistical significance (*p<0.05, **p<0.001) is marked in comparison to non-stimulated control ((−) LPS).

viability than the controls of non-stimulated and stimulated LPS (p<0.05). E1 extract at a concentration of 50 µg × mL^{-1} also decreased cell viability (p<0.001). Only the E2 extract, even in the highest concentration used, did not adversely affect the viability of neutrophils. Co-treatment of cells with dexamethasone at concentrations of 0.01, 0.1, and 1 µM did not affect cell viability (p<0.05).

4.4. *In vitro anti-inflammatory and antioxidant activity*

4.4.1. *Inhibition of LOX activity*

All extracts inhibit lipoxygenase activity in a dose dependent manner (Fig. 6). There were no differences between the extracts at each concentration (p<0.05). At the concentration of 250 µg × mL^{-1}, the extracts inhibited the activity of lipoxygenase by 20–30%, and at a concentration of 500 µg × mL^{-1} by over 60%. The IC_{50} values are shown in Table 1. The IC_{50} value for indomethacin was 53.4 ± 6.5 µg × mL^{-1}.

4.4.2. *Scavenging of $O_2^{-}\cdot$*

All extracts scavenged the superoxide anion in a dose-dependent manner (Fig. 7). At the concentration of 5 µg × mL^{-1}, there were no differences in the activity of the extracts. At concentrations of 10–25 µg × mL^{-1}, ethanolic extracts (E2 and E4) had higher scavenging activity. The extract E3 had the weakest activity against the superoxide anion. Based on the SC_{50} values (Table 1), the E2 extract was the most potent radical scavenger. The SC_{50} value of ascorbic acid used as the positive control was 5.31 ± 0.87 µg × mL^{-1}.

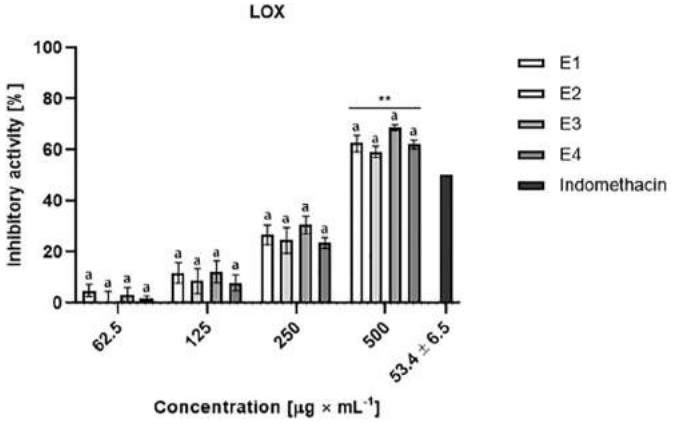

Fig. 6. Inhibition of lipoxygenase activity; a – no differences between extracts activity (p<0.05); statistical significance is marked in comparison to control (**p<0.001).

Table 1
SC_{50} values of scavenging superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), nitric oxide (NO), and DPPH, and IC_{50} values of inhibition of lipoxygenase (LOX) activity.

Extract	SC_{50} ± SD [µg × mL^{-1}]				IC_{50} ± SD [µg × mL^{-1}]
	$O_2^{-}\cdot$	H_2O_2	NO	DPPH	LOX
E1	37.33 ± 4.84	90.90 ± 4.55	8.27 ± 0.99	121.80 ± 5.64	411.62 ± 26.30
E2	27.12 ± 5.40	60.40 ± 1.85	1.29 ± 0.37	60.62 ± 2.73	433.57 ± 22.91
E3	54.73 ± 12.67	71.24 ± 3.10	2.96 ± 0.87	63.65 ± 1.70	376.04 ± 15.08
E4	38.27 ± 9.50	63.74 ± 2.63	0.75 ± 0.35	59.32 ± 2.45	421.04 ± 13.94

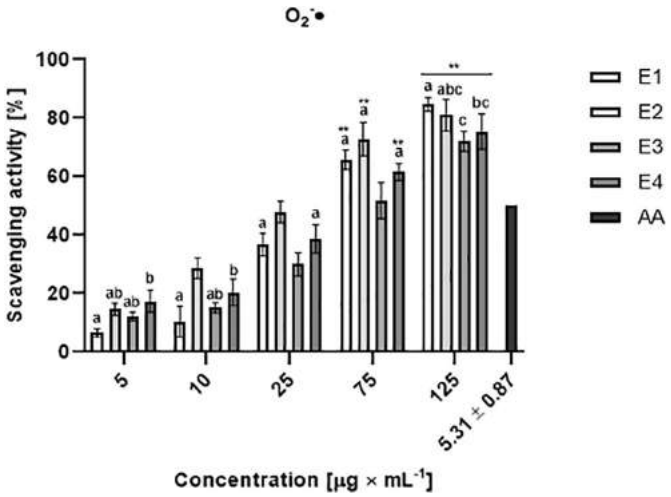

Fig. 7. Scavenging activity against $O_2^{-}\cdot$; a,b,c – no differences between extracts activity (p<0.05); statistical significance is marked in comparison to control (**p<0.001).

The extracts did not affect the activity of xanthine oxidase. Uric acid production, even in the highest concentrations, was 97–100%. For allopurinol, a synthetic xanthine oxidase inhibitor, the IC_{50} value was 1.14 ± 0.14 µg × mL^{-1}.

4.4.3. *Scavenging of H_2O_2*

The hydrogen peroxide scavenging capacity is shown in Fig. 8. At concentrations of 5 and 15 µg × mL^{-1}, there were no differences in the activity of the tested extracts (p<0.05). At the higher concentrations used (50–125 µg × mL^{-1}), the E1 extract showed the weakest effect. At the concentrations of 75 and 125 µg × mL^{-1}, ethanolic extracts (E2 and E4) were more potent than aqueous extracts (E1 and E3), and the E2 extract was the one with the highest H_2O_2 scavenging capacity. The SC_{50} values are shown in Table 1. The positive control was ascorbic acid, which at concentration 1.44 ± 0.06 µg × mL^{-1} scavenged 50% of H_2O_2.

4.4.4. *Scavenging of NO*

The tested extracts strongly scavenged NO (Fig. 9) in a concentration-dependent manner. Even at a concentration of 2.5 µg × mL^{-1}, ethanolic extracts (E2 and E4) scavenged more than 40% of nitric oxide and were significantly more potent (p<0.05) than aqueous extracts (E1 and E3). The SC_{50} values are shown in Table 1. At lower concentrations (2.5–10 µg × mL^{-1}), the E1 extract scavenged NO significantly weaker than the other extracts. In the concentration range of 5–75 µg × mL^{-1}, there were no significant differences in the activity of E2, E3, and E4 extracts. The aforementioned extracts (E2, E3, and E4) scavenged NO statistically significantly stronger than the positive

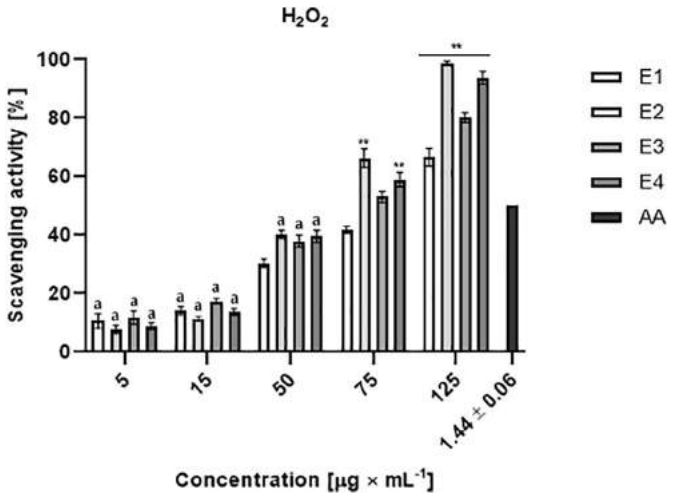

Fig. 8. Scavenging activity against H_2O_2; a – no differences between extracts activity ($p < 0.05$); statistical significance is marked in comparison to control (**p<0.001).

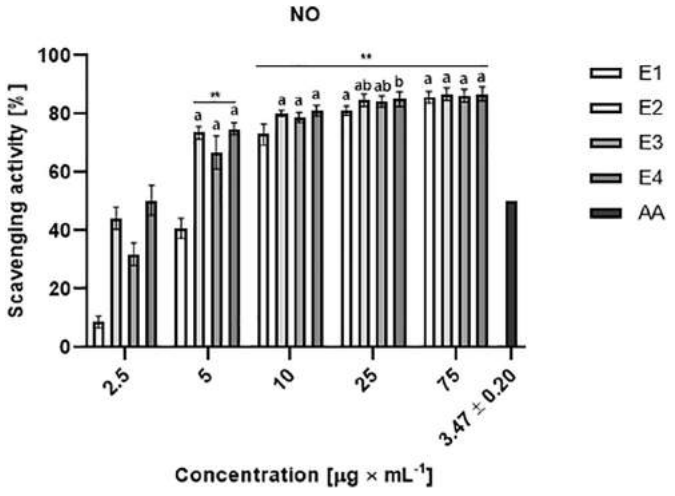

Fig. 9. Scavenging activity against NO; a,b – no differences between extracts activity (p<0.05); statistical significance is marked in comparison to control (**p<0.001).

control – ascorbic acid, for which the calculated SC_{50} value was 3.47 ± 0.20 µg × mL^{-1}.

4.4.5. Scavenging of DPPH

The activity of the extracts against DPPH is shown in Fig. 10. All extracts scavenged DPPH in a dose-dependent manner. The calculated SC_{50} values are presented in Table 1. E1 had the weakest activity against DPPH. There were no statistically significant differences in the activity of E2, E3, and E4 at the concentration of 50 and 75 µg × mL^{-1} (p<0.05). At higher concentrations (125 and 200 µg × mL^{-1}), the activity of E4 was more potent than the other extracts (p<0.05). The calculated SC_{50} value for ascorbic acid (positive control) was 3.99 ± 0.24 µg × mL^{-1}. The E2, E3, and E4 extracts at the concentration of 75 µg × mL^{-1} scavenged more than 50% of the DPPH.

4.5. Phytochemical analysis

4.5.1. The total content of phenolic compounds

The total content of phenolic compounds is presented in Table 3. The ethanolic extracts contained a definitely higher amount of phenolic

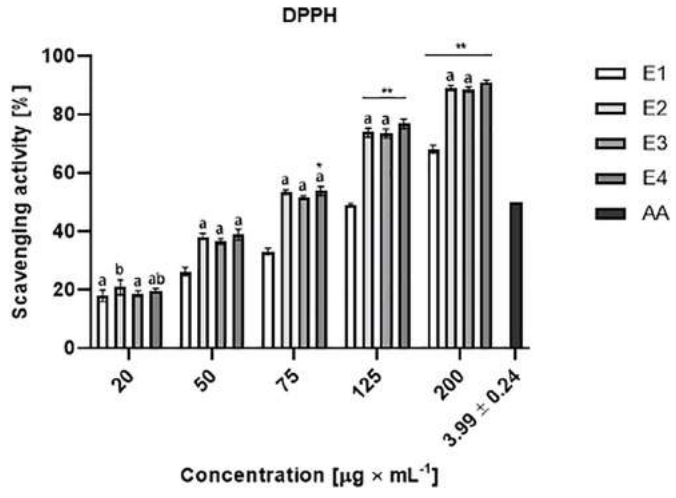

Fig. 10. Scavenging activity against DPPH; a,b – no differences between extracts activity (p<0.05); statistical significance is marked in comparison to control (*p<0.05; **p<0.001).

compounds than the aqueous extracts prepared under the same conditions. The extracts prepared at the solvent boiling point had a higher content of phenolic compounds than the extracts prepared at room temperature.

4.5.2. Qualitative analysis of the composition of extracts

The HPLC-DAD-MS^n analysis of all extracts was performed. The chromatograms of extracts at 350 nm are shown in Fig. 11 in addition to data for major compounds, which are provided in Table 2. The compounds are numbered according to the retention times for the analysis conditions, and the identification was made based on MS spectra in negative ion mode. Caffeoylthreonic acid isomers (compounds 1, 2, and 4) were identified based on their exact molecular weights, fragmentation in MS^2, and UV–Vis maxima compared with the literature (Kim et al., 2019). These isomers were identified in elderberry leaves for the first time. Compound 3 has the MS^2 fragmentation characteristic of caffeoylquinic acids. Based on a comparison with a chemical standard, it was identified as 5-*O*-caffeoylquinic acid. The spectra of compounds 5 (*m/z* 755), 6 (*m/z* 609), and 7 (*m/z* 593) showed absorption maxima at wavelengths characteristic for flavonoids. The MS^2 fragmentation patterns of compounds 5 and 6 showed a signal at *m/z* 301. Therefore, they were tentatively qualified as quercetin derivatives. Compound 7, which was tentatively assigned as kaempferol or luteolin derivative, showed a signal at *m/z* 285. Based on the primary ions in the MS spectrum, MS^2 and MS^3 patterns in negative ESI mode, absorption maxima, and comparison with chemical standards, they were identified as quercetin 7-*O*-rhamnoside-3-*O*-rutinoside, quercetin 3-*O*-rutinoside, and kaempferol 3-*O*-rutinoside, respectively.

4.5.3. Quantitative analysis of the composition of extracts

The prepared calibration curves for chlorogenic acid and quercitrin enabled the calculation of an amount of phenolic acids and flavonoids, respectively. The results are shown in Table 3. The most abundant compounds in the extracts were 5-*O*-caffeoylquinic acid and quercetin 3-*O*-rutinoside. The content of the three isomers of caffeoylthreonic acid, quercetin 7-*O*-rhamnoside-3-*O*-rutinoside, and kaempferol 3-*O*-rutinoside was also calculated. Significantly higher contents of particular phenolic compounds were established in ethanolic extracts compared to aqueous extracts. In the case of water extracts, the increase in the extraction temperature of the plant material allowed to obtain a higher amount of phenolic compounds (E3) than in the extracts prepared at room temperature (E1). In the case of ethanolic extracts, increasing the extraction temperature did not result in obtaining a higher content of the

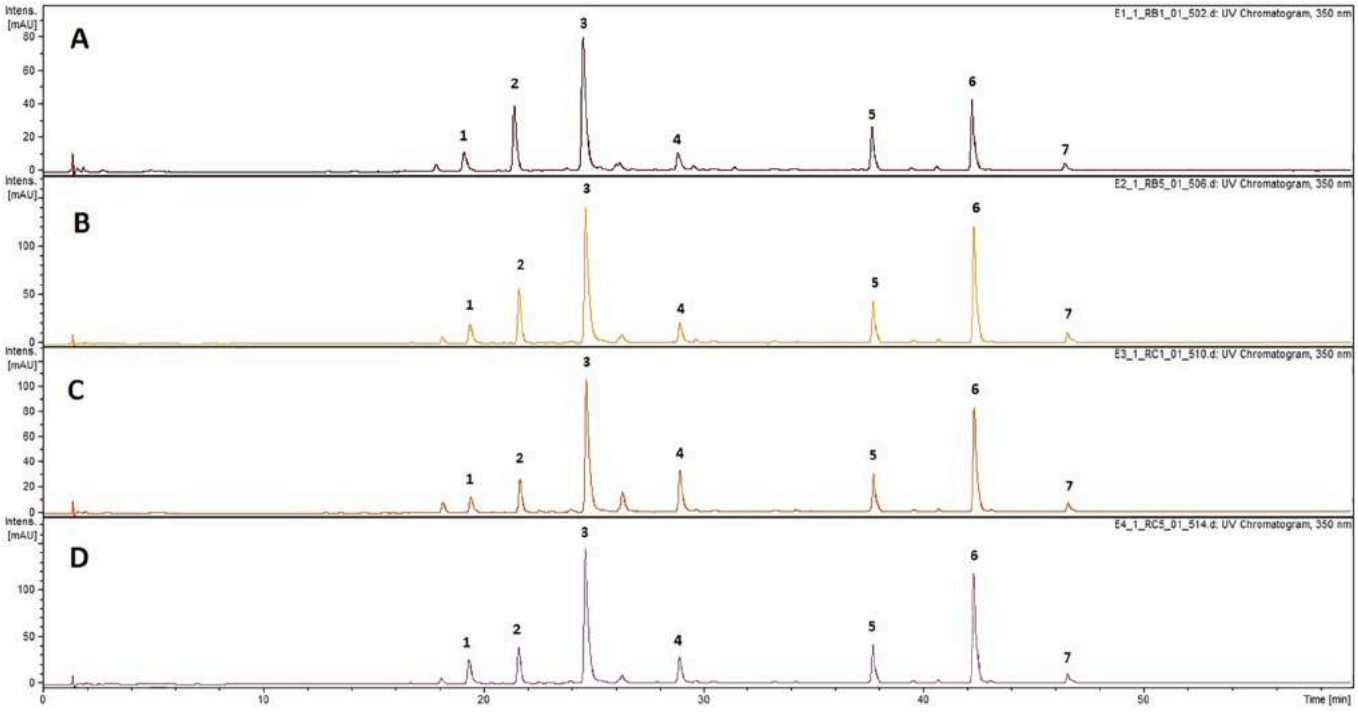

Fig. 11. HPLC chromatogram at 350 nm of extracts (A: E1; B: E2, C: E3; D: E4) from leaves of *Sambucus nigra* L.

Table 2
UV–Vis and MS data of major compounds in extracts of *Sambucus nigra* leaves.

No.	Compound	Rt [min]	UV–Vis maxima [nm]	$[M-H]^-$ *m/z*	MS^2 ions	MS^3 ions	NL [amu]
1	caffeoylthreonic acid isomer I[a]	19.3	211, 285, 325	297	279, 252, 179, **135b**	135	162
2	caffeoylthreonic acid isomer II[a]	21.5	216, 301sh, 326	297	279, 179, **135b**	135	162
3	5-*O*-caffeoylquinic acid[b]	24.6	216, 238sh, 300sh, 325	353	**191b**, 179	191	162
4	caffeoylthreonic acid isomer III[a]	29.1	213, 296, 322	297	279, 252, 179, **135b**	135	162
5	quercetin 7-*O*-rhamnoside-3-*O*-rutinoside[b]	37.8	216, 256sh, 353	755	**609**, 591, 489, 300b	445, 373, 343b, 301	146
6	quercetin 3-*O*-rutinoside[b]	42.3	213, 254, 353	609	343, 301b, 179		
7	kaempferol 3-*O*-rutinoside[b]	46.6	216, 262, 293, 343	593	327, 285b, 257, 229		

[a] – Identification based on literature (Kim et al., 2019).
[b] – Comparisons with chemical standards have been made; b – base peak (the most abundant ion in the recorded spectrum); in bold – ions subjected to MS^3 fragmentation; NL – neutral loss detected corresponding to the cleavage of sugar or phenolic acid.

Table 3
The total content of phenolic compounds and content of phenolic acids and flavonoids in extracts of *Sambucus nigra* leaves.

	Content [μg × mg^{-1}]			
	E1	E2	E3	E4
Total phenolic content	57.40 ± 7.81	72.74 ± 6.89	69.69 ± 7.86	88.87 ± 11.21
caffeoylthreonic acid isomer I	2.18 ± 0.04	3.33 ± 0.05	2.17 ± 0.02	4.52 ± 0.06
caffeoylthreonic acid isomer II	5.19 ± 0.06	7.72 ± 0.13	3.55 ± 0.08	5.10 ± 0.17
3-*O*-caffeoylquinic acid	16.78 ± 0.21	29.48 ± 0.16	21.05 ± 0.05	28.65 ± 0.20
caffeoylthreonic acid isomer III	2.07 ± 0.09	3.85 ± 0.07	6.15 ± 0.06	4.83 ± 0.09
quercetin 7-*O*-rhamnoside-3-*O*-rutinoside	2.12 ± 0.02	3.44 ± 0.03	2.42 ± 0.02	3.17 ± 0.03
quercetin 3-*O*-rutinoside	3.60 ± 0.06	10.14 ± 0.05	6.94 ± 0.03	9.63 ± 0.05
kaempferol 3-*O*-rutinoside	0.38 ± 0.01	1.04 ± 0.01	0.72 ± 0.01	0.95 ± 0.02

analyzed chemical substances in the extracts.

5. Discussion

Sambucus nigra leaves have been used in folk medicine in the treatment of various inflammatory diseases such as dermatitis, eye inflammation, or rheumatism. They are an easily available plant material that can be obtained at a low cost, because they are the main biomass of the plant. We decided to investigate the effect of elderberry leaf extracts on the inflammatory process that occurs locally in affected tissue due to injury. Injury causes a series of processes involving inflammation, proliferation, and maturation to restore skin integrity.

Neutrophils produce ROS, primarily the superoxide anion radical, in huge quantities in a process described as a 'respiratory burst'. The superoxide anion radical is dismutated by superoxide dismutases (SODs) to molecular oxygen and H_2O_2, which can easily penetrate cell membranes and be converted by Fenton's reaction to the hydroxyl radical – a highly reactive molecule capable of damaging proteins, DNA nucleic acids, and polyunsaturated fatty acids contained in cell membranes and intracellular organelles. The process, a non-specific immune response, is used to clean the wound with bacteria intruding into the body via damaged skin integrity. However, it is not a selective process. In addition to the disposal of pathogens, a respiratory burst has many negative

consequences for the surrounding tissue (Shukla et al., 2019; Wilgus et al., 2013). The prolonged inflammation associated with the overproduction of ROS is characteristic of chronic wounds in which the process of reconstructing damaged tissues is stopped (Las Heras et al., 2020). Regulation of the level of ROS is therefore essential for the effective repair of skin damage, which can be supported by the antioxidants contained in plant extracts (Ibrahim et al., 2018).

Our research on biological activity shows that aqueous and ethanolic extracts from *Sambucus nigra* leaves strongly scavenge ROS both in the f-MLP-stimulated human neutrophil model and cell-free systems. The ability to scavenge free radicals (H_2O_2, NO, and DPPH) is much higher for our extracts than for methanolic extracts, which activity was previously described (Azari et al., 2015). The extracts tested by us scavenged nitric oxide more potent than ascorbic acid, one of the strongest antioxidants. The effect on the secretion of pro-inflammatory cytokines by LPS-stimulated neutrophils also differs for aqueous, ethanolic, and methanolic extracts (Yeşilada et al., 1997). The secretion of TNF-α, after incubating the cells with ethanolic extracts at the concentration of 50 μg × mL^{-1}, was more relevant than the aqueous extracts, decreased by about 40%, while the methanolic extract at a concentration of 10 μg × mL^{-1} reduced the secretion by 47%. The methanolic extract does not significantly affect the secretion of interleukin 1β, while the aqueous and ethanolic extracts stimulate its release (Yeşilada et al., 1997).

The most common groups of polyphenols with antioxidant activity are phenolic acids and flavonoids. In the extracts of *Sambucus nigra* leaves, aqueous and ethanolic, we confirmed the presence of phenolic acids, caffeic acid derivatives, and flavonoids, such as quercetin and kaempferol derivatives. In addition, we determined their content. Water and ethanol are the most commonly used solvents in traditional medicine, inexpensive and non-toxic. To examine the influence of temperature on the activity and chemical composition, aqueous and 70% (*v/v*) ethanolic macerates were prepared at room temperature and decoctions at the solvent boiling point. The highest amounts of polyphenols were found in ethanolic extracts. The total content of phenolic compounds was statistically significantly higher in the extracts prepared at 100 °C than those prepared at room temperature. The content of phenolic compounds determined in our research is much higher than in methanolic extracts prepared by the liquid pressurized extraction method (Dawidowicz et al., 2006). The content of quercetin 3-*O*-rutinoside was 0.67 ± 0.2 and 1.67 ± 0.26 mg × g^{-1} for aqueous extracts prepared at 20 and 100 °C, respectively. The quercetin 3-*O*-rutinoside content in ethanolic extracts was 10.14 ± 0.05 and 9.63 ± 0.05 mg × g^{-1}, respectively. However, the total content of phenolic compounds in the aqueous and ethanolic extracts prepared in our study is lower than in the methanolic extracts (Azari et al., 2015). The total content of phenolic compounds in the extracts prepared at room temperature was 57.40 ± 7.81 and 72.74 ± 6.89 mg × g^{-1} for the aqueous and ethanolic, respectively. Meanwhile, the content in the methanolic extracts was 185.2 ± 5.8, 132.3 ± 7.6 and 115.9 ± 4.1 mg × g^{-1}, for the extract made in the ultrasonic bath, by the percolation method and in the Soxhlet apparatus, respectively (Azari et al., 2015). The application of various solvents and extraction methods drastically affects the content of polyphenolic compounds in plant extracts.

During the inflammatory phase, the expression of 5-lipoxygenase increases, primarily in leukocytes, which catalyzes the conversion of arachidonic acid to leukotrienes, a group of pro-inflammatory lipid mediators. They increase the permeability of blood vessels and are a strong chemotactic factor for neutrophils that appear at the site of injury (Rådmark et al., 2007). As the inflammatory phase of the healing process sustains, regulation of lipoxygenase activity may be beneficial to the healing process. Elderberry leaf extracts have the ability to inhibit the activity of lipoxygenase, which proves their anti-inflammatory potential.

During normal wound healing, the highest levels of TNF-α are observed within 12–24 h after injury. After the end of the proliferation phase, TNF-α levels return to baseline. TNF-α levels are continuously elevated in the presence of a prolonged inflammatory phase and overexposure to oxidative stress, such as in a diabetic wound or non-healing venous leg ulcers (Xu et al., 2013). TNF-α has been shown to induce the NF-κB pathway, which plays a crucial role in the cellular immune response. This leads to an increase in the inflammatory response of cells such as keratinocytes and the induction of endothelial cell apoptosis and inhibition of their proliferation and migration (Liu et al., 2017). Therefore, reduction of TNF-α secretion may be beneficial, especially in treating chronic and difficult to heal wounds. The traditional use of elderberry leaf extracts in the treatment of skin inflammation may be related to their inhibitory effect on TNF-α release.

The role of neutrophils is also to fight pathogens. They are activated by endogenous factors such as the interleukin 8 (named CXCL8) and exogenous factors such as N-formylated bacterial peptides. One of them is f-MLP, by which neutrophils release ROS. They help to fight microorganisms that enter the body, but they are not selective. They destroy surrounding tissues, which is why prolonged inflammation has such a negative effect. Therefore, maintaining the prooxidative and antioxidant balance can be supported by the using *Sambucus nigra* leaves extract.

During the studies on the release of cytokines IL-1β and IL-8 by neutrophils, an increase in their secretion was observed after incubation with some of the extracts at the concentrations of 50 or 100 μg × mL^{-1}. It was observed that some of the extracts used at these concentrations exerted a cytotoxic effect on the cells. Therefore it is supposed that cell damage was possible. As a result of cell damage or death, molecules called Damage-Associated Molecular Patterns (DAMPs) are released from the extracellular or intracellular space. They are endogenous molecules, recognized for example by macrophages, capable of triggering and prolonging an immune response through various pathways, including NF-κB by binding to Toll Like Receptors (Roh and Sohn, 2018). Many of the released DAMPs induce the secretion of pro-inflammatory cytokines, including IL-1β and IL-8, which may explain their increased secretion in the case of extracts at the concentration of 50 or 100 μg × mL^{-1}, which decreased cells viability. Moreover, the specific chemical compounds contained in the extracts may also have an influence on the cytotoxic effect and the increase in the secretion of pro-inflammatory cytokines. The presence of cyanogenic glycosides, which could affect the viability of cells, was confirmed in the elderberry leaves (DellaGreca et al., 2000a). In the other hand, cyanogenic glycosides are degraded after increasing the extraction temperature of the plant material. For this reason, we do not suppose they can be found in the extracts prepared at the boiling solvents point. In the case of the secretion of IL-8, it was observed that the aqueous extract prepared at room temperature statistically significantly induced the secretion of this cytokine, while the aqueous extract prepared at higher temperature did not have such an effect.

In the case of chronic wounds, the balance of cytokine secretion is disturbed and the cellular response is sometimes inadequate to the impulses. Therefore, due to the multitude of studies, it is difficult to conclude which cytokines will have a beneficial effect on the initiation of the healing process. One example of impaired wound healing leading to a chronic inflammatory process in the affected tissue is diabetic wounds. Cells present at the site of injury almost from the beginning are monocytes, which are activated and transformed into macrophages. They are responsible, among others, for the secretion of vascular epithelial growth factors, such as VEGF-A and VEGF-C, which affect the formation of lymphatic vessels. Correct formation of the lymphatic system in the injured tissue reduces the tissue pressure that causes the swelling and allows the tissue to be rebuilt. Elevated blood glucose levels, for example in diabetes mellitus, inhibit the secretion of interleukin 1β by the RAW 264.7 macrophage-like cells. In addition, it stimulates these cells to increase the production of nitric oxide. As a result of hyperglycemia, suppression of VEGFR3 and VGFR-A expression has also been observed, leading to impaired formation of lymphatic vessels and inhibition of tissue reconstruction (Maruyama et al., 2007). It has been shown that the stimulation of macrophages of diabetic mice

with interleukin 1β leads to an increased expression of markers of the epithelial lymphatic system, and hence the formation of granulation tissue (Zykova et al., 2000). Therefore, in chronic, non-healing wounds, increased IL-1β release may have a beneficial effect in restoring macrophages' ability to regenerate damage.

Interleukin 8 is one of the most potent chemotactic agents, produced by macrophages and neutrophils and many others, such as epithelial cells. It is also mentioned as a factor promoting angiogenesis. In studies on HaCaT cells, it was found that the presence of IL-8 significantly increased the migration and adhesion of these cells to the wound site (Jiang et al., 2012). Moreover, it has been shown that the amount of keratinocytes under the influence of IL-8 increases and the wound contraction in an *in vivo* model after surface administration of IL-8 decreases, which may be beneficial in the case of impaired wound healing (Rennekampff et al., 2000). Therefore, the increased production of these pro-inflammatory cytokines under the influence of the extracts used by us can be considered a kind of beneficial phenomenon. However, we realize that it is necessary to conduct further research on the influence of elderberry leaves on the wound healing process. Our research should be considered as fundamental, giving the opportunity to continue and further explore the topic. More studies on the activity and toxicity of *Sambucus nigra* leaves extracts are needed.

6. Conclusion

The biological activity and chemical composition of traditional *Sambucus nigra* leaves extracts were investigated. The ethanolic extracts contained more polyphenols than the aqueous extracts and showed a higher anti-inflammatory and antioxidant activity. Our research has successfully confirmed that elderberry leaves are a plant material with high anti-inflammatory activity, especially against reactive oxygen species, and a potentially rich source of antioxidants. However, more detailed studies of the chemical composition and biological activity in cellular and animal models are necessary to qualify *Sambucus nigra* leaves as a plant material supporting the treatment of wounds and burns.

CRediT authorship contribution statement

Weronika Skowrońska: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Sebastian Granica:** Software, Resources, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Monika E. Czerwińska:** Methodology, Investigation, Writing – review & editing, Supervision. **Ewa Osińska:** Resources. **Agnieszka Bazylko:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The project was carried out using CePT infrastructure financed by the European Regional Development Fund within the Operational Programme "Innovative economy" for 2007–2013.

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Manuscript co-author statement

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Additionally, I confirm that the scope presented below corresponds to my contribution to the project.

Oświadczenie współautora manuskryptu

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Jednocześnie potwierdzam, że przedstawiony poniżej zakres odpowiada mojemu wkładowi w realizację projektu.

Co-author's name	**Percentage of contribution**	**Scope of contribution**
Imię i nazwisko współautora	**Udział procentowy**	**Zakres wkładu**
Weronika Skowrońska	60%	Preparation of extracts and their qualitative and quantitative phytochemical analysis, performing *in vitro* and *ex vivo* tests, statistical analysis of the obtained results and their visualization, writing the original draft of the manuscript, corresponding author.
		Przygotowanie wyciągów oraz ich jakościowa i ilościowa analiza fitochemiczna, wykonanie badań *in vitro* i *ex vivo*, analiza statystyczna uzyskanych wyników i ich wizualizacja, pisanie oryginalnego tekstu manuskryptu, autor korespondencyjny.
Sebastian Granica	8%	Methodology of the phytochemical research, project supervision, manuscript editing
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Monika E. Czerwińska	10%	Methodology of the *ex vivo* research, manuscript editing
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Ewa Osińska	2%	Providing plant material
		Dostarczenie materiału roślinnego
Agnieszka Bazylko	20%	Conceptualization of the project, methodology of the *in vitro* research, manuscript editing, project supervision.
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27.02.2024 Weronika Skowrońska

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Weronika Skowrońska	60%	Preparation of extracts and their qualitative and quantitative phytochemical analysis, performing *in vitro* and *ex vivo* tests, statistical analysis of the obtained results and their visualization, writing the original draft of the manuscript, corresponding author.
		Przygotowanie wyciągów oraz ich jakościowa i ilościowa analiza fitochemiczna, wykonanie badań *in vitro* i *ex vivo*, analiza statystyczna uzyskanych wyników i ich wizualizacja, pisanie oryginalnego tekstu manuskryptu, autor korespondencyjny.
Sebastian Granica	8%	Methodology of the phytochemical research, project supervision, manuscript editing
		Metodologia badań fitochemicznych, nadzór merytoryczny nad projektem, redakcja manuskryptu
Monika E. Czerwińska	10%	Methodology of the *ex vivo* research, manuscript editing
		Metodologia badań *ex vivo*, redakcja manuskryptu
Ewa Osińska	2%	Providing plant material
		Dostarczenie materiału roślinnego
Agnieszka Bazylko	20%	Conceptualization of the project, methodology of the *in vitro* research, manuscript editing, project supervision.
		Koncepcja projektu, metodologia badań *in vitro*, redakcja manuskryptu, nadzór merytoryczny nad projektem

Date and legible signature

Data i czytelny podpis

04.03.2024 Agnieszka Bazylko

Journal of Ethnopharmacology 320 (2024) 117423

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Wound healing potential of extract from *Sambucus nigra* L. leaves and its fractions

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ARTICLE INFO

Handling Editor: V Kuete

Keywords:
Sambucus nigra
Elderberry
Keratinocytes
Wound healing
Skin
Enzymes

ABSTRACT

Ethnopharmacological relevance: Sambucus nigra L. (Viburnaceae), commonly known as black elderberry, is a native species throughout Europe and Western Asia. Elderberry leaves have been used in traditional European medicine, mainly externally, to treat skin and mucosal diseases. They are usually used as decoctions for washing hemorrhoids, ulcers, insect bites, wounds, scrapes, and gingivitis.

Aim of the study: Our work aimed to scientifically verify the traditional use of the plant material in treating wounds and skin inflammations accompanied by its phytochemical characterization.

Materials and methods: The effect of 70% (*v/v*) ethanolic extract and its fractions of different polarities on the inflammatory response of cells involved in wound healing (fibroblasts, keratinocytes, and neutrophils) was investigated. In addition, their effect on the migration of keratinocytes to the scratch site in an *in vitro* wound healing assay and their impact on the activity of the enzymes involved in skin inflammation, were assessed. The chemical composition was analyzed by UHPLC-DAD-MS^n, and the structure of the isolated compounds was determined by NMR.

Results: The supportive effect of the elderberry leaves extract towards wound healing and modulation of the inflammatory response was demonstrated by induction of the keratinocytes. Additionally, the extract was shown to affect the cellular secretion of TNF-α and interleukins -1β, −6, and −8. Examinations of fractionated extract have shown that active principles were mainly contained in dichloromethane fraction. Eleven chemical constituents belonging to flavonoids, cyanogenic glycosides, and lignans were isolated and characterized.

Conclusion: The beneficial effect of *S. nigra* leaves in treating skin diseases has been partially verified and supports its traditional use in skin ailments of different etiologies.

1. Introduction

The skin's key role is to protect the body against all external factors (physical, chemical, and biological). In case of an injury, a series of repair processes occur, including hemostasis, inflammation, proliferation, and remodeling, to restore the tissue and create a scar. Neutrophils are the first to appear at the site of damage and release several cytokines, chemokines, and pro-inflammatory factors responsible for the fight against pathogens entering the body and mobilizing the other skin cells to repair the injury (Phillipson and Kubes, 2019). Keratinocytes are activated and change their phenotype to migratory, proliferative, and pro-inflammatory. They signal tissue and cell trauma through the Toll-like receptors (TLRs) that activation leads to NF-κB-mediated transcription of enzymes and other healing-promoting agents (Juráňová et al., 2017). However, the prolonged inflammatory phase leads to the transformation of the wound into a chronic injury, in which the inflammatory processes persist and fuel themselves in an uncontrolled manner. Maintaining balance is, therefore, important for the proper healing of a wound (Shukla et al., 2019).

The number of patients suffering from chronic wounds, including diabetic foot ulcers or pressure ulcers, increases every year. The global market for wound care products in 2021 was estimated at nearly 17.5

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https://doi.org/10.1016/j.jep.2023.117423
Received 5 September 2023; Received in revised form 4 November 2023; Accepted 11 November 2023
Available online 16 November 2023

billion USD. It is predicted that by 2029, it will reach over 28 billion USD (Fortune Business Insights, 2022). At the same time, we are observing a growing interest in natural skin care products (Grand View Research, 2018). These reports may suggest a demand for natural products for irritated, injured, or damaged skin. Therefore, research for alternatives of natural origin with potentially beneficial effects on the skin is highly desired.

In traditional European medicine, *Sambucus nigra* L. (Viburnaceae) leaves were used mainly externally to treat and alleviate dermatological conditions. Compresses of fresh, whole, or crumbled leaves were used to treat wounds and scratches (Pieroni and Gray, 2008; Savo et al., 2011), insect bites (Pieroni et al., 2004), erysipelas (Mazzei et al., 2020; Menendez-Baceta et al., 2014), burns, bruises, as well as joint and muscle pain (Menendez-Baceta et al., 2014). Decoctions and infusions were applied to heal hemorrhoids (Menendez-Baceta et al., 2014), abscesses (Cornara et al., 2009), eyes rheum (Menendez-Baceta et al., 2014) or inflammation (Ballero et al., 2001), toothache, and gingivitis (Ballero et al., 2001; Cornara et al., 2009). In veterinary medicine, they were used for scabs in sheep, cattle, and horses (Menale and Muoio, 2014). When used internally, the infusions had a laxative effect (Tuzlacı and Sadıkoğlu, 2007). In the form of inhalation, they relieved headaches and lowered fever (Menendez-Baceta et al., 2014).

The activity studies conducted so far show that elderberry leaves extracts have antioxidant (Azari et al., 2015; Dawidowicz et al., 2006; Tundis et al., 2019), anti-inflammatory (Yeşilada et al., 1997), adaptogenic (Neekhra et al., 2021), anticonvulsant (Ataee et al., 2016), and antidepressant (Mahmoudi et al., 2014) effects. Moreover, they show antimicrobial (Cybulska et al., 2011) and anti-toxoplasma (Daryani et al., 2015) properties. Despite widely described traditional uses in skin-associated ailments, the activities of elderberry leaves were never evaluated on *in vitro* models directly related to skin and mucosal diseases.

Research on the chemical composition of elderberry leaves showed the presence of phenolic compounds, cyanogenic glycosides, and compounds from the groups of allelochemicals or triterpenes. From the group of phenolic compounds, phenolic acids have been identified, such as chlorogenic (Kiprovski et al., 2021), caffeic, ferulic, *p*-coumaroylquinic acids and their derivatives (Kiprovski et al., 2021; Senica et al., 2019), as well as flavonoids, mainly derivatives of quercetin (Dawidowicz et al., 2006), kaempferol, and isorhamnetin (Senica et al., 2019). From the group of cyanogenic glycosides, prunasin, sambunigrin, holocalin, 6-acetylholocalin, and zierin have been identified (DellaGreca et al., 2000a), as well as cyanohydrins (DellaGreca et al., 2000b). In addition, lipophilic compounds (e.g., palmitic acid and methyl linoleate) (Szymański et al., 2020), and terpenoids (e.g., ursolic and oleanolic acid) have also been identified (Inoue and Sato, 1975). High levels of aromatic compound, benzoic aldehyde, were also present (Szymański et al., 2020).

The research aim was to investigate the influence of elderberry leaves on the processes involved in wound healing. Based on our previous research, a 70% ethanolic extract prepared at room temperature was selected due to its excellent anti-inflammatory and antioxidant activity and high biocompatibility on human neutrophils model (Skowrońska et al., 2022). This work investigated the influence of the selected extract and its fractions on the inflammatory response of cells directly involved in the wound healing process (neutrophils, keratinocytes, and fibroblasts). Physical (UV radiation), internal biochemical (TNF-α/IFN-γ), and external microbial (bacterial-derived products) factors were used to induce the inflammatory response. The potential effect on the regeneration of epidermal damage was determined using the *in vitro* scratch assay. Moreover, the influence on the activity of enzymes involved in repair processes, invasion of microbes, and inflammation was investigated. Finally, the chemical composition of extract and fractions was characterized.

2. Materials and methods

2.1. Chemicals

Collagenase type I (from *Clostridium histolyticum*) and elastase (from the porcine pancreas) were purchased from Alfa Aesar (Kandel, Germany). DMEM High glucose w/stable glutamine w/sodium pyruvate, Dulbecco's Phosphate Buffered Saline (DPBS) without Ca^{2+} and Mg^{2+}, fetal bovine serum (FBS), Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}, Lymphosep, penicillin-streptomycin solution, RPMI 1640 w/stable glutamine w/25 mM HEPES and Trypsin-EDTA solution were purchased from Biowest (Nuaillé, France). Acetic acid, citric acid, glacial acetic acid, glucose, hydrochloric acid, potassium chloride, sodium chloride, sodium citrate, sodium hydroxide, sodium tetraborate decahydrate ($Na_2B_4O_7 \times 10H_2O$), and zinc chloride were purchased from Chempur (Piekary Śląskie, Poland). Recombinant human interferon-gamma (IFN-γ) and recombinant human tumor necrosis factor-alpha (TNF-α) were purchased from InvivoGen (Toulouse, France). Chromatographic grade acetonitrile and lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Merck Millipore (Billerica, MA, USA). Dichloromethane, diethyl ether, dimethyl sulfoxide, ethanol, ethyl acetate, formic acid, methanol, and n-butanol were purchased from POCh (Gliwice, Poland). Calcium chloride dihydrate ($CaCl_2 \times 2H_2O$), 4-(dimethylamino)benzaldehyde, dexamethasone, dextran from *Leuconostoc mesenteroides*, 3,4-dihydroxy-L-phenylalanine, ethylenediaminetetraacetic acid (EDTA), *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (f-MLP), gallic acid, gelatine, hyaluronidase, kojic acid, lipoteichoic acid from *Staphylococcus aureus*, luminol, methanol-D4 (CD_3OD), neutral red, ninhydrin, nordihydroguaiaretic acid, polyethylene glycol, propidium iodide, *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide, stannous chloride dihydrate ($SnCl_2 \times 2H_2O$), tannic acid, Triton X-100, tris base, and ursolic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Linoleic acid, lipoxygenase from Soybean, and sodium hyaluronate from Cockscomb were purchased from Tokyo Chemical Industry (Portland, OR, USA). Sets of enzyme-linked immunosorbent assay (ELISA) for interleukins (IL-1β, IL-6, IL-8) and TNF-α and Reagent Set B were purchased from BD Biosciences (Franklin Lakes, NY, USA).

2.2. Plant material and extract preparation

Elderberry leaves were collected after the flowering period (August 2020) from the agricultural field of the Warsaw University of Life Sciences (Poland, 52°16′04.0″N; 21°10′37.1″E). The raw material specimen (SNL202008) is stored at the Department of Pharmaceutical Biology, Medical University of Warsaw, Poland. The plant material was dried in a Leśniczanka type dryer (Hamech, Hajnówka, Poland) with constant airflow at 25–30 °C. The extract was made from 2.0 kg of dry, comminute raw material by threefold maceration with 70 % (*v/v*) ethanol. The plant material was covered each time with 15 L of solvent per 1 kg of leaves and left for 24 h at room temperature with occasional stirring. The extract was filtered through gauze and then through a paper filter grade 41 (Whatman, Marlborough, MA, USA). The purified extracts were combined, the ethanol evaporated, and the residue was concentrated under reduced pressure at 30 °C (Laboranta 4000 WB, Heidolph, Schwabach, Germany with chemistry pumping unit PC 3001 VARIO pro, Vacuubrand, Essex, CT, USA). The concentrated extract was frozen and lyophilized using the laboratory freeze-dryer Cryodos (Telstar, Terrassa, Spain). The obtained dry extract (EX, 598 g) was homogenized in a mortar and stored in a sealed container at 2–8 °C.

2.3. Fractionation and isolation of chemical compounds

Part of the extract was left for testing (5.5 g), and the remaining amount (592.5 g) was dissolved in distilled water (ca. 1.5 L) and fractionated with solvents of increasing polarity as follows:

dichloromethane (7 × 1 L), diethyl ether (5 × 1 L), ethyl acetate (5 × 1 L), and water-saturated *n*-butanol (6 × 1 L). Standard solvents used in phytochemistry were used for fractionation, which made it possible to divide the chemical compounds present in the extract depending on their polarity, starting with dichloromethane (FR1) and ending with the water residue (FR5). After evaporation to dryness under reduced pressure, five fractions were obtained: dichloromethane residue (FR1, 77.5 g), diethyl ether residue (FR2, 1.2 g), ethyl acetate residue (FR3, 5.4 g), *n*-butanol residue (FR4, 96.7 g), and aqueous residue (FR5, 335.5 g). The samples were stored in sealed containers at 2–8 °C.

The ethyl acetate residue (5.2 g) was adsorbed onto silica gel (ca. 15 g), separated on a silica gel 60 column (40 × 4.5 cm), and eluted with dichloromethane: methanol gradient with an Intechim PuriFlash 430evo medium pressure instrument. The gradient program 0–40% methanol from 0 to 540 min, flow rate 30 mL × min^{-1}, and the maximum pressure of 2 bar were established. In total, 704 samples of 20 mL were obtained, which were combined into 16 sub-fractions (FR3_1–16) based on the TLC profile (TLC conditions for control of the composition of all fractions and sub-fractions: silica gel 60 with F_{254}; ethyl acetate: formic acid: water, 18:1:1, *v/v/v*, with visualization at 254 nm). The *n*-butanol residue (96.4 g) was adsorbed onto silica gel (ca. 45 g) and separated similarly to the ethyl acetate residue. A different gradient was used: 5–55% methanol from 0 to 540 min and a 45 mL × min^{-1} flow. Other parameters have not changed. In total, 678 samples of approximately 20 mL each were collected and combined into 21 subfractions based on the TLC profile (FR4_1–21). The FR4_4 sub-fraction was separated on a Sephadex LH-20 column (145 × 3 cm) and eluted with methanol. In total, 100 samples of approximately 10 mL were collected and combined into seven sub-fractions based on the TLC profile (FR4_4_1–7). All subfractions obtained after column chromatography were evaporated to dryness under reduced pressure and stored in sealed vials at 2–8°.

Based on the UHPLC-DAD-MS^n spectra (conditions described in section 2.4.1) of the obtained sub-fractions, eight were selected, dissolved in DMSO, and subjected to further separations using Shimadzu preparative HPLC (LC-20AP solvent delivery unit, SPD-10A VP UV–Vis detector, SIL-10AF autosampler, CTO-10AS VP column oven, CBM-20A communication bus module, and FRC-10A fraction collector) equipped with a Kinetex XB-C_{18} column (150 × 21.2 mm × 5 μm). Pure chemicals were eluted in a gradient of 0.1% HCOOH in H_2O (A): 0.1% HCOOH in acetonitrile (B) (3–26% B from 0 to 60 min; flow 20 mL × min^{-1}, 25 °C, detection at 254, 280 and 350 nm). Compounds **23** (28.2–29.3 min) and **41** (40.0–40.6 min) were obtained from the sub-fraction FR3_3. Compound **32** (14.1–14.9 min) was obtained from the sub-fraction FR3_5. Compound **6** (13.3–14.9 min) was obtained from the sub-fraction FR3_6. Compound **29** (41.5–42.3 min) was obtained from the sub-fraction FR3_7. Compound **30** (42.3–43.3 min) was obtained from the sub-fraction FR3_10. Compounds **28** (37.3–28.1 min) and **33** (43.8–45.1 min) were obtained from the combined sub-fractions FR3_13–14. Compounds **13** (17.3–18.8 min) and **26** (31.5–32.3 min) were obtained from the combined sub-fractions FR4_13–16. Compound **31** (41.4–42.3 min) was obtained from the sub-fraction FR4_4_3.

As a result of the isolation, 11 pure chemical compounds were obtained, the yield of which was: **6** (111 mg), **13** (401 mg), **23** (6 mg), **26** (49 mg), **28** (10 mg), **29** (6 mg), **30** (34 mg), **31** (14 mg), **32** (4 mg), **33** (5 mg), and **41** (2 mg).

2.4. Phytochemical analysis

2.4.1. UHPLC-DAD-MS^n analysis

UHPLC-DAD-MS^n was performed using the Ultimate 3000 series system (Dionex, Idstein, Germany) equipped with a DAD device and splitless connection with an AmaZon SL ion trap mass spectrometer with an ESI interface (Bruker Daltonik GmbH, Germany). The separation of compounds in the tested samples was carried out on a Kinetex XB-C_{18} (150 × 2.1 mm × 1.7 μm) column (Phenomenex, Torrance, CA, USA) at 25 °C. Compounds were eluted using a gradient of 0.1% (*v/v*) formic acid in water (A) and 0.1% (*v/v*) formic acid in acetonitrile (B) as follows: 1–26% B in 0–60 min and 26–95% B in 60–90 min. The flow was 0.3 mL × min^{-1}, and the injection volume was 3 μL. The samples were dissolved in 50% (*v/v*) methanol with 0.1% (*v/v*) formic acid to obtain a concentration of 5 mg × mL^{-1}. The UV–Vis spectra were recorded in the 200–450 nm range. The 254, 280, 325, and 350 nm chromatograms were retained. The nebulizer pressure was 40 psi, the dry gas flow was 9 L × min^{-1}, the dry temperature was 300 °C, and the capillary voltage was 4.5 kV. The MS spectra were registered by scanning from m/z 70 to 2200. Compounds were analyzed in a negative ion mode. The MS^2 fragmentation was obtained for two of the most abundant ions. The identification was based on analytical standards, comparison with literature data, and ^{1}H NMR spectra.

2.4.2. NMR identification

Elucidation of the structures was based on one-dimensional NMR spectra recorded in CD_3OD. ^{1}H NMR data were acquired using a Bruker Avance III 600 MHz spectrometer with 600.13 MHz resonance frequency. The obtained spectra were analyzed and compared with literature data.

2.5. Studies on skin cell lines

2.5.1. Cell cultures

The spontaneously immortalized human keratinocytes (HaCaT) cell line and Normal Human Dermal Fibroblasts (NHDF) cell line (both Lonza Group Ltd, Basel, Switzerland) were cultured in DMEM High Glucose medium (w/L-Glutamine, w/Sodium Pyruvate) supplemented with 10% of fetal bovine serum and 1% of penicillin and streptomycin solution in a humidified incubator at 37 °C with 5% CO_2.

2.5.2. Wound healing assay

HaCaT cells ($1.75 \times 10^5 \times mL^{-1}$) were grown in a 12-well plate until forming a confluent monolayer (18h). The medium was changed to without FBS, and cells were incubated for another 4 h. Subsequently, the single layer of cells was scratched with a 200 μL pipette tip, the medium was aspirated, and the cells were washed with DPBS. Then, the FBS-free medium containing the extract or fractions at the concentration of 100 μg × mL^{-1} was applied to the cells. Photographs were taken under the microscope with a Nikon camera (Nikon Eclipse TS100, Nikon Instruments Inc., Melville, NY, USA, supported by NIS-Elements BR 3.22 Software) immediately after the extract or fractions had been applied, and then 12 and 24 h later. The results are presented as a wound closure ratio. For this purpose, the surface area of the scratch at time 0 and after 24 h of incubation with the tested samples was compared. The control was the FBS-free medium.

2.5.3. UVB irradiation

HaCaT cells ($2 \times 10^4 \times mL^{-1}$) were grown in a 24-well plate until 80% confluent (48–72h). After incubation, the medium was aspirated, and cells were washed with DPBS. Then, a thin layer of DPBS (100 μL per well) was applied, and cells were irradiated with UVB in a dose of 20 mJ × cm^{-2} in a CL-1000 Ultraviolet Crosslinker (Analytik Jena US LLC, Upland, CA, USA) with the Ushio G8T5E UVB linear lamp (Bulbman, Reno, NV, USA) which emits ultraviolet rays between 280 nm and 360 nm (peak at 305–315 nm). DPBS was replaced with medium (stimulated control – ST) or medium containing tested samples at the appropriate concentration. Supernatants were collected after 24 h of incubation and stored at −20 °C until use. The non-stimulated control (NST) was cells not exposed to UVB radiation.

2.5.4. TNF-α and IFN-γ stimulation

HaCaT cells ($2 \times 10^4 \times mL^{-1}$) were grown to 80% confluence (48–72h) in 24-well plates. The medium was aspirated, the cells were rinsed with DPBS, and a fresh medium or medium containing samples at the appropriate concentration was applied. After 4 h of incubation, cells

were stimulated with a mixture of TNF-α and IFN-γ (10 ng × mL^{-1} each) in DPBS. After 24 h of incubation, supernatants were collected and stored at −20 °C until use. Cells to which only DPBS was added were used as a non-stimulated control.

2.5.5. LTA stimulation

NHDF cells were seeded in 24-well plates and incubated until 80% confluent (5–7 days). Then, the medium was gently aspirated, and the cells were washed with DPBS. Subsequently, the medium or the medium with the dissolved samples at the appropriate concentration was applied, and the cells were stimulated with a solution of LTA from *S. aureus* (10 μg × mL^{-1}) in DPBS. DPBS was added to the non-stimulated control wells. Supernatants were collected after 24 h of incubation and stored at −20 °C until use.

2.5.6. Evaluation of IL-6 and IL-8 secretion

To assess the secretion of cytokines, supernatants from HaCaT and NHDF cells were used after exposure to UVB radiation, stimulation with TNF-α and IFN-γ, and stimulation with LTA. The concentration of cytokines was measured by Enzyme-Linked Immunosorbent Assay (ELISA) tests according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). As a positive control of the inhibition of IL-6 and IL-8 secretion by skin cells, urolithin A was used, which showed a strong inhibitory effect in our previous studies (Piwowarski et al., 2022).

2.5.7. Viability (NRU)

HaCaT (2.5 × 10^3 per well) or NHDF (5 × 10^4 per well) cells were cultured in 96-well plates for 48–72 h to reach a confluence of between 50 and 80%. Then, the test samples with the appropriate concentration dissolved in the medium were added and left for 24 h of incubation. Subsequently, the medium was aspirated, the cells were rinsed with DPBS, and freshly prepared neutral red dye dissolved in the medium (33 μg × mL^{-1}) was added. The cells were incubated with the stain for 3 h. They were then rinsed with DPBS and added 1% glacial acetic acid in 50% (*v/v*) ethanol. The mixture was shaken on a shaker for 20–45 min in the dark until a homogenous mixture was obtained. The absorbance was then measured at 530 nm with a correction at 645 nm. The results are presented as a percentage of the control (medium without tested samples).

2.6. Studies on human neutrophils

2.6.1. Isolation of human neutrophils

Buffy coats, prepared from the peripheral venous blood of healthy donors, were purchased at the Regional Centre of Blood Donation and Blood Treatment in Warsaw. The study was conducted under the approval of the local bioethics committee at the Medical University of Warsaw (AKBE/31/2023). All donors had to meet the following requirements: male, 18–35 years of age, drug-free, routine laboratory test results within the normal range, and clinical confirmation of being healthy. Isolation of neutrophils was performed based on the developed method (Böyum, 1968) by sedimentation of dextran and centrifugation on a Lymphosep, a medium for the separation of lymphocytes. After isolation, cells used for viability and ELISA assays were resuspended in RPMI 1640 medium, and cells for reactive oxygen species (ROS) secretion were resuspended in HBSS.

2.6.2. Evaluation of ROS production

ROS production by f-MLP-stimulated neutrophils was analyzed by the previously described method (Skowrońska et al., 2022). Briefly, to 50 μL of samples at the appropriate concentration, 70 μL of cell suspension (3 × 10^5 × mL^{-1}) and 50 μL of luminol (0.4 mg × mL^{-1}) were added. Before reading the luminescence, 30 μL of the f-MLP (1.5 μg × mL^{-1}) solution was added. Everything was dissolved or suspended in HBSS. Changes in luminol-dependent chemiluminescence were measured every 2 min for 40 min in 96-well white plates. Based on the values recorded in the chemiluminescence maximum, the percent of inhibition of ROS production by the tested samples against the stimulated control was calculated. Quercetin at 5, 10, and 20 μM was a positive control for inhibiting ROS production (Granica et al., 2015).

2.6.3. Evaluation of TNF-α, IL-1β and IL-8 secretion

Immediately after isolation, neutrophils were resuspended in the RPMI 1640 medium. The suspension was incubated with the tested samples for 30 min, and then a solution of LPS (0.1 μg × mL^{-1}) in DPBS, or DPBS for the unstimulated control, was added. After 24h incubation, cells were centrifuged (2000 RPM, 10 min, 4 °C), and supernatants were collected and stored at −20 °C until use. The level of cytokines in the harvested supernatants was determined using ELISA tests according to the manufacturer's instructions. Dexamethasone (DEX) at 1, 0.1, and 0.01 μM was used as a positive control for inhibiting cytokine secretion (Zielińska et al., 2020).

2.6.4. Viability

The effect of test samples on neutrophils' viability was examined using propidium iodide (PI) staining. After collecting the supernatants, the cells were washed twice with DPBS and then incubated for 15 min at room temperature without access to light with a PI solution (0.5 μg × mL^{-1}) in DPBS. Cells were analyzed in BD FACSCalibur flow cytometer, recording 10,000 events for each sample. Triton X-100 (0.1% solution in DPBS), a membrane disruptor, was used as a control substrate (Zanette et al., 2011).

2.7. Inhibition of enzymes in vitro

2.7.1. Elastase

The sample solution (100 μL) in a proper concentration was mixed with 25 μL of elastase solution (0.052 mg × mL^{-1}) (Marijan et al., 2023). After 5 min, 70 μL of N-succinyl-Ala-Ala-Ala-*p*-nitroanilide solution (0.41 mg × mL^{-1}) was added. All solutions were made in Tris-HCl buffer (0.1 M; pH 8.0). The absorbance was measured at 410 nm after 30 min. Ursolic acid in the 3.25–60 μg × mL^{-1} concentration range was used as a positive control.

2.7.2. Collagenase

The sample solution (40 μL) in appropriate concentration was mixed with 20 μL collagenase solution (0.1 mg × mL^{-1}) (Ciganović et al., 2023). After 5 min, 40 μL gelatin solution (3.44 mg × mL^{-1}) was added. Samples, collagenase, and gelatin solutions were made in Tris-HCl buffer (0.1 M; pH 7.5). The mixture was incubated for 40 min at 37 °C in the dark, mixing simultaneously at 180 RPM. After incubation, 40 μL of EDTA solution (25 mM in 12% aqueous PEG solution) and 90 μL of ninhydrin (25 mg × mL^{-1} in DMSO with 0.78 mg × mL^{-1} $SnCl_2 \times 2H_2O$) solution were added, and the mixture was allowed to incubate at 80 °C in the dark for 15 min. After the mixture was cooled to room temperature, 90 μL of citric buffer (0.5 M; pH 4.9) was added, and the absorbance was measured at 545 nm. As a positive control, gallic acid in the concentration range of 78.125–625 μg × mL^{-1} was used.

2.7.3. Lipoxygenase

One hundred μL of sample in the appropriate concentration was mixed with 25 μL of the lipoxygenase solution (3 μg × mL^{-1}) (Jakupović et al., 2023). After 5 min, the 70 μL of linoleic acid solution (0.36 μg × mL^{-1}) was added. All solutions were made in PBS (0.1 mM; pH 8). The absorbance was measured at 234 nm after 40 min on plates without self-absorbance. The positive control was the lipoxygenase inhibitor – nordihydroguaiaretic acid (West et al., 2004) in the concentration range of 0.71–11.4 μg × mL^{-1}.

2.7.4. Hyaluronidase

For anti-hyaluronidase activity determination (Jakupović et al., 2023), 25 μL of sample in the appropriate concentration was combined

with 20 μL of enzyme solution (4 mg × mL^{-1}) and the plate was placed for incubation at 37 °C in the dark with simultaneously mixing (80 RPM) for 20 min. Subsequently, 40 μL $CaCl_2$ (12 mM) was added and re-incubated under the same conditions. Then 50 μL of sodium hyaluronate (3.5 mg × mL^{-1}) was added, and the plate was incubated at 37 °C in the dark with simultaneously stirring (250 RPM) for 40 min. After incubation, 20 μL of NaOH (0.9 m) and 40 μL of sodium tetraborate (0.2 M) were added successively, and the plate was heated for 3 min at 100 °C. After cooling the plate for 5–10 min at room temperature, 130 μL of 5% DMABA, prepared in a mixture of acetic acid (4.4 mL CH_3COOH) and hydrochloric acid (0.6 mL 10 M HCl), was added, and then the plate was placed at 37 °C in the dark for 10 min. The absorbance was measured at 580 nm. Tannic acid was used as a positive control for inhibition of hyaluronidase activity in the concentration range of 2.5–20 μg × mL^{-1}. The sample, enzyme, and sodium hyaluronate solutions were made in an acetate buffer (0.1 M; pH 3.6).

2.7.5. Tyrosinase

One hundred twenty μL of the sample and 40 μL of the freshly prepared *Agaricus bisporus* tyrosinase extract were mixed (Jakupović et al., 2023). After 5 min in the dark, 60 μL of L-3,4-dihydroxyphenylalanine solution (0.83 mg × mL^{-1}) was added. All solutions were made in PBS (16 mM; pH 6). Absorbance was measured at 492 nm after 30 min. Kojic acid in the concentration range of 3.4–54.5 μg × mL^{-1} was used as a tyrosinase inhibitor.

3. Statistical analysis

Three independent experiments in triplicates were performed for all determinations. Results are presented as mean ± standard deviation (SD). The results were analyzed in the Statistica program, version 13 (TIBCO Software INC., 2017; Palo Alto, CA, USA). Normal distribution and homogeneity of variance were analyzed by the Shapiro-Wilk and Brown-Forsythe tests, respectively. Statistical significance was determined using one-way ANOVA, with Dunnett's and Tuckey's post-hoc tests or the Kruskal-Wallis test. Statistical significance was determined at the significance level of $p < 0.05$ and $p < 0.001$. IC_{50} values were calculated based on concentration-inhibition curves.

4. Results

4.1. Phytochemical analysis

4.1.1. UHPLC-DAD-MS^n analysis

UV chromatograms at 254 nm are shown in Fig. 1. Numbers indicate chemical compounds according to retention time. The results and identification of the compounds are summarized in Table 1. Identification of the compounds was based on the analysis of the UV–Vis spectra at which the maximum absorbance occurs and the masses and fragmentations from the mass spectra. Then, the obtained values were compared with standard substances or available literature. Some of the compounds were identified based on ^{1}H NMR spectra.

4.1.2. NMR identification

Spectra of isolated chemical compounds are included in Supplementary materials.

Holocalin ***(6)****:* white needle-like crystals, $C_{14}H_{17}NO_7$, ESI-MS, *m/z* 310 [M-H]$^-$. ^{1}H NMR spectrum (500 MHz, CD_3OD) δ 7.28 (1H, t, $J = 8.0$ Hz, H-5), 7.05–7.03 (1H, m, H-2), 7.02–7.01 (1H, m, H-6), 6.87 (1H, ddd, $J = 8.2$, 2.5, 1.0 Hz, H-4), 5.84 (1H, s, H-7), 4.26 (1H, dd, $J = 5.4$, 2.2 Hz, H-1′), 3.93 (1H, dd, $J = 12.0$, 2.3 Hz, H-6′a), 3.71 (1H, dd, $J = 12.0$, 6.2 Hz, H-6′b), 3.31–3.21 (4H, m, H-2′,3′,4′,5′) (DellaGreca et al., 2000a).

5-O-Caffeoylquinic acid ***(13)****:* white powder, $C_{16}H_{18}O_9$, ESI-MS, *m/z* 353 [M-H]$^-$. ^{1}H NMR spectrum (500 MHz, CD_3OD) δ 7.56 (1H, d, $J = 15.9$ Hz, H-3′), 7.05 (1H, d, $J = 2.1$ Hz, H-2″), 6.96 (1H, dd, $J = 8.5$, 2.1 Hz, H-6″), 6.78 (1H, d, $J = 8.2$ Hz, H-5″), 6.26 (1H, d, $J = 15.9$ Hz, H-2′), 5.33 (1H, td, $J = 9.3$, 4.4 Hz, H-5), 4.17 (1H, dt, $J = 5.5$, 3.3 Hz, H-3), 3.73 (1H, dd, $J = 8.5$, 3.2 Hz, H-4), 2.23 (1H, ddd, $J = 13.3$, 4.4, 2.1 Hz, H-2a), 2.18 (1H, dd, $J = 14.1$, 3.3 Hz, H-2b), 2.11–2.02 (2H, m, H-6a,6b) (Ayeni et al., 2022).

4,6-Dihydroxy-2-O-glucosyl-benzophenone ***(23)****:* white powder, $C_{19}H_{20}O_9$, ESI-MS, *m/z* 391 [M-H]$^-$. ^{1}H NMR spectrum (500 MHz, CD_3OD) δ 7.73 (2H, d, $J = 7.1$ Hz, H-2′,6′), 7.55 (1H, t, $J = 7.4$ Hz, H-4′),

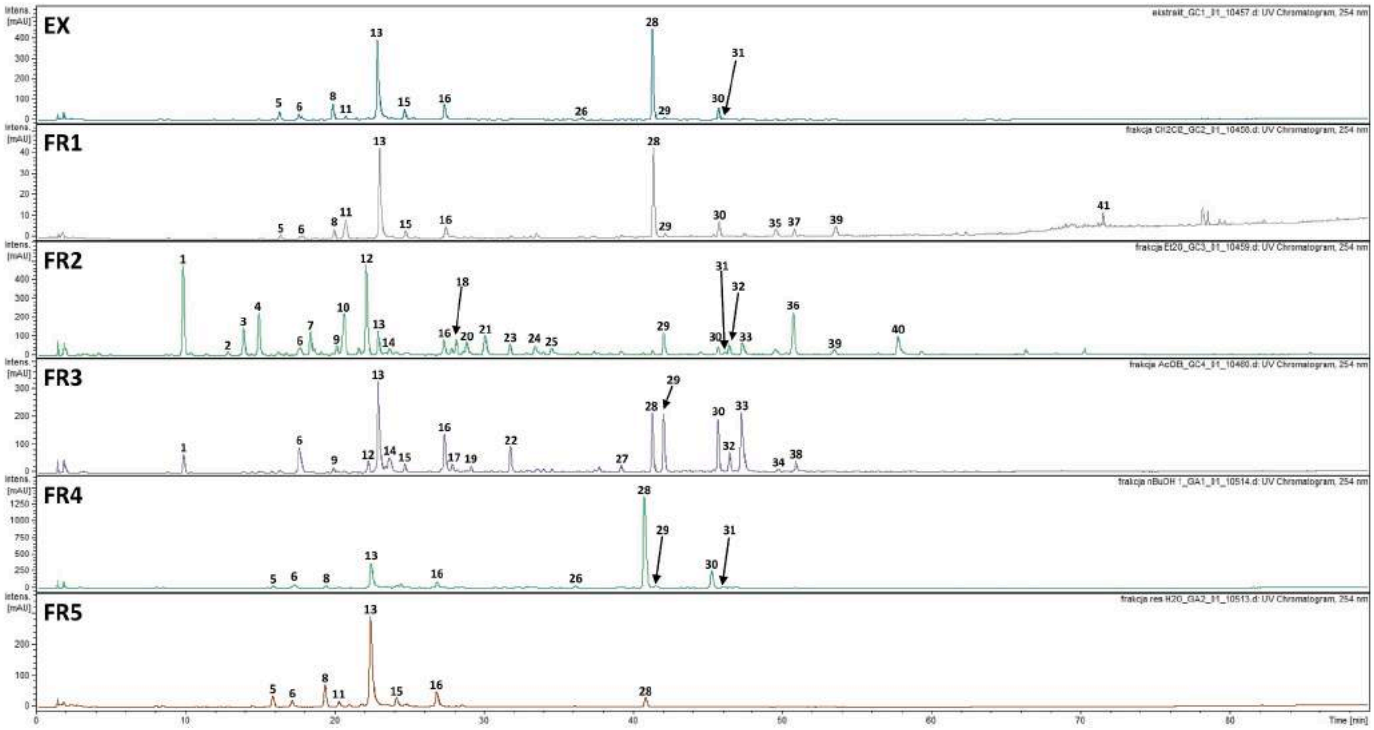

Fig. 1. UV chromatograms of extract (EX) and its fractions (FR1-5) at 254 nm.

Table 1
UHPLC-DAD-MS^n data of compounds detected in extract and fractions of *S. nigra* L. leaves.

No	Compound	Rt [min]	UV–Vis [nm]	[M-H]$^-$ *m/z*	MS^2 ions	MS^3 ions	NL	Ref.
1	protocatechuic acid	9.9	202, 215sh, 259, 293	153	153			Smeriglio et al. (2016)
2	caffeic acid [s]	12.6	214, 262, 282sh	179	179			
3	hydroxybenzoic acid dimer	13.9	202, 228, 279, 310	328	137			
4	dihydroferulic acid	14.9	196, 253	194	194			
5	3-*O*-caffeoylquinic acid	16.4	240, 305sh, 324	353	**191b**, 179, 135	191	162	Clifford et al. (2003)
6	holocalin [n]	17.8	278	356	310			
7	4-hydroxy-propiophenone	18.3	206, 225, 269, 327	149	149b, 121, 93			Attygalle et al. (2006)
8	caffeic acid derivative	19.9	248, 325	297	251, 179, **135b**	135	162	
9	5-*p*-coumaroylquinic acid [+HCOO$^-$]	20.1	198, 282	383	337b, 319, 277			Clifford et al. (2003)
10	undefined	20.5	198, 216, 254, 314	329	283b, 207, 121			
11	dihydroferulic acid hexoside	20.7	245sh, 321	357	339, **195b**, 177	195b, 136	162	Guy et al. (2009)
12	caffeic acid dimer	22.1	216, 233, 303sh, 321	359	179			
13	5-*O*-caffeoylquinic acid [n]	22.9	243, 301sh, 324	353	215, **191b**, 179	191	162	
14	prunasin/sambunigrin [+HCOO$^-$]	23.9	206, 261	340	294b, **187**, 161	187b, 161	152	DellaGreca et al. (2000b)
15	4-*O*-caffeoylquinic acid	24.6	302sh, 322	353	**191**, 179, 173b, 135	191	162	Clifford et al. (2003)
16	caffeoylquinic acid derivative	27.3	244, 303sh, 321	651	514, 458, **353b**, 296	215, 191b, 179		
17	undefined	27.8	198, 215, 274	295	251b, 189			
18	undefined	28.1	216, 260, 295	239	239b, 195			
19	*p*-coumaric acid	28.9	224, 309	163	163			
20	esculin	29.1	215, 305sh, 317	339	277, 179b, **177**, 161	177	162	Jiménez-López et al. (2017)
21	undefined	30.4	193, 225, 281	421	375			
22	5-*O*-feruoylquinic acid	31.8	302sh, 325	367	191			Clifford et al. (2003)
23	4,6-dihydroxy-2-*O*-glucosyl-benzophenone [n]	31.8	252, 297	391	253, **229b**	229	162	
24	undefined	33.5	212, 287, 308sh	281	163b, **135**	135	146	
25	caffeic acid derivative	34.5	202, 231, 305	325	279, **179b**, 164	179	146	
26	quercetin 3-*O*-(2″,6″-di-O-rhamnosyl)-glucopyranoside [n]	36.6	263sh, 350	755	**609b**, 591, 300	373, 343, 301b	146	
27	undefined	39.2	202, 281	431	385, 292, 205, 179b, 161, 143			
28	quercetin 3-*O*-rutinoside (rutin) [n]	41.3	255, 354	609	343, 301b, 271, 179			
29	quercetin 3-*O*-glucopyranoside (isoquercitrin) [n]	42.1	252sh, 355	463	**301b**, 179	271, 179b, 151	162	
30	kaempferol 3-*O*-rutinoside [n]	45.7	265, 344	593	285			
31	syringaresinol 4-*O*-glucopyranoside [n]	46.4	218, 269	579	533, 417b, 255, 179		162	
32	kaempferol 3-*O*-glucopyranoside (astragalin) [n]	46.5	217, 265, 285, 335	447	327, 285b, 255			
33	3,5-di-*O*-caffeoyl-quinic acid [n]	47.4	302sh, 326	515	**353b**, 191	191b, 179, 173		
34	rosmarinic acid	49.8	217, 287	359	**197b**, 179, 161	197	162	Lau et al. (2015)
35	syringaresinol pentoside	49.9	220	549	461, **417b**, 235, 161	255b, 161, 159	132	
36	abscisic acid	50.7	223, 263	263	219, 153			Lin et al. (2021)
37	syringaresinol [+HCOO$^-$]	51.7	223, 283	463	417			
38	dicaffeoylquinic acid	51.0	218, 308sh, 326	515	**353b**, 335, 299, 203, 173	191, 179, 173b	162	Clifford et al. (2005)
39	dimethoxycinnamic acid	53.6	220, 298, 325	207	207			Bottamedi et al. (2021)
40	quercetin [s]	57.8	213, 254, 293, 368	301	257, 179b, 151			
41	pinoresinol 4-*O*-glucopyranoside [n]	71.8	282	519	357			

b – base peak (the most abundant ion in the recorded spectrum); in bold – ions subjected to MS^3 fragmentation; sh – shoulder in UV–Vis spectrum; n – determined by ^{1}H NMR; s – comparison with chemical standard have been made.

7.44 (2H, t, J = 7.7 Hz, H-3′,5′), 6.24 (1H, d, J = 2.0 Hz, H-5), 6.10 (1H, d, J = 2.1 Hz, H-3), 4.84 (1H, d, J = 7.7 Hz, H-1″), 3.88 (1H, dd, J = 12.0, 2.2 Hz, H-6″a), 3.69 (1H, dd, J = 12.1, 5.7 Hz, H-6″b), 3.39–3.36 (2H, m, H-3″,5″), 3.27–3.22 (1H, m, H-4″), 2.86 (1H, dd, J = 9.1, 7.8 Hz, H-2″) (Demirkiran et al., 2009).

Quercetin 3-O-(2″,6″-di-O-rhamnosyl)-glucopyranoside ***(26):*** yellow powder, $C_{33}H_{40}O_{20}$, ESI-MS, *m/z* 755 [M-H]$^-$. ^{1}H NMR spectrum (500 MHz, CD_3OD) δ 7.62 (1H, dd, J = 10.1, 4.0 Hz, H-6′), 7.61 (1H, d, J = 1.7 Hz, H-2′), 6.88 (1H, d, J = 8.3 Hz, H-5′), 6.38 (1H, d, J = 2.1 Hz, H-8), 6.20 (1H, d, J = 2.1 Hz, H-6), 5.60 (1H, d, J = 7.7 Hz, H-1″), 5.23 (1H, d, J = 1.5 Hz, H-1‴‘), 4.52 (1H, d, J = 1.6 Hz, H-1‴), 4.09 (1H, dq, J = 9.7, 6.2 Hz, H-5‴‘), 4.02 (1H, dd, J = 3.3, 1.6 Hz, H-2‴‘), 3.83 (1H, dd, J = 11.2, 1.6 Hz, H-6″a), 3.81 (1H, dd, J = 9.6, 3.4 Hz, H-3‴‘), 3.66 (1H, dd, J = 9.1, 7.7 Hz, H-2″), 3.60 (1H, dd, J = 3.4, 1.7 Hz, H-2‴), 3.55 (1H, t, J = 8.9 Hz, H-3″), 3.50 (1H, dd, J = 9.5, 3.4 Hz, H-3‴), 3.45–3.42 (1H, m, H-5‴), 3.42–3.40 (1H, m, H-6″b), 3.39–3.36 (1H, m, H-4″), 3.36–3.34 (1H, m, H-5″), 3.31–3.27 (1H, m, H-4‴‘), 3.24 (1H, d, J = 9.5 Hz, H-4‴), 1.09 (3H, d, J = 6.2 Hz, H-6‴), 1.01 (3H, d, J = 6.2 Hz, H-6‴‘) (Kazuma, 2003).

Quercetin 3-O-rutinoside ***(28):*** yellow powder, $C_{27}H_{30}O_{16}$, ESI-MS, *m/z* 609 [M-H]$^-$. ^{1}H NMR spectrum (500 MHz, CD_3OD) δ 7.68 (1H, d, J = 2.2 Hz, H-2′), 7.64 (1H, dd, J = 8.5, 2.2 Hz, H-6′), 6.88 (1H, d, J = 8.5 Hz,

H-5′), 6.41 (1H, d, J = 2.1 Hz, H-8), 6.22 (1H, d, J = 2.1 Hz, H-6), 5.12 (1H, d, J = 7.7 Hz, H-1″), 4.53 (1H, d, J = 1.6 Hz, H-1‴), 3.81 (1H, dd, J = 11.0, 1.5 Hz, H-6″a), 3.64 (1H, dd, J = 3.4, 1.7 Hz, H-2‴), 3.55 (1H, dd, J = 9.5, 3.4 Hz, H-6″b), 3.49–3.33 (5H, m, H-2″,3″,4″,3‴,4‴), 3.31–3.26 (2H, m, H-5″,5‴), 1.13 (3H, d, J = 6.2 Hz, H-6‴) (Mouthé Happi et al., 2022).

Quercetin 3-O-glucopyranoside ***(29)****:* yellow powder, $C_{21}H_{20}O_{12}$, ESI-MS, *m/z* 463 [M-H]$^-$. ^{1}H NMR spectrum (500 MHz, CD_3OD) δ 7.72 (1H, d, J = 2.2 Hz, H-2′), 7.59 (1H, dd, J = 8.5, 2.2 Hz, H-6′), 6.87 (1H, d, J = 8.5 Hz, H-5′), 6.40 (1H, d, J = 2.1 Hz, H-8), 6.21 (1H, d, J = 2.1 Hz, H-6), 5.26 (1H, d, J = 7.6 Hz, H-1″), 3.72 (1H, dd, J = 11.9, 2.4 Hz, H-6″a), 3.58 (1H, dd, J = 11.9, 5.4 Hz, H-6″b), 3.49 (1H, dd, J = 9.1, 7.7 Hz, H-2″), 3.43 (1H, t, J = 8.9 Hz, H-3″), 3.35 (1H, t, J = 9.2 Hz, H-4″), 3.23 (1H, ddd, J = 9.7, 5.3, 2.4 Hz, H-5″) (Kim et al., 2004).

Kaempferol 3-O-rutinoside ***(30)****:* yellow powder, $C_{27}H_{30}O_{15}$, ESI-MS, *m/z* 593 [M-H]$^-$. ^{1}H NMR spectrum (500 MHz, CD_3OD) δ 8.07 (2H, d, J = 9.0 Hz, H-2′,6′), 6.90 (2H, d, J = 9.0 Hz, H-3′,5′), 6.42 (1H, d, J = 2.1 Hz, H-8), 6.22 (1H, d, J = 2.1 Hz, H-6), 5.14 (1H, d, J = 7.6 Hz, H-1″), 4.52 (1H, d, J = 1.6 Hz, H-1‴), 3.81 (1H, dd, J = 10.9, 1.4 Hz, H-6″a), 3.64 (1H, dd, J = 3.4, 1.7 Hz, H-2‴), 3.53 (1H, dd, J = 9.5, 3.4 Hz, H-6″b), 3.48–3.36 (5H, m, H-2″,3″,4″,3‴,4‴), 3.27 (2H, dd, J = 9.2, 4.7 Hz, H-5″,5‴), 1.13 (3H, d, J = 6.2 Hz, H-6‴) (Hassan et al., 2014).

Syringaresinol 4-O-glucopyranoside ***(31)****:* white powder, $C_{28}H_{36}O_{13}$, ESI-MS, *m/z* 579 [M-H]$^-$. ^{1}H NMR spectrum (500 MHz, CD_3OD) δ 6.73 (2H, s, H-2,6), 6.67 (2H, s, J = 0.4 Hz, H-2′,6′), 4.88 (1H, d, J = 1.2 Hz, H-1″), 4.78 (1H, d, J = 4.3 Hz, H-7), 4.73 (1H, d, J = 4.5 Hz, H-7′), 4.30 (2H, td, J = 9.0, 7.0 Hz, H-9), 3.93 (2H, dd, J = 9.2, 2.7 Hz, H-9′), 3.87 (6H, s, H-3,5 (2xOCH_3)), 3.86 (6H, s, H-3′,5' (2xOCH_3)), 3.79 (1H, dd, J = 12.0, 2.4 Hz, H-6″a), 3.68 (1H, dd, J = 12.0, 5.2 Hz, H-6″b), 3.50–3.40 (3H, m, H-2″,3″,4″), 3.21 (1H, ddd, J = 9.6, 5.2, 2.4 Hz, H-5″), 3.17–3.12 (2H, m, H-8,8') (Zhang et al., 2022).

Kaempferol 3-O-glucopyranoside ***(32)****:* yellow powder, $C_{21}H_{20}O_{11}$, ESI-MS, *m/z* 447 [M-H]$^-$. ^{1}H NMR spectrum (500 MHz, CD_3OD) δ 8.06 (2H, d, J = 8.8 Hz, H-2′,6′), 6.89 (2H, d, J = 8.8 Hz, H-3′,5′), 6.40 (1H, s, H-8), 6.21 (1H, d, J = 1.5 Hz, H-6), 3.70 (1H, dd, J = 11.9, 2.3 Hz, H-6″a), 3.54 (1H, dd, J = 11.9, 5.5 Hz, H-6″b), 3.48–3.39 (3H, m, H-2″,3″,4″), 3.23–3.20 (1H, m, H-5″) (Kim et al., 2004).

3,5-Di-O-caffeoylquinic acid ***(33)****:* white needle-like crystals, $C_{25}H_{24}O_{12}$, ESI-MS, *m/z* 515 [M-H]$^-$. ^{1}H NMR spectrum (500 MHz, CD_3OD) δ 7.62 (1H, d, J = 15.9 Hz, H-7′), 7.58 (1H, d, J = 15.9 Hz, H-7″), 7.07 (2H, t, J = 2.1 Hz, H-2′,2″), 6.99–6.96 (2H, m, H-6′,6″), 6.79 (2H, dd, J = 8.2, 1.2 Hz, H-5′,5″), 6.36 (1H, d, J = 15.9 Hz, H-8′), 6.27 (1H, d, J = 15.9 Hz, H-8″), 5.43 (2H, dt, J = 12.3, 4.4 Hz, H-3,5), 3.98 (1H, dd, J = 7.5, 3.3 Hz, H-4), 2.32 (2H, dd, J = 13.9, 3.9 Hz, H-6), 2.19 (2H, ddd, J = 25.9, 13.0, 7.0 Hz, H-2) (Abudurexiti et al., 2017).

Pinoresinol 4-O-glucopyranoside ***(41)****:* white powder, $C_{26}H_{32}O_{11}$, ESI-MS, *m/z* 519 [M-H]$^-$. ^{1}H NMR spectrum (500 MHz, CD_3OD) δ 7.18 (1H, d, J = 8.3 Hz, H-5′), 7.06 (1H, d, J = 1.5 Hz, H-2′), 6.98 (1H, d, J = 1.6 Hz, H-2), 6.95 (1H, dd, J = 8.4, 1.8 Hz, H-6), 6.85 (1H, dd, J = 8.1, 1.6 Hz, H-6′), 6.80 (1H, d, J = 8.1 Hz, H-5), 4.91 (1H, dd, J = 7.4, 2.6 Hz, H-1″), 4.80 (1H, d, J = 3.6 Hz, H-7′), 4.74 (1H, d, J = 4.3 Hz, H-7), 4.32–4.25 (2H, m, H-9a,9'a), 3.91 (3H, s, H-3' (OCH_3)), 3.89 (3H, s, H-3 (OCH_3)), 3.88 (2H, s, H-9b,9'b), 3.86–3.83 (1H, m, H-6″a), 3.74–3.70 (1H, m, H-6″b), 3.54–3.48 (2H, m, H-2″,3″), 3.42 (2H, d, J = 5.3 Hz, H-4″,5″), 3.17 (2H, s, H-8,8′) (Kim et al., 2015).

4.2. Studies on HaCaT cell line

4.2.1. Wound healing assay

A significant increase in the migration of keratinocytes of the HaCaT cell line to the scratch site compared to the control was observed for the extract (EX) and the dichloromethane (FR1), diethyl ether (FR2) and butanol (FR4) fractions (Fig. 2). The wound closure ratios were 50.03 ± 4.05, 52.77 ± 5.28, 52.85 ± 6.34, and 47.29 ± 9.13%, respectively, while in control it was 23.74 ± 7.32%. The ethyl acetate (FR3) and aqueous residue (FR5) fractions did not increase cell migration, and the

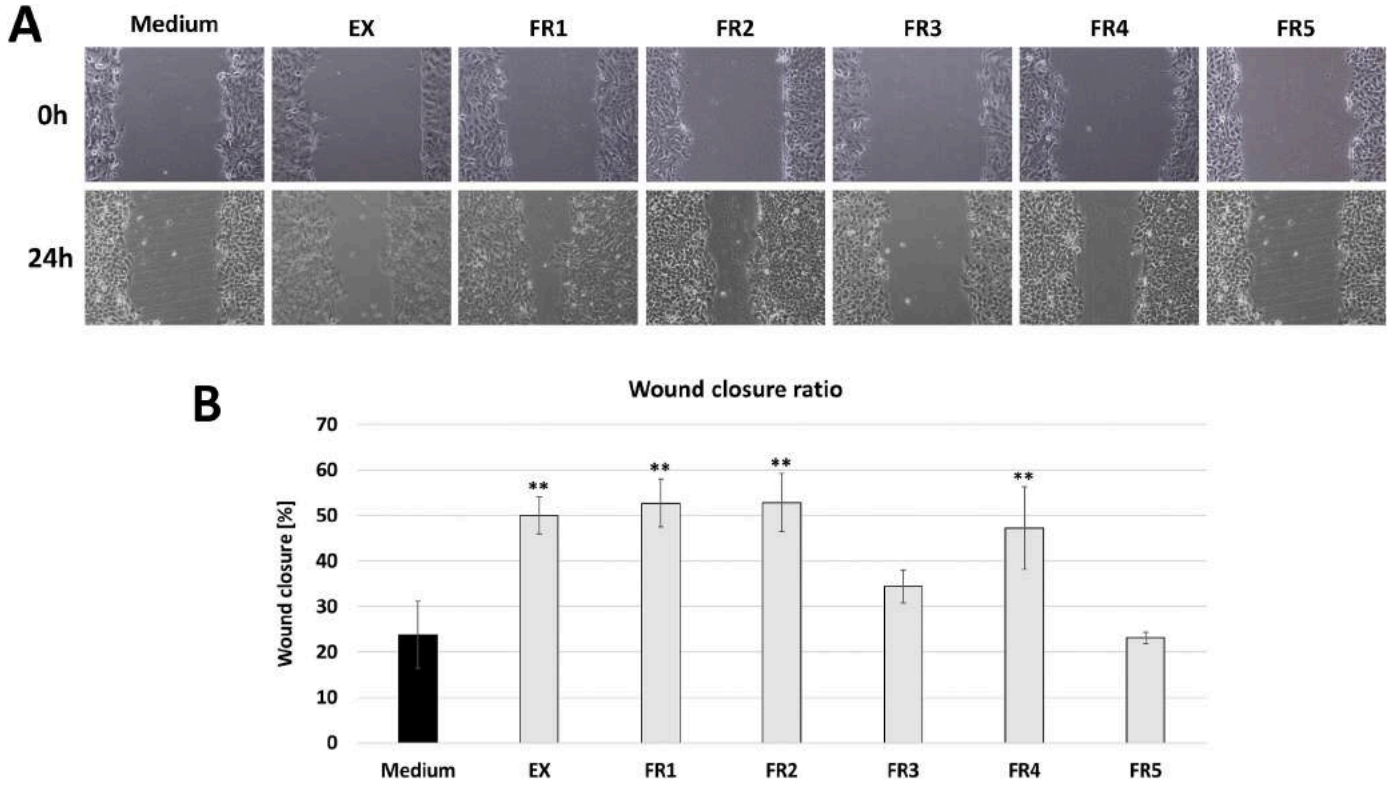

Fig. 2. The effect of *S. nigra* leaves extract and its fractions on migrating HaCaT keratinocytes to the scratch site. Samples were tested at 100 μg × mL^{-1}. **A** – representative photographs for each tested group at the beginning (0h) and after 24 h of incubation (24h). **B** – the ratio of wound closure. The results are presented as the mean ± SD of three experiments. ** – statistically significant impact on cell migration compared to medium ($p < 0.01$).

wound closure ratios were 34.37 ± 3.67 and 23.11 ± 1.25%, respectively.

4.2.2. UVB stimulation

After irradiation of HaCaT cells with UVB radiation at the dose of 20 mJ × cm^{-2}, the level of their secretion of interleukin 6 (IL-6) and interleukin 8 (IL-8) increased (Fig. 3). Incubation of irradiated cells with EX, FR2, FR3, and FR4 at 50 and 100 μg × mL^{-1} increased IL-6 secretion. However, incubation with FR1 and FR5 at concentrations of 50 and 100 μg × mL^{-1} did not affect IL-6 release but slightly reduced it at 5 μg × mL^{-1} (Fig. 3A). In contrast, incubation of irradiated cells with the extract and its fractions significantly decreased the secretion of IL-8 (Fig. 3B). FR1 and FR2 were the most potent, reducing IL-8 secretion by 43 and 45%, respectively, at a concentration of 100 μg × mL^{-1}. EX, FR3, FR4, and FR5 at 100 μg × mL^{-1} reduced IL-8 secretion by 28, 29, 24, and 31%, respectively. Urolithin A (UroA), used at concentrations of 6.25, 12.5, and 25 μM, lowered both IL-6 and IL-8 secretion in a concentration-dependent manner.

4.2.3. TNF-α and IFN-γ stimulation

Adding a mixture of TNF-α and IFN-γ increased the secretion of IL-6 and IL-8 by HaCaT keratinocytes (Fig. 4). After 24 h of incubation of the cells with the tested samples, a significant increase in the release of IL-6 by the cells was observed in a concentration-dependent manner (Fig. 4A). The highest increase in secretion was observed for FR3 and FR4, which at a concentration of 100 μg × mL^{-1}, stimulated release by 120 and 140%, respectively, relative to the stimulated control. TNF-α and IFN-γ incubated cells with EX, FR1, and FR2, slightly decreasing IL-8 secretion (Fig. 4B). However, incubation with FR4 at 50 and 100 μg × mL^{-1} increased IL-8 secretion by 41 and 45% over the control, respectively. Incubation with FR3 and FR5 did not affect IL-8 secretion. Urolithin A significantly and dose-dependently decreased the secretion of both IL-6 and IL-8.

4.2.4. Viability

The extract, fractions, and urolithin A did not adversely affect the viability of HaCaT cells in the concentrations used. Triton X-100 was used as a positive control, which, as a detergent, damages cell

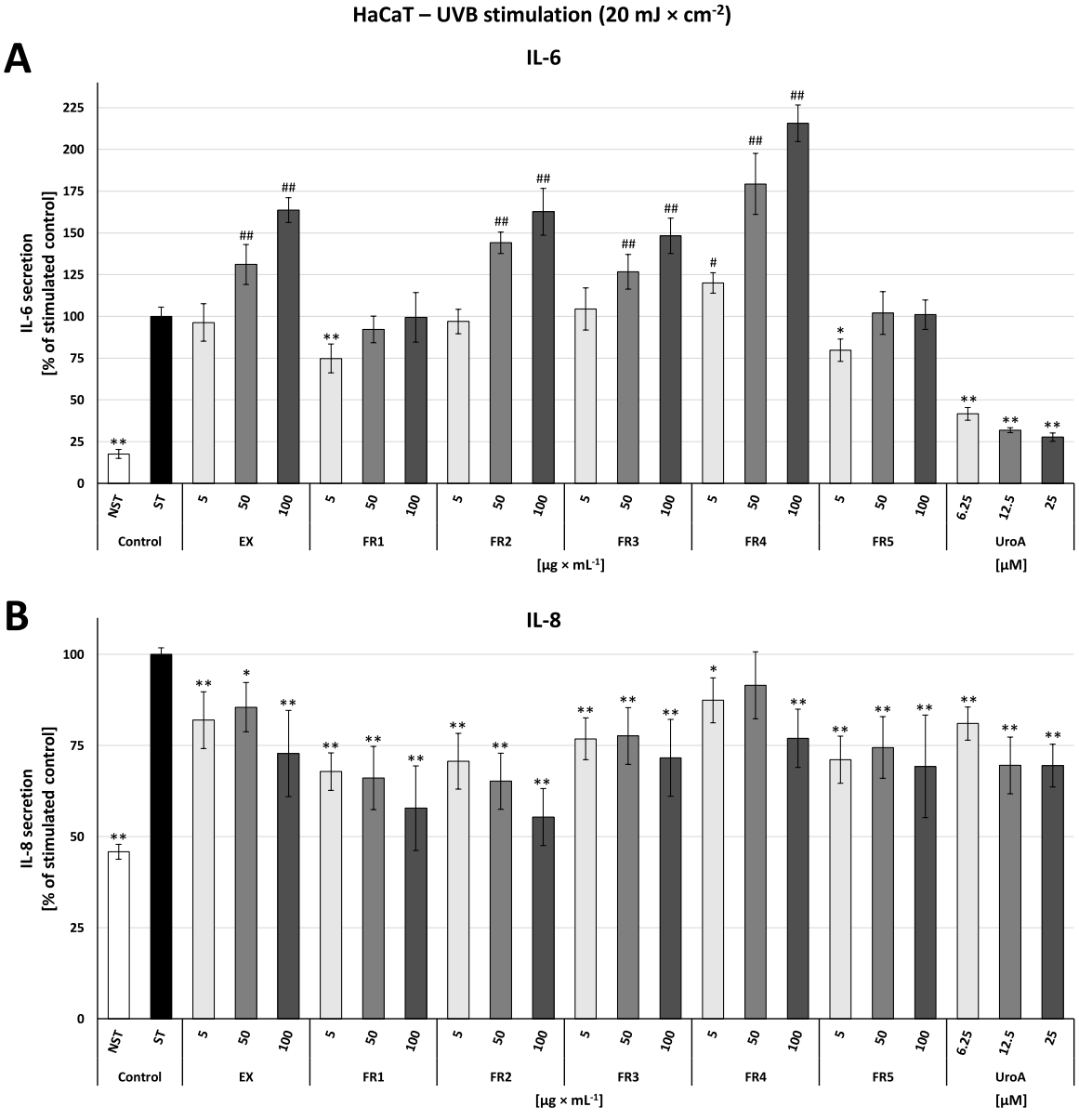

Fig. 3. Effect of 24 h incubation with *S. nigra* extract or its fractions on the secretion of IL-6 (**A**) and IL-8 (**B**) by HaCaT keratinocytes irradiated with UVB at a dose of 20 mJ × cm^{-2}. Statistically significant higher (#p < 0.05, ##p < 0.001) or lower (*p < 0.05, **p < 0.001) secretion relative to ST is marked.

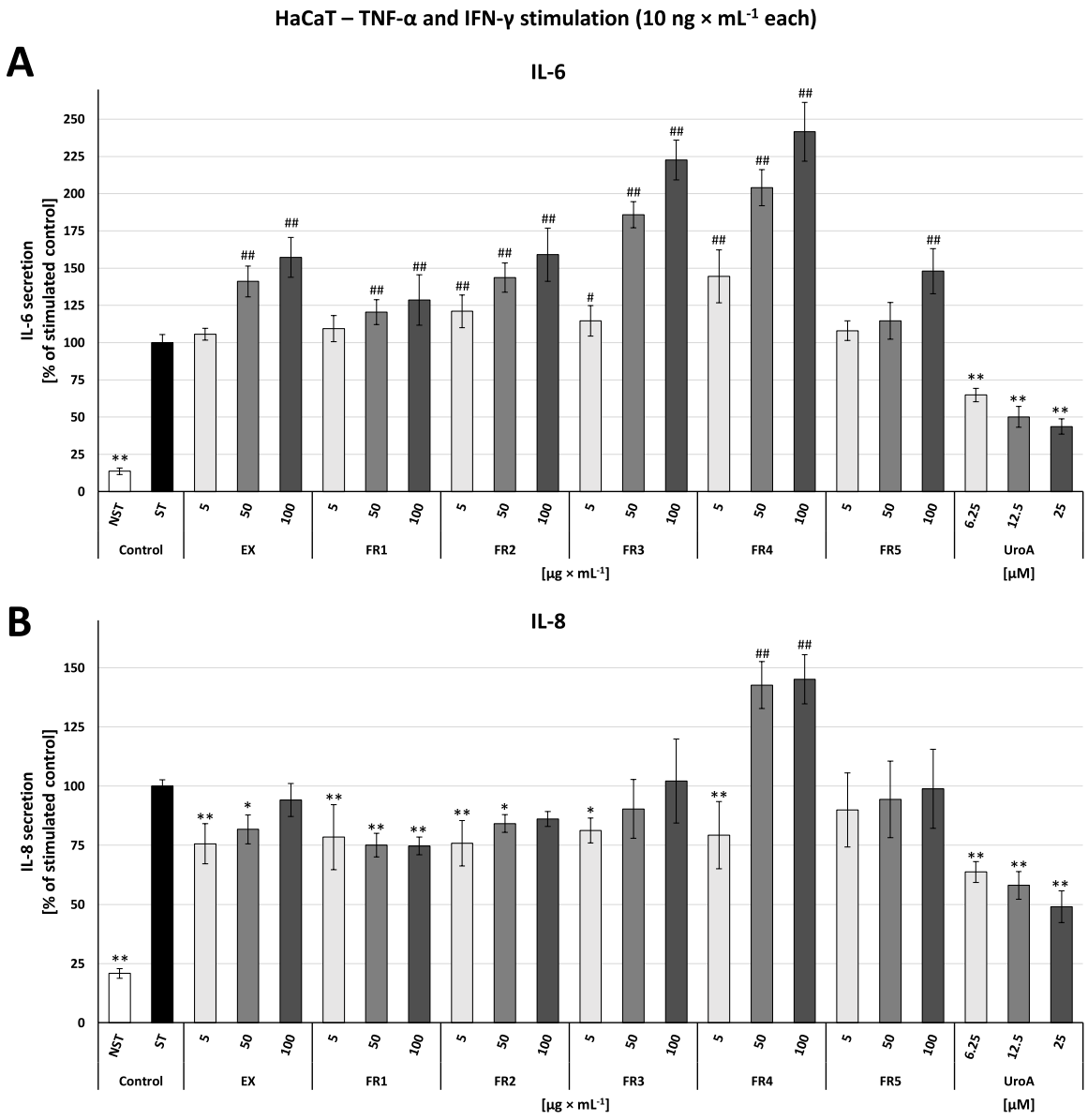

Fig. 4. Effect of 24 h incubation with *S. nigra* extract or its fractions on the secretion of IL-6 (**A**) and IL-8 (**B**) by HaCaT keratinocytes stimulated with TNF-α and IFN-γ (10 ng × mL^{-1} each). Statistically significant higher (#$p < 0.05$, ##$p < 0.001$) or lower (*$p < 0.05$, **$p < 0.001$) secretion relative to ST is marked.

membranes and leads to their death.

4.3. Studies on NHDF cell line

4.3.1. LTA stimulation

When NHDF are exposed to lipoteichoic acid, which is present in the cell walls of *S. aureus*, the cells secrete increased amounts of the cytokines IL-6 and IL-8. Incubation with the extract, fractions, and urolithin A significantly reduced their secretion (Fig. 5). IL-6 secretion was most strongly inhibited by EX, FR1, and FR2, which at a concentration of 100 µg × mL^{-1}, reduced its secretion by 67, 70, and 75%, respectively. FR3 and FR5 at this concentration decreased IL-6 secretion by 52% and 43%, respectively. FR4 had the weakest effect, inhibiting the release of IL-6 by about 20%, regardless of the concentration. In contrast, IL-8 secretion was most strongly inhibited by FR3. At a concentration of 100 µg × mL^{-1}, FR3 and FR5 reduced IL-8 secretion by 47 and 48%, respectively. EX, FR1, FR2, and FR4 at this concentration reduced IL8 release by 31, 30, 28, and 24%, respectively. Urolithin A inhibited the secretion of both IL-6 and IL-8 in a concentration-dependent manner.

4.3.2. Viability

The effect on viability was tested using neutral red. Neither the extract and fractions nor urolithin A do not adversely affect NHDF viability in the concentration range of 5–100 µg × mL^{-1}.

4.4. Studies on human neutrophils (PMNs)

4.4.1. Effect on ROS production

The extract and all fractions strongly inhibited ROS secretion by f-MLP-stimulated human neutrophils in a concentration-dependent manner (Fig. 6). The FR1-FR4 fractions were the most effective, scavenging over 50% of ROS at a concentration of 5 µg × mL^{-1} and almost 100% ROS at a concentration of 100 µg × mL^{-1}. The aqueous fraction (FR5) was the least active. Quercetin was a potent ROS scavenger, depending on the concentration.

4.4.2. Effect on TNF-α, IL-1β and IL-8 release

After stimulation of human neutrophils with LPS from *E. coli* (0.1 µg × mL^{-1}), the secretion of TNF-α, IL-1β, and IL-8 increased (Fig. 7).

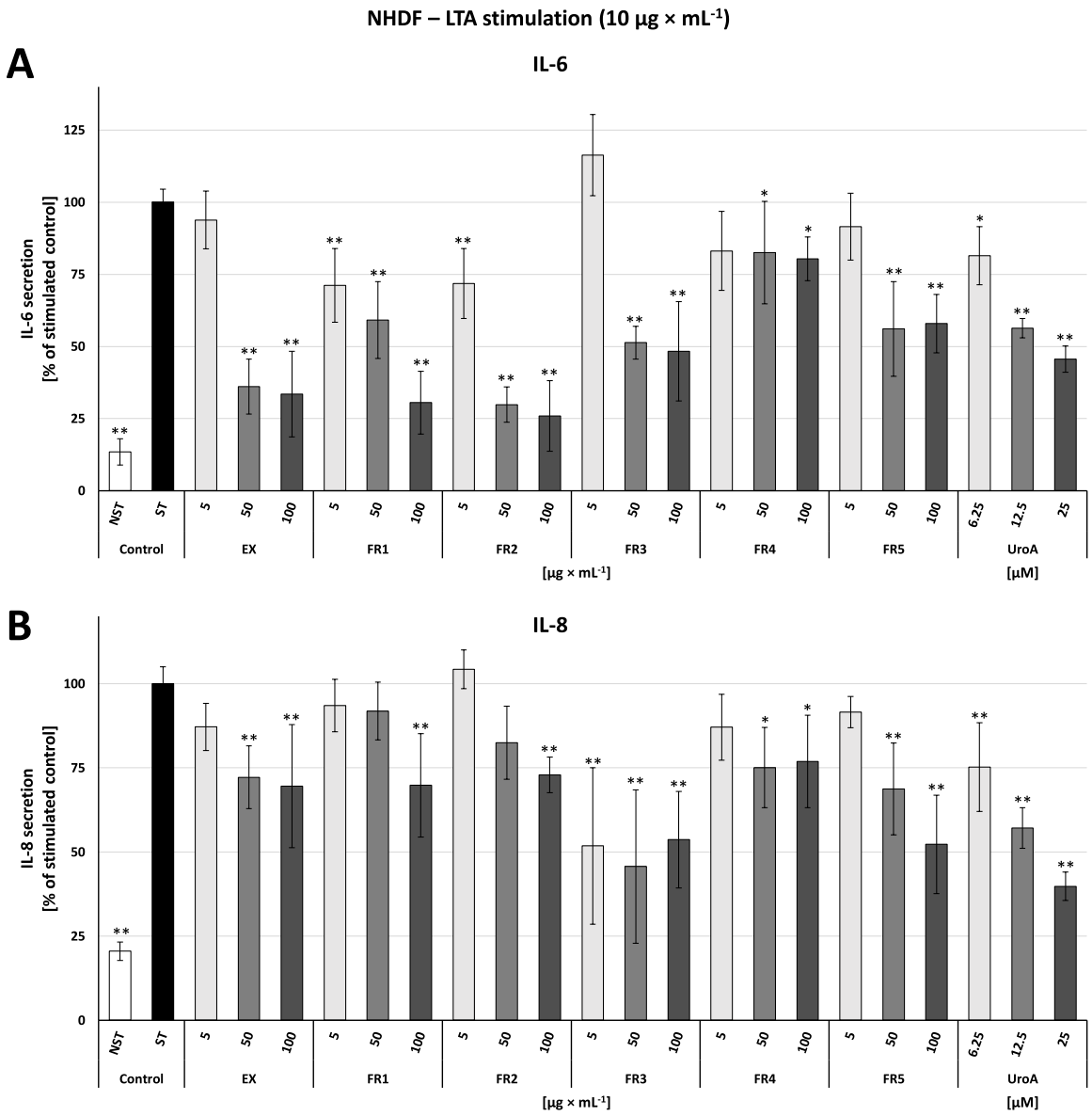

Fig. 5. Effect of 24 h incubation with *S. nigra* extract or its fractions on the secretion of IL-6 (**A**) and IL-8 (**B**) by NHDF stimulated with LTA from *S. aureus* (10 µg × mL^{-1}). Statistically significant lower secretion than ST is marked (*$p < 0.05$, **$p < 0.001$).

Incubation with the tested samples, except for FR1, resulted in a significant decrease in TNF-α secretion in a concentration-dependent manner (Fig. 7A). The extract at a concentration of 50 and 100 µg × mL^{-1} reduced TNF-α secretion by 39 and 69%, respectively. FR3 and FR4 had the most potent effect of the fractions, inhibiting TNF-α secretion by 79 and 81%, respectively, at the concentration of 100 µg × mL^{-1}. FR2 and FR5 fractions at this concentration inhibited TNF-α release by 66 and 70%, respectively. On the other hand, FR1 at concentrations of 50 and 100 µg × mL^{-1} increased TNF-α secretion by 100 and 70%, respectively. Secretion of IL-1β was inhibited only by FR2 at concentrations of 50 and 100 µg × mL^{-1} by 27 and 45%, respectively (Fig. 7B). However, the extract and other fractions stimulated the secretion of this cytokine. The highest level was recorded for FR1 - respectively 127 and 182% more than the stimulated control for concentrations of 50 and 100 µg × mL^{-1}. IL-8 secretion was strongly encouraged by FR1 – more than 560% at 100 µg × mL^{-1} (Fig. 7C). Additionally, FR2 at this concentration increased IL-8 secretion by 170%. However, the extract and the remaining fractions did not affect the release of IL-8. Dexamethasone (DEX), used as a positive control, decreased cytokine secretion in a concentration-dependent manner and following previous experiments (Skowrońska et al., 2022).

4.4.3. Viability

The extract, FR3-FR5 fractions, and dexamethasone did not adversely affect the viability of neutrophils in the tested concentration range. FR1 at 100 µg × mL^{-1} and FR2 at 50 and 100 µg × mL^{-1} reduced cell viability to 95.98 ± 2.17, 95.23 ± 1.73, and 92.44 ± 3.47%, respectively. 0.1% Triton X-100 was cytotoxic, reducing cell viability to 5.51 ± 3.22%. No significant differences were observed in the viability of the LPS-stimulated and non-stimulated control cells.

4.5. Enzymes inhibition

The extract and its fractions affected the activity of the tested enzymes: elastase, collagenase, lipoxygenase, hyaluronidase, and tyrosinase (Table 2.), but to various extent. Elastase activity was most strongly inhibited by FR4, for which the IC_{50} was 154.55 ± 7.88 µg × mL^{-1}. FR1 and FR2 most strongly inhibited collagenase and lipoxygenase activity.

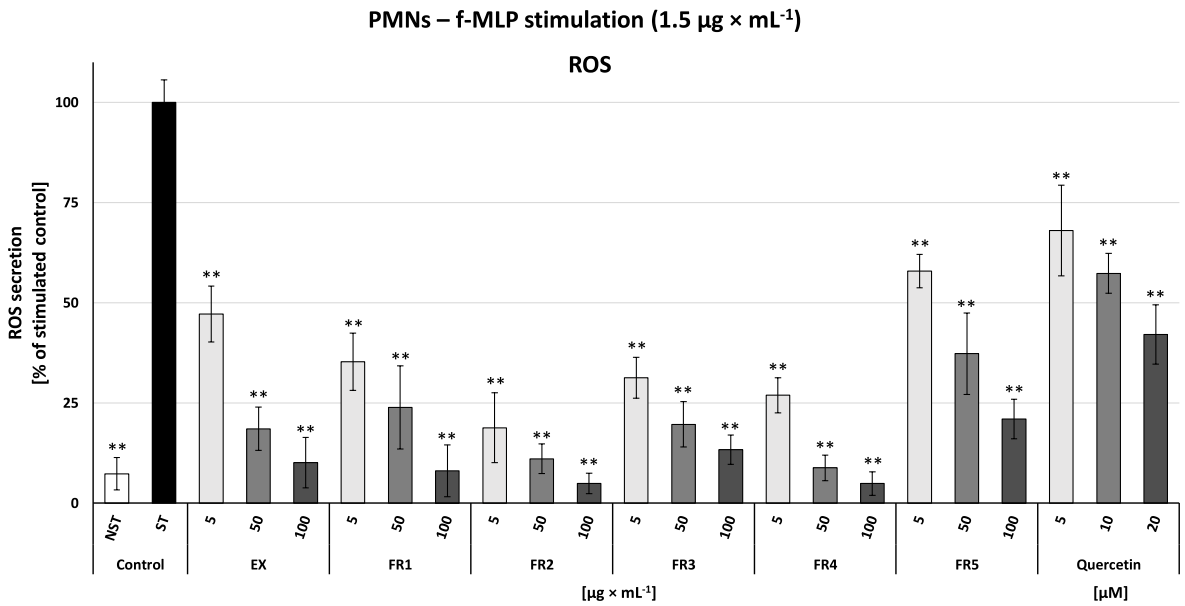

Fig. 6. Effect of 24 h incubation with *S. nigra* extract or its fractions on the secretion of ROS by human neutrophils stimulated with f-MLP (1.5 µg × mL^{-1}). Statistically lower secretion than ST is marked (**$p < 0.001$).

It is interesting to note that the anti-lipoxygenase activity of FR2 was equal to the activity of the positive control, nordihydroguaiaretic acid. On the other hand, EX, FR1, and FR2 inhibited hyaluronidase activity, albeit weaker than the positive control, tannic acid. The tested samples did not affect tyrosinase activity.

5. Discussion

Herbal products have been used for centuries to treat dermatological diseases, alleviate their symptoms, and heal wounds. In recent decades, we have observed increased interest in natural medicine and using natural products in therapy and cosmetology. Applied herbal preparations were often used in folk medicine to treat skin diseases. For many plants and their ingredients, numerous biological and clinical studies are conducted to scientifically verify their activity and mechanism of action (Yazarlu et al., 2021). In our work, we examined the properties of *Sambucus nigra* leaves, which in traditional medicine have been used to treat and soothe insect bites, burns, wounds, scratches, hemorrhoids, and ulcers. We analyzed the effect of a 70% (*v/v*) ethanolic extract of elderberry leaves and its fractions: dichloromethane (FR1), diethyl ether (FR2), ethyl acetate (FR3), *n*-butanol (FR4), and water residue (FR5), on the inflammation of cells directly involved in the wound healing process, i.e., neutrophils, keratinocytes, and fibroblasts. In addition, we investigated their effect on the migration of keratinocytes to the scratch site and the activity of enzymes related to repair processes. The chemical composition was also analyzed, and NMR confirmed the structure of the isolated compounds.

Inflammation is an essential step in the normal physiological process of wound healing. Inflammatory cells, such as neutrophils and pro-inflammatory macrophages, fight pathogens and mobilize other tissue cells to repair the damage by secreting several cytokines and chemokines. The reactive oxygen species secreted in this process, mainly hydrogen peroxide and superoxide anion radical, may benefit the healing process, mainly in the re-epithelialization stage (Wlaschek et al., 2019). However, the overproduction of ROS can disturb cellular homeostasis, damage cellular structures, and lead to severe diseases. Examples include non-healing chronic wounds such as diabetic foot, chronic venous leg ulcers, and pressure ulcers. The pathophysiology of these diseases is not entirely understood, but maintaining the inflammatory balance is an essential therapeutic component (Yao et al., 2019). In chronic wounds associated with diabetes, it has been observed that hyperglycemia can cause the downregulation of epithelial lymphoid markers, including VEGFR3 and its ligand VEGF-C, by macrophages (Maruyama et al., 2007).

On the other hand, increasing the concentration of IL-1β led to increased expression of these markers in diabetic *db/db* mice (Zykova et al., 2000). Increased concentration of IL-1β results in an increase in the formation of lymphatic vessels, thus accelerating the formation of granulation tissue in chronic wounds associated with hyperglycemia. Elevated levels of TNF-α have also been found in chronic injuries related to hyperglycemia. Studies have shown a relationship between TNF-α levels and wound healing in hyperglycaemic and hypercholesterolemic *ob/ob* mice. Treatment with the anti-TNF-α antibody reduced overall inflammation, improved wound contraction, and formed granulation tissue, leading to complete re-epithelialization. It has been shown that anti-inflammatory pharmacological strategies affecting the reduction of such inflammatory mediators as TNF-α can restore tissue sensitivity to insulin and thus improve the healing of chronic wounds (Goren et al., 2006). In the studies presented above, we showed that the *S. nigra* leaves extract and its fractions significantly reduced the level of ROS and TNF-α and modulated the secretion of IL-1β by human neutrophils. These results may indicate their beneficial effect on the healing of chronic wounds, primarily related to diabetes and hyperglycemia. Fractions FR1 and FR2 strongly stimulated IL-8 secretion by neutrophils. Consistent with previous studies (Jiang et al., 2012), the increase in IL-8 secretion correlates with increased migration of IL-8R-expressing keratinocytes to the site of injury, which is beneficial in regenerating damage.

Intensified inflammatory processes and impaired immune response characterize atopic dermatitis (AD). The acute phase of AD is accompanied by infiltration of $CD4^+$ T cells and secretion of cytokines such as IL-4, IL-5, and IL-13. However, in chronic conditions, large amounts of TNF-α and IFN-γ are secreted by Th1 cells (Choi et al., 2013). An increase in the concentration of TNF-α/IFN-γ leads to the activation of several pathways, such as STAT-1/Jak-2, MAPK, and NF-κB, and then to a synergistic increase in the secretion of other molecules such as RANTES, TARC, IL-6, and IL-8. There is an exacerbation of the inflammatory response, which may turn into a chronic state due to the complex mechanism of formation involving a number of receptors and biochemical changes (Yang et al., 2018).

In our research, we analyzed the effect of *S. nigra* extract and fraction

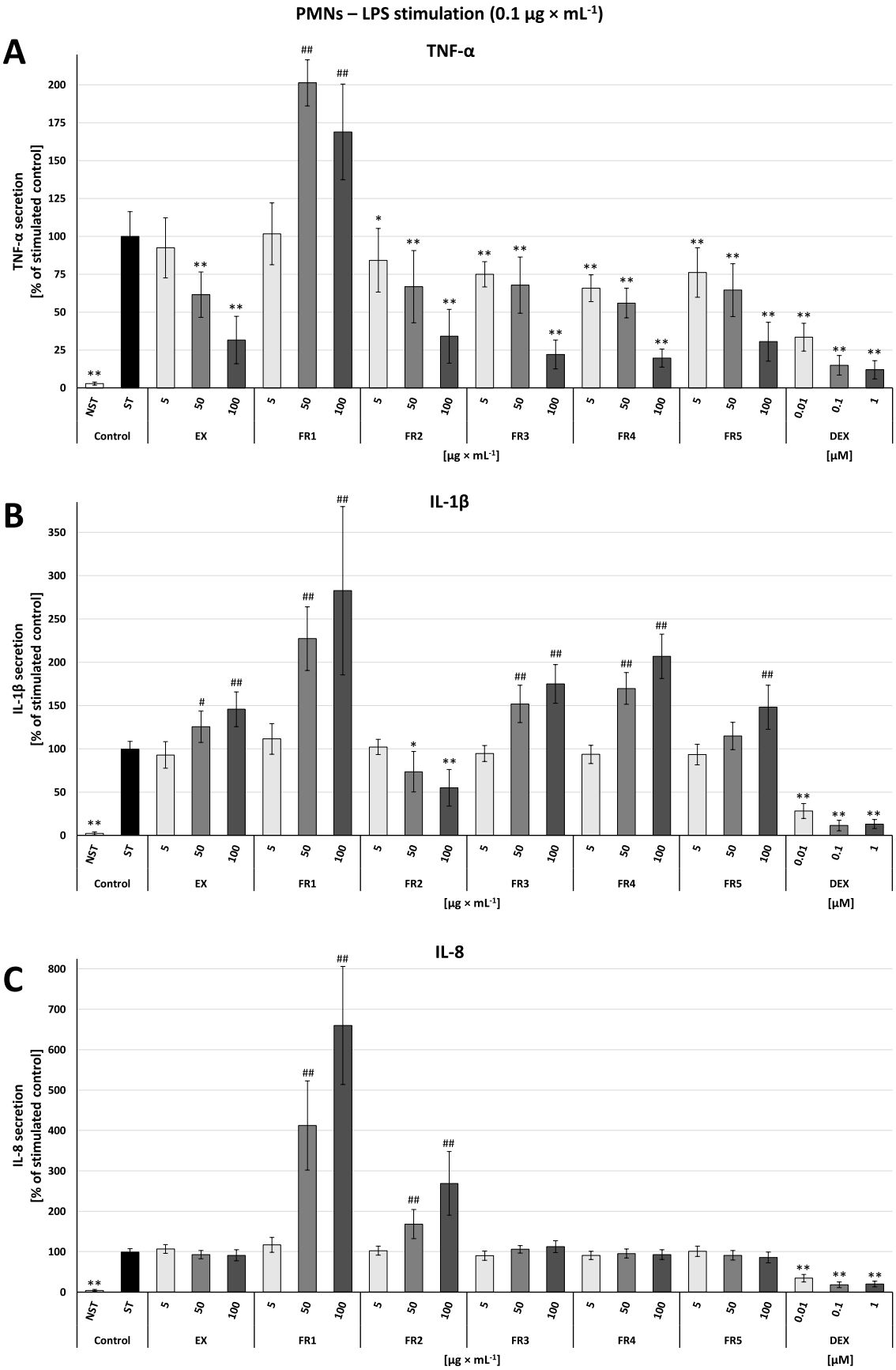

Fig. 7. Effect of 24 h incubation with tested samples on the secretion of TNF-α (**A**), IL-1β (**B**), and IL-8 (**C**) by LPS-stimulated human neutrophils. Statistically significant higher ($\#p < 0.05$, $\#\#p < 0.001$) or lower ($*p < 0.05$, $**p < 0.001$) secretion relative to ST is marked.

Table 2
IC_{50} values [μg × mL^{-1}] of inhibition of enzymes.

	Elastase	Collagenase	Lipoxygenase	Hyaluronidase	Tyrosinase
EX	>500*	428.74 ± 64.89^{abx}	294.34 ± 43.49^{a}	129.29 ± 2.85^{a}	>500*
FR1	431.64 ± 5.69^{a}	236.94 ± 58.75^{ax}	170.05 ± 30.71^{bc}	136.81 ± 5.72^{a}	>500*
FR2	>500*	255.71 ± 42.58^{abx}	75.88 ± 13.79^{bx}	173.66 ± 4.46^{b}	>500*
FR3	>500*	383.94 ± 98.43^{abx}	221.09 ± 55.07^{ac}	>500*	>500*
FR4	154.55 ± 7.88^{b}	414.36 ± 82.05^{abx}	342.51 ± 65.95^{a}	>500*	>500*
FR5	>500*	458.55 ± 85.12^{bx}	>500*	>500*	>500*
Control	Ursolic acid	Gallic acid	Nordihydro-guaiaretic acid	Tannic acid	Kojic acid
	21.95 ± 2.36^{x}	356.59 ± 63.96^{x}	2.07 ± 0.47^{x}	18.31 ± 2.86^{x}	2.69 ± 0.08^{x}

^{a,b,c} – differences between samples within a column ($p < 0.05$), ^{x} – no differences with the positive control ($p < 0.05$). Columns not connected with the same letter are statistically different. * - value not included in statistical analysis.

on the secretion of the main cytokines – IL-6 and IL-8 by keratinocytes stimulated with a mixture of TNF-α and IFN-γ. The tested samples caused a decrease in IL-8 secretion but also a concentration-dependent stimulation of IL-6 secretion. After incubating keratinocytes exposed to UVB radiation with the extract and fractions, a similar effect was achieved. UVB radiation is essential for the synthesis of vitamin D in human skin. However, prolonged exposure or too high doses of UVB radiation cause damage to lipid molecules, proteins, and nucleic acids, causing inflammatory damage to the skin, including infiltration of inflammatory cells and increased production of pro-inflammatory factors. This results in redness, swelling, and an increase in temperature, and the long-term effect of increased exposure may be skin aging, apoptosis, or carcinogenesis (Lee et al., 2013). After exposure to UVB radiation, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are released, and increased activity of cyclooxygenase-2 (COX-2) or inducible nitric oxidase synthase (iNOS) is observed. In addition, an inflammatory response is triggered, including the p38 MAPK and JNK pathways. The dominant cytokines released from skin cells are IL-6 and IL-8 (Jaisin et al., 2020). The studies carried out so far have shown that some plant extracts inhibit the secretion of these cytokines, which proves their photoprotective effect and could be used in cosmetic formulations (Cavinato et al., 2017).

The skin of patients with chronic skin diseases such as AD is characterized by impaired skin barrier function, impaired immune and inflammatory skin responses, and dysbiosis of the skin microbiota, resulting in reduced diversity and susceptibility to colonization by pathogens (Iwamoto et al., 2019). It is estimated that the skin of 60–100% of patients suffering from AD is colonized by *Staphylococcus aureus*, while its colonization affects 5–30% of healthy people. Colonization by *S. aureus* exacerbates AD symptoms and a worse prognosis (Kim et al., 2019). The secretion of factors by this pathogen and components of its cell wall leads to a drastic increase in the body's inflammatory and immune response, exacerbating symptoms. In addition, during damage to the epidermis caused by, for example, scratching, abrasions, or cracking caused by drying, which is typical during AD, *S. aureus* can penetrate the deeper layers of the skin, impairing its functions (Hwang et al., 2021; Iwamoto et al., 2019). In our study, we subjected normal human skin fibroblasts of the NHDF cell line to lipoteichoic acid isolated from the cell wall of *S. aureus*. We observed a significant increase in the secretion of IL-6 and IL-8 by cells, which was inhibited by the tested samples. These results prove that *S. nigra* leaves extract and its components may help maintain the inflammatory balance in the deeper layers of the skin.

The proteins present in the skin, such as collagen and elastin, are responsible for the proper condition and the appearance of the skin, including its integrity, elasticity, and flexibility. Aging, stress, or UV radiation leads to increased inflammation and accumulation of ROS, which increase the activity of enzymes, such as collagenase and elastase, that break down structural proteins. The activity of hyaluronidase, which breaks down hyaluronic acid, a polysaccharide responsible for proper hydration and turgor of the skin, also increases. Inhibiting the activity of these enzymes has, therefore, a positive effect on skin function and appearance (Jiratchayamaethasakul et al., 2020). In our research, we have shown that *S. nigra* leaf extract and several of its fractions may effectively reduce the activity of the enzymes, albeit to various extents. Overall, the dichloromethane fraction showed the most potent effect on the tested enzymes, followed by the diethyl ether fraction. In addition, exposure to UV radiation and other noaxe may produce inflammatory reactions that impair the physiological function of the skin (Nichols and Katiyar, 2010). Under the influence of several among the tested samples, especially the diethyl eter fraction, the activity of lipoxygenase was reduced, proving the anti-inflammatory activity of elderberry leaves. The tested samples did not affect the activity of tyrosinase, the enzyme responsible for the synthesis of skin pigment melanin.

6. Conclusion

Sambucus nigra leaves have been used in traditional European medicine externally in the treatment of wounds and dermatological diseases of the skin and mucous membranes, such as erysipelas, acne, abscesses, eye inflammation, or gingivitis. The effect of the extract was verified for the first time on *in vitro* models of wound healing and inflammation of cells involved in repair processes. The extract and some of its fractions accelerated the healing of the scratch and reduced the secretion of IL-8 by keratinocytes, IL-6 and IL-8 by fibroblasts, and ROS, TNF-α, and IL-8 by neutrophils. In addition, they affected the activity of enzymes present in the skin. The phytochemical analysis showed the presence of chemical compounds, including phenolic acids, flavonoids, lignans, and cyanogenic glycosides. Due to the content of cyanogenic glycosides in both the extract and all its fractions, it is possible to apply these preparations only topically.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Weronika Skowrońska: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization. **Sebastian Granica:** Conceptualization, Methodology, Software, Resources, Writing – review & editing, Supervision. **Jakub P. Piwowarski:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Lejsa Jakupović:** Investigation, Writing – review & editing. **Marijana Zovko Končić:** Methodology, Resources, Writing – review & editing, Supervision. **Agnieszka Bazylko:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, All the listed authors have read and approved the submitted manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2023.117423.

Abbreviations

AD	atopic dermatitis
COX	cyclooxygenase
DEX	dexamethasone
DMEM	' 'Dulbecco's Modified Eagle Medium
DMSO	- dimethyl sulfoxide
DPBS	' 'Dulbecco's Phosphate Buffered Saline
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EX	*Sambucus nigra* L. leaves extract
FBS	foetal bovine serum
FR	fraction of *Sambucus nigra* L. extract
f-MLP	- *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine
HaCaT	spontaneously immortalized human keratinocytes cell line
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	concentrationn required to inhibit 50% activity of enzyme
IFN-γ	interferon gamma
IL	interleukin
iNOS	inducible nitric oxidase synthase
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHDF	normal human dermal fibroblasts
NRU	neutral red uptake
NST	non-stimulated control
PBS	Phosphate Buffered Saline
PI	propidium iodide
RANTES	regulated on activation, normal T cell expressed and secreted
ROS	reactive oxygen species
RNS	reactive nitrogen species
RPMI	Roswell Park Memorial Institute
ST	stimulated control
STAT	signal transducer and activator of transcription
TARC	thymus and activation-regulated chemokine
TLC	thin layer chromatography
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
UHPLC-DAD-MS^n	ultra-high-performance liquid chromatography with diode array detection coupled with mass spectrometry
UroA	urolithin A
USD	United States dollar
UV	ultraviolet
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor.

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SUPPLEMENTARY MATERIALS

Wound healing potential of extract from *Sambucus nigra* L. leaves and its fractions

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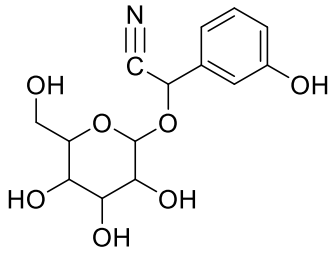
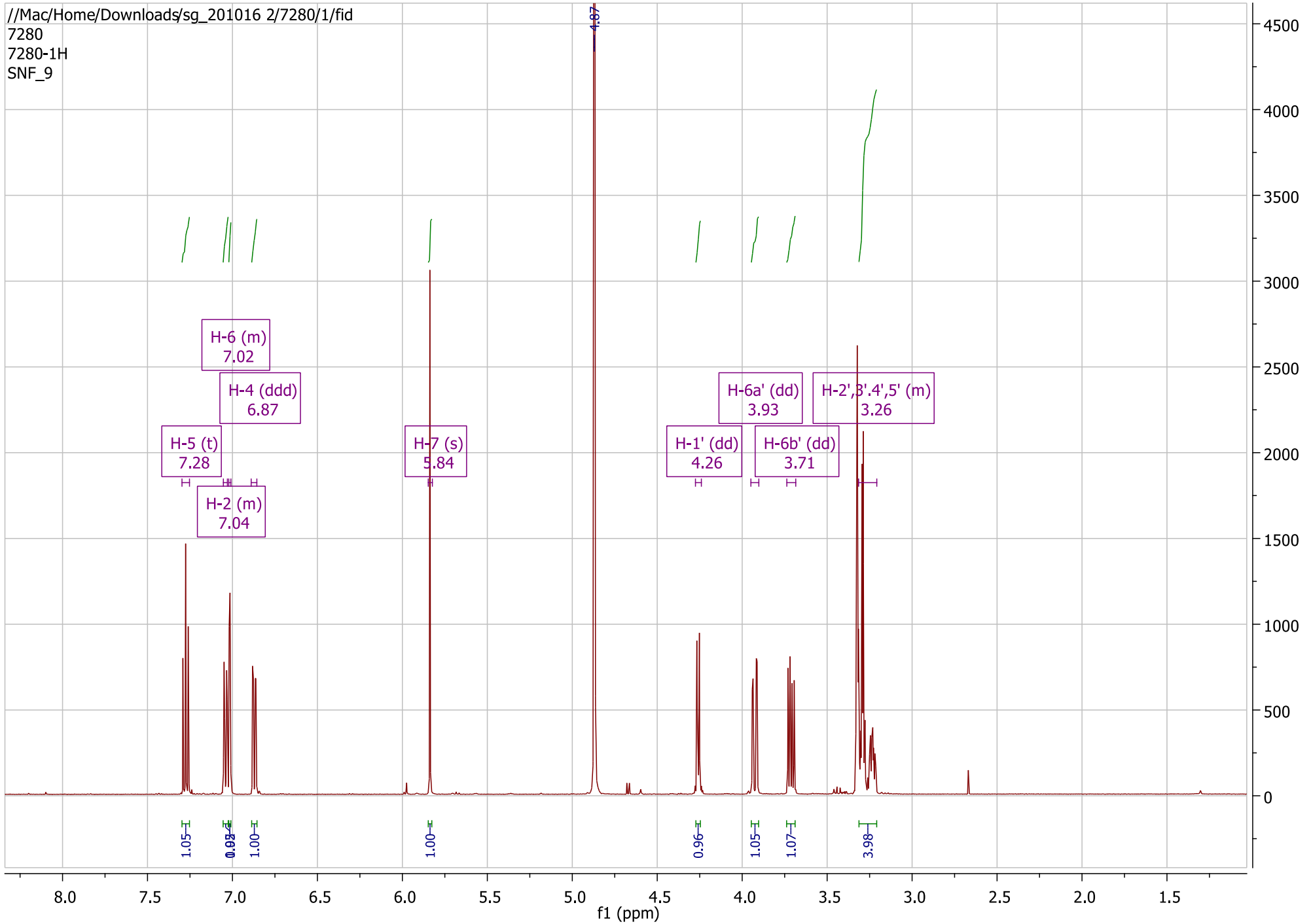
Compound 6

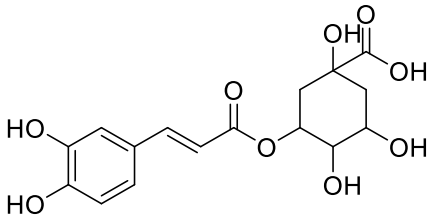
^{1}H NMR (500 MHz, CD_3OD) δ 7.28 (t, *J* = 8.0 Hz, 1H), 7.05 – 7.03 (m, 1H), 7.02 – 7.01 (m, 1H), 6.87 (ddd, *J* = 8.2, 2.5, 1.0 Hz, 1H), 5.84 (s, 1H), 4.26 (dd, *J* = 5.4, 2.2 Hz, 1H), 3.93 (dd, *J* = 12.0, 2.3 Hz, 1H), 3.71 (dd, *J* = 12.0, 6.2 Hz, 1H), 3.31 – 3.21 (m, 4H).

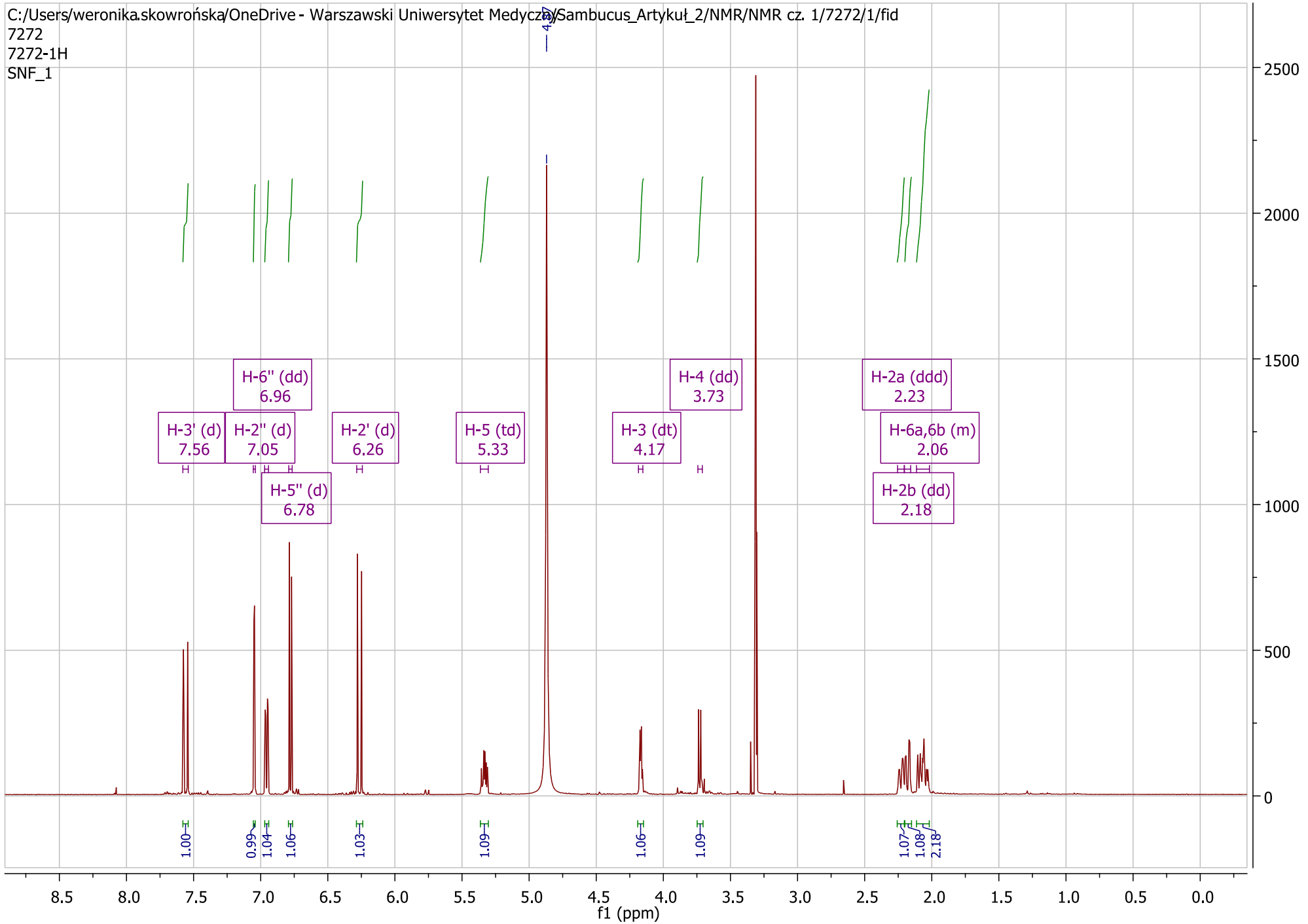




Compound 13

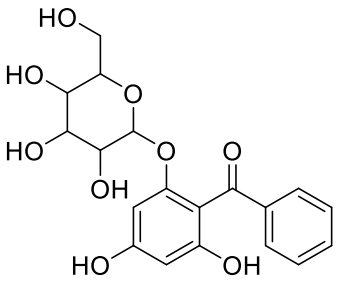
^{1}H NMR (500 MHz, CD_3OD) δ 7.56 (d, *J* = 15.9 Hz, 1H), 7.05 (d, *J* = 2.1 Hz, 1H), 6.97 – 6.94 (m, 1H), 6.78 (d, *J* = 8.2 Hz, 1H), 6.26 (d, *J* = 15.9 Hz, 1H), 5.33 (td, *J* = 9.3, 4.4 Hz, 1H), 4.17 (dt, *J* = 5.5, 3.3 Hz, 1H), 3.73 (dd, *J* = 8.5, 3.2 Hz, 1H), 2.23 (ddd, *J* = 13.3, 4.4, 2.1 Hz, 1H), 2.18 (dd, *J* = 14.1, 3.3 Hz, 1H), 2.11 – 2.02 (m, 2H).

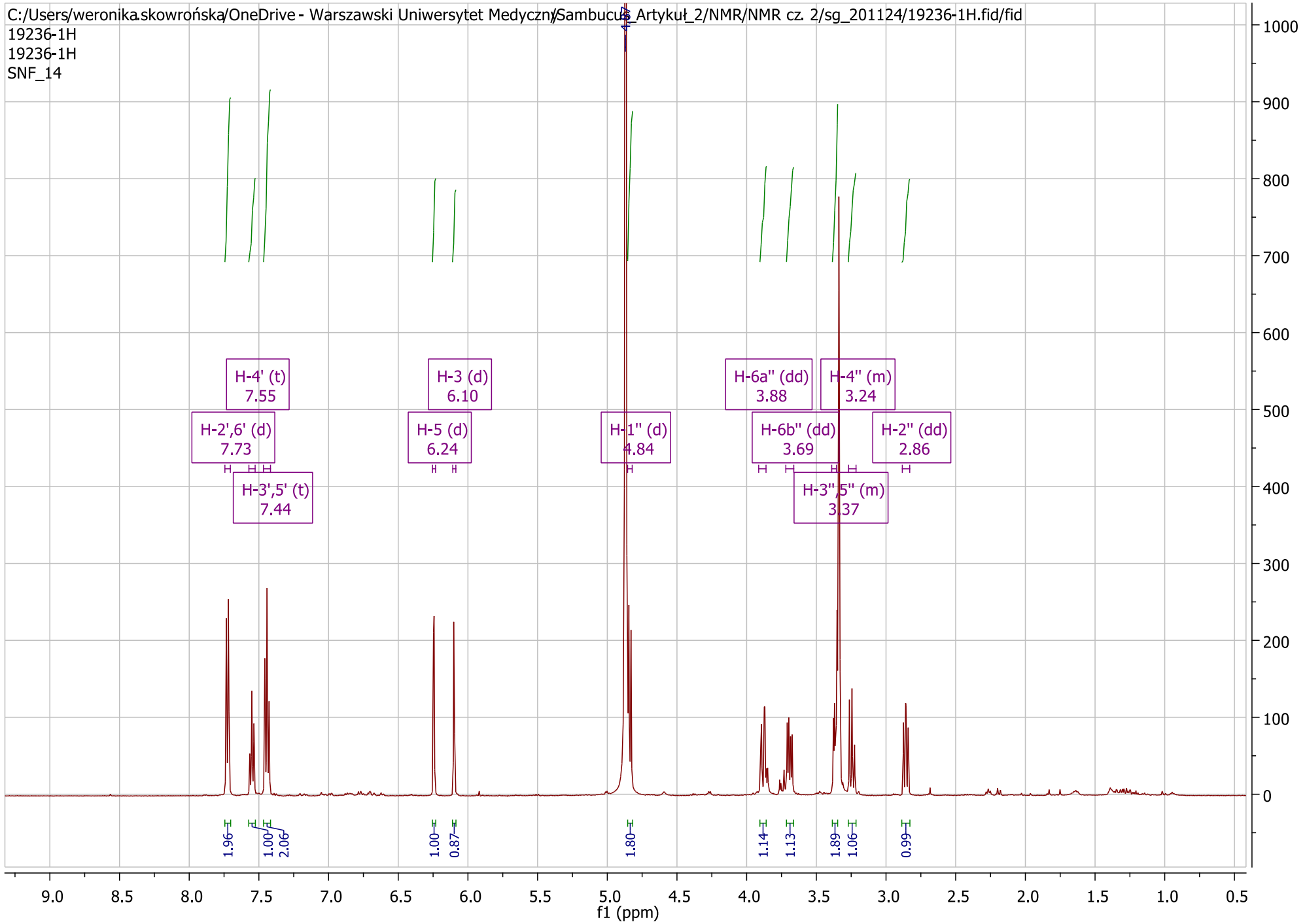




Compound 23

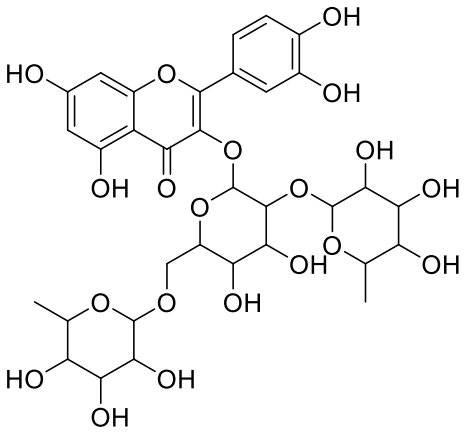
^{1}H NMR (501 MHz, CD_3OD) δ 7.73 (d, *J* = 7.1 Hz, 2H), 7.55 (t, *J* = 7.4 Hz, 1H), 7.44 (t, *J* = 7.7 Hz, 2H), 6.24 (d, *J* = 2.0 Hz, 1H), 6.10 (d, *J* = 2.1 Hz, 1H), 4.84 (d, *J* = 7.7 Hz, 1H), 3.88 (dd, *J* = 12.0, 2.2 Hz, 1H), 3.69 (dd, *J* = 12.1, 5.7 Hz, 1H), 3.39 – 3.36 (m, 2H), 3.27 – 3.22 (m, 1H), 2.86 (dd, *J* = 9.1, 7.8 Hz, 1H).

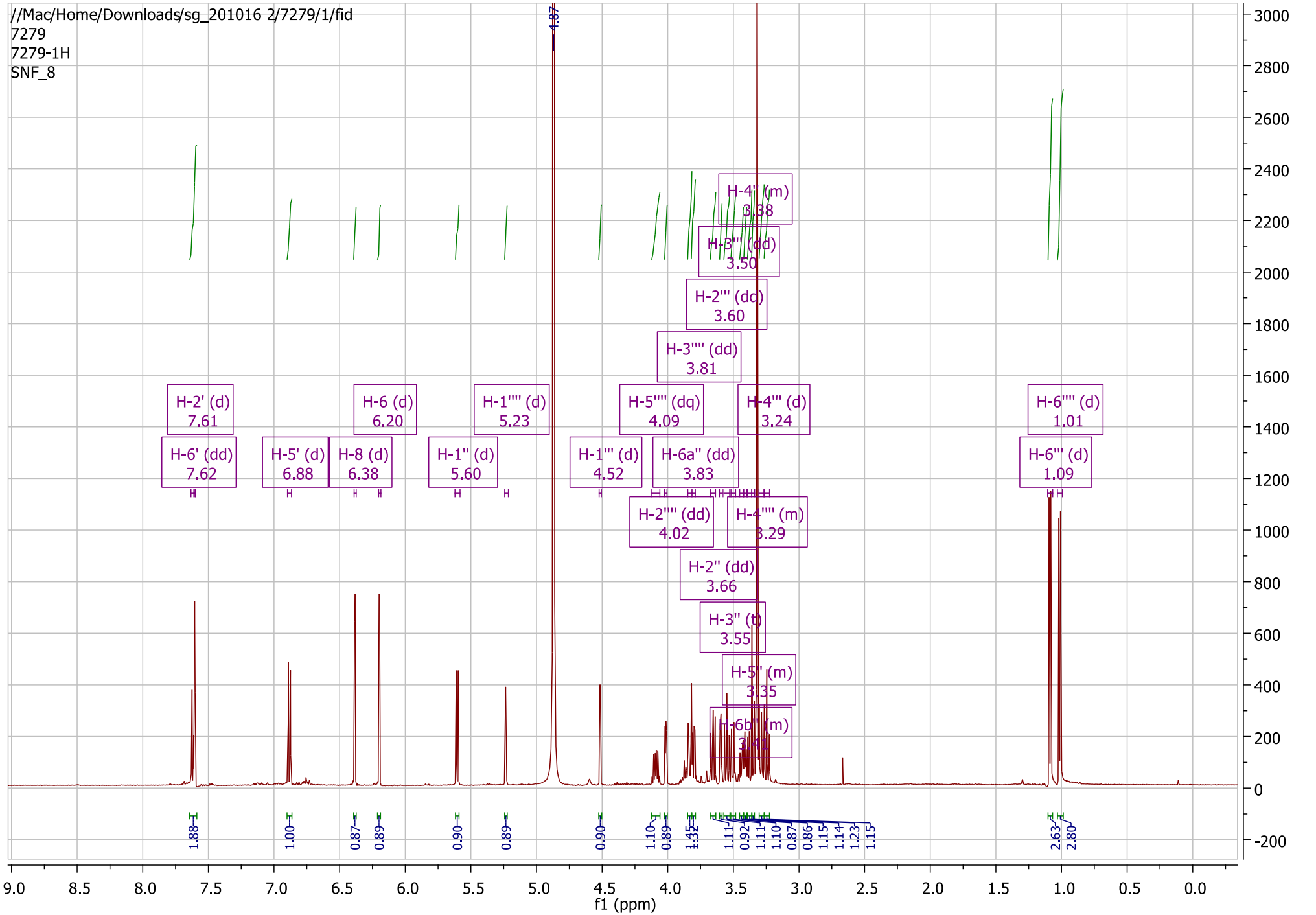




Compound 26

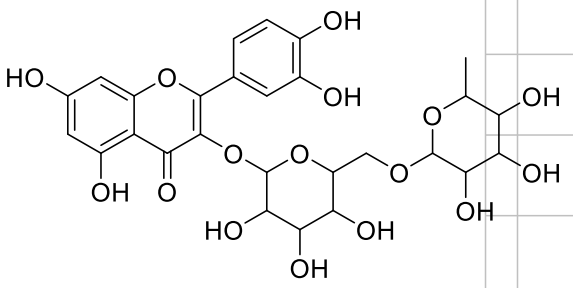
^{1}H NMR (500 MHz, CD_3OD) δ 7.62 (dd, *J* = 10.1, 4.0 Hz, 1H), 7.61 (d, *J* = 1.7 Hz, 1H), 6.88 (d, *J* = 8.3 Hz, 1H), 6.38 (d, *J* = 2.1 Hz, 1H), 6.20 (d, *J* = 2.1 Hz, 1H), 5.60 (d, *J* = 7.7 Hz, 1H), 5.23 (d, *J* = 1.5 Hz, 1H), 4.52 (d, *J* = 1.6 Hz, 1H), 4.09 (dq, *J* = 9.7, 6.2 Hz, 1H), 4.02 (dd, *J* = 3.3, 1.6 Hz, 1H), 3.83 (dd, *J* = 11.2, 1.6 Hz, 2H), 3.81 (dd, *J* = 9.6, 3.4 Hz, 1H), 3.66 (dd, *J* = 9.1, 7.7 Hz, 1H), 3.60 (dd, *J* = 3.4, 1.7 Hz, 1H), 3.55 (t, *J* = 8.9 Hz, 1H), 3.50 (dd, *J* = 9.5, 3.4 Hz, 1H), 3.45 – 3.42 (m, 1H), 3.42 – 3.40 (m, 1H), 3.39 – 3.36 (m, 1H), 3.36 – 3.34 (m, 1H), 3.31 – 3.27 (m, 1H), 3.24 (d, *J* = 9.5 Hz, 1H), 1.09 (d, *J* = 6.2 Hz, 3H), 1.01 (d, *J* = 6.2 Hz, 3H).

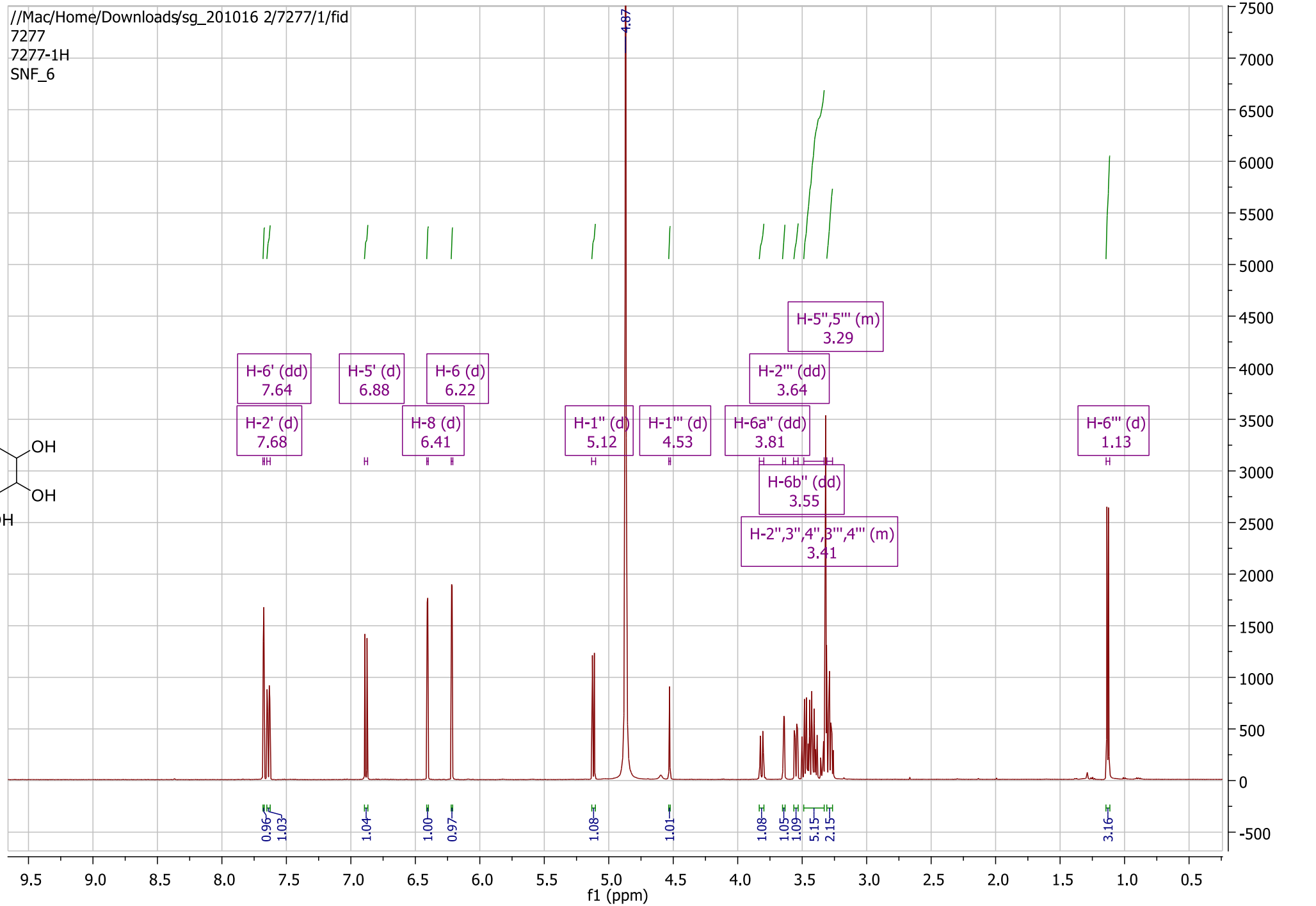




Compound 28

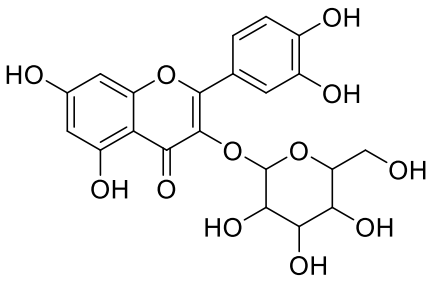
^{1}H NMR (500 MHz, CD_3OD) δ 7.68 (d, *J* = 2.2 Hz, 1H), 7.64 (dd, *J* = 8.5, 2.2 Hz, 1H), 6.88 (d, *J* = 8.5 Hz, 1H), 6.41 (d, *J* = 2.1 Hz, 1H), 6.22 (d, *J* = 2.1 Hz, 1H), 5.12 (d, *J* = 7.7 Hz, 1H), 4.53 (d, *J* = 1.6 Hz, 1H), 3.81 (dd, *J* = 11.0, 1.5 Hz, 1H), 3.64 (dd, *J* = 3.4, 1.7 Hz, 1H), 3.55 (dd, *J* = 9.5, 3.4 Hz, 1H), 3.49 – 3.33 (m, 5H), 3.31 – 3.26 (m, 2H), 1.13 (d, *J* = 6.2 Hz, 3H).

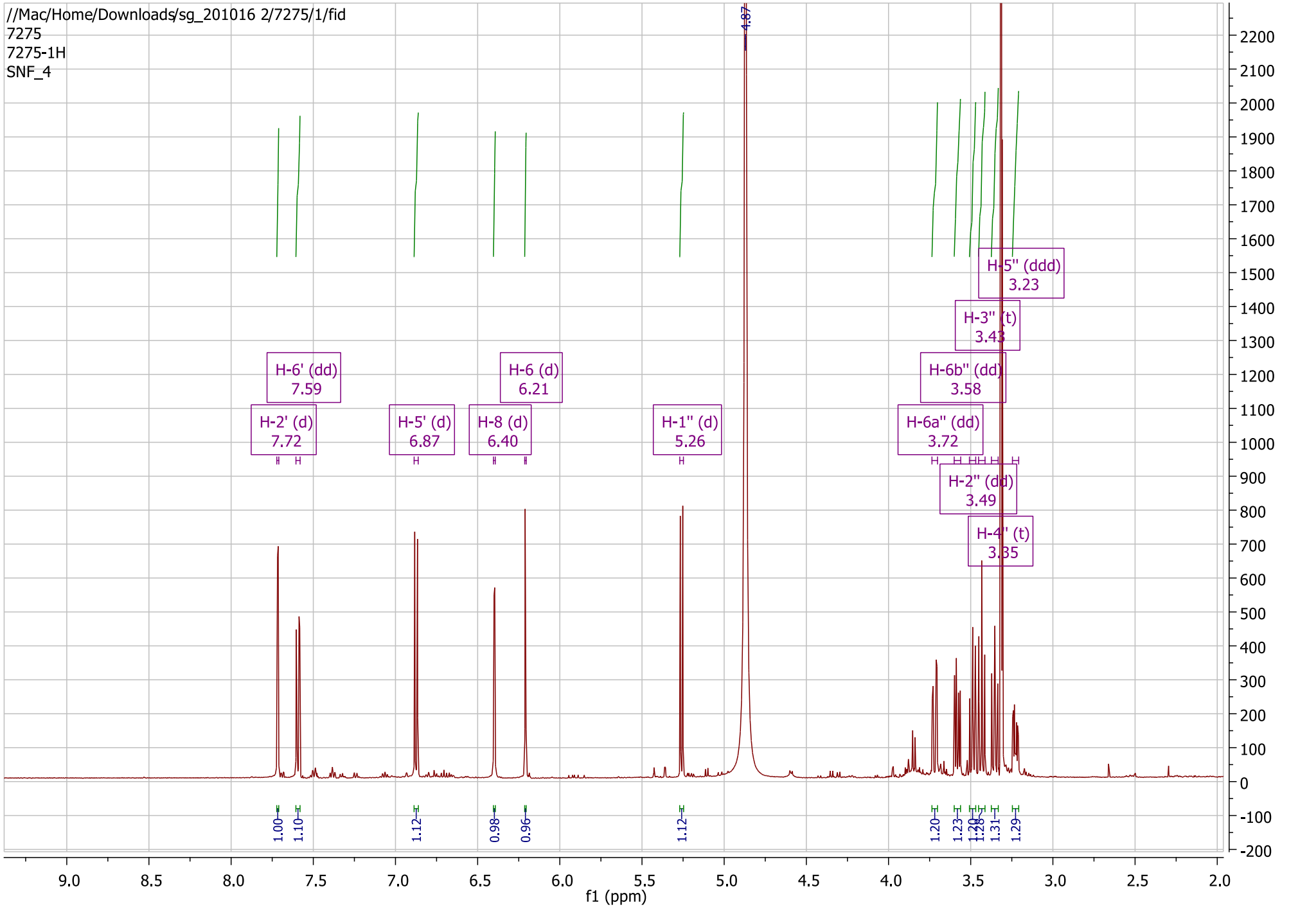




Compound 29

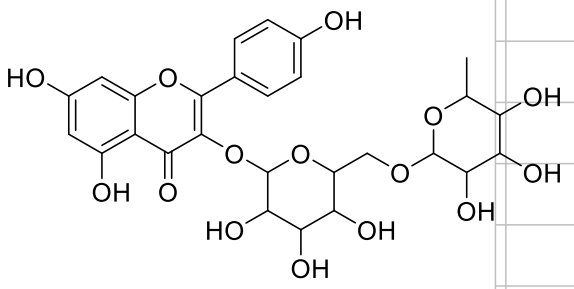
^{1}H NMR (500 MHz, CD_3OD) δ 7.72 (d, *J* = 2.2 Hz, 1H), 7.59 (dd, *J* = 8.5, 2.2 Hz, 1H), 6.87 (d, *J* = 8.5 Hz, 1H), 6.40 (d, *J* = 2.1 Hz, 1H), 6.21 (d, *J* = 2.1 Hz, 1H), 5.26 (d, *J* = 7.6 Hz, 1H), 3.72 (dd, *J* = 11.9, 2.4 Hz, 1H), 3.58 (dd, *J* = 11.9, 5.4 Hz, 1H), 3.49 (dd, *J* = 9.1, 7.7 Hz, 1H), 3.43 (t, *J* = 8.9 Hz, 1H), 3.35 (t, *J* = 9.2 Hz, 1H), 3.23 (ddd, *J* = 9.7, 5.3, 2.4 Hz, 1H).

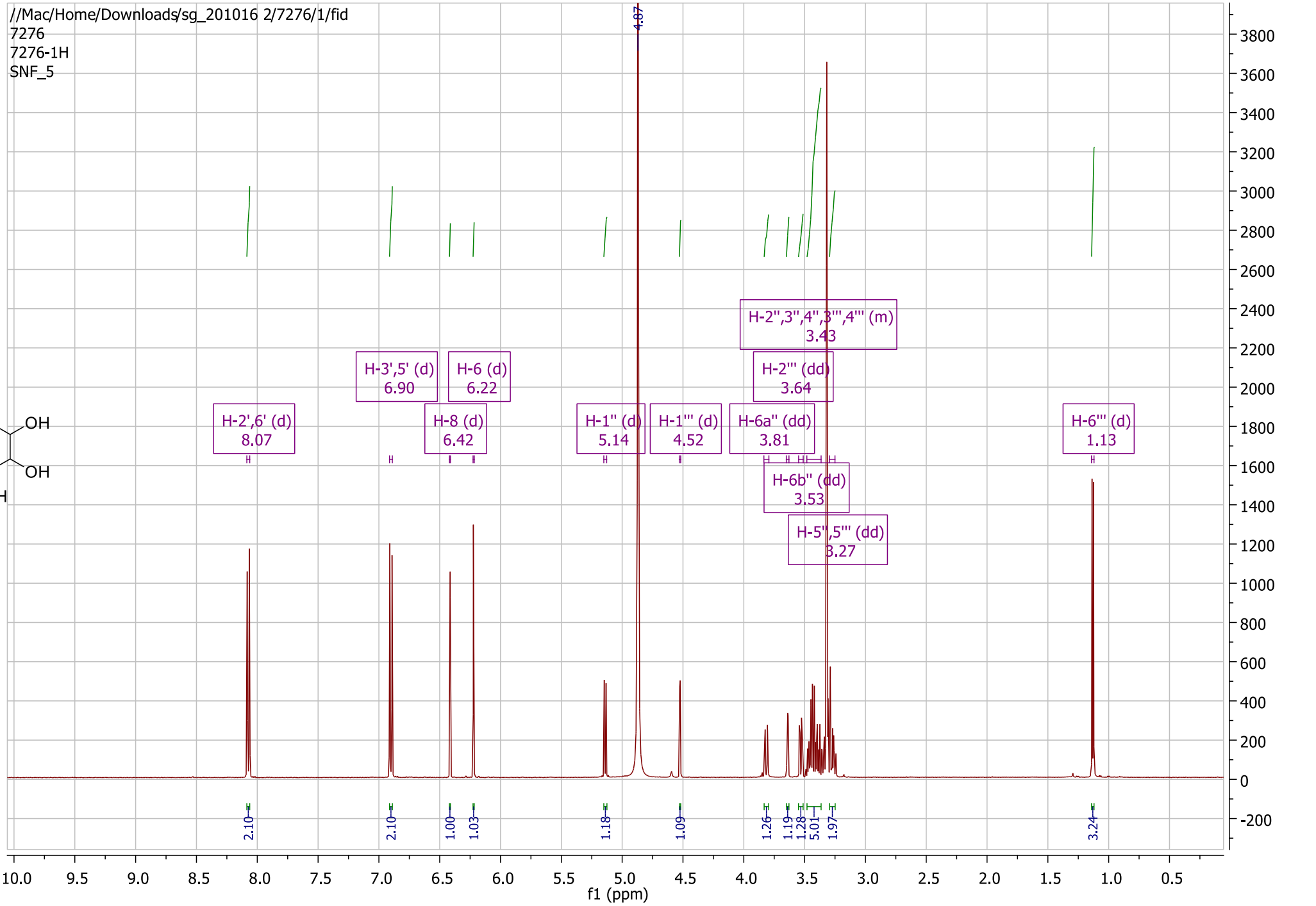




Compound 30

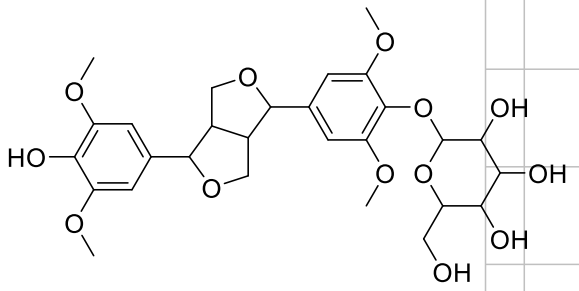
^{1}H NMR (500 MHz, CD_3OD) δ 8.07 (d, *J* = 9.0 Hz, 2H), 6.90 (d, *J* = 9.0 Hz, 2H), 6.42 (d, *J* = 2.1 Hz, 1H), 6.22 (d, *J* = 2.1 Hz, 1H), 5.14 (d, *J* = 7.6 Hz, 1H), 4.52 (d, *J* = 1.6 Hz, 1H), 3.81 (dd, *J* = 10.9, 1.4 Hz, 1H), 3.64 (dd, *J* = 3.4, 1.7 Hz, 1H), 3.53 (dd, *J* = 9.5, 3.4 Hz, 1H), 3.48 – 3.36 (m, 5H), 3.27 (dd, *J* = 9.2, 4.7 Hz, 2H), 1.13 (d, *J* = 6.2 Hz, 3H).

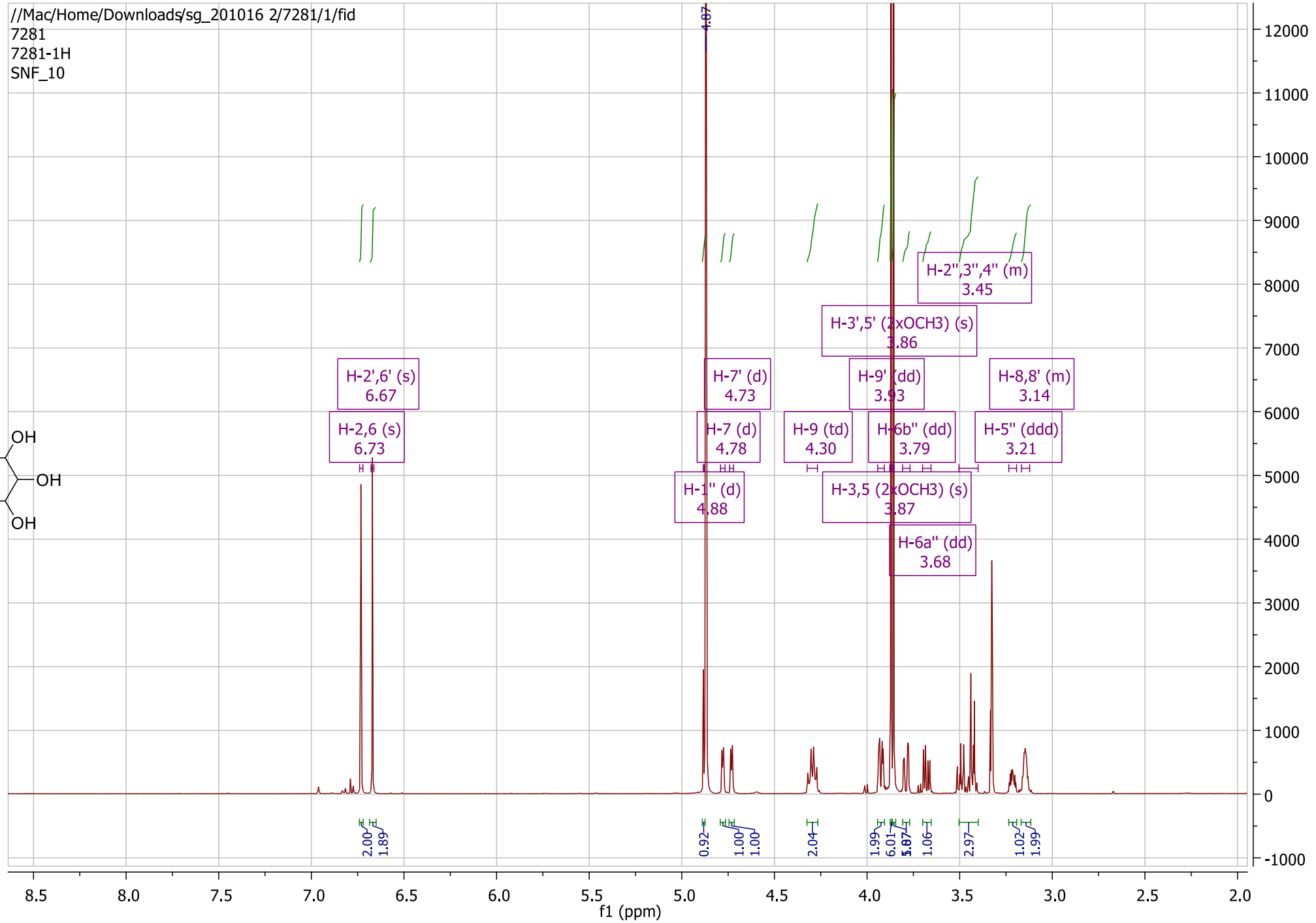




Compound 31

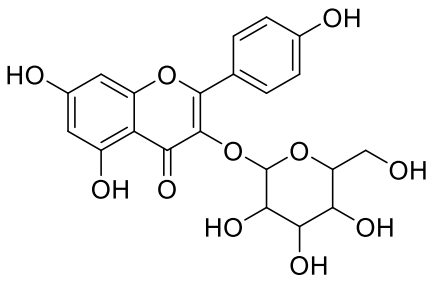
^{1}H NMR (500 MHz, CD_3OD) δ 6.73 (s, 2H), 6.67 (s, *J* = 0.4 Hz, 2H), 4.88 (d, *J* = 1.2 Hz, 1H), 4.78 (d, *J* = 4.3 Hz, 1H), 4.73 (d, *J* = 4.5 Hz, 1H), 4.30 (td, *J* = 9.0, 7.0 Hz, 2H), 3.93 (dd, *J* = 9.2, 2.7 Hz, 2H), 3.87 (s, 6H), 3.86 (s, 6H), 3.79 (dd, *J* = 12.0, 2.4 Hz, 1H), 3.68 (dd, *J* = 12.0, 5.2 Hz, 1H), 3.50 – 3.40 (m, 3H), 3.21 (ddd, *J* = 9.6, 5.2, 2.4 Hz, 1H), 3.17 – 3.12 (m, 2H).

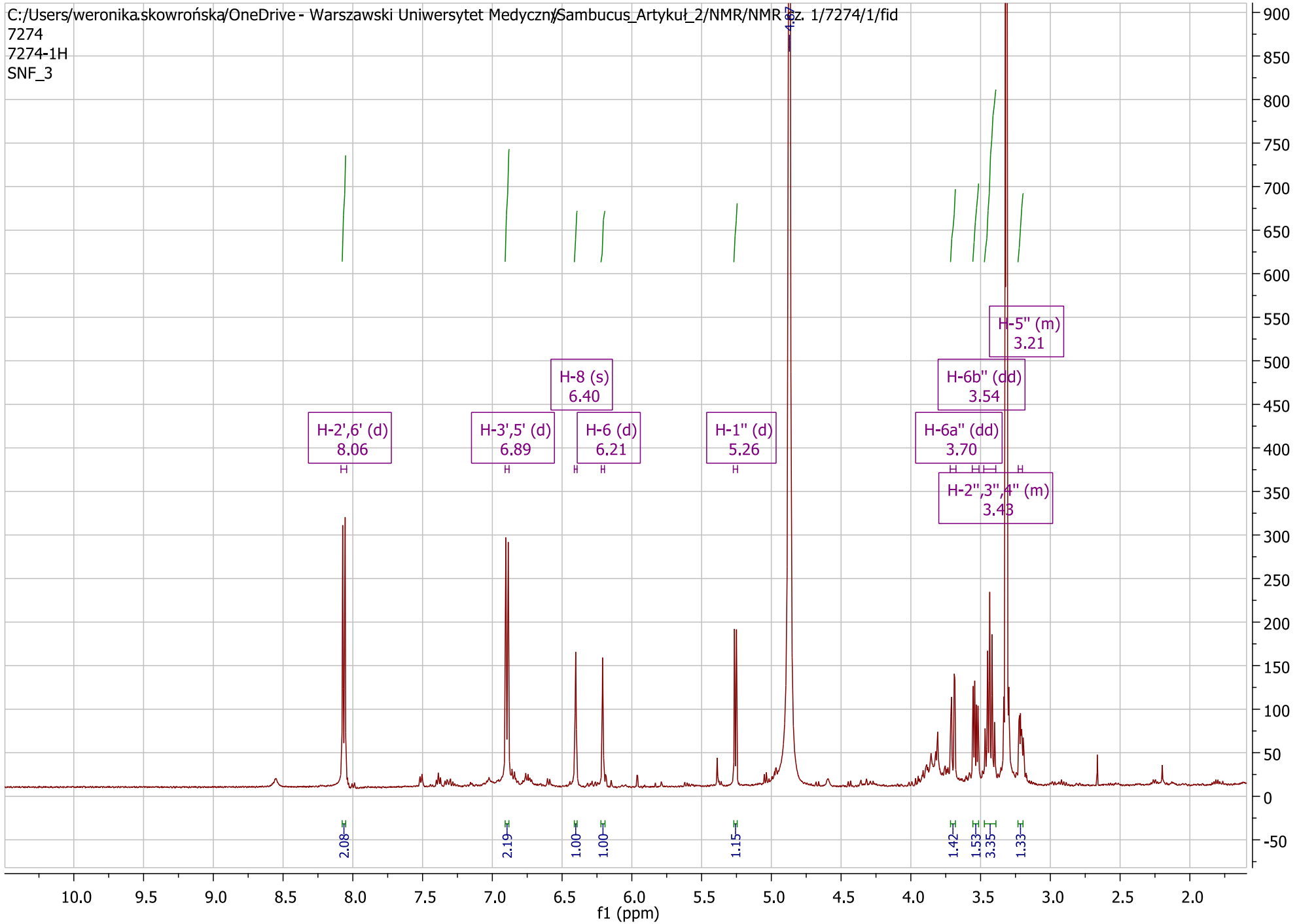




Compound 32

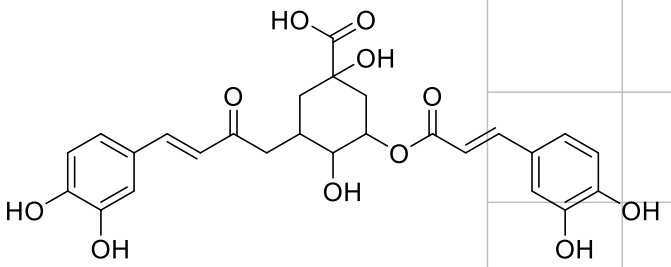
^{1}H NMR (500 MHz, CD_3OD) δ 8.06 (d, *J* = 8.8 Hz, 2H), 6.89 (d, *J* = 8.8 Hz, 2H), 6.40 (s, 1H), 6.21 (d, *J* = 1.5 Hz, 1H), 3.70 (dd, *J* = 11.9, 2.3 Hz, 1H), 3.54 (dd, *J* = 11.9, 5.5 Hz, 1H), 3.48 – 3.39 (m, 3H), 3.23 – 3.20 (m, 1H).

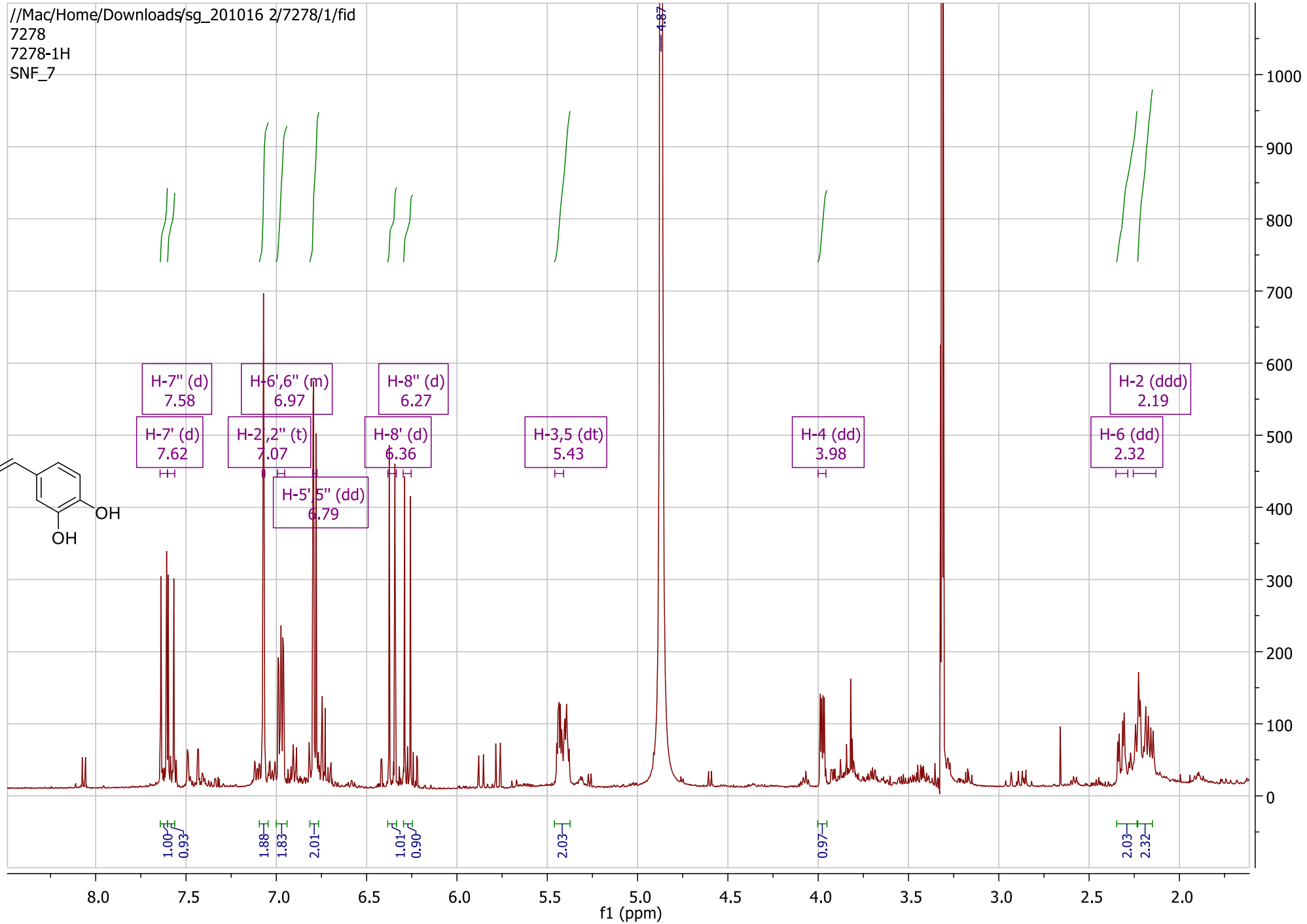




Compound 33

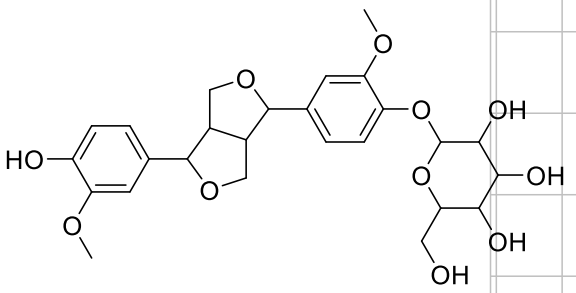
^{1}H NMR (500 MHz, CD_3OD) δ 7.62 (d, *J* = 15.9 Hz, 1H), 7.58 (d, *J* = 15.9 Hz, 1H), 7.07 (t, *J* = 2.1 Hz, 1H), 6.99 – 6.96 (m, 2H), 6.79 (dd, *J* = 8.2, 1.2 Hz, 2H), 6.36 (d, *J* = 15.9 Hz, 1H), 6.27 (d, *J* = 15.9 Hz, 1H), 5.43 (dt, *J* = 12.3, 4.4 Hz, 2H), 3.98 (dd, *J* = 7.5, 3.3 Hz, 1H), 2.32 (dd, *J* = 13.9, 3.9 Hz, 1H), 2.19 (ddd, *J* = 25.9, 13.0, 7.0 Hz, 3H).

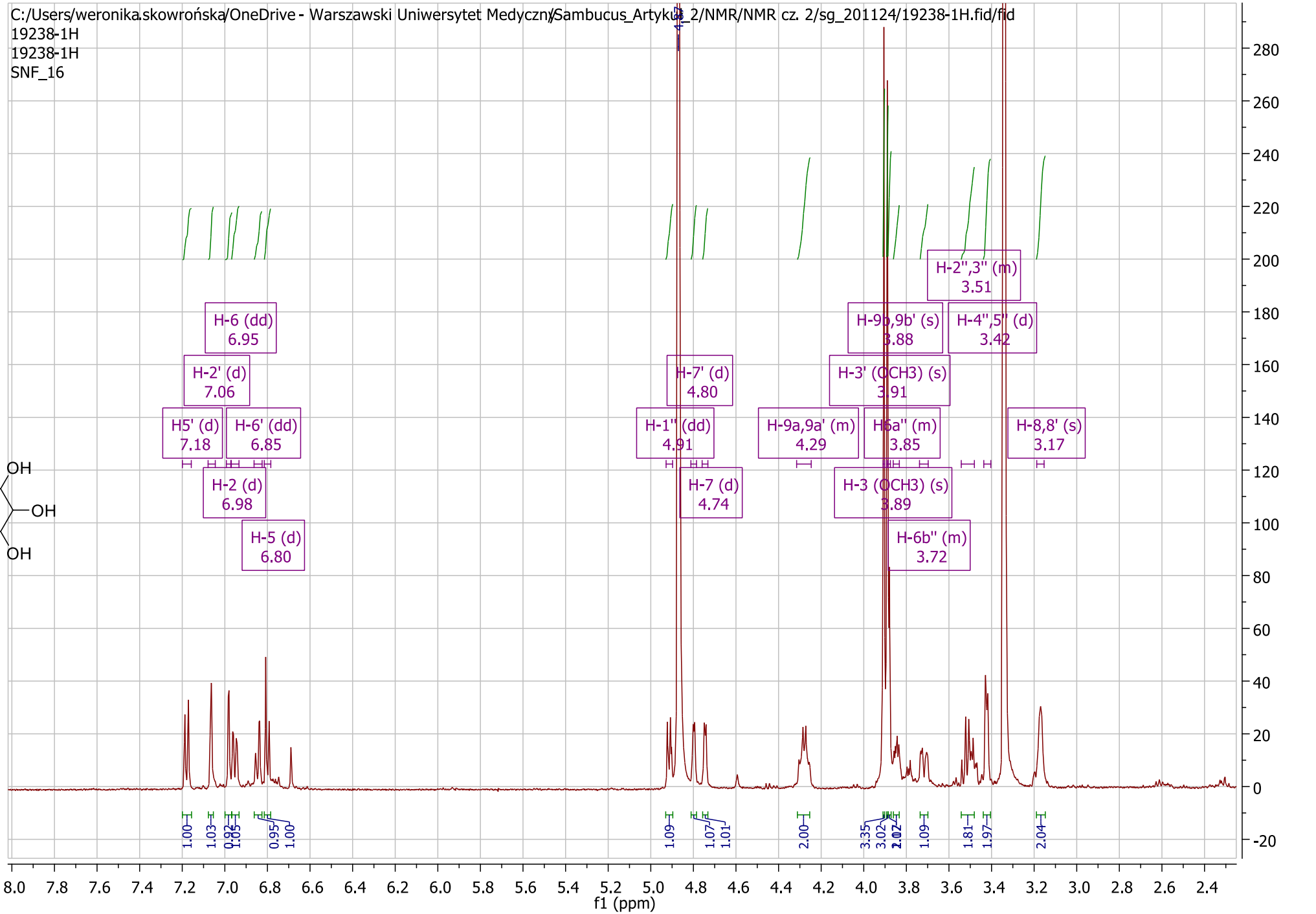




Compound 41

^{1}H NMR (501 MHz, CD_3OD) δ 7.18 (d, *J* = 8.3 Hz, 1H), 7.06 (d, *J* = 1.5 Hz, 1H), 6.98 (d, *J* = 1.6 Hz, 1H), 6.95 (dd, *J* = 8.4, 1.8 Hz, 1H), 6.85 (dd, *J* = 8.1, 1.6 Hz, 1H), 6.80 (d, *J* = 8.1 Hz, 1H), 4.91 (dd, *J* = 7.4, 2.6 Hz, 1H), 4.80 (d, *J* = 3.6 Hz, 1H), 4.74 (d, *J* = 4.3 Hz, 1H), 4.32 – 4.25 (m, 2H), 3.91 (s, 3H), 3.89 (s, 3H), 3.88 (s, 2H), 3.86 – 3.83 (m, 1H), 3.74 – 3.70 (m, 1H), 3.54 – 3.48 (m, 2H), 3.42 (d, *J* = 5.3 Hz, 2H), 3.17 (s, 2H).





Manuscript co-author statement

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Sebastian Granica	8%	Methodology of the phytochemical and cellular research, project supervision, manuscript editing
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Agnieszka Bazylko	10%	Conceptualization of the project, manuscript editing, project supervision
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Date and legible signature

Data i czytelny podpis

27.02.2024 Weronika Skowrońska

Manuscript co-author statement

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Date and legible signature

Data i czytelny podpis

01.03.2024

Piwowarski

Manuscript co-author statement

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Data i czytelny podpis

27.02.2024. Lejsa Jakupović

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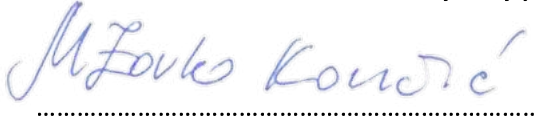

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... 06.03.2024.

Manuscript co-author statement

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		Metodologia badań komórkowych, redakcja manuskryptu
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04.03.2024 Agnieszka Bazylko

pharmaceutics

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Review

The Potential of Medicinal Plants and Natural Products in the Treatment of Burns and Sunburn—A Review

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Abstract: Treating burns remains a challenge for modern medicine, especially in developing countries that cannot afford expensive, advanced therapies. This review article summarises clinical and animal model studies of botanical preparations and their mixtures in treating burn wounds and sunburn. Articles available in electronic databases such as PubMed, Scopus, Web of Science, Science Direct and Google Scholar, published in English in 2010–2022, were considered. In the described clinical trials, it was shown that some herbal preparations have better effectiveness in treating burn wounds, including shortening the healing time and reducing inflammation, than the conventional treatment used hitherto. These herbal preparations contained extracts from *Albizia julibrissin*, *Alkanna tinctoria*, *Aloe vera*, *Arnebia euchroma*, *Betula pendula* and *Betula pubescens*, *Centella asiatica*, *Hippophaë rhamnoides*, *Juglans regia*, *Lawsonia inermis*, and mixtures of *Matricaria chamomilla* and *Rosa canina*. Research on animal models shows that many extracts may potentially benefit the treatment of burn wounds and sunburn. Due to the diverse mechanism of action, antibacterial activity, the safety of use and cost-effectiveness, herbal preparations can compete with conventional treatment. The growing interest in alternative medicine and herbal medicine encourages further research. Not only single preparations but also their mixtures should be taken into account because the research conducted so far often suggests a synergistic effect of the ingredients.

Keywords: burn; sunburn; wound; plant extracts; *Albizia julibrissin*; *Aloe vera*; *Arnebia euchroma*; *Betula*; *Centella asiatica*; *Hippophaë rhamnoides*

Citation: Skowrońska, W.; Bazylko, A. The Potential of Medicinal Plants and Natural Products in the Treatment of Burns and Sunburn—A Review. *Pharmaceutics* **2023**, *15*, 633. https://doi.org/10.3390/pharmaceutics15020633

Academic Editors: Beata Nowak and Sylwia Zielińska

Received: 16 January 2023
Revised: 4 February 2023
Accepted: 10 February 2023
Published: 13 February 2023

1. Introduction

According to the World Health Organisation, burn injuries affect more than 11 million people yearly. More than 180,000 people die, and approximately 95% of deaths occur in low-income and developing countries. Due to high costs, modern therapies are available almost exclusively in developed countries. Poorly designed healthcare systems and low health expenditures per capita in low-income countries correlate with higher post-injury complications, which can lead to disability and death. Therefore, treating burn wounds remains challenging, particularly as cost-effective therapy [1,2].

Burns can be defined as tissue damage due to the action, in most cases, of high temperature, electricity, chemicals, and radiation. The classification of burns is based on their depth and size. There are four degrees based on the severity of the injury. Superficial burns, also known as first-degree burns, involve only the epidermis. They are characterised by redness and discomfort, sometimes pain, but usually do not require medical intervention. Second-degree burns can be divided into two subcategories, superficial partial-thickness burns and deep partial-thickness burns. A superficial partial-thickness burn covers the epidermis and part of the dermis. It is usually manifested by the appearance of blisters filled with serous fluid. It is painful and requires wound care and dressing but usually does not cause scarring. A deep partial-thickness burn covers the epidermis and dermis. It is deeper but usually less painful due to damage to the pain receptors. It leads to scarring

and sometimes requires surgical intervention. Third-degree burns, or full-thickness burns, involve the entire skin. They are usually not painful because the nerve endings are damaged. Blood vessels and subcutaneous tissue are also damaged. Treatment is long and requires surgical removal of necrotic tissue, administration of antibiotics and usually a skin graft. In addition to the skin and subcutaneous tissue, fourth-degree burns involve muscles and bones. Charring is a characteristic picture. The changes are irreversible. They usually lead to amputation of the affected limb or death of the patients [3].

Burns are characterised by high susceptibility to bacterial infections. A damaged skin barrier, easy access to nutrients in the wound environment, damage to the wound vascularisation, lack of epithelialisation of the basal epidermal tissue, or systemic disorders leading to immunosuppression facilitate the entry of pathogens [4]. Therefore, topical antimicrobial agents are still very often used in treating burns. The most commonly used topical formulation and considered the standard therapy is 1% silver sulfadiazine (SSD) cream. However, in recent years, numerous disadvantages of this preparation have been described, including delaying healing or a cytotoxic effect on various host cells [5,6]. Therefore, new preparations containing, or no silver ions are introduced to the market, mainly in ready-to-use dressings. Studies show that burn wounds treated with new dressings heal faster and are easier to use. In addition, burns treated with the new dressings are less susceptible to secondary infection than treatment with 1% SSD cream [7,8].

Superficial and partial-thickness burns are the most common in pharmaceutical practice. The ideal dressing that we would like to recommend to a patient with a burn wound should, in addition to healing properties, have the following features: infection prevention, pain relief, moisture control, exudation removal, gas exchange, low skin adhesion, mechanical stable, reducing wound necrosis, cost-effective, non-toxic, biocompatible, and biodegradable [9]. However, individual regional needs or desires and the patient's perspective and economic constraints must also be considered [10]. The answer to the requirements could be dressings or pharmaceutical preparations containing products of natural origin [11].

This review aimed to summarize knowledge on the use of plant preparations in the treatment of burn wounds. The paper includes articles from 2010 to 2022 describing clinical trials for single herbal preparations or their mixtures and animal studies of plants or plant extracts and their mixtures.

2. Materials and Methods

Electronic databases, including PubMed, Scopus, Web of Science, Science Direct, and Google Scholar, were searched for materials for this review. Articles published in English in the years 2010–2022 were selected. Search terms were "burn" and "sunburn" in the title or abstract, and "plant extract", "plant", and "herbal" in the abstract and full text.

Original papers describing the effect of individual plant preparations and mixtures on treating burns and sunburn were included. The paper describes clinical trials and studies on animal models in separate sections. In addition, the results for single formulations and mixtures are described separately. The descriptions or tables provide the scientific names of individual plant species or plants included in the mixture and the plant part used. Studies on single chemical compounds isolated from plants were not taken into account.

3. Results

3.1. Clinical Trials—Single Preparations

In 2010–2022, several clinical trials were conducted to check the effectiveness of single-plant preparations in treating burns. Studies have been conducted on the Persian silk tree (*Albizia julibrissin*), aloe (*Aloe vera*), pink Arnebia (*Arnebia euchroma*), silver birch (*Betula pendula*) and downy birch (*Betula pubescens*), tea plant (*Camelia sinensis*), gotu kola (*Centella asiatica*), sea buckthorn (*Hippophaë rhamnoides*), and common walnut (*Juglans regia*). Studies for individual species are described below. A summary of the results is provided in Supplementary Materials in Table S1.

3.1.1. *Albizia julibrissin*

Albizia julibrissin from the Fabaceae family was originally found in South and East Asia, from Iran and Azerbaijan to China and Korea. In local folk medicine, the *Albizia* species has been used to treat melancholy, insomnia, fever, headaches and abdominal pain, diabetes, and rheumatism, but also to treat wounds, snake bites, haemorrhoids, abscesses, erysipelas, and leprosy. The main chemical components are triterpene saponins, but flavonoids, lignans, alkaloids, and phenolic glycosides are also present [12].

A prospective, randomised, double-blind clinical trial investigated the efficacy of a gel containing 5% (*w*/*w*) *Albizia julibrissin* extract in treating burns [13]. The extract was prepared by extracting the stem bark of *Albizia julibrissin* with 60% (*v*/*v*) ethanol. Forty patients with second and third-degree burns participated in the study. Patients were randomly assigned to two equal groups where 20 were treated with 5% *A. julibrissin* gel and the remaining 20 with 1% SSD cream. The wounds were washed once daily with a saline solution, and treatment was applied. The treatment was continued for 30 days. The study compared clinical parameters such as inflammation, pain, itching, erythema, oedema, purulent discharge, and skin discolouration. Before the beginning of the study, there were no significant differences in those parameters between patients from the two groups.

After 15 days of treatment, a statistically significant reduction in pain, inflammatory reaction and purulent discharge were observed in the group treated with 5% *A. julibrissin* gel compared to the group treated with 1% SSD cream. There were no significant differences between groups in itching, erythema, oedema, and skin discolouration. At the end of treatment, after 30 days, a reduction in inflammation and pain sensation was observed in the group treated with the 5% *A. julibrissin* gel. However, the other assessed parameters found no statistically significant differences between the groups. Treatment with 5% *A. julibrissin* gel shortened epithelialisation time in second and third-degree burns by 33.3 and 43.78%, respectively, compared to treatment with 1% SSD cream. The patients rated the colour, odour, and stability of 5% *A. julibrissin* gel worse than the 1% SSD cream.

3.1.2. *Aloe vera*

Aloe vera (Asphodelaceae) has a long history of medicinal use, dating back to ancient cultures such as Chinese, Egyptian, and Indian. Over the years, several studies have been conducted on its pharmacological use. Its therapeutic activity includes, among others, antibacterial, antiviral, anticancer, antioxidant, anti-inflammatory, skin protective, wound healing, and regulating blood glucose and cholesterol levels. *Aloe vera* is known primarily for its beneficial effects on the skin, mainly its mucous gel filling the leaves, which is used in many cosmetic and pharmaceutical preparations [14].

The effectiveness of *Aloe vera* cream in treating second-degree burns was tested in a randomised and controlled clinical trial [15]. The study involved 30 patients with two thermal burns of similar size and depth on two different but similar body areas (such as hands or feet). The burn must have occurred within 24 h of treatment initiation and not affected more than 40% of the total body surface area. After cleaning the wound with saline solution, a base cream containing 0.5% pure spray-dried aloe powder (Zarband Phytopharmaceuticals, Teheran, Iran) was applied to one burn. A 1% SSD cream was applied to the second burn. Dressings were changed, and the cream was applied twice a day. Treatment was continued until complete epithelialisation of the burn.

Mean times to complete healing were 15.9 $\pm$ 2 and 18.73 $\pm$ 2.65 days for a burn treated with aloe cream and 1% SSD cream, respectively. The time to complete healing was statistically significantly shorter in the case of treatment with aloe cream. Additionally, the size of the wound treated with the aloe cream was significantly smaller after 10, 13, and 16 days. After days 3, 7, and 13, no microbial contamination was observed. This clinical study showed that aloe cream might be more effective in treating burns than 1% SSD cream, significantly reducing wound healing time and surface area.

Another randomised clinical trial compared the effectiveness of 98% *Aloe vera* gel with that of 1% SSD cream in treating second-degree burns [16]. The study involved 50 patients

with second-degree heat burns that developed within 24 hours of starting treatment and did not exceed 25% of the total body surface area. The wound was washed with pyodine scrub and saline, and then aloe gel or 1% SSD cream was applied. Dressings were changed twice a day. Treatment was continued until the wound was completely healed and re-epithelialised.

The study compared the size and area of the wound as well as the patients' subjective perception of pain. The mean wound epithelialisation time was 11 ± 4.18 and 24.24 ± 11.16 days for the aloe gel and 1% SSD cream treated groups, respectively, and was significantly shorter for those treated with aloe vera gel. There were no differences in the infection of the wounds of both groups. In the group treated with aloe vera gel, the time to complete pain relief was 21 days and was significantly shorter than in the group treated with 1% SSD cream, which was 26 days. The study showed that aloe vera gel significantly shortens the re-epithelialisation time, alleviates pain symptoms, and is more cost-effective.

3.1.3. *Arnebia euchroma*

Arnebia euchroma from the Boraginaceae family occurs naturally in high mountain regions, mainly in the Himalayas and other regions of Asia and North Africa. It is a source of many promising chemical compounds from the group of naphthoquinones, mainly ester derivatives of shikonin, alkannin, and isohexenylnaphthazarin. Potential medicinal properties include wound-healing, antibacterial, antiviral, antifungal, anti-inflammatory, and anticancer effects [17].

The effectiveness of *Arnebia euchroma* ointment was tested in a prospective, randomised, single-blind clinical study compared to the effectiveness of 1% SSD cream [18]. To prepare the ointment, chopped dried roots of *A. euchroma* were heated in goat fat, cow butter, and glycerin at 95–100 °C for 30 min. The mixture was sterilised, filtered, and then Eucerin, methylparaben, and propylene paraben were added. The weight ratio of *A. euchroma* roots to primary materials was 10%.

The study involved 45 patients who suffered second-degree burns on two parts of their body, covering no more than 15% of their total body surface area. The burn was to appear within 24 h of starting treatment. The injured parts were randomly assigned to treatment with *A. euchroma* ointment (AEO) or 1% SSD cream, which was continued until complete wound healing. The wound was washed daily with saline solution, and appropriate treatment was applied. On days 1, 3, 5, 7, 10, 13, 15, 20, 25, and 30, the wound was measured, and photographs were taken prior to the application of the cream.

By the fifth day of treatment, no significant differences were observed in the size of the wound treated with AEO and 1% SSD cream. In the following days, the area of the wound treated with AEO was significantly smaller than that of the wound treated with SSD. The average wound healing time was 13.9 ± 5.3 days for AEO and 17.5 ± 6.9 days for 1% SSD cream. It was significantly lower for wounds treated with AEO. Physicians' treatment preferences overwhelmingly favoured AEO from day 15 of treatment. The mean global assessment of wound appearance by the experienced nurse did not differ between the groups. Burning sensation and pain sensation were lower, while warming of the injury area was higher for the part treated with AEO than the part treated with 1% SSD cream. Patients' satisfaction with AEO treatment was significantly higher than with 1% SSD cream treatment.

3.1.4. *Betula pendula, Betula pubescens*

The leaves of *Betula pendula* and *Betula pubescens*, species of the Betulaceae family, rich in flavonoid compounds, have been used in traditional medicine as diuretics that increase urine flow, flush the urinary tract, and prevent the development of infections [19]. However, now researchers are interested in birch bark. Betulin, the main component of birch bark extract, was first described in 1788. Only in recent years has it gained importance as a pharmaceutical ingredient. Betulin has been found to have the ability to stabilize water-in-oil emulsions but not as a surfactant. In addition, it gels oils, thanks to which it creates thixotropic gels, the durability of which is higher at body temperature than at

room temperature. After receiving excellent results in toxicology and pharmaceutical safety studies, the era of clinical trials began to clarify the indications where triterpene birch bark extract could be used [20].

Oleogel-S10 (tradename Episalvan®) is a sterile gel containing 10% birch bark extract and 90% sunflower oil. Triterpene birch extract, obtained from *Betula pendula*, *Betula pubescens* and mixtures of these species, is standardised for the content of betulin (72–88%), and it also contains, among others, betulinic acid, lupeol, oleanolic acid, and erythrodiol.

An open, blindly evaluated, randomised clinical trial was conducted to test the effectiveness of Oleogel-S10 in treating superficial partial-thickness burn wounds [21]. Based on the study set out below, the European Medicines Agency decided to approve Episalvan® for treating second-degree burns. The obtained results were compared to treatment with octenidine hydrochloride gel (Octenilin® wound gel, Schülke & Mayr GmbH, Germany). Patients with one superficial second-degree burn >80 cm^2 and <25% of total body surface area or two comparable burns >40 cm^2 and <12.5% of total body surface area were eligible for the study. After washing the wound with octenidine hydrochloride or polyhexanide, Oleogel-S10 was applied to one wound or half of a large burn, and Octenilin® was applied to the other, both approx. 1 mm thick, and then covered with gauze. The wounds were washed and dressed every 2 days for 21 days. Finally, treatment parameters were compared in 57 patients.

The study showed that 35 patients out of 57 had differences in the time required for wound closure. Among them, 30 patients (85.7%) treated with Oleogel-S10 had earlier healing than 5 (14.3%) treated with Octenilin®. The statistical analysis showed the advantage of treatment with Oleogel-S10 over treatment with Octenilin®. The percentage of wound epithelialisation on each analysed day was significantly higher for burns treated with Oleogel-S10 compared to Octenilin®. Oleogel-S10 was rated "better" or "significantly better" than Octenilin® by 73.7% of the investigators and 71.9% of the patients. Treatment with these preparations was considered comparable by 8.8% of the investigators and 12.3% of the patients. Only 1.8% of the investigators and none of the patients considered that treatment with Octenilin® was "better" or "much better". At the end of the treatment, the tolerability of treatment with Oleogel-S10 and Octenilin® was assessed. Oleogel-S10 was rated as "better" or "much better" by 65.6% of the investigators and 65.6% of the patients. The treatment was considered comparable by 19.7% of the investigators and 18.0% of the patients. Only 1.6% of patients and none of the investigators considered the treatment with Octenilin® "better" or "much better".

After 3 months of continuation in which 43 patients participated, the treatment with Oleogel-S10 was superior to treatment with Octenilin®. After 12 months of follow-up with 25 patients, the same result was obtained.

3.1.5. *Camellia sinensis*

Camellia sinensis from the Theaceae family is a rich source of compounds from the polyphenol group, mainly catechin, epicatechin, and their derivatives. It is known primarily for their strong antioxidant and anti-inflammatory properties. Many studies have proven its beneficial effects on the skin, including photoprotection, anti-ageing, and anti-cellulite. Moreover, they have been shown to improve the condition of hair and skin and its blood supply [22].

The effectiveness of a cream with 10% water extract from green tea leaves containing 85% catechins in the treatment of burns was tested in a clinical study compared to a 1% SSD cream [23]. The study involved 50 patients who developed second-degree thermal burns to less than 5% of their total body surface area within 24 hours of starting treatment. The patients were divided equally into two groups so that each group included patients with a similar body surface area affected by the injury. The wounds were cleaned with a saline solution, a cream with green tea extract (GT) or 1% SSD cream was applied directly to the burn, and a dressing was applied. The patients and those changing the dressing and making records did not know which cream had been applied. The wounds

were cleaned, and cream was applied daily. Photographs of the burned area were taken just before applying the cream. Treatment and photographic documentation continued until complete epithelisation. Treatment progress was assessed daily using the Bates-Jensen assessment tool, which includes 13 parameters rated on a 5-point Likert scale. The parameters assessed included wound size, wound depth, wound edge, undermining, necrotic tissue type, necrotic type amount, exudate type, amount of exudate, surrounding skin colour, peripheral tissue induration, peripheral tissue oedema, granulation tissue, and epithelialisation.

There were no statistically significant differences in the assessment of treatment effectiveness in the GT cream and the 1% SSD cream groups, comparing the results between 2 and 14 days. Although in the 1% SSD cream group, only 2 patients had complete epithelialisation after 8 days, and as many as 7 patients in the GT cream group, finally, after 14 days of treatment, the number and time of complete epithelialisation did not differ. Moreover, slightly better (the difference was not statistically significant) patients from the GT cream group assessed the effect of treatment on peripheral oedema, the presence of granulation tissue and epithelialisation on days 8 to 12.

3.1.6. *Centella asiatica*

Centella asiatica is a plant from the Apiaceae family derived from traditional Chinese medicine. The main chemical compounds responsible for its action are terpenoids, mainly asiaticoside, asiatic acid, madecassoside, and madecassic acid. The potential therapeutic effect is mainly related to the influence on the nuclear factor kappa-light-chain-enchancer of activated B cells (NF-κB), migoten-activated protein kinase (MAPK), glycogen synthase kinase 3β (GSK-3β), phosphoinositide 3-kinases/protein kinase B (PI3K/AKT), transforming growth factor β1/Smad (TGF-β1/Smad), and Janus kinases/signal transducer and activator of transcription proteins (JAK/STAT) pathways. Clinical studies have proven, among others, the effect on improving cognitive functions, alleviating anxiety, supporting wound healing or having a beneficial effect on skin care [24].

In a prospective randomised clinical trial, Centiderm® ointment and 1% SSD cream were compared in parallel to treat second-degree burns [25]. Centiderm® ointment contains the butanolic fraction of ethanolic extract (approx. 3%) from *Centella asiatica* leaves and is made with Vaseline and glycerine. Patients with second-degree burns on a limb that covered no more than 10% of the total body surface and occurred within 48 hours of the start of treatment were eligible for the study. Finally, 60 patients randomly assigned to two equal groups were analysed. Centiderm® ointment or 1% SSD cream was applied to the burn once a day until complete healing. On days 0, 3, 7 and 14 of the study, objective (pliability, vascularity, pigmentation, heigh, visual acuity score (VAS) and scoring according to Vancouver Scar Scale (VSS)) and subjective (dryness, itching and irritation) indices were assessed. In addition, the time needed for re-epithelialisation and complete healing was assessed.

Statistically, significantly more favourable effects of Centiderm® ointment were observed compared to 1% SSD cream from the 3rd day of treatment. Pliability, height, vascularity, VAS and VSS were rated significantly better in the group treated with Centiderm® ointment. The exception was pigmentation, for which no differences in the assessment were observed on the seventh day. However, on the 3rd and 14th day, it was assessed more favourably in the Centiderm® group. Also, according to the patients' subjective assessment of dryness, irritation and itching, the use of Centiderm® ointment was more effective and prevailed over 1% SSD cream. The mean time to re-epithelialisation of 13.7 ± 1.48 and 20.67 ± 2.02 days for the Centiderm® group and the 1% SSD cream group, respectively, was significantly shorter for the Centiderm® group. On average, complete wound healing was 14.67 ± 1.78 days in Centiderm® versus 21.53 ± 1.65 days in 1% SSD cream, which was a statistically significant difference.

3.1.7. *Hippophaë rhamnoides*

Hippophaë rhamnoides is a plant of the Elaeagnaceae family that has been cultivated and harvested for its nutritional and medicinal properties since ancient times. It is used mainly due to its anti-diabetic, anti-obesity, and cardiovascular-improving properties. When used topically on the skin, its protective effect against solar radiation is emphasised [26,27].

In a randomised, triple-blind clinical trial, the effectiveness of sea buckthorn cream and 1% SSD cream in treating burns was investigated and compared [28]. The study involved 30 patients with second-degree thermal burns that affected no more than 10% of their total body surface area and occurred within 6 hours of arrival at the hospital. Patients were randomly assigned to two equal groups. The burns of the first group were treated with 1% SSD cream, and the burns of the second group with sea buckthorn cream. Sea buckthorn cream in 100 g contained 40 g of active ingredients from fresh fruits of *Hippophaë rhamnoides*. Once a day, after washing the wound with sterile normal saline, the cream was applied to a thickness of 3 mm. The study was completed by 27 patients in the first group and 28 in the second group.

The average wound healing time in the group treated with sea buckthorn cream was 6.7 $\pm$ 2.1 days and was statistically significantly lower than in the group treated with 1% SSD cream, which was 11.2 $\pm$ 2.3 days.

3.1.8. *Juglans regia*

Walnuts (*Juglans regia*) from the Juglandaceae family are valued primarily in Asia and Europe for their nutritional properties. They are a rich source of unsaturated fatty acids, proteins, vitamins, and minerals. Phytosterols, flavonoids, and polyphenols are also present. Due to their antioxidant, anti-inflammatory, and antibacterial properties, walnuts have been used in folk medicine to treat acne and eczema [29].

The Department of Burns and Plastic Surgery of the General Hospital of Ningxia Medical University (Ningxia, China) developed a walnut-based ointment decades ago that was successfully used to treat non-healing burn wounds. In a retrospective evaluation of cases, it was decided to compare the effectiveness of treatment with walnut ointment, conventional treatment, and surgery [29]. The study enrolled 411 patients with burn wounds covering 0.1 to 7% of the total body surface area, which were classified as non-healing. In the experimental group (49 patients), the burns were covered with a layer of 1–2 mm thick walnut ointment, prepared by crushing the nuts, heating them for 30 min, and grinding them into a paste. In 165 patients receiving conventional treatment, wounds were treated with an antimicrobial agent and recombinant human epidermal growth factor (rhEGF), 88 patients were treated with silver ion dressing + rhEGF, 42 patients were treated with Polymyxin B + rhEGF, and 35 patients were treated with Gentamicin + rhEGF. Patients qualified for the surgical group (197 people) received wound debridement and skin autograft.

The successful cure was reported for 76.60% of cases in the experimental group, 75.13% in the surgical group, and only 9.70% in the conventional treatment group. Treatment with walnut ointment was statistically as effective as surgery, and both treatments were superior to conventional treatment. The time necessary for complete wound closure was 19.87 $\pm$ 9.10 days for the experimental group and 22.71 $\pm$ 11.77 days for the surgical group, and it was significantly shorter than in the conventional treatment group, where it was 36.67 $\pm$ 10.18 days.

3.2. Clinical Trials—Mixtures of Natural Products

From 2010–2022, six clinical trials were also conducted to test the effectiveness of treating burn wounds with mixtures of plant-origin preparations. They are described below and summarised in Table S2 in Supplementary Materials.

3.2.1. *Alkanna tinctoria*, Olive Oil, and Beeswax

The effectiveness of a mixture of *Alkanna tinctoria*, beeswax and olive oil in the treatment of burn wounds was tested in a clinical study [30]. The mixture was prepared by adding 30 g of beeswax to 1000 mL of medical olive oil brought to the boiling point (200–210 °C), and then, after its complete melting, 50 g of *Alkanna tinctoria* (the part of the plant used was not specified) was added and heated for 5 min. Afterwards, the mixture was filtered and dispensed into bottles which were then sterilised. Dressings were prepared immediately before application by saturating a sterile sponge with the mixture.

The study ultimately compared the results of 64 patients (33 in the control group and 31 in the experimental group) with thermal burns caused, in most cases, by boiling liquids within 24 h of admission to the hospital. There were no statistically significant differences in injury characteristics at the start of the study. Dressings were changed every two days using aseptic techniques under sterile conditions. The wound was washed with normal saline and 0.1% chlorhexidine digluconate, and dressings were applied. In the experimental group, it was a dressing saturated with a previously prepared mixture, while in the control group, a standard dressing used in this hospital for burns with nitrofurazone and rifamycin was used.

The time to start re-epithelialisation was 3.0 ± 0.85 days in the experimental group and was significantly shorter than in the control group, which was 6.79 ± 1.77 days. The average pain experienced by patients was significantly lower in the experimental group (8.12 ± 1.38 points) than in the control group (9.39 ± 1.05 points). In addition, the use of treatment with a natural mixture significantly reduced the duration of hospitalisation. In the control group, it was 14.42 ± 7.79 days, and in the experimental group, it was only 8.22 ± 3.05 days.

3.2.2. *Aloe vera* and *Centella asiatica*

A randomised, prospective clinical trial compared the effectiveness of treatment of second-degree burns with a dressing containing *Aloe vera* and *Centella asiatica* and a commercial Bactigras® (Smith & Nephew, Hull, UK) dressing [31]. Thirty-five patients with second-degree burns covering at least 20% of their total body surface area were randomly divided into two groups. The experimental group was treated with a dressing impregnated with lipocolloids, 5% of *Centella asiatica* cream (Cosmelene®), 2.5% spray-dried powder of *Aloe vera* gel, and the standard group with a dressing impregnated with soft paraffin and 0.5% chlorhexidine acetate. Dressings were changed every 3 days until complete wound healing. Each time, the wound surface was measured, and the patient's pain was assessed 30 minutes after applying a new dressing.

The time to complete healing in the group treated with dressings with herbal extracts was 18.53 ± 1.66 days and was significantly shorter than in the group treated with a standard dressing (20.06 ± 2.51 days). Moreover, the hospital patients' stay was significantly shortened, from 22.78 ± 2.58 days in the standard group to 21.12 ± 1.83 days in the experimental group. The percentage of epithelialisation was significantly higher in the experimental group from day 15, and the mean pain the patient experienced was lower than the standard group.

There was one *Pseudomonas aeruginosa* infection in the experimental group on day 7. Therefore, the patient received standard treatment and dropped out of the clinical trial. No alarming symptoms or side effects of treatment with *Aloe vera* and *Centella asiatica*-impregnated dressings were observed.

3.2.3. *Aloe vera*, *Lavandula stoechas*, and *Pelargonium roseum*

A randomised, double-blind clinical trial investigated the effectiveness of a herbal mixture containing *Aloe vera* gel, *Lavandula stoechas*, and *Pelargonium roseum* essential oils in treating burns [32]. The exact composition of the preparation has not been provided. The results were compared with standard treatment with 1% SSD cream. The study enrolled 120 patients with second-degree burns that developed within 48 h of treatment

and covered less than 5% of the total body surface area. The treatment consisted of daily dressing changes preceded by cleaning the wound with an antimicrobial solution and applying a cream (5 g per 10 cm^2 of the injured area). Patients were assessed for pain intensity, skin dryness, and infection.

The study was completed by 111 patients. There were 56 people in the experimental group and 55 in the standard group. There were no statistically significant differences in the occurrence of dry skin between the groups at any time. Both groups had a reduction in pain compared to the first day. In the experimental group, pain intensity was significantly lower on the 7th day than in the standard group. There was only one case of infection in the experimental group that resolved during continued treatment.

3.2.4. *Azadirachta indica* Oil and *Hypericum perforatum* Oil

In a retrospective, non-controlled study, the effectiveness of a plant preparation in a spray (1 Primary Wound Dressing®; Phytoceuticals AG, Zurich, Switzerland) in treating burn wounds was checked [33]. The product contains hypericum oil (*Hypericum perforatum*) and neem oil (*Azadirachta indica*), which, when applied directly to the wound, creates a mist that provides an appropriate wound healing environment and does not adhere to the wound. The review was performed on 9 paediatric patients with 18 wounds in total. Granulation tissue formation, epithelisation, wound surface, pain sensation, and time to healing were assessed.

After a few days of using the preparation, granulation tissue formation and epithelisation were induced. The average time needed for wound healing was 16.6 ± 4.69 days. In six patients, a strong relief of pain was observed (from about 7–8 out of 10 points to 0) in the first week of using the preparation. In the remaining patients, the pain subsided within the second or third week. No adverse effects of the therapy, such as an allergic reaction or infection, were observed.

3.2.5. *Lawsonia inermis* and Beeswax

Originating from Iran, the herbal ointment Fundermol, which contains *Lawsonia inermis* and beeswax, has been used to treat severe burns. The exact composition of the preparation has not been provided. Its effectiveness was tested in a clinical study for treating second-degree burns compared to 1% SSD cream [34]. The study involved 50 patients with burns covering 1 to 10% of the total body surface, which resulted from contact with a heater or hot liquid within 6 hours of arrival at the clinic. Patients were randomly assigned to two equal groups, treated once daily with Fundermol ointment or 1% SSD cream, respectively.

The average wound healing time in the group treated with Fundermol ointment was 4.4 ± 1.87 days and was significantly shorter than in the group treated with 1% SSD cream, which was 5.9 ± 2.20 days.

3.2.6. *Matricaria chamomilla*, *Rosa canina* and Beeswax

Adibderm® ointment is a herbal preparation which includes chamomile and rose extracts, ascorbic acid, beeswax, and oleic and linoleic acids. The formulation activity was tested in a randomised clinical trial involving 60 patients with second-degree burns covering 1–10% of the total body surface area, which occurred within 2 h of admission to the emergency room [35]. Patients were randomly assigned to two groups where patients applied herbal ointment and 1% SSD cream every six hours. The average wound healing time was 7.53 ± 2.28 days in the herbal ointment group and 11.83 ± 2.32 days in the 1% SSD cream group. The difference was statistically significant.

Patients' satisfaction with herbal treatment was significantly higher than conventionally treated patients. In the group treated with Adibderm® ointment, there were no cases of infection, but 7 patients developed irritation, while in the group treated with 1% SSD cream, there was one case of infection and no irritation.

3.3. In Vivo Studies on Animal Models of Burn

In order to better understand the physiological and pathophysiological mechanisms associated with burn injury, in vivo models are used in which animals, mainly mice, rats, guinea pigs, rabbits, hamsters, and sometimes pigs, are used. None of these could be considered better than the others. Rather, they should be considered complementary and show basic mechanisms that may not always reflect the pathology of a burn in humans. Mice and rats are the most commonly used models as they are cheap and have a high reproductive rate. However, there are many differences compared to humans, including their size, anatomy, and metabolic characteristics. It has been shown that, compared to mice or rats, guinea pig skin is anatomically and physiologically more similar to human skin. Due to, among other things, the thickness of the epidermis, the guinea pig burn model better reflects the thermal skin of a human wound [36]. The model closest to humans is pigs. However, they are very expensive to maintain, require increased care and carry a higher risk of infections [37].

Burns are usually formed on the previously hairless backs of animals and cover 5 to 30% of the total body surface area [36]. Animals are obligatorily anaesthetised with pharmacological agents such as ketamine, xylazine, diazepam, midazolam, thiopental and others, or mixtures thereof, and a burn is induced [38]. The most important animal models of burn wound formation include the gas flame burn model, burning ethanol bath burn model, pre-heated single metal plate/bar burn models, boiling or hot water burn models, and pre-heated double brass blocks burn model [36]. The assessment of burn healing is based on the time of epithelisation, wound closure, and histopathological analysis. Biochemical parameters can also be assessed, including the activity of superoxide dismutase, catalase, glutathione S-transferase, hydroxyproline content, or total protein content. Occasionally, the degree of hair regrowth in the injured area can be assessed [38].

In vivo studies in animal models of the effectiveness of preparations of plant origin in treating burn wounds in the single form are presented in Table 1, while mixtures of preparations are presented in Table 2.

Table 1. In vivo studies on animal models—single preparations.

Plant Material	Animal Model	Burn Wound	Treatment Schedule and Study Groups *	Results	Ref.
Aegialitis rotundifolia leaves ethanolic extract	Wistar albino rats, male	Chemical burn—a few drops of concentrated hydrochloric acid Thermal burn—metal rod heated over the open flame for 30 s	Once a day for 18 days E: 2.5% and 5% (*w*/*w*) in the simple ointment S: 1% SSD cream C: simple ointment	The experimental and standard groups significantly increased the percentage of wound closure and decreased epithelisation time after chemical and thermal burns compared to the control group.	[39]
Achillea millefolium aerial parts ethanolic extract	New Zealand white rabbits, male	Third-degree thermal burn—heated metal plate (170 °C) applied for 10 s	Once a day for 21 days E: 5 mL of extract C: 5 mL of normal saline	From the 7th day of treatment, the wound area in the experimental group was significantly smaller than in the control group. After histopathological analysis in the experimental group, complete filling with granulation tissue, an increased amount of collagen fibres and a decrease in the number of inflammatory cells were observed, while in the control group, only fresh granulation tissue was observed. The number of isolated microorganisms decreased in the experimental group.	[40]
Achyranthes aspera leaves methanolic extract	Albino rats, either sex	Third-degree thermal burn—metal rod heated to 85 °C, pressed for 20 s	Twice a day for 7 days E: 5.0% (*w*/*w*) of extract in soft white petroleum S: Himax® (traditional Ayurvedic ointment) C: soft white petroleum	On the 8th day, the wound area of the experimental group was significantly smaller than that of the control and standard groups. From the biochemical parameters, a higher content of protein, vitamin C, glutathione, catalase, superoxide dismutase, and hydroxyproline were noted in the experimental group than in the control and standard group. The concentration of matrix metalloproteinase 9 and matrix metalloproteinase 2 was higher in the experimental than in the control group. Moreover, there were more collagen fibres, and the proliferation of fibroblasts increased.	[41]

Table 1. *Cont.*

Plant Material	Animal Model	Burn Wound	Treatment Schedule and Study Groups *	Results	Ref.
Actinida deliciosa fruits freshly sliced	Wistar albino rats, male	Second-degree thermal burn—hot plate warmed up to 110 °C, placed for 10 s	Once a day for 21 days E: sliced fresh kiwifruit (3 mm thick) S: 1% SSD cream C: Vaseline	The wound area in the experimental group was significantly smaller, and complete wound closure was observed faster than in the standard and control groups. In the macroscopic evaluation, in the experimental group, compared to other groups, an increase in hyperaemia was observed in the first days, and after 11 days, a significant decrease, moreover after 5 days, a reduction in oedema, as well as earlier epithelisation. Histopathological analysis showed significantly less inflammation and a higher score of vascularisation than the other groups, as well as a score of granulation similar to the standard group. Fewer bacteria were isolated from the wound of the experimental group. It has been shown that applying kiwi to a wound results in its enzymatic debridement.	[42]
Actinida deliciosa fruits freshly cut kiwifruit mixed with kiwi juice	Sprague-Dawley rats, male	Third-degree thermal burn—copper stamp, kept at 90 °C for 15 min	Once a day for 30 days E: freshly cut kiwifruit mixed with kiwi juice C: neutral ointment	After 20 days, the eschar separation was significantly accelerated in the experimental group. In addition, a significantly reduced wound surface area and better wound closure were observed than in the control group. No differences were observed in the microscopic assessment of the degree of vascularisation, collagen precipitation and acute and chronic inflammation level.	[43]
Allium cepa bulbs poultice	Holtzman albino rats, male	Second-degree thermal burn—hot 60-watt bulb, applied 3 times for 20 s	Once a day for 21 days E: 4 g of poultice S: 1% SSD cream C: w/o treatment	The wound area after 21 days in the experimental and control groups was similar and significantly smaller than in the control group. In the experimental group, the histopathological examination showed the presence of skin composed of a reticular stratum of fibroblasts, collagen, and several blood vessels. In contrast, only fibroblasts and collagen were present in the standard group; in the control group, a hyperaemic chorion was.	[44]
Aloe vera fresh leaves dry powder of leaf gel	Wistar albino rats, male	Second-degree thermal burn—hot water (90 °C), applied for 6 s	Twice a day for 25 days E: 0.5% of *Aloe* gel powder in base cream S: 1% SSD cream C: base cream N: w/o treatment	After 25 days, the mean wound size was 0.78 ± 1.3, 4.1 ± 3.6, 4 ± 2.3, and 5.5 ± 3 for the experimental, standard, control, and negative control groups, respectively, and was significantly lowest in the experimental group. The biopsy showed that epidermal re-epithelialisation and skin fibrosis were observed in the experimental group. Inflammation and granulation tissue were minimal, and no bacteria were found. Wound healing was significant in this group compared to the standard group. In the control groups, healing was minimal or negligible, and bacteria were present in the wounds.	[45]
Aloe vera fresh leaves dry powder of leaf juice	Wistar albino rats, female	Second-degree thermal burn—a piece of the aluminium heated to 100 °C, applied for 15 s	Once a day for 30 days E: 1 mL of 2% *Aloe* gel NC: w/o treatment	After 24 days, significant differences in the degree of wound closure and epithelialisation were observed between the groups. In the experimental group, wound closure was $95.64 \pm 1.99\%$, and the time of complete epithelisation was 27 days, while in the control group, it was $80.15 \pm 2.80\%$ and 32.5 days, respectively. Histopathological analysis showed that after 18 days of treatment in the experimental group, the number of inflammatory cells was significantly lower, and the degree of epithelialisation and neo-angiogenesis was significantly higher compared to the control group.	[46]
Aloe vera leaves 30% (*v*/*v*) methanolic extract	Wistar albino rats, male	Second-degree thermal burn—metal plate heated in boiling water for 5 min, applied for 10 s	Twice a day for 21 days E: 0.5, 1, 1.5 or 2% of extract in Eucerin S: 1% SSD cream C: Eucerin N: w/o treatment	The degree of wound closure in the experimental groups was higher than in the control groups. After 21 days, the wound area of the group treated with 1.5 and 2% extract was smaller than in the standard group. Histopathological analysis showed better wound healing parameters for the experimental and standard groups than in the control group, such as the number of hair follicles, sebaceous glands, fibroblasts, macrophages, neutrophils, blood vessels, and the thickness of the epidermal layer.	[47]

Table 1. *Cont.*

Plant Material	Animal Model	Burn Wound	Treatment Schedule and Study Groups *	Results	Ref.
Anredera cordifolia leaves ethanolic extract	White rats (*Rattus norvegicus*), male	Thermal burn—iron plate soaking in boiling water for 5 min, applied for 30 s	Three times a day for 14 days E: 2.5, 5 or 7.5% of extract in Vaseline S: 1% SSD cream	Statistically, the best results were obtained in the experimental group treated with 5% of the extract. The highest degree of collagen deposition, the lowest infiltration of polymorphonuclear cells, mild angiogenesis, and moderate fibrosis were observed there. In the standard group, the degree of healing was the lowest.	[48]
Arnebia euchroma whole plant 50% (*v*/*v*) ethanolic extract	Sprague-Dawley rats, female	Third-degree thermal burn—iron plate heated to 100 °C, applied for 40 s	Once a day for 17 days E: 10 or 20% of extract in a carboxymethylcellulose (CMC) gel C: CMC gel N: w/o treatment	The degree of wound closure after 18 days was significantly higher in the group treated with 20% of the extract. However, the histopathological analysis showed the highest content of fibroblasts, collagen, and blood vessels in both experimental groups.	[49]
Arnebia euchroma leaves and root unspecified extract	Wistar albino rats, female	Second-degree thermal burn—aluminium plate heated to 60 °C, applied for 5 s	Once a day for 28 days E: 10 or 20% (*v*/*v*) of extract in vehicle gel S: 1% SSD cream N: w/o treatment	Compared to the control group, the treatment groups showed an increase in the amount of granulation tissue, degree of epithelisation, reduction of the wound area, fibroblast proliferation, volume of collagen fibres, and length and diameter of blood vessels. Wound closure was the fastest in the group treated with 10% extract, and fibroblast proliferation was the highest in the group treated with 20%.	[17]
Azadirachta indica leaves 90% (*v*/*v*) ethanolic extract	Wistar albino rats, male	Second-degree thermal burn—hot wax (80 °C), applied until solidified	Once a day (0.5 g per wound) for 15 days E: 1% of extract in base gel S: 1% SSD cream C: base gel	The average wound closure after 15 days in the experimental group was 81.25 ± 0.7822%, and in the standard group, 90.43 ± 0.7691%, which were higher values than in the control group (68.58 ± 0.7791%). The average re-epithelialisation time was 28.77 ± 1.22 days in the experimental group and 25.20 ± 2.10 days in the standard group, while in the control group, it was significantly longer–38.36 ± 1.77 days.	[50]
Bauhinia purpurea leaves methanolic extract	Sprague-Dawley rats	Second-degree thermal burn—hot molten wax (80 °C), applied until solidified	Once a day for 22 days E: 2.5 or 5% of extract in a simple ointment S: 5% of *Aloe vera* extract C: simple ointment	The wound epithelialisation period was 15.83 ± 0.30, 14.33 ± 0.49, 12.16 ± 0.40 and 13.13 ± 0.40 days for the control, treated with 2.5% extract, treated with 5% extract and standard groups, respectively. The period was statistically significantly shorter for the group treated with 5% extract and the standard group compared to the control.	[51]
Bauhinia purpurea leaves chloroform extract	Sprague-Dawley rats	Second-degree thermal burn—hot molten wax (80 °C), applied until solidified	Once a day for 22 days E: 2.5 or 5% of extract in Carbopol base S: 5% of *Aloe vera* extract C: Carbopol base	The period of wound epithelialisation in the group treated with 5% of the extract and the standard group was 14.50 ± 0.42 and 13.13 ± 0.40 days, respectively, and was statistically significantly shorter than in the control group and the group treated with 2.5% extract, in which it was 16.50 ± 0.50 and 16.33 ± 0.49 days, respectively.	[51]
Brassica oleracea leaves aqueous extract	Sprague-Dawley rats, female	Second-degree thermal burn—hot metal stamp (80 °C), applied for 10 s	Once a day for 28 days E: 1 g of extract per 1 mL base cream (70% sorbitol in glycerin) S: 1% SSD cream C: base cream NC: w/o treatment	Compared to other groups, the experimental group showed a decrease in the fibrinoleukocytic layer and an increase in granulation tissue, as well as a decrease in the number of macrophages and neutrophils in the experimental group at the end of the 2nd week. However, at the end of the 3rd week, the stratum keratinosum was formed, the number of fibroblasts and macrophages increased, and the number of neutrophils decreased. At the end of the 4th week, the epidermis was fully developed.	[52]
Camelia sinensis leaves 70% (*v*/*v*) ethanolic extract	Wistar albino rats, male	Second-degree thermal burn—hot metal plate (120 °C), applied for 5 s	Once a day (1 g per wound) for 21 days E: 0.6% of extract in Vaseline C: Vaseline NC: normal saline	The average healing time was significantly shorter in the experimental group compared to the control group. In histopathological analysis, a significantly lower number of inflammatory cells was observed in the experimental group during the entire study, and no significant differences in epidermal regeneration and angiogenesis were observed. On the 21st day, angiogenesis was significantly higher, and there were no significant differences in other parameters.	[53]

Table 1. *Cont.*

Plant Material	Animal Model	Burn Wound	Treatment Schedule and Study Groups *	Results	Ref.
Camelia sinensis leaves 70% (*v*/*v*) ethanolic extract	Wistar albino rats, male	Second-degree thermal burn—metal cube heated to 100 °C, applied for 15 s	Once a day for 14 days E: 2% of extract in normal saline S: 1% SSD cream C: normal saline	The average burn area was significantly smaller in the experimental group compared to the negative control group. No significant differences in burn size, vascularisation, number of inflammatory cells, and epithelisation were observed between the experimental, standard, and control groups.	[54]
Carissa spinarum roots methanolic extract	Swiss albino mice, either sex	Second-degree thermal burn—hot aluminium plate (85 °C), applied for 5 s	Once a day for 36 days E: 1 or 2.5% of extract in an ointment base S: 1% SSD cream C: ointment base NC: w/o treatment	From day 20, the experimental and standard groups observed a significant increase in wound closure. Reepithelialisation time was significantly reduced compared to controls in the group treated with 2.5% extract and 1% SSD cream. In the histological examination in the group treated with 2.5% of the extract compared to the other groups, healing was advanced. There was a complete renewal of the epidermis, an increase in the amount of collagen and a decrease in the number of inflammatory cells. In addition, the hydroxyproline content was significantly higher.	[55]
Centella asiatica herb 70% (*v*/*v*) ethanolic extract	albino mice, male	Chemical burn—50% phenol solution, applied for 30 s	Once a day for 10 days E: 2% of extract in base gel S: Bioplacenton® jelly (neomycin + placenta extract) C: base gel	Treatment with *C. asiatica* gel improves wound healing compared to the control group but less than in the standard group. Complete wound closure in the experimental group was observed after 8 days, in the standard group after 6 days, while in the control group, after 10 days, the wound closure was only 75.34 ± 20.709%.	[56]
Centella asiatica aerial parts n-hexane, ethyl acetate, methanolic and aqueous extracts	Sprague-Dawley rats, male	Second-degree thermal burn—hot plate heated to 75 °C, applied for 10 s	Once a day (0.5 mL per wound) for 14 days E: 10% of appropriate extract in vehicle C: vehicle or normal saline NC: w/o treatment	The degree of wound healing was statistically significantly higher in the experimental groups than in the control groups. After 14 days, it was 53.87 ± 4.64, 57.53 ± 5.68, 60.31 ± 5.70, and 59.82 ± 8.31% for the hexane, ethyl acetate, methanolic, and aqueous extracts, respectively, and 38.07 ± 5.15, 31.85 ± 2.66, and 25.36 ± 1.81% for the vehicle, normal saline, and untreated controls, respectively. In the histopathological analysis of the experimental groups, in contrast to the control groups, fully developed epithelisation and keratinisation were observed without necrosis and inflammation.	[57]
Cleistocalyx operculatus leaves hydrodistillation	Swiss albino mice, male	Second-degree thermal burn—aluminium bar heated to 100 °C, applied for 15 s	Once a day (50 µL per wound) for 20 days E: 1% of essential oil (in 0.1% DMSO and Tween 20 solution) S: Tamanu oil C: normal saline	The wound area of the experimental group after 10 and 20 days was significantly smaller than in the control and standard groups. In the histopathological analysis in the experimental group, in contrast to the other groups, re-epithelialisation was completed. Fewer inflammatory cells, thick, neatly arranged fibres, and mature hair follicles were observed.	[58]
Copaifera officinalis oleoresin	Swiss mice, male	UVB radiation-induced paw burn model (0.61 mW/cm^2, 0.75 J/cm^2)	Once a day (15 mg per paw) for 6 days E: 3% of oleoresin in base cream S: 1% SSD cream C: base cream NC: w/o treatment	Mechanical allodynia lasting 6 days in the untreated group was significantly reduced from the second day of treatment in the experimental group and the third day in the standard group. Irradiation-induced thermal hyperalgesia was abolished more strongly in the experimental group than in the other groups. Infiltration of inflammatory cells after irradiation was significantly reduced in the experimental and standard groups, while increased skin thickness was significantly reduced only in the standard group.	[59]

Table 1. *Cont.*

Plant Material	Animal Model	Burn Wound	Treatment Schedule and Study Groups *	Results	Ref.
Crocus sativus stigmas 70% (*v*/*v*) ethanolic extract	Sprague-Dawley rats, male	Third-degree thermal burn—aluminium bar boiled in water for 30 s, applied for 10 s	Once a day for 28 days E: 20% of extract in 1% SSD cream S: 1% SSD cream NC: w/o treatment	The percentage of wound closure was significantly higher in the experimental group after 7 days compared to the others. After 14 days, the wounds of the experimental and the standard groups were almost completely closed. In the experimental group, inflammation and redness were significantly reduced, and scarring was minimal. Histopathological analysis after 14 days showed an increase in the number of cells, blood vessels, fibroblasts, and fibrocytes in the experimental group compared to the standard, as well as a comparable, lower than in control, number of inflammatory cells. After 28 days, the number of inflammatory cells, fibrocytes, fibroblasts and total cells significantly decreased in the experimental group compared to the control. In addition, the secretion of interleukin 1β and tumour growth factor β1 was reduced, and the hydroxyproline content was increased.	[60]
Cucurbita moschata fruit peel 70% (*v*/*v*) ethanolic extract	Wistar albino rats, male	Second-degree thermal burn—electrical heater (110 °C), applied for 10 s	Once a day for 14 days E: 10 or 20% of extract in Eucerin S: 1% SSD cream C: Eucerin	The degree of wound closure after 14 days was 57.80 ± 5.71, 78.80 ± 3.96, 77.60 ± 5.41, and 90.80 ± 5.86% for the group, standard, treated with 10% extract and treated with 20% extract groups, respectively. It was significantly lowest in the control group and significantly highest in the group treated with 20% of the extract. Tissue analysis of oxidative stress biomarkers showed that lipid peroxidation in the standard and experimental groups was significantly reduced compared to the control group. The total antioxidant power and total thiol molecules content were significantly higher in the standard group and the group treated with 20% extract than in the control group. In histopathological analysis, the group treated with 20% extract showed better signs of wound healing. Inflammatory cells were absent, collagen fibres were well organised, and the basal epithelial layer reached a normal level.	[61]
Cucurbita moschata oil	BALB/c albino mice, male	Third-degree thermal burn—coin heated for 3 min with a spirit lamp, applied for 8 s	Once a day for 28 days E: 30 or 40% of oil NC: w/o treatment	Compared to the negative control, the group treated with sesame oil showed better wound healing, higher total antioxidant power, and lower malondialdehyde levels than the control.	[62]
Ephedra alata whole plant 2-step extraction with n-hexane and 50% (*v*/*v*) ethanol	Syrian hamsters (*Mesocricetus auratus*), male	Third-degree thermal burn—metal plate boiled in water for 5 min	Once a day for 15 days E: 1.5% of extract in an ointment base C: ointment base NC: w/o treatment	In the macroscopic assessment, the burn wound of the experimental group healed faster and better than in the other groups. However, in the histopathological analysis, no statistically significant differences in the degree of fibrosis and collagen fibres density were observed compared to the control group.	[63]
Globularia alypum leaves methanolic extract	Wistar albino rats, male	Second-degree thermal burn—electric heater (110 °C), applied for 10 s	Once a day for 16 days E: extract in glycerol (unspecified concentration) S: Cytol Centella® cream (with *Centella asiatica*) C: glycerol NC: normal saline	From day 12, the wound closure of the experimental group was significantly better compared to the untreated group. The hydroxyproline level in the experimental group was significantly higher than in the other groups. In the histopathological analysis after 16 days, the appearance of the epidermis and skin in the experimental group was normal, while in the standard group, there were signs of inflammation. In the untreated groups, there was a massive infiltration of inflammatory cells without a developed epidermal layer.	[64]
Glycyrrhiza glabra roots 75% (*v*/*v*) ethanolic extract	Sprague-Dawley rats, male	Third-degree thermal burn—hot plate	Once a day for 29 days E: 10% of extract in base gel S: 1% SSD cream C: base gel NC: w/o treatment	After 14 days, no signs of wound closure were observed macroscopically in the experimental group. Histopathological evaluation of this group showed complete re-epithelialisation, minimal granulation tissue formation, mild inflammation, and irregular collagen distribution. In contrast, in the standard group, only granulation tissue and severe inflammation were present. Coagulative necrosis of the epidermis without granulation tissue was noted in the untreated groups. After 28 days, there were no differences between the groups in tensile strength, maximum stress, yield strength and stiffness.	[65]

Table 1. *Cont.*

Plant Material	Animal Model	Burn Wound	Treatment Schedule and Study Groups *	Results	Ref.
Gundelia tournefortii aerial parts unspecified extract	Wistar albino rats, male	Second-degree thermal burn—metal plate heated in a flame for 5 min, applied for 8 s	Once a day for 21 days E: extract with milk-cream (4:1) S: 1% SSD cream NC: w/o treatment	From day 7, the wound dimensions of the experimental group and the standard were significantly smaller than those of the controls. From day 14, significantly greater wound closure was observed in the experimental group than in the other groups. After 21 days, the histopathological analysis in the experimental group showed a reduction in inflammation and an increase in the degree of re-epithelialisation and the length of blood vessels compared to the standard group. In addition, there was a significantly higher total volume of collagen fibres, and the scab covered a larger wound area than in the control group.	[66]
Hippophaë rhamnoides leaves aqueous extract	Sprague-Dawley rats, male	Third-degree thermal burn—metal rod heated to 85 °C, applied for 20 s	Twice a day for 7 days E: 2.5, 5, 7.5 or 10% of extract in soft white petroleum S: 1% SSD cream C: soft white petroleum	The wound area on days 4 and 8 was the smallest in the group treated with 5% extract. Also in this group, significantly higher contents of hydroxyproline, hexosamine, protein, matrix metalloproteinase 9, vascular endothelial growth factor, type-III collagen, and antioxidants (glutathione, vitamin c, superoxide dismutase, catalase, glutathione S-transferase and malondialdehyde) were found in the granulation tissue than in control. The epidermis thickness in this group was comparable to the standard group and significantly higher than in the control group, and the density of blood vessels was significantly higher than in the other groups.	[67]
Hippophaë rhamnoides seeds oil	Merino sheep, female	Third-degree flame burns—applied with a Bunsen gas burner	Every 6 days for 18 days E: 20 mL of oil C: w/o treatment	The re-epithelialisation time was significantly shortened in the treated group, and the degree of epithelialisation was significantly higher than in the control group. There were no statistically significant differences between the groups in the mean peripheral blood flow and the mean content of malondialdehyde and superoxide dismutase in the wound.	[68]
Hippophaë rhamnoides leaves aqueous extract	Sprague-Dawley rats, male	Third-degree thermal burn—metal probe heated to 85 °C, applied for 20 s	Twice a day for 7 days E: 2.5% of extract in soft white petroleum S: 1% SSD cream C: soft white petroleum	In the macroscopic evaluation, the experimental group showed the best wound repair and improvement of the peri-wound skin condition. Compared to the control, the wound of the experimental group had a significantly reduced level of reactive oxygen species and the inflammatory response (3-nitrotyrosinase, nitric oxide synthase-2, tumour necrosis factor α, interleukin 1β, interleukin 6 and NF-κB), as well as significantly increased expression of markers responsible for cell proliferation, epithelial migration, angiogenesis, skin hydration, and cytoprotection (proliferating cell nuclear antigen, cytokeratin-14, cluster of differentiation 31, aquaporin 3, hypoxia-inducible factor 1α, glucose-regulated protein 78, and transient receptor potential vanilloid 3). In contrast to the control and standard groups in the treated group, the tissue was characterised by a well-organised orientation with increased mature collagen and rapid epithelial migration towards the wound bed. Increased activity of hexokinase, citrate synthase, glucose-6-phosphate dehydrogenase, mitochondrial enzyme cytochrome c oxidase and an increase in adenosine triphosphate levels were noted, and lower activity of lactate dehydrogenase.	[69]
Hypericum perforatum seeds oil	Sprague-Dawley rats, male	Second-degree thermal burn—iron plate heated in boiling water for 5 min, applied for 20 s	Once a day for 20 days E: 2 mL of oil NC: w/o treatment	In the treatment group, re-epithelialisation was complete after 21 days, while in the control group, no epidermal layer was formed. In the histopathological analysis of the experimental group, significantly fewer inflammatory cells and increased angiogenesis were observed than in the control group.	[70]

Table 1. *Cont.*

Plant Material	Animal Model	Burn Wound	Treatment Schedule and Study Groups *	Results	Ref.
Juglans regia seeds grounded into ointment	Guangxi Bama mini-pigs, female	Third-degree thermal burn—aluminium plate heated in boiling water for 10 min, applied for 45 s. The wounds were left unhealed for 3 weeks.	After 3 weeks, the wound was cleaned and treated twice daily for 28 days. E: walnut ointment S: recombinant human Epidermal Growth Factor C: normal saline	Compared to the control group, the experimental and standard groups showed a significant reduction in the wound area and improved healing from day 7 to day 28. The total wound closure time was 18.44 ± 3.09, 23.56 ± 4.85 and 32.56 ± 5.36 days in the experimental, standard and control groups, respectively. After 28 days, the histopathological analysis showed significantly thinner proliferative and differentiating epidermis layers in the experimental group. The positive staining of P63 (epidermal proliferation marker) and CK10 (epidermal differentiation marker Cytokeratin 10) was stronger in the standard group compared to the others, but in the experimental group, the positive staining of P63 was stronger than in control.	[29]
Linum usitatissimum seeds oil	New Zealand rabbits, male	Second-degree thermal burn—stainless steel cylinder heated in boiling water for 3 min, applied for 15 s	Once a day (1 g per wound) for 28 days E: linseed oil S: Cicatryl-bio® ointment (sodium hyaluronate + allantoin) C: Vaseline NC: w/o treatment	The degree of wound closure was significantly higher in the experimental and standard groups from day 16 than in the other groups. The total wound healing time was 26 ± 5.8, 32.5 ± 2.8, 35.6 ± 3.9 and 35 ± 1.1 days for the experimental, standard, control, and negative control group, respectively. In the histopathological analysis of the experimental group, compared to the other groups, a smaller number of inflammatory cells, complete re-epithelialisation, reduced thickness and fibrosis of the epidermis, and an increase in the number of capillaries, collagen fibres, fibroblasts, and myofibroblasts were observed.	[71]
Lobelia alsinoides whole plant ethanolic extract	Wistar albino rats, male	Third-degree thermal burn—metal plate, heated red hot, applied for 30 s	Once a day for 16 days E: 5% or 10% of extract in a simple ointment S: 1% SSD cream C: simple ointment NC: w/o treatment	In the group treated with 10% of the extract, complete macroscopic re-epithelialisation was visible after 12 days, and in the group treated with 5% of the extract after 16 days. In the other groups, after 16 days, the wound was still open and red. In the histopathological analysis after 16 days, the experimental groups, compared to the others, showed neovascularisation, complete re-epithelialisation, fibroblast proliferation, neutrophil infiltration, angiogenesis, increased amount of collagen and decreased inflammation.	[72]
Malva sylvestris flowers 70% (*v*/*v*) ethanolic extract	Albino rats, male	Second-degree thermal burn—metal plate heated in boiling water for 5 min, applied for 10 s	Once a day for 35 days E: 5% or 10% of extract in base cream S: 1% SSD cream C: base cream NC: normal saline	There was a significant increase in the percentage of wound closure in the experimental groups from the 7th day. After 8 days, in the experimental groups, an increase in the thickness of the epidermis and granulation tissue was observed, and the organisation of squamous cell maturation and orthokeratin improved. In addition, after 21 days, a higher degree of scar formation, collagen organisation, formation of hair follicles and lymphatic vessels, and the degree of innervation was observed in the experimental groups.	[73]
Michelia champaca flowers ethanolic extract	Wistar albino rats, male	Second-degree thermal burn—hot molten wax (80 °C), applied for 8 min	Once a day until healed E: 10% of extract in an ointment base S: 1% SSD cream	The epithelisation period in the experimental group was 18.33 ± 2.42 days and was shorter than in the standard group, which was 21.67 ± 3.01 days.	[74]
Musa paradisiaca stems methanolic extract	Wistar albino rats	Third-degree thermal burn—red hot steel rod	Once a day for 14 days E: methanolic extract (unspecified concentration and preparation) C: Vaseline	Compared to the control group, the experimental group showed an increase in wound closure percentage, epithelisation, and faster tissue regeneration.	[75]
Myrtus communis leaves ethanolic extract	Wistar albino rats	Third-degree thermal burn—exposed to 90 °C water bath for 10 s	Twice a day for 48 h E: 5% (*w*/*w*) of extract in simple ointment (0.5 g) NC: w/o treatment	In the skin affected by a burn injury, an increase in superoxide dismutase and catalase activity was observed, as was an increase in malondialdehyde and a decrease in glutathione and nitric oxide levels. After the topical application of 5% ointment, a significant reduction in malondialdehyde level, an increase in nitric oxide level, and an increase in superoxide dismutase and catalase were observed. There was no effect on glutathione level and total tissue protein.	[76]

Table 1. *Cont.*

Plant Material	Animal Model	Burn Wound	Treatment Schedule and Study Groups *	Results	Ref.
Nigella sativa seeds oil	Wistar albino rats, male	Second-degree thermal burn—brass probe heated in boiling water, applied for 20 s	Twice a day for 14 days E: 50% of oil + 50% of cold cream S: 1% SSD cream C: cold cream	The experimental and standard groups observed a reduction in the clinical signs of inflammation, such as warmth, redness, and swelling. The histopathological analysis showed a significant increase in granulation tissue thickness in these groups. After 14 days, the wound appearance of the experimental group was the most normal.	[77]
Olea europaea seeds oil	domestic pigs, female	Second-degree thermal burn—aluminium bar preheated to 400 °C, applied for 20 s, the necrotic epidermis was removed using bromelain-derived agent, Debridase®	Once a day for 14 days E: purified olive oil S: 1% SSD cream NC: w/o treatment	After 14 days, it was observed that treatment efficiency was significantly better in the standard group. There were no significant differences in wound healing between the experimental and no-treatment groups.	[78]
Olea europaea leaves 70% (*v*/*v*) ethanolic extract	Wistar albino rats, male	Third-degree thermal burn—metal plate, heated in 94 °C water for 20 min, applied for 30 s	Twice a day for 21 days E: 10% of extract in Eucerin S: 1% SSD cream NC: w/o treatment	In the macroscopic evaluation from day 14, it was observed that the treated groups had significantly less wound area than the untreated group. Moreover, the wound area of the experimental group was significantly smaller than that of the standard group. In the histopathological evaluation after 14 days, it was observed that the number of neutrophils and macrophages in the wound significantly decreased in the treatment groups, and the number of fibroblasts increased. There were no differences between the groups in the number of endothelial cells.	[79]
Onosma dichroanthum roots acetone extract	Wistar albino rats, female	Second-degree thermal burn—metal rod heated in boiling water to 95 °C, applied for 10 s	Once a day for 14 days E: 2% of extract in a base ointment S: 1% SSD cream C: base ointment NC: w/o treatment	After 14 days in the standard group, 2 rats had the wound completely healed, and in the remaining rats, the area was significantly reduced compared to the control groups. On the other hand, in the experimental group, the surface area not only did not decrease but increased its surface area.	[80]
Onosma bulbotrichum roots n-hexane and dichloromethane (1:1) extract	rabbits, either sex	Second-degree thermal burn—steel plate heated to 150 °C, applied for 20 s	Twice a day until healed E: 1, 2 or 5% of extract in cold cream S: 1% SSD cream C: cold cream NC: w/o treatment	In the study, it was observed that in the standard group and the group treated with 5% of the extract, the time to complete wound healing was significantly shortened to 16 and 17 days, respectively. In contrast, in the control group and the negative control, it was 24 and 26 days, respectively. Compared to the control groups, the wounds of the treatment groups had a significantly higher content of collagen and non-collagen proteins. The histopathological analysis observed the best wound healing in the group treated with the ointment with 5% extract.	[81]
Phyllanthus niruri whole plant ethanolic extract	Wistar albino rats, male	Second-degree thermal burn—hot wax (80 °C), applied for 8 min	Once a day until healed E: 10% of extract in a simple ointment S: 1% SSD cream	Compared to the group treated with 1% silver sulfadiazine cream, topical administration of an emulgel containing 10% of the extract does not reduce the wound area or shorten the epithelisation period.	[82]
Pistacia atlantica resin hydrodistillation	Wistar albino rats, female	Third-degree thermal burn—aluminium plate heated to 100 °C, applied for 10 s	Once a day for 14 days E: 5, 10 or 20% of resin oil in the ointment base C: ointment base	There were no significant differences in the size of the wound in the study groups in the macroscopic assessment. In the microscopic and histopathological evaluation, the groups treated with the emulgel with 5 or 10% of the extract developed more capillaries, and significantly higher concentrations of basic fibroblast growth factor and platelet-derived growth factor were observed than in the control group.	[83]
Pistacia atlantica mastic gum ethanolic extract and essential oil	albino rabbits, male	Second-degree thermal burn—metal plate	Once a day for 21 days E: 30% (9.12 mL of extract + 24.15 mL of essential oil) or 60% (18.24 mL of extract + 48.3 mL of essential oil) of composition in Eucerin C: 30 or 60% of distilled water in Eucerin	After 21 days, the degree of wound closure was significantly higher and amounted to 65% and 94%, respectively, for the group treated with 30% and 60% of the extract, and for the corresponding control groups, 8% and 10%, respectively. Blood concentrations of glutathione peroxidase, superoxide dismutase, catalase, and HDL in the treatment groups were significantly elevated compared to controls. Contrary to control groups, glucose concentration was not elevated. However, there were no significant differences between the groups in malondialdehyde and LDL concentrations.	[84]

Table 1. *Cont.*

Plant Material	Animal Model	Burn Wound	Treatment Schedule and Study Groups *	Results	Ref.
Pistacia atlantica resin oil purchased	Sprague-Dawley rats, male	Third-degree thermal burn—aluminium plate heated to 100 °C, applied for 15–20 s	Once a day (200 mg/kg body weight) for 14 days E: resin oil S: 1% SSD cream NC: w/o treatment	The degree of wound closure in the experimental group after 14 days was 98.6 ± 2.5% and was comparable to the standard group (94.7 ± 4.1%) and significantly higher than in the negative control group (71.2 ± 3.4%). The level of superoxide dismutase, glutathione peroxidase, total antioxidant status, vascular endothelial growth factor, and hydroxyproline was significantly higher in the experimental group compared to the negative control group and the standard group. The malondialdehyde level was comparable to the standard group and higher than the control group.	[85]
Pistacia lentiscus oil cold pressed	New Zealand rabbits, male	Third-degree thermal burn—metal cylinder heated for 3 min in boiling water, applied for 15 s	Once a day until healed E: 1 mL of oil S: 1% Madecassol® (with *Centella asiatica*) C: Vaseline NC: w/o treatment	The period of complete re-epithelisation in the treatment groups was 30 ± 3.94 and 33.5 ± 3.78 days in the experimental and standard groups, respectively. It was significantly shorter than in the negative control group, which was 37.16 ± 3.54 days, but it was not considerably different from the group receiving Vaseline (34.66 ± 3.88 days).	[86]
Plantago major seeds aqueous extract	Sprague-Dawley rats, male	Third-degree thermal burn—hot metal plate	Once a day for 21 days E: 20 or 50% of extract in Eucerin S: 1% SSD cream C: Eucerin	There were no statistically significant differences between the groups in wound size. However, in the histopathological analysis of the wound in the experimental group, in contrast to the control group, good re-epithelialisation and organisation of the granulation tissue were observed. Parallel-oriented fibroblasts were present in the well-structured layer of the epidermis, and the number of inflammatory cells and capillaries was high.	[87]
Pothos scandens leaves ethanolic extract	Wistar albino rats, either sex	Thermal burn—iron plate heated in a flame to red hot, applied for 10 s	Once a day for 20 days E: 2, 4, 6, 8 or 10% of extract in glycerol C: glycerol NC: w/o treatment	The extract's application significantly reduced the re-epithelialisation time compared to the control groups. The shortest time was obtained in the group treated with 4% extract (22 ± 2.43 days). In the control group, the time was 35 ± 1.69 days, and in the negative control group, 40 ± 1.06 days.	[88]
Punica granatum peel standardised pomegranate rind extract (13% of ellagic acid)	Wistar albino rats, male	Third-degree thermal burn—metal rod heated to 100 °C in boiling water, applied for 20 s	Once a day (0.5 g per wound) for 12 days E: 1, 2.5 or 5% of extract in formulation base S: 1% SSD cream C: formulation base	Significant differences in the percentage of wound closure between the treatment and control groups were observed from day 4 onwards. The extract reduced the wound surface area in a concentration-dependent manner and was comparable to 1% silver sulfadiazine cream. In addition, in the standard group and, depending on the concentration, in the experimental groups, a significant reduction in myeloperoxidase activity was observed, which is an indicator of inflammatory neutrophil infiltration.	[89]
Punica granatum fruits standardised pomegranate extract (40% of ellagic acid)	albino rats (*Rattus norvegicus*), male	Second-degree thermal burn—steel plate heated to 85 °C, applied for 5 s	Twice a day for 14 day E: 2.5, 5 or 10% of extract in a cream base S: 1% SSD cream C: cream base	The experimental groups showed higher re-epithelialisation and collagen levels and reduced neutrophil infiltration and angiogenesis than the control and standard groups.	[90]
Punica granatum fruits methanolic extract	minipigs, either sex	Second-degree thermal burn—fuel smeared on the skin and lit by an open fire for 45 s	Twice a day for 28 days E: 5% of extract in base gel S: 1% SSD cream or 20 g/kg Jing Wan Hong herbal ointment C: base gel NC: w/o treatment	After 28 days of treatment, the skin morphology in the experimental group improved and was close to normal skin. Compared to the control, comparable acceleration of healing, shortening of healing time, and elimination of scab and fur growth were observed in the experimental and standard groups. From day 7, the treatment groups showed an increase in vascular endothelial growth factor A and transforming growth factor β1 levels compared to the control groups.	[91]
Rubus caesius leaves 70% (*v*/*v*) ethanolic extract	Wistar albino rats, male	Third-degree thermal burn—steel device heated in boiling water (100 °C), applied for 5 s	Once a day for 21 days E: 10% of extract in cold cream S: 1% SSD cream C: cold cream	After 7 days, compared to the control and standard group, a higher number of capillaries was observed in the experimental group, and their number decreased in the following days. After 21 days, an almost complete wound healing with well-rebuilt granulation tissue and no inflammatory cells were observed in the experimental group and incomplete epithelisation and infiltration of inflammatory cells in the control and standard groups.	[92]

Table 1. *Cont.*

Plant Material	Animal Model	Burn Wound	Treatment Schedule and Study Groups *	Results	Ref.
Sambucus nigra flowers, leaves 70% (*v*/*v*) ethanolic extract	Wistar albino rats, male	Third-degree thermal burn—steel device heated in boiling water (100 °C), applied for 5 s	Once a day for 21 days E: 10% of extract of flowers or leaves in cold cream S: 1% SSD cream C: cold cream	Compared to the control group and the standard, the experimental group had a higher number of capillaries after 7 days, and their number decreased in the following days. After 21 days, an almost complete wound healing with well-rebuilt granulation tissue and no inflammatory cells were observed in the experimental group and incomplete epithelisation and infiltration of inflammatory cells in the control and standard groups.	[92]
Sauromattum guttatum tubers 70% (*v*/*v*) methanolic extract	BALB/c mice, either sex	Second-degree thermal burn—metal bar heated on opened flame, applied for 9 s	Three times a day for 15 days E: 2% of extract in petroleum jelly S: 1% SSD cream C: petroleum jelly NC: w/o treatment	After 15 days, the experimental and standard groups showed a reduction in wound area compared to the control group. In the histopathological analysis, in the treated groups, in contrast to the control groups, a normal healing process was observed, i.e., normal regeneration of the epidermis, the presence of new capillaries, granulation tissue, sebaceous glands and hair follicles. In addition, the treatment groups increased the expression of platelet-derived growth factor, epidermal and fibroblast growth factor.	[93]
Sanguisorba officinalis roots 70% (*v*/*v*) ethanolic extract	Sprague-Dawley rats, male	Second-degree thermal burn—electrical scald instrument (75 °C), applied for 15 s	Twice a day for 14 days E: 1 mL of extract (100 mg/mL) S: 0.3 g of 1% SSD cream NC: w/o treatment	After 14 days, the experimental and standard groups' wounds was significantly smaller than the wound of the control group. In the histopathological analysis in the treatment groups, rapid progression of reepithelialisation, formation of granulation tissue, collagen fibres and blood vessels, and disappearance of inflammatory cells were observed in contrast to the negative control.	[94]
Senna podocarpa leaves 50% (*v*/*v*) ethanolic extract	Wistar albino mice, either sex	Second-degree chemical burn—hydrochloric acid (0.2 mL, 37%), applied for 15 s	Once a day for 14 days E: 2.5 or 7.5% of extract in base emulgel or extract poultice S: 1% SSD cream C: base emulgel NC: w/o treatment	The degree of wound closure after 14 days was 64, 87, 50 and 66% for the group treated with 2.5% extract, 7.5% extract, extract poultice and 1% SSD cream, respectively. In contrast, in the control groups, the degree of wound closure was below 10%. The group treated with 7.5% extract in emulgel had the best results in histopathological analysis, with significantly higher levels of keratin, epidermal cells, gland cells, adipocytes, and collagen than in the control and standard groups. However, more inflammatory cells and fewer elastin fibres were also observed.	[95]
Sesamum indicum oil cold pressed	BALB/c albino mice, male	Third-degree thermal burn—coin heated for 3 min with a spirit lamp, applied for 8 s	Once a day for 28 days E: 30 or 40% of oil NC: w/o treatment	Compared to the negative control, the group treated with sesame oil showed better wound healing. The level of total antioxidant power was significantly higher, and the level of malondialdehyde significantly lower than in control.	[62]
Terminalia chebula fruits 70% (*v*/*v*) ethanolic extract	Wistar albino rats, male	Second-degree thermal burn—hot water (90 °C), applied for 6 s	Once a day for 30 days E: 5 or 10% of extract in base cream S: 1% SSD cream C: base cream NC: normal saline	The wound size decreased the most in the group treated with the cream with 10% extract. Significant differences in wound size between this group and the other groups were evident from day 10. Complete wound closure was seen after 20 days in the 10% extract cream group, 25 days in the 5% extract cream group, and 33–35 days in the standard and control groups. In the morphological and histopathological analysis, inflammatory cell infiltration, neovascularisation, fibroblast proliferation, mucopolysaccharide deposition in the matrix, degree of inflammation, the extent of bacterial colonisation, and degree of granulation tissue formation were scored. Statistically, the best results, indicating an advanced degree of healing, were obtained for the group treated with cream with 10% of the extract. The group treated with the cream with 5% extract obtained as good results as the standard group.	[96]

Table 1. *Cont.*

Plant Material	Animal Model	Burn Wound	Treatment Schedule and Study Groups *	Results	Ref.
Tragopogon graminifolius aerial parts 80% (*v*/*v*) ethanolic extract	Wistar albino rats, male	Second-degree thermal burn—aluminium rod, heated to 110 °C, applied for 10 s	Once a day for 14 days E: 5 or 10% of extract in Eucerin S: 1% SSD cream C: Eucerin	After 14 days, the wound area was reduced by 80, 73, 78, and 58% in the 10% extract, 5% extract, standard, and control groups, respectively. Significant differences were observed in the standard group and the group treated with 10% extract compared to the control. In these two groups, also in the histopathological analysis, re-epithelialisation of the epidermis and a significantly better profile of biomarkers of tissue oxidative stress, including a higher content of total thiol molecules and lower lipid peroxidation, were visible. The control and experimental groups observed no differences in total antioxidant power.	[97]
Tridax procumbens leaves 90% (*v*/*v*) ethanolic extract	Wistar albino rats, male	Second-degree thermal burn—hot wax (80 °C), applied until solidified	Once a day (0.5 g per wound) for 15 days E: 1% of extract in base gel S: 1% SSD cream C: base gel	Wound closure after 15 days was 69.14 ± 0.7497% in the experimental group, 90.43 ± 0.7691 % in the standard group, and 68.58 ± 0.7791% in the control group. The mean re-epithelisation time was 32.10 ± 0.86 days in the experimental group, 25.20 ± 2.10 days in the standard group and 38.36 ± 1.77 days in the control group. There were no statistical comparisons.	[50]
Viola tricolour flowers 70% (*v*/*v*) ethanolic extract	Wistar albino rats, male	Sunburn—UVB radiation: 0.27 mW/cm^2, 0.5 J/cm^2	Once a day for 6 days E: 1, 3 or 10% of extract in base gel S: 1% SSD cream C: base gel	In the groups treated with 3 and 10% of the extract, static and dynamic allodynia and paw oedema were significantly reduced, and the increase in myeloperoxidase activity was inhibited compared to the control, showing effectiveness comparable to that in the standard group.	[98]
Vitis vinifera leaves 30% (*v*/*v*) methanolic extract	Wistar albino rats, male	Second-degree thermal burn—metal plate heated in boiling water for 5 min, applied for 10 s	Twice a day for 21 days E: 0.5, 1, 1.5 or 2% of extract in Eucerin S: 1% SSD cream C: Eucerin NC: w/o treatment	Wound healing in the experimental groups was better than in the control groups. Wound healing in the experimental group was worse (for 0.5, 1 and 1.5%) or comparable (for 2%) than in the standard group. The groups treated with 1.5 and 2% of the extract showed better healing parameters than the control groups, such as the number of hair follicles, sebaceous glands, fibroblasts, macrophages, neutrophils, blood vessels, and the thickness of the epidermal layer.	[47]
Zanthoxylum bungeanum seeds oil (expeller pressed)	Sprague-Dawley rats, male	Second-degree thermal burn—hot water (100 °C), applied for 12 s	Twice a day for 7 days, then once a day until healed E: 0.5 or 1 mL of oil per wound S: 1% SSD cream NC: w/o treatment	From day 7 of treatment, a dose-dependent increase in wound healing was observed in the experimental groups compared to the controls. The increase for the group treated with 1000 µL of oil was comparable to the standard group. Histopathological analysis showed a statistically significantly thicker epidermal layer in the treated groups compared to the controls, but no significant differences in skin thickness were found. Levels of superoxide dismutase, hydroxyproline, and type-III collagen were significantly higher in experimental and standard groups compared to controls. However, the level of malondialdehyde, matrix metalloproteinase 2, matrix metalloproteinase 9, tumour necrosis factor α, interleukin 6, interleukin 1β, phosphor-nuclear factor-κB p65, and phosphor-inhibitor of nuclear factor-κB subunit α was significantly lower in these groups compared to controls.	[99]

* C—control group; E—experimental groups; NC—negative control group; S—standard/positive control group; SSD— silver sulfadiazine; w/o—without.

Table 2. In vivo studies on animal models—a mixture of preparations.

Composition of the Mixture	Animal Model	Burn Wound	Treatment Schedule *	Results	Ref.
Allium sativum (bulbs; squeezed juice)Euphorbia honey	Wistar albino rats, either sex	Thermal burn—metal plate, heated in boiling water for 10 min, applied for 20 s	Once a day until healed E: Euphorbia honey or a mixture of Euphorbia honey and *Allium sativum* juice (amount and concentration not given) S: Betadine solution or 1% SSD cream	The shortest time needed for complete epithelisation and wound closure was noted for the group treated with the mixture, 1% SSD cream, and then for the group treated with Euphorbia honey. The longest time was recorded in the group treated with betadine. In the histological examination, the group treated with the mixture was characterised by a thicker layer of epidermis and skin than the other groups. There were no differences between the groups in the interdigitation index and the orientation of collagen fibres.	[100]
Aloe vera leaves *Vitis vinifera* leaves 30% (*v*/*v*) methanolic extracts	Wistar albino rats, male	Second-degree thermal burn—metal plate heated in boiling water for 5 min, applied for 10 s	Twice a day for 21 days E: the combination of leaf extracts in a ratio of 1.5%:1.5% in Eucerin S: 1% SSD cream C: Eucerin NC: w/o treatment	The wound area of the experimental group after 7, 14 and 21 days was significantly smaller than the wounds of the control groups but comparable to the standard group. It has been observed that treatment with the composition has better healing effects than treatment with the individual components. Compared to the control and standard groups, the experimental group showed a higher degree of tissue maturation and organisation and re-epithelialisation, a fully formed epidermis, more hair follicles, sebaceous glands, fibroblasts and capillaries, and a decrease in neutrophil and macrophage infiltration.	[47]
Arctocarpus heterophyllus, fruits *Murraya koenigii* leaves *Nerium indicum*, leaves *Punica granatum*, bark 70% (*v*/*v*) ethanolic extracts	albino rats, either sex	Third-degree chemical burn—sulphuric acid, applied for 10 s	Once a day until healed E: 10% or 15% of the combination of extracts (1:1:1:1) in ointment base or base gel S: Povidone-iodine C: ointment base or base gel NC: w/o treatment	The period of epithelialisation in the experimental groups with the basic ointment, the basic gel, and the standard group was significantly shorter than in the corresponding control groups. In addition, it was shorter than in the groups treated with single components. Hydroxyproline level and tensile strength were higher in the experimental and standard groups than in the controls. In the histopathological analysis, better wound healing parameters were observed in the experimental groups with base ointment than with base gel.	[101]
Azadirachta indica leaves *Tridax procumbens* leaves Honey 90% (*v*/*v*) ethanolic leaf extracts	Wistar albino rats, male	Second-degree thermal burn—hot molten wax (80 °C), applied until solidified	Once a day for 15 days E: herbal gel (unspecified concentration) S: 1% SSD cream C: base gel	The degree of wound closure after 15 days was 89.35 ± 0.4155, 90.43 ± 0.7691 and 68.58 ± 0.7791%, and the period of epithelisation was 26.32 ± 2.22, 25.20 ± 2.10 and 38.36 ± 1.77 days in the experimental, standard and control groups, respectively. The results obtained in the experimental group were as good as in the standard group and significantly better than in the control group. Moreover, the formulation has been shown to have synergistic activity in wound healing as the results obtained are better than those of the single components of the formulation.	[50]
Calendula officinalis *Rosa damascena* Beeswax	Wistar albino rats, male	Second and third-degree thermal burn—metal plate heated in boiling water for 5 min, applied for 10 or 30 s	Once a day for 40 days E: commercial herbal ointment Robacin® (the exact composition is not given) S: 1% SSD cream or *Aloe vera* cream	In the second-degree burn group treated with herbal ointment, wound healing was significantly fastest for the first two weeks and comparable to the standard groups. In the third-degree burn group, wound closure was the fastest in the experimental group. In both burns (second and third degree), a much smaller extent of angiogenesis and fibrosis was observed in the experimental group than in the standard groups. In third-degree burns, epithelialisation in the experimental group was as good as in the *Aloe vera* cream treatment.	[102]
Cannabis sativa *Juglans regia* *Pistacia atlantica* *Sesamum indicum* cold pressed oils	albino mice, male	Third-degree thermal burn—boiling water (100 °C), applied for 10 s	Twice a day for 21 days E: the combination of sesame oil (60%), pistachio oil (20%), hemp oil (12%) and walnut oil (8%) S: 1% SSD cream NC: w/o treatment	The degree of wound closure was 99.5 ± 0.8, 78.0 ± 4.0 and 88.4 ± 2.5% in the experimental, standard and control groups. The differences between the groups were statistically significant. In the experimental, standard and control groups, the time to complete epithelisation was 20.5 ± 1.37, 26.33 ± 0.81 and 25.5 ± 0.83 days. It was significantly shorter in the group treated with the oil composition.	[103]

Table 2. *Cont.*

Composition of the Mixture	Animal Model	Burn Wound	Treatment Schedule *	Results	Ref.
Centella asiatica, herb, 70% (*v*/*v*) ethanolic extract Papaya latex, dried and powdered	albino mice, male	Chemical burn—50% phenol solution, applied for 30 s	Once a day for 10 days E: *C. asiatica* and papaya latex in a ratio of 1%:1%, 0.5%:1.5% and 1.5%:0.5% in a base gel S: Bioplacenton® jelly (neomycin + placenta extract) C: base gel	Complete wound closure was achieved after 6 days in the experimental group in the ratio of 1:1 and in the standard group. After 7 days, it was achieved in the groups in the ratio of 1.5:0.5 and 0.5:1.5. After 10 days, in the control group, the wound closure was only 75.34 ± 20.709%. The combination of *C. asiatica* and papaya latex showed a synergistic effect on wound healing, as in the case of the components used alone. Complete wound closure was observed after 8 days.	[56]
Cucurbita moschata *Sesamum indicum* oils	BALB/c albino mice, male	Third-degree thermal burn—coin heated for 3 min with a spirit lamp, applied for 8 s	Once a day for 28 days E: the combination of oils (1:1) NC: w/o treatment	In the experimental groups, wound healing was significantly better than in the negative control and the groups treated separately with sesame oil and pumpkin oil. In addition, a significantly higher level of total antioxidant power and a lower level of malondialdehyde were obtained than in the other groups.	[62]
Malva sylvestris, leaves, aqueous extract *Rosa damascena*, petal powder, sesame oil extract *Solanum nigrum*, leaves, aqueous extract	Wistar albino rats, male	Second-degree thermal burn—electrical heater heated to 110 °C, applied for 10 s	Once a day for 14 days E: herbal ointment (5% of each aqueous extract and 33% of oily extract in base ointment) S: 1% SSD cream C: base ointment NC: without treatment	After 14 days of treatment, the wound closure was the highest in the group treated with herbal ointment and amounted to 87.0 ± 2.1%. The remaining groups were 70.8 ± 3.5, 57.0 ± 5.3 and 32.2 ± 1.6% in the standard, control, and negative control groups. Compared to the other groups, the histopathological analysis in the experimental group showed a significant improvement in wound healing with complete re-epithelialisation, well-formed granulation tissue and mild infiltration of inflammatory cells. In addition, advanced neovascularisation, and irregular distribution of myofibroblasts, fibroblasts and collagen fibres were present.	[104]
Momordica charantia, fruits *Piper nigrum*, fruits *Pongamia glabra*, leaves aqueous extracts	albino rats, either sex	Third-degree chemical burn—sulphuric acid, applied for 10 s	Once a day for 21 days E: 10% or 15% of the combination of extract (1:1:1) in the ointment base S: Povidone-iodine ointment C: ointment base NC: w/o treatment	The period of epithelialisation was 14.97 ± 0.256, 14.77 ± 0.207 and 15.5 ± 0.315 days in the group treated with 10% ointment, 15% ointment and standard, respectively, and was significantly shorter in these groups than in the control groups, where it was 18.18 ± 0.345 and 18.88 ± 0.259 days in the control and negative control groups, respectively. Hydroxyproline levels and tensile strength were significantly higher in experimental and standard groups compared to controls. In the histopathological analysis in the experimental and standard groups, better wound healing, fewer inflammatory cells, a thicker layer of granulation tissue and epidermis, and more dermal fibrosis were observed than in control.	[105]
Rhododendron macrophyllum *Thymus serpyllum*	Wistar albino rats, male	Second and third-degree thermal burn—metal plate heated in boiling water for 5 min, applied for 10 or 30 s	Once a day for 40 days E: commercial herbal ointment Rimojen® (the exact composition is not given) S: 1% SSD cream or *Aloe vera* cream	In both the second and third-degree burn groups, wounds treated with herbal ointment healed much more slowly than those treated with *Aloe vera* cream and 1% silver sulfadiazine cream.	[102]
Sesamum indicum oil Camphora Honey	Wistar albino rats, male	Second-degree thermal burn—hot metal plate, applied for 10 s	Once a day for 28 days E: herbal ointment (the exact composition is not given) C: Vaseline	The percentage of wound healing from day 7 was higher for the group treated with herbal ointment than Vaseline. Moreover, neovascularisation was higher in the group treated with herbal ointment than in the control group.	[106]

* C—control group; E—experimental groups; NC—negative control group; S—standard/positive control group; SSD—silver sulfadiazine; w/o—without.

4. Discussion

Burns significantly affect the quality of life of patients. Although they are common worldwide, they are a significant problem in developing countries, mainly because health care in every country cannot provide access to the latest, most effective therapies. Treatment,

for example using only creams with antibacterial agents such as 1% silver sulfadiazine cream, can prolong healing time, lead to complications, and increase antibiotic resistance. Therefore, the search for new, safe, effective, and cost-effective preparations supporting burn healing is ongoing [2,107]. Burn injury affects the patient's physical health, quality of life, and mental health. Therefore, the challenge is not only the wound treatment itself, which should be effective, but also possible in the patient and long-term care and rehabilitation in more serious cases [3].

Burn is accompanied by an inflammatory and immune reaction, metabolic changes, and distributive shock, especially in the case of severe burns. These symptoms can be difficult to manage, leading to multiple organ failure. An important factor in assessing the treatment needs of a burn injury patient is the wound's depth [108]. In severe burns, highly deregulated inflammation develops, characterised by the release of inflammatory cytokines, chemokines, and acute phase proteins. During this violent phase, the immune system is stimulated and reacts inadequately to stimuli. Therefore, the anti-inflammatory effect of preparations on burn wounds is highly recommended [109,110]. The inflammatory phase is followed by the proliferation phase, during which primarily keratinocytes and fibroblasts are stimulated to rebuild tissue and vessels. In the final phase of healing, the wound remodels. Collagen and elastin are deposited, and fibroblasts are transformed into myofibroblasts. In the case of incorrect and fibrous location of collagen fibres and imbalance in the re-epithelialisation process, scar formation may occur [3,111].

Although several therapeutic activities have been demonstrated for plant-derived products, which are also beneficial in the treatment of burn wounds and sunburn, their preparation is associated with certain limitations. The chemical composition of plants and extracts made from them may be subject to certain deviations due to many factors. The place and time of harvesting, the level of insolation, the geographical altitude, the method of drying and fragmentation are some of the factors that have a significant impact on the chemical composition of the plant material [112,113]. Then, the method of extraction, selection of solvents, the ratio of plant material to solvent, time, and temperature of extraction, as well as the method of its drying and purification affect the composition of the extract. Therefore, it is necessary to introduce certain procedures for standardisation and assessment of the chemical composition when working with plant material [114].

The next step that has a significant impact on the activity is the appropriate formulation of the finished product that will achieve the intended effect in the biological system. Most of the formulations presented in this review are traditional products, such as ointments and creams, containing previously prepared extracts. However, in recent decades, the approach to wound care and dressing has completely changed. Patients more and more often use ready-made dressings, e.g., polymer or hydrogel, which contain incorporated active substances released in a controlled manner. This method of the formulation is also increasingly used for herbal products [115,116]. Another new way of preparing natural preparations is nanoformulation. The development of methods using nanotechnology can support the effectiveness of herbal products. In the treatment of dermatological diseases, various novel drug delivery systems can be used, which contain compounds or extracts of interest in the form of, among others, nanoparticles, liposomes, nanoparticle polymers, nanohydrogels, and nanofibres. Some studies have confirmed that they may be more effective than conventional systems [117,118]. Plant extracts prepared in the form of nanoparticles are characterised by higher bioavailability and penetration of biological membranes, as well as a controlled release at the target site [119]. The role of natural products in different stages of wound healing is presented in Figure 1. It can be assumed that soon, natural compounds used in the form of nanopreparations will be the basis for the creation of new pharmaceutical drugs. However, it is necessary to conduct clinical trials that would verify these theses. In addition, studies are needed to compare the effectiveness compared to standard treatment, as well as to check whether isolated chemical compounds or extracts work better in specific clinical situations.

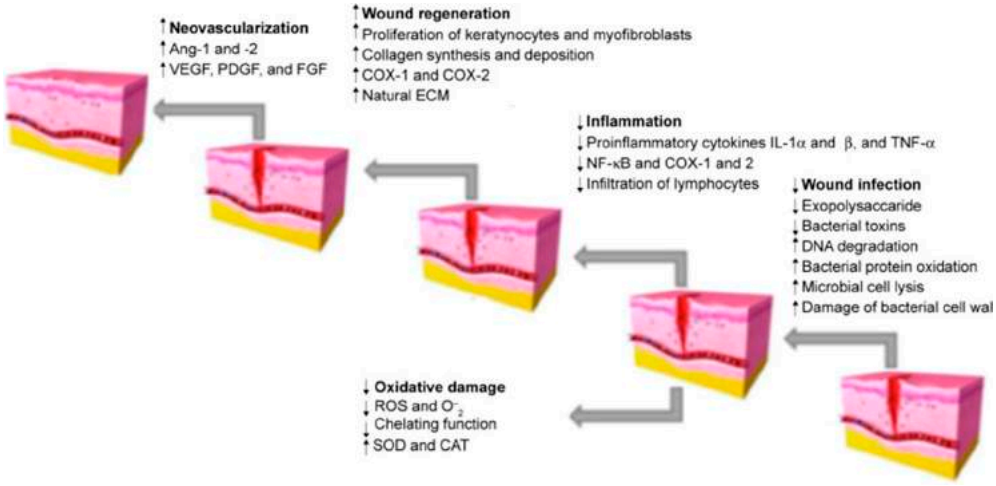

Figure 1. Stages of wound healing. Adapted from Hajialyani, M.; Tewari, D.; Sobarzo-Sánchez, E.; Nabavi, S.M.; Farzaei, M.H.; Abdollahi, M. Natural Product-Based Nanomedicines for Wound Healing Purposes: Therapeutic Targets and Drug Delivery Systems [119].

This review focuses on plant extracts and their mixtures that have been tested in animal models and clinical trials. Extracts, as mixtures of chemical compounds, can show different directions of action affecting the various stages of wound healing (Figure 1). In the inflammation phase, compounds with antibacterial, antioxidant, and anti-inflammatory effects will be particularly important. These include mainly polyphenols and flavonoid compounds, as well as essential oils [120,121]. They may have an antibacterial effect, as well as modulate the inflammatory response by regulating the secretion of cytokines and chemokines, such as interleukin 1β or interleukin 8, or tumour necrosis factor α (TNF-α). Studies on the effect of extracts and chemical compounds on the inflammatory phase are quite popular and there are more and more publications describing this issue [122]. However, less is known about the impact of individual chemical compounds on the proliferation and remodelling phase of wound healing. It is assumed that compounds from the groups of alkaloids, tannins, flavonoids and terpenes are of the greatest importance [11,120]. Important for the proliferative phase will be compounds that affect the formation of the extracellular matrix and stimulate re-epithelialisation, angiogenesis, or the formation of granulation tissue through, among others, stimulating cell proliferation and increasing the expression of proteins such as transforming growth factor β (TGF-β) or vascular endothelial growth factor (VEGF). Finally, in the remodelling phase, chemicals that affect, for example, stimulation of collagen deposition and elastin fibres [11,123] will be necessary. Thanks to the diverse mechanisms of action, potential antibacterial effect and safety of use, natural preparations compete with conventional treatment, all the more that the public's interest in traditional medicine and herbal medicine is growing [124]. The chemical structures of the compounds involved in the wound-healing process are presented in Figure 2.

The results of clinical trials show that herbal preparations may work as well or better than conventional drugs. Preparations containing extracts from *Albizia julibrissin*, *Aloe vera*, *Arnebia euchroma*, *Centella asiatica*, *Hippophaë rhamnoides*, *Lawsonia inermis*, and the mixture of *Matricaria chamomilla* and *Rosa canina* work significantly better than 1% silver sulfadiazine cream, which was considered the "gold standard" of burn treatment until recently. Preparations containing extracts from *Betula*, *Juglans regia*, *Alkanna tinctoria*, *Aloe vera*, and *Centella asiatica* have shown in clinical trials an advantage over other conventional drugs, mainly containing antimicrobial agents. Considering the above results, the possibility of using herbal preparations, such as the marketed drug Episalvan® containing birch bark extract, should be considered to be on par with standard drugs. Additionally,, in many studies of herbal formulations in animal models listed in Tables 1 and 2, their superiority over treatment with antimicrobial agents, mainly 1% silver sulfadiazine

cream, was demonstrated. Therefore, it is important to continue searching for new plant substances that potentially benefit burn wound healing. First, it is necessary to conduct clinical trials for promising formulations. Such research can contribute to introducing new drugs with documented beneficial effects that are safer and more affordable and would be available to all patients worldwide. In addition, considering the results in Tables 2 and S2 (Supplementary Materials), the benefits of combining several plant substances in preparations should be considered. Many studies suggest a synergistic effect and may be more beneficial in treating burn wounds.

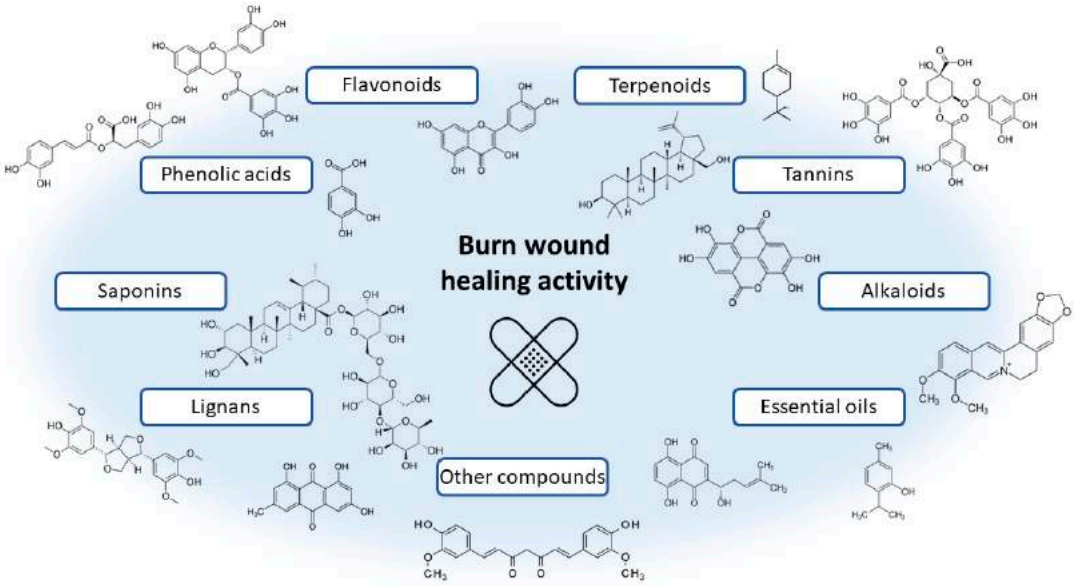

Figure 2. Examples of chemical structures of naturally occurring compounds involved in the wound healing process.

Scientific, clinical, and animal studies should be well-planned. In some of the studies presented above, a positive control group treated with a standard preparation with known therapeutic activity was not designed. That makes it difficult to interpret the results and unambiguously assess the tested preparation's effectiveness. In addition, the tests performed should be described in detail. The lack of information about the research model, such as the conditions of creating a burn wound, make it impossible to reproduce the study in another centre, for example, to compare the results. In the case of market preparations, some authors did not provide information on their exact composition and manufacturer. Any deficiencies were noted in the individual studies in the tables. Appropriate interpretation of the obtained results and a correctly performed statistical analysis is essential. Some of the studies lacked statistical comparisons between the individual study groups, which made it difficult to analyse the results.

Researchers studying preparations of natural origin should pay particular attention to the accurate representation of the plant material under study. The chemical composition of plants changes depending on many factors, such as geographical altitude or insolation in the natural site [113]. Changes in chemical composition determine changes in therapeutic activity, which is why it is important to standardize the obtained extracts. Not all authors paid sufficient attention to describe and characterize the tested products adequately. Some studies lacked basic information, such as the final concentration or the amount of preparation applied to the burn.

What is more, sometimes the authors did not provide the method of preparation and did not even specify the part of the plant used. Such errors and oversights lead to a loss of credibility and trust among other scientists and doctors [125]. In conclusion, new

research is needed for promising herbal products, but they should be carried out following all guidelines for this type of research.

5. Conclusions

Due to the diverse mechanism of action, antibacterial activity, and safety, herbal preparations compete with conventional treatment in treating burns and sunburn. The growing interest in alternative therapies and herbal medicine is also generating demand for such products. However, there is still a lack of clinical trials that would check the effectiveness of preparations showing beneficial effects on animal models of burns. Creating an ideal dressing for burn wounds that could replace the common use of antibacterial agents is a challenge for modern medicine, and the research presented in this review suggests that formulations based on herbal products are a strong competition for synthetic compounds.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pharmaceutics15020633/s1, Table S1: Summary of clinical trials—single preparations; Table S2: Summary of clinical trials—mixtures of preparations.

Author Contributions: Conceptualisation, W.S.; methodology, W.S.; investigation, W.S.; resources, W.S.; data curation, W.S.; writing—original draft preparation, W.S.; writing—review and editing, A.B.; visualisation, A.B. and W.S.; supervision, A.B.; project administration, A.B.; All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

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SUPPLEMENTARY MATERIALS

The Potential of Medicinal Plants and Natural Products in the Treatment of Burns and Sunburn—A Review

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Table S1. Clinical trials – single preparations

Plant material	Patients	Test groups*	The course of the clinical trial	Results compared to the control group	Ref.
Albizia julibrissin stem bark 60% (*v*/*v*) ethanolic extract	40 patients with second-degree thermal burn	E: 5% (w/w) of extract in base gel C: 1% SSD cream	Dressing changed once a day for 30 days.	After 30 days, pain and inflammation were reduced in the experimental group. There were no significant differences in itching, redness, swelling, purulent discharge, and skin discolouration.	[13]
Aloe vera inner gel spray-dried	30 patients with two similar burns	E: 0.5% of powder in a base cream C: 1% SSD cream	Dressing changed twice a day until complete healing.	Using a cream with *Aloe vera* significantly shortened the time to complete wound healing and resulted in a faster reduction of its surface.	[15]
Aloe vera inner gel	50 patients with second-degree burn	E: 98% *Aloe vera* gel C: 1% SSD cream	Dressing changed twice a day until complete healing.	The study showed that using *Aloe vera* gel significantly shortens the re-epithelialization time, alleviates pain symptoms, and is more cost-effective.	[16]
Arnebia euchroma roots heated with goat fat, cow butter and glycerin	45 patients with two similar burns	E: 10% *Arnebia euchroma* ointment C: 1% SSD cream	Dressing changed once a day until complete healing.	*Arnebia euchroma* ointment reduces the burn surface, shortens the healing time, reduces the burning sensation and pain by patients, and increases the warming of the wound. Moreover, it was better tolerated by patients and physicians.	[18]
Betula pendula *Betula pubescens* bark triterpene extract (standardized for 72-88% of betulin)	57 patients with two similar burns or one major burn divided into two halves	E: Oleogel-S10 (10% of extract in sunflower oil) C: Octenilin®	Dressing changed every 2 days for 21 days.	The study showed that using Oleogel-S10 significantly shortened the time to complete wound closure and increased the percentage of epithelialization. Moreover, it was better tolerated by patients.	[21]
Camelia sinensis leaves aqueous extract	50 patients with second-degree thermal burn	E: 10% of extract in a base cream C: 1% SSD cream	Dressing changed once a day until complete healing.	After 14 days, there were no significant differences in the treatment effects between groups.	[23]
Centella asiatica leaves butanolic fraction of ethanolic extract	60 patients with second-degree thermal burn	E: Centiderm® ointment (containing 3% of the fraction) C: 1% SSD cream	Dressing changed once a day until complete healing.	After 14 days, all analyzed parameters, i. e. pliability, vascularity, pigmentation, height, scoring according to Visual Acuity Scale and Vancouver Scar Scale, dryness, itching, and irritation, were assessed statistically significantly better. In addition, the mean time to re-epithelialization and complete healing was significantly shorter.	[25]

Hippophaë rhamnoides fruits (unspecified preparation)	60 patients with second-degree thermal burn	E: sea buckthorn cream (containing 40% of active ingredient) C: 1% SSD cream	Cream (3 mm thick) was applied once a day until complete healing.	The average wound healing time was statistically significantly lower.	[28]
Juglans regia seeds heated and grounded into an ointment	411 patients with non-healing burn wounds	E: walnut ointment C1: wound debridement and skin autograft C2: antimicrobial agent + recombinant human Epidermal Growth Factor	Daily application of a 1-2 mm thick layer to the wound or surgery.	The effectiveness was comparable to the C1 group and was significantly better than in the C2 group. In E and C1 groups, the time to complete wound healing was significantly shorter.	[29]

* E – experimental; C – control; SSD – silver sulfadiazine

Table S2. Clinical trials – mixtures of natural preparations

Composition	Patients	Test groups*	The course of the clinical trial	Results compared to the control group	Ref.
Alkanna tinctoria (unspecified part) Beeswax Olive oil	64 patients with thermal burn	E: dressing saturated with the herbal mixture (3% of beeswax and 5% of *Alkanna tinctoria* in olive oil) C: dressing saturated with nitrofurazone and rifamycin	The dressings were changed every two days until complete healing.	A significant reduction in the time to start re-epithelialization and decreased pain experienced by patients was observed. In addition, the time of their hospitalization was significantly shortened.	[30]
Aloe vera (spray-dried powdered gel) *Centella asiatica* (commercial cream Cosmelene®)	35 patients with second-degree thermal burn	E: herbal dressing (impregnated with lipocolloids, 5% of Cosmelene®, and 2.5% of aloe powder) C: Bactigras® dressing (impregnated with soft paraffin and 0.5% chlorhexidine acetate)	The dressings were changed every three days until complete healing.	The time to complete healing and the time of the patient's stay in the hospital were significantly shortened. Moreover, the average pain experienced by patients was lower.	[31]
Aloe vera gel *Lavandula stoechas* essential oil *Pelargonium roseum* essential oil	111 patients with second-degree thermal burn	E: herbal cream (unspecified composition) C: 1% SSD cream	The dressings were changed every day until complete healing.	Significantly less pain sensation was observed from the 7th day. There was no change in the prevalence of dry skin.	[32]
Azadirachta indica oil *Hypericum perforatum* oil	9 paediatric patients with second and third-degree thermal burn, keloid ulceration and partial failures of skin graft	E: 1 Primary Wound Dressing® spray composed of neem oil and hypericum oil (unspecified proportions)	The dressings were changed every two days until complete healing.	The preparation induces the formation of granulation tissue and epithelialization and relieves pain.	[33]
Lawsonia inermis (unspecified part) Beeswax	50 patients with second-degree thermal burn	E: herbal ointment (unspecified composition) C: 1% SSD cream	The dressings were changed every day until complete healing.	The average wound healing time was statistically shorter in the experimental group.	[34]
Matricaria chamomilla (unspecified part) *Rosa canina* (unspecified part) Beeswax	60 patients with second-degree thermal burn	E: Adibderm® ointment (unspecified composition) C: 1% SSD cream	The dressings were changed every 6 hours until complete healing.	The average wound healing time was statistically shorter in the experimental group.	[35]

* E – experimental; C – control; SSD – silver sulfadiazine

Manuscript co-author statement

As a one of co-authors of the manuscript: "The potential of medicinal plants and natural products in the treatment of burns and sunburn – a review" published in *Pharmaceutics* (Volume 15(2), 13 February 2023, 633; DOI: 10.3390/pharmaceutics15020633) I agree to include this publication into a collection of thematically related articles constituting a doctoral dissertation prepared by mgr farm. Weronika Skowrońska.

Additionally, I confirm that the scope presented below corresponds to my contribution to the project.

Oświadczenie współautora manuskryptu

Jako jeden z współautorów manuskryptu: „The potential of medicinal plants and natural products in the treatment of burns and sunburn – a review" opublikowanego w *Pharmaceutics* (Tom 15(2), 13 lutego 2023, 633; DOI: 10.3390/pharmaceutics15020633) wyrażam zgodę na włączenie tej publikacji do zbioru powiązanych tematycznie artykułów naukowych stanowiącego rozprawę doktorską mgr farm. Weroniki Skowrońskiej.

Jednocześnie potwierdzam, że przedstawiony poniżej zakres odpowiada mojemu wkładowi w realizację projektu.

Co-author's name	**Percentage of contribution**	**Scope of contribution**
Imię i nazwisko współautora	**Udział procentowy**	**Zakres wkładu**
Weronika Skowrońska	90%	Conceptualization of the project, data collection, methodology, writing the original text of the manuscript
		Koncepcja projektu, gromadzenie danych, metodologia, pisanie oryginalnego tekstu manuskryptu
Agnieszka Bazylko	10%	Manuscript editing, corresponding author
		Redakcja manuskryptu, autor korespondencyjny

Date and legible signature

Data i czytelny podpis

27.02.2024 Weronika Skowrońska

Manuscript co-author statement

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Data i czytelny podpis

04.03.2024 Agnieszka Bazylko