UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU

Wydział Farmaceutyczny
Collegium Medicum w Bydgoszczy

Bydgoszcz 2024

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Jakub Gębalski

ROŚLINNE INHIBITORY HIALURONIDAZ I TYROZYNAZY – ASPEKT CHEMICZNY I FARMAKOLOGICZNY ORAZ IMPLEMENTACJE PRZEMYSŁOWE

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

Promotor:

dr hab. n. farm. Daniel Załuski, prof. UMK

Bydgoszcz 2024

Panu dr. hab. Danielowi Załuskiemu, prof. UMK składam serdeczne podziękowania za okazane wsparcie oraz przychylność i nieocenioną pomoc w realizacji niniejszej rozprawy doktorskiej

Serdecznie dziękuję Koleżankom i Kolegom z Katedry Botaniki Farmaceutycznej i Farmakognozji Collegium Medicum w Bydgoszczy, Uniwersytetu Medycznego w Toruniu za przyjazną atmosferę

Mojej Ukochanej Narzeczonej Milenie, Rodzicom, Babci i Rodzeństwu serdecznie dziękuję za okazanie wsparcia w powstawaniu niniejszej pracy

Pracę dedykuję mojemu Dziadkowi, który do końca we mnie wierzył.

Przedstawiona rozprawa doktorska została zrealizowana m.in. dzięki następującym źródłom finansowania:

1) 2021-2022: *The search for new plant-based tyrosinase inhibitors*, Grants4NCUStudents w ramach pragramu Inicjatywa Doskonałości – Uczelnia Badawcza, UMK, granty dla uczestników Szkoły Doktorskiej. Gębalski J., Załuski D.

2) 2021-2022: *The search for new plant-based hyaluronidase inhibitors*, Grants4NCUStudents w ramach pragramu Inicjatywa Doskonałości – Uczelnia Badawcza, UMK, granty dla uczestników Szkoły Doktorskiej. Gębalski J., Załuski

Spis treści

1. Wykaz osiągnięć doktoranta

1.1. Wykształcenie

10.2020 – 09.2024

Studia doktorskie w Szkole Doktorskiej Nauk Medycznych i Nauk o Zdrowiu Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy Uniwersytetu Mikołaja Kopernika w Toruniu.

10.2013 – 03.2019

Studia farmaceutyczne na Wydziale Farmaceutycznym Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy Uniwersytetu Mikołaja Kopernika w Toruniu.

1.2. Zatrudnienie

10.2023 - obecnie

Asystent

Katedra Botaniki Farmaceutycznej i Farmakognozji CM UMK, Wydział Farmaceutyczny

10.2021 - obecnie

Specjalista inżynieryjno-techniczny

Katedra Botaniki Farmaceutycznej i Farmakognozji CM UMK, Wydział Farmaceutyczny

10.2019 – 09.2020

Asystent

Katedra Farmakologii i Terapii CM UMK, Wydział Lekarski

04.2019 - obecnie

Magister farmacji

Apteka Alba

04.2019-06.2019

Mikrobiolog w dziale R&D

Boruta Zachem Biochemia

1.3. Publikacje

Publikacje*			
Prace w języku angielskim	**12**		
Prace w języku polskim	**1**		
Publikacje jako pierwszy autor	**4**		
Sumaryczny IF	**58,266**		
Sumaryczna punktacja MEiN	**1545**		
Cytowania*		**Hirsh index***	
Web of Science	**301**	Web of Science	**5**
Scopus	**346**	Scopus	**6**
Google Scholar	**497**	Google Scholar	**6**

*dane na dzień 20.09.2024 r.

Wykaz publikacji współautorskich

1. Graczyk F, **Gębalski J**, Sulejczak D, Małkowska M, Wójciak M, Gawenda-Kempczyńska D, Piskorska E, Krolik K, Markiewicz M, Kondrzycka-Dąda A, Lepianka W., Borowski G., Feldo M., Verporte R., 9 Załuski D. UHPLC-DAD/ESI-TOF-MS Phytochemical characterization and evaluation of the impact of *Eleutherococcus senticosus* fruit intractum on biochemical, hepatological, and blood Parameters in Balb/c Mice, Int. J. Mol. Sci. 2024; 25(17):9295. IF **4.9**
2. Graczyk F., **Gębalski J.**, Piskorska E., Małkowska M., Słomka A., Gawenda-Kempczyńska D., Załuski D. The *Eleutherococcus senticosus* fruits' intractum affects changes in the transepithelial electric potential in the distal section of the rabbit's large intestine and inhibits hyaluronidase, J. Ethnopharmacol. 2024; 117847. IF **5.4**
3. **Gębalski J.**; Małkowska M.; Graczyk F.; Słomka A.; Piskorska E.; Gawenda-Kempczyńska D.; Kondrzycka-Dąda A.; Bogucka-Kocka A.; Strzemski M.; Sowa I.; Wójciak M., Grzyb S., Krolik K., Ptaszyńska A.A., Załuski D. Phenolic compounds and antioxidant and anti-enzymatic activities of selected adaptogenic plants from south america, asia, and africa, Molecules, 2023; 28, 6004. IF **4.2**
4. Król A, Kokotkiewicz A., Gorniak M., Naczk A., Zabiegala B., **Gębalski J.**, Graczyk F., Zaluski D., Bucinski A., Luczkiewicz M. Evaluation of the yield, chemical

composition and biological properties of essential oil from bioreactor-grown cultures of *Salvia apiana* microshoots, Sci. Rep. 2023; 13 (1), 7141 IF **4.997**

5. Graczyk F., **Gębalski J**., Makuch-Kocka A., Gawenda-Kempczyńska D., Ptaszyńska, A. A., Grzyb S., Bogucka-Kocka A., Załuski, D. (2022). Phenolic profile, antioxidant, anti-enzymatic and cytotoxic activity of the fruits and roots of *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim. Molecules, 27(17), 5579. IF **4.927**
6. Przekwas J., **Gębalski J**., Kwiecińska-Piróg J., Wiktorczyk-Kapischke N., Wałecka-Zacharska E., Gospodarek-Komkowska E., Rutkowska D., Skowron K. The effect of fluoroquinolones and antioxidans on biofilm formation by *Proteus mirabilis* strains. Annals of Clinical Microbiology and Antimicrobials, 2023; 21(1), 1-10. IF **6.12**
7. Wiciński M.; **Gębalski J**.; Gołębiewski J.; Malinowski B. Probiotics for the treatment of overweight and obesity in humans—a review of clinical trials, Microorganisms, 2020; 8, 1148. IF **4.128**
8. Wiciński M.; **Gębalski J.**; Mazurek E.; Podhorecka M.; Śniegocki M.; Szychta P.; Sawicka E.; Malinowski B. The influence of polyphenol compounds on human gastrointestinal tract microbiota, Nutrients 2020; 12, 350. IF **6.706**
9. Wiciński M.; Sawicka E.; **Gębalski J.**; Kubiak K.; Malinowski B. Human milk oligosaccharides: health benefits, potential applications in infant formulas, and pharmacology, Nutrients 2020; 12, 266. IF **6.706**
10. Wiciński M., Socha M., **Gębalski J.**, Malinowski B., Czerwiński M., Liczner G., Panek M., Pawlak-Osińska, K. Wpływ koenzymu Q10 na insulinooporność, Lek w Polsce, 2019; 29 (1), 16-24

1.4. Wykaz doniesień konferencyjnych

- **Tyrosinase and hyaluronidase inhibitors in *Eleutherococcus divaricatus***, International Congress on Natural Products Research, Kraków, 13-17.07.2024 - poster
- **Inhibitory tyrozynazy i hialuronidazy w gatunkach *Eleutherococcus***, during the XXIV Congress of the Polish Pharmaceutical Society, Lublin, 22-24.09.2021- poster
- **Inhibitory tyrozynazy i hialuronidazy w gatunkach *Araliaceae***, during the National Scientific Conference, Natural medicinal and cosmetic raw materials, Lublin, 15-16.11.2022 – prezentacja ustna

Udział w badaniach prezentowanych na konferencjach

- **Extracts from the roots of *Scutellaria baicalensis* reduce honeybee death caused by nosemosis,** International Congress on Natural Products Research, Kraków, 13-17.07.2024 – poster
- **Analiza składu chemicznego i właściwości biologicznych wybranych roślin adaptogennych Afryki, Azji i Ameryki Południowej**, XV Interdisciplinary Scientific Conference TYGIEL 2023, Lublin/online, 23-26.03.2023 r. – prezentacja ustna
- **Właściwości przeciwstarzeniowe wybranych gatunków z rodziny *Araliaceae***, National Scientific and Training Conference entitled 'Cosmetology in a holistic approach', Lublin, 20-21.04.2023 r. - poster
- ***Salvia apiana* in vitro shoot system as a source of volatile fraction with acetylcholinesterase inhibitory potential**, Trends in Natural Products Reserach: A Young Scientists' Meeting, 23-26.05.2022, Kolymbari, Grecja – prezentacja ustna

1.5. Organizacja konferencji naukowych

- Komitet Organizacyjny Ogólnopolskiej Konferencji Opieka nad pacjentem onkologicznym wyzwaniem dla lekarzy i farmaceutów, Bydgoszcz, 18-20.05.2018 r.
- Komitet Pomocniczy XXVIII Zjazd Polskiego Towarzystwa Mikrobiologów „Mikrobiologia - nowe wyzwania, nowe możliwości", Bydgoszcz, 25-27.09.2016 r.

1.6. Granty

12.6.1. Kierownictwo grantów

Debiuty – Inicjatywa Doskonałości Uczelni Badawczej

- Kierownik projektu badawczego pt. ***AralDiab - new multifunctional compounds in the treatment and prevention of diabetes in the genus Aralia***

Grants4NCUStudents – Inicjatywa Doskonałości Uczelni Badawczej

- Kierownik projektu badawczego pt. ***The search for new plant-based hyaluronidase inhibitors*** (2 edycja)
- Kierownik projektu badawczego pt. ***The search for new plant-based tyrosinase inhibitors*** (3 edycja)

- Kierownik projektu badawczego pt. ***PolyBaic – in a direction of the new baicalein polymers*** (5 edycja)
- Kierownik projektu badawczego pt. ***The search for new plants in treatment civilization diseases*** (6 edycja)
- Kierownik projektu badawczego pt. ***Effect of fruits with adaptogenic properties from Polish crops on inflammation in neurodegenerative diseases*** (7 edycja)
- Kierownik projektu badawczego pt. ***Study of the anti-inflammatory properties of selected species from the Aralia genus*** (8 edycja)

1.6.1. Udział w projektach badawczych

- Realizacja projektu Biotransformacja i rafinacja kaskadowa śruty oleistej w celu uzyskania surfaktyny, polimerów i komponentów paszowych w firmie biotechnologicznej InventionBio sp. z o.o., Europejski Fundusz Rozwoju Regionalnego, Sektorowe programy B+R - INNOCHEM

Debiuty – Inicjatywa Doskonałości Uczelni Badawczej

- The significance of *Rhizophagus irregularis* in the production of phenolic compounds and modulating the immunostimulating activity of the aerial parts of *Echinacea purpurea* (L.) Moench
- Spectroscopic analysis of medicinal plants*: Eleutherococcus senticosus* and *Scutellaria baicalensis* - a pilot study

1.7. Wykaz staży

- Staż naukowy w firmie biotechnologicznej Boruta-Zachem Biochemia, Bydgoszcz, 01-31.09.2018 r.
- Staż w szpitalu Nemocnice Milosrdných sester sv. Karla Boromejského v Praze, 01-12.07.2018 r.
- Staż w szpitalu Orthopedic hospital "Banjicia", Belgrade, 01-30.07.2017 r.

1.8. Zgłoszenia patentowe

- Alkoholowy ekstrakt etanolowy ze świeżego liofilizowanego korzenia *Scutellaria baicalensis* do zastosowania w preparatach zwiększających odporność pszczół i

zwalczających nosemozę, dr hab. Ptaszyńska A. prof. UMCS, dr hab. Załuski D. prof. UMK, **mgr Gębalski J.** Polski Urząd Patentowy, 2024

- Alkoholowy ekstrakt etanolowy z suszonego napowietrznie korzenia *Scutellaria baicalensis* do zastosowania w preparatach zwiększających odporność pszczół i zwalczających nosemozę, dr hab. Ptaszyńska A. prof. UMCS, dr hab. Załuski D. prof. UMK, **mgr Gębalski J.** Polski Urząd Patentowy, 2024

1.9. Wykaz nagród

- Nagroda Zespołowa trzeciego stopnia Rektora UMK za działalność naukowo-badawczą, 2024
- Nagroda Rektora za wysoko punktowane publikacje:
    - „Evaluation of the yield, chemical composition and biological properties of essential oil from bioreactor-grown cultures of *Salvia apiana* microshoots", 2023
    - „The Influence of Polyphenol Compounds on Human Gastrointestinal Tract Microbiota", 2020
    - „Human Milk Oligosaccharides: Korzyści zdrowotne, potencjalne zastosowania w preparatach dla niemowląt i farmakologia", 2020
- Nagroda dla najlepszego koła naukowego na Wydziale Farmaceutycznym, Studenckie Towarzystwo Naukowe, 2022/23
- Nagroda za działalność naukowo-badawczą w toku studiów farmaceutycznych, Bydgoszcz, 2019
- Nagroda za uzyskanie bardzo dobrych wyników w nauce podczas studiów farmaceutycznych, 2019
- Tytuł Najlepszego Studenta Wydziału Farmaceutycznego CM UMK w roku akademickim 2016/17 oraz 2017/18
- II miejsce w wydziałowym Konkursie Prac Magisterskich, CM UMK, 2018
- II miejsce w Ogólnopolskim Konkursie Umiejętności Klinicznych (OKUK), Gdańsk, 2017
- I miejsce w Konkursie Receptury Aptecznej (KRA), Warszawa, 2016

1.10. Recenzowanie artykułów w czasopismach naukowych

- Journal of Enzyme Inhibition and Medicinal Chemistry, **5.75 IF**, (2 recenzje)
- International Journal of Molecular Sciences **4.9 IF** (2 recenzje)
- Molecules, **4.2 IF** (2 recenzje)
- Plants **4.0 IF** (1 recenzje)
- Journal of Food Processing and Preservation, **2.93 IF**, (2 recenzje)
- Natural compounds communication, **1.49 IF** (2 recenzje)
- Nutrition and Dietary Supplements, **1.3 IF** (1 recenzje)
- Indian Journal of Pharmaceutical Sciences, **0.66 IF**, (1 recenzja)
- Current Cosmetic Science (2 recenzje)
- Farmacja Polska (8 recenzji) **140 pkt MNiSW**
- Prospects in Pharmaceutical Sciences **0.1 IF** (1 recenzja**)**

1.11. Kursy i szkolenia

- 2023 r. – ukończenie kursu uprawniającego do uczestnictwa w procedurach na zwierzętach, Wydział Farmaceutyczny, CM UMK
- 2023 r. – udział w kursie „Pozyskiwanie środków na badania, pisanie grantów międzynarodowych z sukcesem" realizowanych przez Poznański Park Naukowo-Technologiczny (PPNT) z siedzibą w Poznaniu, NAWA
- 2023 r. – szkolenie z obsługi aparatury do denzytometrii, CAMAG
- 2022 r. – kurs ze statystyki podstawowej, eTrapez

1.12. Inna działalność naukowa, dydaktyczna oraz organizacyjna

Opieka nad pracami magisterskimi

- Kwasy fenolowe jako inhibitory hialuronidazy; 2024, mgr farm. Milena Małkowska
- Immobilizacja metabolitów wtórnych obecnych w *Scutellaria baicalensis* Georgi do alginianu wapnia; 2023, mgr farm. Paulina Sas
- Immobilizacja metabolitów wtórnych obecnych w *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim. do alginianu wapnia; 2023, mgr farm. Maria Mleczak
- Aktywność biologiczna wybranych gatunków roślin adaptogennych; 2022, mgr farm. Jakub Hubert Siuda

- Analiza fitochemiczna wybranych gatunków roślin adaptogennych; 2022, mgr farm. Paulina Zabrodzka

Opieka nad projektami Studenckiego Koła Naukowego Farmakognozji

- Wykorzystanie naturalnych głębokich rozpuszczalników eutektycznych (NADES) w celu uzyskania ekstraktów bogatych we flawonoidy oraz saponiny z korzenia *Astragalus membranaceus.*
- Kwasy fenolowe jako inhibitory hialuronidazy, tyrozynazy i acetylocholinoesterazy - czy istnieje zależność struktura-aktywność?
- Poszukiwanie nowych inhibitorów elastazy w roślinach adaptogennych.
- Wpływ wybranych roślin z rodziny *Araliaceae* na aktywność kolagenazy i utlenianie kolagenu

Opieka nad studentami z zagranicy

- Opieka nad studentami uczestniczącymi w programie Erasmus

Działalność dydaktyczna

- Prowadzenie ćwiczeń, seminariów oraz wykładów dla studentów III roku farmacji z farmakognozji
- Prowadzenie wykładów dla studentów IV roku analityki medycznej z farmakologii ogólnej
- Prowadzenie ćwiczeń oraz seminariów dla studentów III roku medycyny z farmakologii ogólnej z elementami toksykologii oraz prawa farmaceutycznego

Działalność organizacyjna

- Udział w przygotowaniu zajęć dla uczniów szkół średnich 2022/23 - promocja Wydziału Farmaceutycznego
- Organizacja akcji prozdrowotnej „Rzeka Zdrowia”, Studenckie Towarzystwo Naukowe
- Organizacja Dni Otwartych z Katedrą Chemii Leków, Katedrą Technologii Postaci Leków i Katedrą Farmakognozji w latach 2016/17, 2017/18
- Prowadzenie wykładu *Samoleczenie lekami OTC – szansa czy zagrożenie?* w ramach Bydgoskiego Festiwalu Nauki, 25 maja 2018 r.
- Prowadzenie warsztatów chemicznych "Minichemia - maxisprva" w ramach Bydgoskiego Festiwalu Nauki, 19 maja 2016 r.

- Prowadzenie warsztatów "Biały proszek - jak rozpoznać, jaki to lek" w ramach "Medicalia" Nauka 2016 i 2017 r.

2. Wykaz publikacji stanowiących podstawę postępowania w sprawie o nadanie stopnia naukowego doktora

P.1. Gębalski J., Graczyk F., Załuski D. Paving the way towards effective plant-based inhibitors of hyaluronidase and tyrosinase: A critical review on a structure–activity relationship. J. Enzyme. Inhib. Med. Chem. 2022; 37(1), 1120-1195.

Impact Factor: **5.6**

Punktacja MNiSW: **140.000**

P.2. Gębalski J., Małkowska M., Gawenda-Kempczyńska D., Słomka A., Strzemski M., Styczyński J., Załuski D. *Eleutherococcus divaricatus* fruits decrease hyaluronidase activity in blood serum and protect from antioxidative damages in in vitro model. Int. J. Mol. Sci. 2024; 25(4):2033.

Impact Factor: **4.9**

Punktacja MNiSW: **140.000**

P3. Gębalski J., Małkowska M., Wnorowska S., Gawenda-Kempczyńska D., Strzemski M, Wójciak M., Słomka A., Styczyński J., Załuski D. Ethyl acetate fraction from *Eleutherococcus divaricatus* root extract as a promising source of compounds with anti-hyaluronidase, anti-tyrosinase, and antioxidant activity but not anti-melanoma activity. Molecules. 2024; 29(15):3640.

Impact Factor: **4.2**

Punktacja MNiSW: **140.000**

Łączna wartość wskaźnika Impact Factor (IF) dla prac wchodzących w cykl: **14.7**[*]. Łączna wartość punktów Ministerstwa Edukacji i Nauki (MEiN) dla prac wchodzących w cykl: **420**[*].

*dane na dzień 20.09.2024 r.

3. Wykaz skrótów

Skrót	Pełna nazwa w języku polskim	Pełna nazwa w języku angielskim
ED	*Eleutherococcus divaricatus*	*Eleutherococcus divaricatus*
TCM	Tradycyjna Medycyna Chińska	Traditional Chinese Medicine
HA	kwas hialuronowy	hyaluronic acid
HYAL	hialuronidaza	hyaluronidase
TYR	tyrozynaza	tyrosinase
hTYR	tyrozynaza ludzka	human tyrosinase
mTYR	tyrozynaza grzybowa	fungal tyrosinase
bHYAL	hialuronidaza bydlęca	bovine hyaluronidase
hHYAL	hialuronidaza ludzka	human hyaluronidase
IC50	minimalne stężenie hamujące aktywność enzymu w 50%	minimum inhibitory concentration of 50% of enzyme activity
LPS	lipopolisacharydy	lipopolysaccharides
IL	interleukina	interleukin
SGLT	białka transportujące sód i glukozę	sodium-glucose transport proteins
eNOS	śródbłonkowa syntaza tlenku azotu	endothelial nitric oxide synthase
HPLC-DAD	chromatografia cieczowa z detekcją diodową	Liquid chromatograpy with diode-array detection
TPC	całkowita zawartość polifenoli	the total phenolic contents
TFC	całkowita zawartość flawonoidów	the total flavonoid contents
TPAC	całkowita zawartość kwasów fenolowych	the total phenolic acid contents

4. Streszczenie w języku polskim

Rośliny na przestrzeni dziejów odgrywały kluczową rolę jako źródło związków leczniczych. Napary, odwary, maści i nalewki z substancji roślinnych stanowiły podstawowe leki stosowane w różnych dolegliwościach. Interesującą grupą roślin o szerokim działaniu biologicznym jest rodzaj *Eleutherococcus*, obejmujący 29 gatunków rosnących głównie w Azji (Chiny, Japonia, Korea). Najbardziej znanym przedstawicielem jest *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim., powszechnie stosowany w Tradycyjnej Medycynie Dalekowschodniej. Rośliny te, dzięki zróżnicowanemu składowi fitochemicznemu (triterpeny, flawonoidy, lignany, kwasy fenolowe, chalkony, stilbeny, diterpeny), wykazują szerokie spektrum aktywności biologicznej. Mniej znanym przedstawicielem jest *Eleutherococcus divaricatus* (Siebold & Zucc.) S.Y.Hu..

Celem pracy było uzyskanie odpowiedzi na pytanie, czy *E. divaricatus* zawiera związki o charakterze inhibitorów hialuronidazy oraz tyrozynazy. Obecne na rynku inhibitory tyrozynazy i hialuronidaz mają wiele niekorzystnych właściwości. Hydrochinon i arbutyna wykazują działanie mutagenne i mają skutki uboczne, takie jak pieczenie, kontaktowe zapalenie skóry, podrażnienie i rumień. Kwas kojowy jest rakotwórczy i ma niską stabilność podczas przechowywania. Kwas L-askorbinowy jest niestabilny i wrażliwy na czynniki zewnętrzne (tlen, światło, pH, temperatura). Jedynym dostępnym inhibitorem hialuronidaz jest escyna, która jednak charakteryzuje się niską biodostępnością. Dlatego konieczne jest poszukiwanie nowych, bardziej skutecznych i bezpieczniejszych substancji.

Wyniki badań własnych opublikowałem jako dwie prace oryginalne, które wraz z pracą przeglądową dotyczącą roślinnych inhibitorów hialuronidazy i tyrozynazy, wchodzą w skład monocyklu stanowiącego rozprawę doktorską.

Wyniki tych badań wskazują na obecność inhibitorów hialuronidaz (bHYAL, hHYAL) oraz tyrozynazy (mTYR) w owocach oraz korzeniu *E. divaricatus*. Najsilniejszą inhibicję wykazała frakcja octanu etylu otrzymana z 75% metanolowego ekstraktu z korzenia. Ta sama frakcja hamowała hialuronidazę obecną w surowicy krwi dzieci ze zdiagnozowaną ostrą białaczką limfoblastyczną. Za aktywność odpowiedzialne są związki polifenolowe (głównie kwasy fenolowe oraz pochodne kwasu kawoilochinowego).

Obecność inhibitorów hialuronidazy stwarza możliwość wykorzystania owoców i korzeni *E. divaricatus* w łagodzeniu stanów zapalnych i niedoboru immunologicznego. Uwzględniając wyniki przeprowadzonych badań dostarczyłem naukowych dowodów na

zasadność stosowania *E. divaricatus* w etnomedycynie Chin i Syberii, w chorobach o podłożu immunologicznym i zapalnym.

5. Streszczenie w języku angielskim

Plants throughout history have played a key role as a source of medicinal compounds. Infusions, decoctions, ointments and tinctures of plant substances have been the primary medicines used for various ailments. An interesting group of plants with broad biological activity is the genus *Eleutherococcus*, which includes 29 species growing mainly in Asia (China, Japan, Korea). The best-known representative is *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim., widely used in Traditional Far Eastern Medicine. These plants, thanks to their diverse phytochemical composition (triterpenes, flavonoids, lignans, phenolic acids, chalcones, stilbenes, diterpenes), exhibit a wide spectrum of biological activity. A lesser-known representative is *Eleutherococcus divaricatus* (Siebold & Zucc.) S.Y.Hu.

The purpose of this study was to find out whether *E. divaricatus* contains compounds that are hyaluronidase and tyrosinase inhibitors. Tyrosinase and hyaluronidase inhibitors present on the market have many adverse properties. Hydroquinone and arbutin show mutagenic effects and have side effects such as burning, contact dermatitis, irritation and erythema. Kojic acid is carcinogenic and has low stability during storage. L-ascorbic acid is unstable and sensitive to external factors (oxygen, light, pH, temperature). The only hyaluronidase inhibitor available is escin, which has low bioavailability. Therefore, it is necessary to search for new, more effective and safer substances.

I published the results of my own research as two original papers, which, together with a review paper on plant hyaluronidase and tyrosinase inhibitors, are included in the monocycle constituting my dissertation.

The results of our own research indicate the presence of inhibitors of hyaluronidases (bHYAL, hHYAL) and tyrosinase (mTYR) in the fruit and root of *E. divaricatus*. The ethyl acetate fraction obtained from a 75% methanolic extract of the root showed the strongest inhibition. The same fraction inhibited hyaluronidase present in the blood serum of children diagnosed with acute lymphoblastic leukemia. Polyphenolic compounds (mainly phenolic acids and cavoylquinic acid derivatives) are responsible for the activity.

The presence of hyaluronidase inhibitors creates the possibility of using the fruits and roots of *E. divaricatus* in alleviating inflammation and immune deficiency. Considering the results of my research, I have provided scientific evidence for the legitimacy of the use of *E. divaricatus* in ethnomedicine in China and Siberia, in diseases with immunological and inflammatory causes.

6. Wstęp

6.1. Rośliny jako źródło leków

Rośliny od wieków stanowią kluczowe źródło leków stosowanych w profilaktyce czy leczeniu chorób człowieka. Wykorzystywane były przez ludzi w różnych jednostkach chorobowych, często bez znajomości związków odpowiedzialnych za ich aktywność biologiczną. Przełomem w „świadomym" wykorzystaniu roślin było wyizolowanie morfiny w 1804 roku przez niemieckiego aptekarza Friedricha Sertürnera. Badania te zapoczątkowały nową erę prac nad właściwościami leczniczymi roślin, co umożliwiło leczenie takich chorób jak nadciśnienie (rezerpina), dna moczanowa (kolchicyna) czy nowotwory (paklitaksel, winkrystyna, winblastyna). Po okresie zmniejszonego zainteresowania roślinami, spowodowanego rozwojem chemii kombinatoryjnej, XXI wiek przyniósł „renesans" leków pochodzenia naturalnego. Wynika to z dwóch głównych powodów:

1. rośliny są bogate w metabolity wtórne o różnorodnej budowie chemicznej, co generuje wiele unikalnych farmakoforów,
2. związki pochodzenia naturalnego, w przeciwieństwie do cząsteczek syntetycznych, cechują się większym powinowactwem do białek komórkowych oraz zdolnością przenikania międzykomórkowego.

Należy również wspomnieć, że gwałtowny rozwój metod screeningowych (bardziej skuteczne), metod analitycznych (pozwalające na określenie niedominujących składników ekstraktu, a które ze względu na właściwości biologiczne determinują aktywność substancji roślinnej) oraz metod statystycznych (integrowanie oraz analiza ogromnej ilości danych) pozwolił na dokładniejsze badanie roślin.

6.2. Charakterystyka botaniczna *Eleutherococcus divaricatus* (Siebold & Zucc.) S.Y.Hu

Interesującą grupą roślin o wielokierunkowym działaniu biologicznym jest rodzaj *Eleutherococcus*. Rodzaj ten obejmuje 29 gatunków, rosnących głównie w Azji (Chiny, Japonia, Korea). Najbardziej znanym przedstawicielem tego rodzaju jest *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim., powszechnie stosowany w Tradycyjnej Medycynie Dalekowschodniej. Jednym z mniej znanych przedstawicieli tego rodzaju jest *Eleutherococcus divaricatus* (Siebold & Zucc.) S.Y.Hu, znany również jako oni-ukogi. Gatunek ten występuje w środkowej i południowej Japonii, na Półwyspie Koreańskim oraz w

kontynentalnych Chinach. Jest to krzew, który może osiągać wysokość do 2-3 metrów (Ryc. 1.). Liście są dłoniasto złożone, składające się z 5-7 listków. Listki są eliptyczne, ząbkowane na brzegach (Ryc. 2.). Kwiaty są drobne, zebrane w baldachy, mają kolor żółtawy lub zielonkawy. Owoce są czarne, kuliste, o średnicy około 5 mm (Ryc. 3.). Korzeń jest gruby i rozgałęziony, często z licznymi bocznymi korzeniami. Zewnętrzna warstwa korzenia jest brązowa, podczas gdy wewnętrzna część ma jasny, kremowy kolor. Korzeń jest twardy i włóknisty (Ryc. 4.).

Ryc. 1. Pokrój morfologiczny *Eleutherococcus divaricatus*, Arboretum w Kórniku [fot. Jakub Gębalski].

Ryc. 2. Budowa morfologiczna liści *Eleutherococcus divaricatus*, Arboretum w Rogowie [fot. Daniel Załuski].

Ryc. 3. Budowa morfologiczna owoców *Eleutherococcus divaricatus*, Arboretum w Rogowie [fot. Daniel Załuski].

Ryc. 4. Budowa morfologiczna korzenia *Eleutherococcus divaricatus*, Arboretum w Kórniku [fot. Jakub Gębalski].

6.3. Charakterystyka fitochemiczna *Eleutherococcus divaricatus* (Siebold & Zucc.) S.Y.Hu

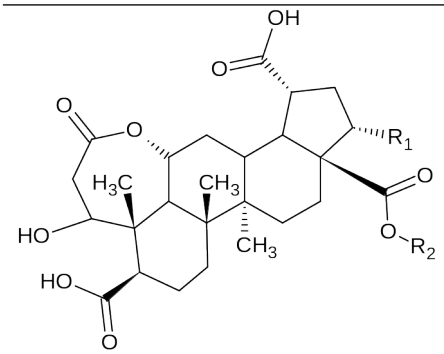
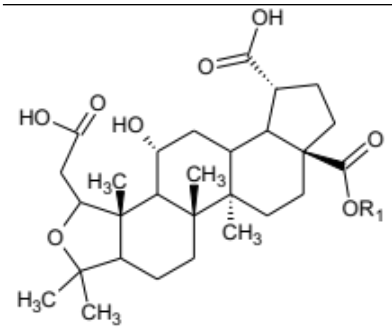
Główne związki czynne obecne w *E. divaricatus* to glikozydy pochodne lignanów i fenylopropanu, tzw. eleuterozydy, w tym eleuterozydy B, E i E1. Najbogatszym źródłem eleuterozydów jest korzeń. Ponadto w korzeniu zidentyfikowano izofraksydynę, kwas ursolowy oraz oleanolowy [1].

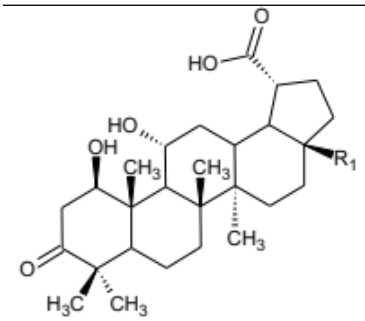
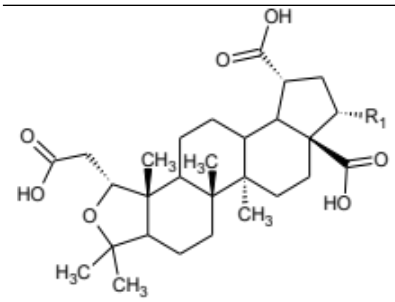
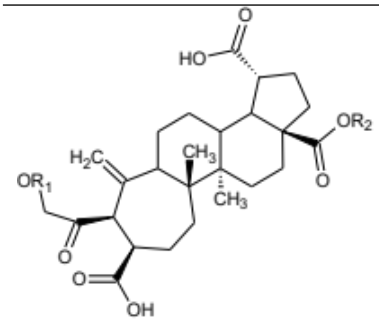
Z kolei owoce są bogate w kwasy fenolowe (kwas *p*-kumarowy, kwas kawowy, kwas protokatechowy, kwas ferulowy oraz kwas salicylowy), flawonoidy (rutyna, hyperozyd, izokwercetyna oraz naryngenina) oraz polisacharydy [2]. W liściach zidentyfikowano głównie saponozydy (sochiisanozyd, 22α-hydroksychiisanogenina, chiisanogenina, chiisanozyd, 24-hydroksychiisanogenina, izochiinasonia, protochiisanozyd) oraz pochodne kwasu kawowego (kwas 5-O-kawoilochinowy, kwas 4-O-kawoilochinowy, kwas 3,4-di-O-kawoilochinowy, kwas 3,5-di-O-kawoilochinowy, kwas 4,5-di-O-kawoilochinowy, kwas 1,5-di-O-kawoilochinowy) [3-8].

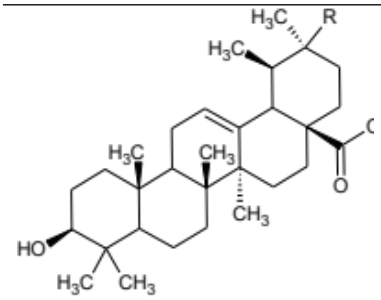
Większość badań nad składem fitochemicznym *E. divaricatus* dotyczyła łodygi, w której zidentyfikowano głównie lignany [(β)-pinoresinol, (β)-medioresinol, (β)-syringaresinol,

akantozyd B, obtusifozyd A, akantozyd D, (β)-sesamina, (β)-larikiresinol-9-O-*β*-D-glukopiranozyd, (β)-alangilignozyd C, (β)-salwadorozyd, cytrusyna B], fenole (4-(3-metoksy-1-propen-1-ylo)-1,2-benzenodiol, alkohol koniferylowy, 4-[(1E)-3-metoksy-1-propenyl]fenol, kwas *trans*-4-hydroksycynamonowy, kwas *trans*-kofeinowy, kofeinian metylu, sinapaldehyd) oraz glikozydy fenolowe (3-metoksy-1-(3-hydroksy-propen-1-ylo)fenylo-4-O-*α*-L-rhamnopyranozylo-(1/6)-*β*-D-glukopiranozyd, (E)-izokoniferyna) [9,10]. Budowa chemiczna poszczególnych metabolitów wtórnych została przedstawiona w Tabeli 1.

Tabela 1. Budowa chemiczna metabolitów wtórnych wyizolowanych z części nadziemnych i podziemnych *Eleutheroccocus divaricatus.*

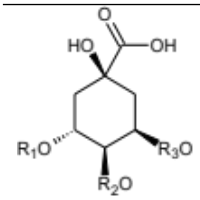
ZWIĄZKI TERPENOWE		
		
	R_1	**R_2**
chiisanozyd	H	α-L-Rha(1→4)-β-D-Glc(1→6))-β-D-Glc
chiisanogenina	H	H
22α-hydroksychiisanozyd	OH	α-L-Rha(1→4)-β-D-Glc(1→6))-β-D-Glc
24α-hydroksychiisanozyd	H	α-L-Rha(1→4)-β-D-Glc(1→6))-β-D-Glc
		

izochiisanozyd	Glc-Glc-Rha	H
		
protochiisanozyd	COO-Glc-Glc-Rha	
		
11-deoksyizochiisanozyd	α-L-Rha(1→4)-β-D-Glc(1→6))-β-D-Glc	
		
sachunozyd	β-D-Glc	α-L-Rha(1→4)-β-D-Glc(1→6))-β-D-Glc

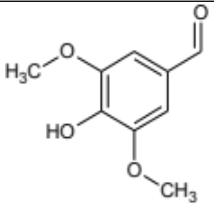


kwas ursolowy	H
kwas oleanowy	CH_3

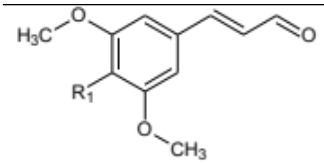
ZWIĄZKI FENOLOWE



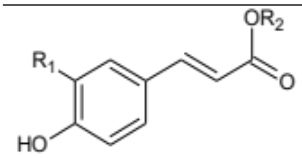
	R_1	**R_2**	**R_3**
kwas 5-O-kawoilochinowy	H	H	kofeinian
kwas 4-O-kawoilochinowy	H	kofeinian	H
kwas 4,5-di-O-kawoilochinowy	H	kofeinian	kofeinian
kwas 3,5-di-O-kawoilochinowy	kofeinian	H	kofeinian
kwas 3,4-di-O-kawoilochinowy	kofeinian	kofeinian	H



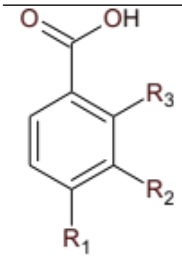
	R_1	**R_2**

aldehyd sinapylowy


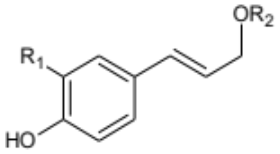
aldehyd sinapinowy	H
glukozyd aldehydu sinapinowego	β-D-Glc

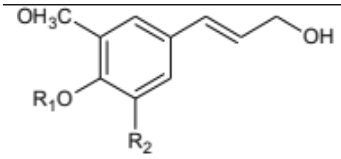
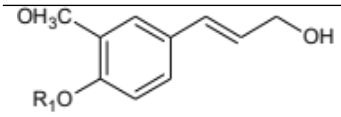
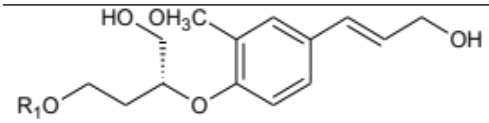


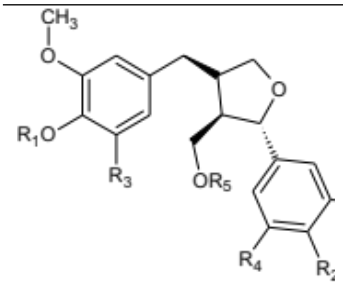
kwas 4-hydroksycynamonowy	H	H
kwas kawowy	OH	H
kwas ferulowy	OCH_3	H
kofeinian metylu	OH	OCH_3



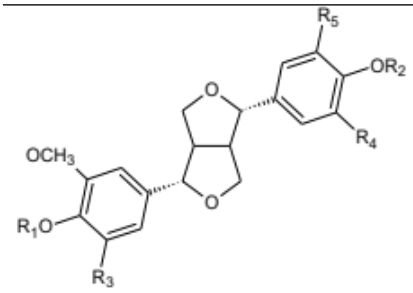
	R_1	**R_2**	**R_3**
kwas protokatechowy	OH	OH	H
kwas salicylowy	H	H	OH



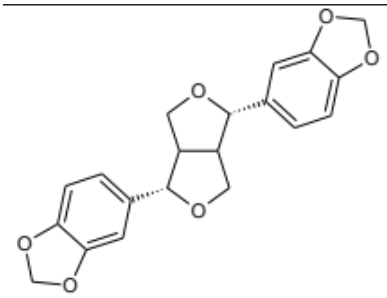
4-(3-metoksy-1-propen-1-yl)-1,2-benzenediol	OH	OCH_3
(E)-izokoniferyna	OCH_3	β-D-Glc
alkohol koniferylowy	OCH_3	H
4-[(1E)-3-metoksy-1-propenyl] fenol	H	OCH_3
		
eleuterozyd B	β-D-Glc	OCH_3
3-metoksy-1-(3-hydroksy-propen-1-ylo)-fenyl-4-O-α-L-rhamnopyranozylo-(1→6)-β-D-glukopiranozyd	α-L-Rha(1→6)-β-D-Glc	H
		
koniferyna	β-D-Glc	
		
3-hydroksy-2-{4-(1E)-3-hydroksyprop-1-en-1-yl]-2-metoksyfenoksy}-propyl-β-D-glukopyranozyd	β-D-Glc	
LIGNANY		



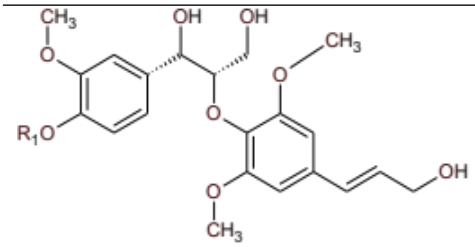
	R₁	**R₂**	**R₃**	**R₄**	**R₅**
(+)-larycyrezynol-9-O-β-D- glukopyranozyd	H	H	H	H	β-D-Glc
(+)-alangilignozyd C	H	H	OCH_3	OCH_3	β-D-Glc
(+)-salvadorazyd	β-D-Glc	β-D-Glc	OCH_3	OCH_3	H



	R₁	**R₂**	**R₃**	**R₄**	**R₅**
(+)-pinoresinol	H	H	H	H	OCH_3
(+)-medioresinol	H	H	H	OCH_3	OCH_3
(+)-syringaresinol	H	H	OCH_3	OCH_3	OCH_3
akantozyd B	H	β-D-Glc	OCH_3	OCH_3	OCH_3
obtusifozyd A	H	β-D-Glc-(1→6)- β-D-Api	OCH_3	OCH_3	OCH_3
eleuterozyd E	β-D-Glc	β-D-Glc	OCH_3	OCH_3	OCH_3

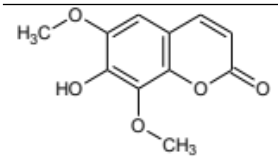


(+)-sesamina



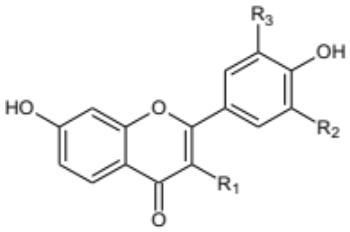
cytrusyna B	β-D-Glc

KUMARYNY

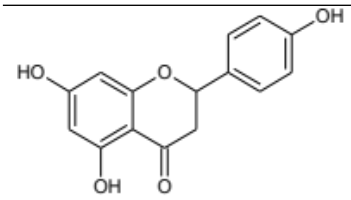


izofraksydyna

FLAWONOIDY



	R_1	**R_2**	**R_3**
hiperyna	O-β-D-Gal	OH	H
rutyna	O-Rut	H	OH

afzelina	O-α-L-Rha	H	H
kwercetyna	OH	H	OH
kempferol	OH	H	H
			
naryngenina			

6.4. Charakterystyka fitofarmakologiczna *Eleutherococcus divaricatus* (Siebold & Zucc.) S.Y.Hu

Korzeń *E. divaricatus* stosowany był w Tradycyjnej Medycynie Chińskiej (TMC) w leczeniu nadciśnienia, cukrzycy, reumatyzmu, zapalenia wątroby, osłabienia mięśni i kości oraz w celu przywrócenia sił witalnych [11-16]. Niemniej jednak istnieje niewiele badań naukowych określających aktywność biologiczną *E. divaricatus*. Wodny ekstrakt z łodygi *E. divaricatus* var. *albeofructus* redukował stany zapalne w płucach. W badaniu zastosowano trzy dawki: 30, 100 i 300 mg/ml. Ekstrakty oraz związki takie jak akantozyd B, 4-[(1E)-3-metoksy-1-propenyl]fenol i kofeinian metylu hamowały syntezę markerów stanu zapalnego, IL-6 i tlenku azotu, w komórkach nabłonka płuc poddanych działaniu IL-1β oraz makrofagach pęcherzykowych poddanych działaniu lipopolisacharydu (LPS). Podawanie myszom z ostrym uszkodzeniem płuc ekstraktów oraz akantozydu D znacząco zmniejszało stan zapalny w płucach. Wyniki tych badań sugerują, że *E. divaricatus* var. *albeofructus* ma duży potencjał w leczeniu chorób płuc związanych ze stanem zapalnym [17].

Ekstrakt wodny z łodygi *E. divaricatus* var. *albeofructus* skutecznie blokował upośledzenie biernego unikania oraz zwiększał immunoreaktywność kwaśnego białka włókienkowego gleju i IL-1α w hipokampie. Ponadto łagodził spadek poziomu acetylocholiny oraz wzrost poziomu aldehydu dimalonowego w korze mózgowej. Ekstrakt znacząco poprawiał wyniki w zadaniu polegającym na rozpoznawaniu nowych obiektów oraz zmniejszał odkładanie się amyloidu i IL-1β w mózgu [18].

Wodny ekstrakt z łodygi i korzenia (1:4) *E. divaricatus* var. *albeofructus* oraz akantozyd D mogą łagodzić uszkodzenia wątroby spowodowane niedokrwieniem i reperfuzją. Działają poprzez hamowanie infiltracji komórek zapalnych, co zmniejsza uwalnianie cytokin zapalnych i równoważy stan oksydacyjno-antyoksydacyjny. Proces ten jest mediowany przez p38 MAPK oraz c-Jun NH2-terminalną kinazę/kinazę białkową aktywowaną stresem [13]. Wodny ekstrakt z *E. divaricatus* var. *chiisanensis* oraz akantozyd D znacząco obniżyły ciśnienie krwi oraz zmniejszyły grubość ściany aorty. Ekstrakt i akantozyd D działały poprzez zwiększenie ekspresji śródbłonkowej syntazy tlenku azotu (eNOS) w błonie wewnętrznej i środkowej. Ponadto, istotny wzrost wewnątrzkomórkowej produkcji tlenku azotu został wywołany po stymulacji wodnym ekstraktem *E. divaricatus* var. *chiisanensis* oraz akantozydem D ludzkich komórek śródbłonka żyły pępowinowej [19,20].

6.5. Hialuronidazy oraz tyrozynaza

6.5.1 Hialuronidaza

Hialuronidaza (HYAL) jest enzymem odpowiedzialnym za hydrolizę kwasu hialuronowego (HA). HA to liniowy, wysokocząsteczkowy, niesiarczanowany polisacharyd, zbudowany z reszt N-acetylo-D-glukozaminy i kwasu D-glukuronowego, połączonych wiązaniami glikozydowymi. Związek ten występuje zarówno u prokariontów, jak i eukariotów, i jest powszechnie obecny w wielu tkankach i płynach ustrojowych, takich jak mięśnie, stawy, płyny maziowe, skóra i ciało szkliste. Dodatkowo, HA bierze udział w procesach gojenia się ran, stanach zapalnych i karcenogenezie.

W oparciu o strukturę i mechanizm działania wyróżniono trzy klasy hialuronidaz.

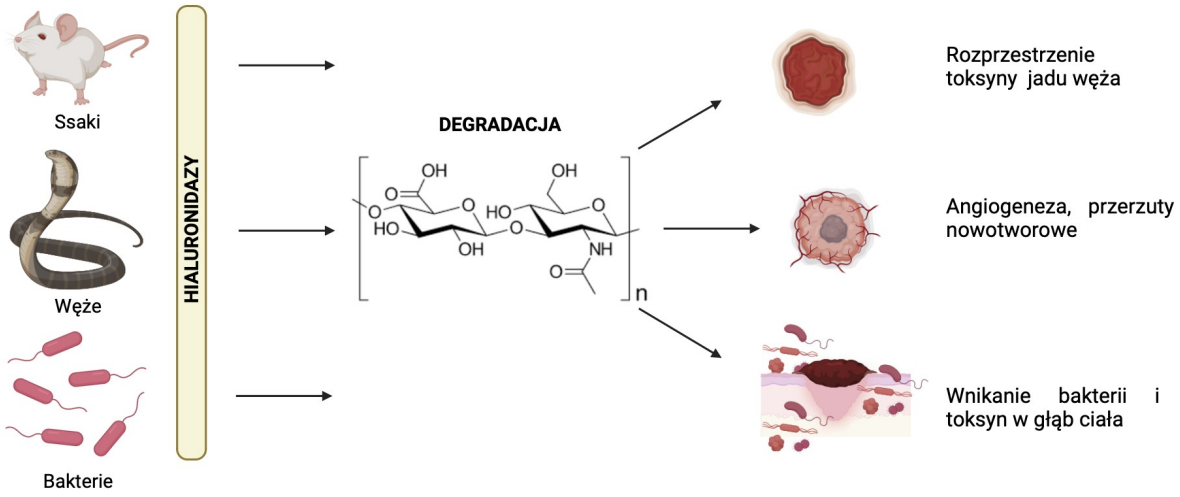
1. Pierwsza klasa obejmuje endo-β-N-acetyloheksozaminidazy (EC 3.2.1.35), które są obecne u ssaków oraz w jadzie węży. Enzymy te są odpowiedzialne za hydrolizę wiązania β-1,4-glikozydowego w kwasie hialuronowym (HA). HA jest rozkładany na mniejsze fragmenty, co może wpływać na różne procesy biologiczne, takie jak przepuszczalność tkanek i migracja komórek.

2. Druga klasa to endo-β-D-glukuronidazy (EC 3.2.1.36), które hydrolizują wiązania β-1,3-glikozydowe w HA. Enzymy te są obecne głównie u bezkręgowców. Ich działanie prowadzi do rozkładu HA, co może mieć znaczenie w regulacji struktury macierzy zewnątrzkomórkowej.

3. Trzecia klasa obejmuje endo-β-N-acetyloheksozaminidazy (EC 4.2.2.1), które są obecne wyłącznie u prokariontów. Enzymy bakteryjne z tej grupy rozrywają wiązania β-1,4-glikozydowe w HA. Proces ten prowadzi do depolimeryzacji HA, co może wpływać na zdolność bakterii do kolonizacji i infekcji tkanek gospodarza.

HYAL biorą udział w patogenezie wielu chorób. HYAL są ważnym czynnikiem zjadliwości dla wielu gatunków bakterii, takich jak *Staphylococcus* spp., *Streptococcus* spp. i *Streptomyces* spp. Rozkład HA zwiększa lepkość płynów ustrojowych i zmniejsza integralność tkanek, ułatwiając przenikanie mikroorganizmów w głąb tkanek. Podobną funkcję HYAL pełnią w jadzie błonkówek, pająków i węży.

Ponadto wykazano udział HYAL we wzroście guza, przerzutach i angiogenezie. Jednak przeprowadzone badania nie dostarczają jednoznacznych wyników pokazujących, że HYAL może funkcjonować zarówno jako supresor, jak i promotor karcenogenezy. HA wspomaga przerzuty do nowotworu, więc enzym, który rozkłada hialuronian hamuje ich wzrost. Z drugiej strony, HA o niskiej masie cząsteczkowej stymuluje angiogenezę, promując wzrost guza i tworzenie przerzutów (Ryc. 5) [21-28].



Ryc. 5. Udział hialuronidaz w patogenezie chorób. BioRender.com

6.5.2. Tyrozynaza

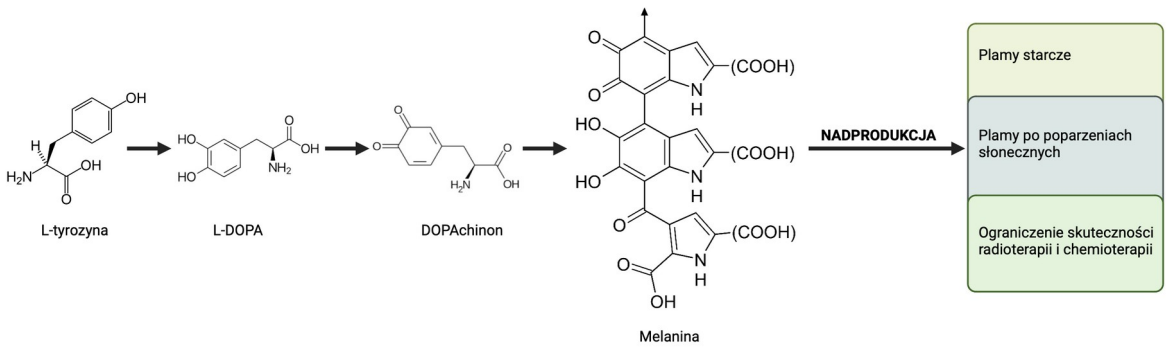
Tyrozynaza jest kluczowym enzymem biorącym udział w melanogenezie, należącym do klasy oksydoreduktaz (EC 1.14.18.1). Odpowiada za katalizę hydroksylacji tyrozyny do L-DOPA oraz oksydacyjną konwersję L-DOPA do dopachinonu. Enzym ten jest szeroko

rozpowszechniony w przyrodzie, występujee w grzybach, bakteriach, glonach, roślinach i zwierzętach.

U wyższych kręgowców melanina jest wytwarzana w melanocytach obecnych w naskórku, mieszkach włosowych, błonie naczyniowej oka (naczyniówka, ciało rzęskowe i tęczówka), uchu wewnętrznym (ślimak) oraz ośrodkowym układzie nerwowym. Melaniny to makrocząsteczkowe pigmenty polimerowe powstające w wyniku utleniania i polimeryzacji związków fenolowych. Synteza melaniny odbywa się w pęcherzykach zwanych melanosomami i jest jednym z najczęstszych procesów pigmentacyjnych zachodzących w przyrodzie.

U ssaków tyrozynaza melanosomalna bierze udział w tworzeniu czarno-brązowej eumelaniny oraz żółto-czerwonawej feomelaniny. Eumelanina ma właściwości fotoprotekcyjne, wynikające ze zdolności do pochłaniania promieniowania ultrafioletowego (UV) oraz neutralizacji wolnych rodników i reaktywnych form tlenu (ROS). Z kolei feomelanina ma właściwości fotouczulające i pod wpływem promieniowania UV może uczestniczyć w wytwarzaniu ROS.

Pigmentacja melaniny u ssaków pełni wiele ważnych funkcji fizjologicznych, takich jak zabarwienie adaptacyjne, ochrona tkanek przed promieniowaniem UV, kontrola termiczna organizmu oraz regulacja biosyntezy witaminy D3. Nieprawidłowa aktywność tyrozynazy może prowadzić do zaburzeń skórnych, takich jak bielactwo czy piegi. Ponadto, tyrozynaza może odgrywać rolę w nowotworzeniu oraz chorobach neurodegeneracyjnych, takich jak choroba Parkinsona (Ryc.6.) [29-35].



Ryc. 6. Udział tyrozynaz w patogenezie chorób. BioRender.com

7. Powody podjęcia badań

Brak skutecznych i bezpiecznych inhibitorów tyrozynazy i hialuronidazy jest podstawą do poszukiwania nowych związków z tej grupy. Nowe inhibitory tyrozynazy mają szczególne zastosowanie w dermatologii i produkcji żywności. Soki przygotowywane z owoców i warzyw z powodu brązowienia mają krótki okres przydatności do spożycia. Aby wydłużyć okres przydatności do spożycia i zachować naturalny, świeży wygląd, przemysł przetwórstwa owoców wymaga opracowania nowych, skutecznych i bezpiecznych inhibitorów tyrozynazy. Z drugiej strony, nadprodukcja melaniny w skórze jest przyczyną różnych chorób i problemów estetycznych. Inhibitory tyrozynazy mogą zmniejszać biosyntezę melaniny i być stosowane w produktach kosmetycznych do profilaktyki przebarwień, w tym piegów i przebarwień po oparzeniach słonecznych. Ponadto inhibitory tyrozynazy mogą być również stosowane w leczeniu przebarwień, takich jak poparzenia słoneczne, przebarwienia pozapalne i plamy starcze [36,37].

Hialuronidazy odgrywają kluczową rolę w wielu procesach fizjologicznych i patologicznych, jednakże ich udział w wielu z tych procesów pozostaje niewyjaśniony. Selektywne inhibitory hialuronidazy są niezbędne do zrozumienia związku między aktywnością hialuronidazy a jej skutkami. Co więcej, inhibitory hialuronidazy mogą być użyteczne jako narzędzia farmakologiczne, np. w połączeniu z antybiotykami w leczeniu infekcji wywołanych przez bakterie wytwarzające hialuronidazę, w tym *Streptococcus pneumoniae*, główny Gram-dodatni patogen człowieka. Identyfikacja i charakterystyka inhibitorów hialuronidazy może również przyczynić się do opracowania nowych leków przeciwnowotworowych, środków antykoncepcyjnych i odtrutek na jady i toksyny. Obecnie znane inhibitory, tj. kwas askorbinowy i jego analogi, a także escyna, wykazują słabą aktywność wobec hialuronidazy, ponadto escyna jest słabo wchłaniana w przewodzie pokarmowym [38-40].

Uwzględniając powyższe argumenty, niezwykle istotne jest poszukiwanie nowych inhibitorów tych enzymów, które będą charakteryzować się większą funkcjonalnością i bezpieczeństwem stosowania.

8. Cel rozprawy doktorskiej

Rośliny od wieków stanowiły ważne źródło leków (przez większość czasu jedyne). Duża różnorodność strukturalna związków naturalnych determinuje ich szeroką aktywność biologiczną. Pierwszym skutecznym lekiem w leczeniu nadciśnienia tętniczego była rezerpina wyizolowana z korzeni rauwolfii żmijowej (*Rauwolfia serpentina* (L.) Bentham ex Kurz). Prekursorem metforminy, leku pierwszego rzutu w leczeniu cukrzycy, była galagina wyizolowana z ruty lekarskiej (*Galega officinalis* L.). Inhibitory SGLT-2, np. empagliflozyna, kanagliflozyna i dapagliflozyna należą do nowej generacji leków przeciwcukrzycowych wywodzących się ze struktury floryzyny wyizolowanej z jabłoni (*Malus domestica* Borkh.). Leczenie bólu paliatywnego oraz pooperacyjnego było możliwe dzięki morfinie wyizolowanej z maku lekarskiego (*Papaver somniferum* L.). Powyższe przykłady pokazują istotność roślin jako źródła nowych leków.

Celem nadrzędnym rozprawy było zidentyfikowanie inhibitorów hialuronidazy oraz tyrozynazy w *Eleutherococcus divaricatus* (Siebold & Zucc.) S.Y.Hu, określenie ich cytotoksyczności wobec wybranych linii komórkowych czerniaka i właściwości antyoksydacyjnych.

Realizacja celu została oparta na dwóch strategiach (A i B):

A: identyfikacji ekstraktów/frakcji/związków czynnych o pożądanej funkcjonalności w kierunku stymulacji aktywności hialuronidazy i tyrozynazy z uwzględnieniem tych enzymów obecnych w surowicy krwi dzieci ze zdiagnozowaną ostrą białaczką limfoblastyczną, przed rozpoczęciem leczenia,

B: ocenie bezpieczeństwa na modelowych liniach czerniaka w skojarzeniu z chemioterapeutykiem doksorubicyną.

9. Wyniki badań

P.1. Charakterystyka naturalnych oraz półsyntetycznych inhibitorów hialuronidazy oraz tyrozynazy

Praca przeglądowa

P.1. Gębalski J., Graczyk F., Załuski D. Paving the way towards effective plant-based inhibitors of hyaluronidase and tyrosinase: A critical review on a structure–activity relationship. J. Enzyme. Inhib. Med. Chem. 2022; 37(1), 1120-1195.

Przed rozwojem współczesnej medycyny, ziołolecznictwo było szeroko stosowane w leczeniu wielu chorób. Współczesna medycyna wykorzystuje przede wszystkim roślinne składniki aktywne, które ze względu na swoją różnorodność strukturalną stanowią dobre źródło nowych leków.

Jednym z mechanizmów działania leków jest inhibicja białek enzymatycznych, których aktywność często wzrasta w rozwoju stanów patologicznych. Na szczególną uwagę zasługują tyrozynaza i hialuronidaza. Hamowanie aktywności tyrozynazy może zostać wykorzystane w leczeniu chorób skóry (plamy starcze, przebarwienia słoneczne), czy nowotworach skóry (czerniak). Z kolei inhibitory hialuronidaz mogą zostać wykorzystane w leczeniu infekcji bakteryjnych, ukąszeń węży, ograniczeniu przerzutów nowotworowych, czy jako doustna antykoncepcja u mężczyzn.

Celem tej pracy było poszukiwanie korelacji pomiędzy budową chemiczną związków roślinnych a ich hamującym wpływem na aktywność hialuronidazy (ludzkiej, bakteryjnej, wężowej) i tyrozynazy (grzybowej i ludzkiej). W celu potwierdzenia mojej hipotezy, czyli wykazania, że związki roślinne hamują powyższe enzymy, dokonałem krytycznej analizy artykułów oryginalnych, korzystając z następujących baz danych: ScienceDirect, PubMed, Scopus, Web of Science, Google Scholar i ClinicalTrials. W pracy uwzględniono publikacje z okresu od stycznia 1990 r. do grudnia 2021 r. W strukturze pracy można wyróżnić dwie części: pierwsza poświęcona jest charakterystyce enzymów (klasyfikacja, występowanie, funkcja), a druga przedstawia zależność struktura-aktywność związków naturalnych wobec tyrozynazy oraz hialuronidazy (polifenole, alkaloidy, związki izoprenowe).

W przypadku tyrozynazy dużą aktywnością hamującą charakteryzowały się związki polifenolowe, szczególnie flawonoidy o strukturze podobnej do kwasu kojowego. Obecność grup hydroksylowych (OH) w położeniu rezorcynolu lub katecholu zwiększała aktywność

tych związków. Natomiast metylacja grup OH oraz wprowadzenie reszty cukrowej zmniejszały ich działanie. Podobne wyniki uzyskano dla innych polifenoli, takich jak kumaryny, lignany, chalkony, stilbeny oraz garbniki.

Przechodząc do drugiego enzymu, mianowicie do hialuronidazy, okazało się, że grupą inhibitorów szczególnie aktywną są również polifenole. Obecność wiązania podwójnego między drugim a trzecim atomem węgla, grupy OH w położeniu C-4', C-5 oraz C-7 oraz grupy ketonowej w pozycji C-4 we flawonoidach korzystnie wpływały na aktywność tych związków. Wysoką aktywnością charakteryzowały się również kwasy fenolowe, szczególnie pochodne kwasu cynamonowego. Wzrost liczby grup OH oraz estryfikacja grupy karboksylowej (COOH) alkoholami o długim łańcuchu węglowodorowym korzystnie wpływały na aktywność kwasów fenolowych. Zarówno, w przypadku flawonoidów jak i kwasów fenolowych, przyłączenie reszty cukrowej oraz metylacja grup OH zmniejszały aktywność inhibicyjną.

Przechodząc do konkluzji, z całą pewnością można stwierdzić, że związki naturalne są skutecznymi inhibitorami hialuronidaz i tyrozynazy. Na szczególną uwagę zasługują związki polifenolowe, których budowa chemiczna umożliwia interakcje z aminokwasami enzymów w centrum aktywnym. W przyszłości związki te mogą zostać wykorzystane do opracowania skutecznych leków w leczeniu różnych schorzeń. Jednakże należy mieć na uwadze, że większość badań została wykonana na enzymach zwierzęcych, bakteryjnych lub grzybowych. W celu uzyskania bardziej wiarygodnych wyników, które można skorelować z organizmem człowieka, w przyszłości enzymy ludzkie powinny być uwzględnione w analizach.

P.2. Określenie właściwości anty-hialuronidazowych oraz anty-tyrozynazowych owoców *Eleutherococcus divaricatus* (Siebold & Zucc.) S.Y.Hu

Praca oryginalna

P.2. Gębalski J., Małkowska M., Gawenda-Kempczyńska D., Słomka A., Strzemski M., Styczyński J., Załuski D. *Eleutherococcus divaricatus* fruits decrease hyaluronidase activity in blood serum and protect from antioxidative damages in in vitro model. Int. J. Mol. Sci. 2024; 25(4):2033.

Owoce są kluczowymi składnikami diety i źródłem związków biologicznie czynnych, które znajdują zastosowanie w farmakologii żywieniowej. Ze względu na obecność związków polifenolowych, owoce odgrywają istotną rolę w zapobieganiu chorobom cywilizacyjnym. Z tego względu ważne jest badanie składu fitochemicznego i aktywności biologicznej owoców, zwłaszcza tych, które od dawna są wykorzystywane w etnomedycynie.

Celem pracy było określenie zawartości związków fenolowych oraz hamującego potencjału 75% ekstraktu metanolowego z owoców *Eleutherococcus divaricatus* wobec tyrozynazy (ludzkiej i grzybowej) oraz hialuronidazy (ludzkiej i bydlęcej). Wykorzystując chromatografię cieczową z detekcją diodową (HPLC-DAD) zbadano skład ekstraktu. Testy oparte na spektrofotometrii posłużyły do oceny aktywności antyenzymatycznej oraz antyoksydacyjnej ekstraktu (chelatowanie jonów żelaza (II), DPPH, ABTS oraz FRAP).

Na podstawie wyników analizy metodą HPLC-DAD zidentyfikowano 3 związki wobec 14 wzorcowych, mianowicie eleuterozyd E (0,23 mg/g), kwas chlorogenowy (0,13 mg/g) oraz kwas protokatechowy (1,47 mg/g). Przechodząc do aktywności antyenzymatycznej, zauważono, że ekstrakt wykazał umiarkowaną aktywność wobec hialuronidazy z jąder bydlęcych (9,06–37,70%). W ramach współpracy z prof. Janem Styczyńskim (Katedra Pediatrii, Hematologii i Onkologii, CM UMK, Bydgoszcz) pozyskaliśmy krew od pacjentów onkologicznych przed leczeniem (dzieci ze zdiagnozowaną ostrą białaczką limfoblastyczną, mediana wieku 7 lat). Hialuronidaza jest enzymem, którego aktywność wzrasta w chorobach nowotworowych czy chorobach przebiegających z rozwojem stanu zapalnego. Uzyskane wyniki potwierdziły znaczącą inhibicję hialuronidazy na poziomie 82,51% (wartość średnia dla grupy), wobec escyny zastosowanej jako kontrola, inhibicja 71,04% (wartość średnia dla grupy). Różnica w hamowaniu hialuronidazy w surowicy ludzkiej może wynikać ze zmienności osobniczej między pacjentami.

Nie zauważono znacznej aktywności antytyrozynazowej ekstraktu (tyrozynaza grzybowa, 2,94–12,46%). Przeprowadzone badania wskazują na umiarkowane działanie antyoksydacyjne (IC_{50} – ABTS 0,28 mg/mL, DPPH 1,30 mg/mL, chelatowanie Fe^{2+} 1,45 mg/mL oraz rodnik $O_2^{\bullet -}$ 1,51 mg/mL).

Na podstawie analizy uzyskanych wyników badań wstępnych, szczególnie w przypadku inhibicji hialuronidazy obecnej w surowicy krwi, można z całą pewnością stwierdzić, że wyniki te uzasadniają tradycyjne stosowanie *E. divaricatus* w leczeniu chorób zapalnych oraz związanych z układem odpornościowym. Hialuronidaza, enzym biorący udział w degradacji kwasu hialuronowego, odgrywa istotną rolę w procesach zapalnych i immunologicznych. Inhibicja tego enzymu przez ekstrakty z *E. divaricatus* wskazuje na potencjalne korzyści terapeutyczne tej rośliny w leczeniu schorzeń, w których hialuronidaza może odgrywać kluczową rolę.

P.3. Określenie właściwości anty-hialuronidazowych oraz anty-tyrozynazowych korzenia *Eleutherococcus divaricatus* (Siebold & Zucc.) S.Y.Hu
Praca oryginalna

P3. Gębalski J., Małkowska M., Wnorowska S., Gawenda-Kempczyńska D., Strzemski M, Wójciak M., Słomka A., Styczyński J., Załuski D. Ethyl acetate fraction from *Eleutherococcus divaricatus* root extract as a promising source of compounds with anti-hyaluronidase, anti-tyrosinase, and antioxidant activity but not anti-melanoma activity. Molecules. 2024; 29(15):3640.

Eleutherococcus divaricatus to roślina, która od wieków jest wykorzystywana w tradycyjnej medycynie dalekowschodniej. Korzeń posiada różne właściwości farmakologiczne, takie jak działanie przeciwzapalne, przeciwnowotworowe, przeciwdepresyjne, przeciwcukrzycowe, neuroprotekcyjne, hepatoprotekcyjne i immunostymulujące. Jednakże, pomimo długiej historii stosowania w etnofarmakologii w leczeniu wyżej wymienionych chorób, mechanizm jego działania pozostaje w dużej mierze nieznany.

Celem trzeciej pracy było określenie składu chemicznego oraz aktywności farmakologicznej ekstraktów z korzenia *E. divaricatus*, w kontekście ich wpływu na tyrozynazę (ludzką i grzybową) oraz hialuronidazę (ludzką i bydlęcą). W tym celu przygotowałem cztery ekstrakty, stosując rozpuszczalniki o różnej polarności: 75% metanol, chloroform, octan etylu oraz mieszaninę metanolu, chloroformu i wody (7:3:0,4). Ekstrakty te zostały wstępnie przebadane pod kątem inhibicji hialuronidazy i tyrozynazy. Najaktywniejszy ekstrakt (75% metanolowy) poddano frakcjonowaniu metodą ekstrakcji ciecz-ciecz. Następnie określono skład chemiczny frakcji octanu etylu, która wykazała najwyższą inhibicję wobec enzymów. Ponadto, frakcja ta została przebadana *in vitro* na trzech liniach czerniaka, aby ocenić jej potencjalne działanie cytotoksyczne. Dodatkowo, określono aktywność antyoksydacyjną ekstraktów.

Wstępna analiza fitochemiczna frakcji octanu etylu, którą przeprowadziłem z wykorzystaniem prostych reakcji chemicznych (TPC, TFC, TPAC), wykazała, że frakcja octanu etylu zawiera najwięcej polifenoli. Analiza chromatograficzna przy użyciu UHPLC-DAD/ESI-TOF-MS potwierdziła obecność głównie kwasów fenolowych. Intensywny sygnał zaobserwowano dla jonów o *m/z* 353.08835 (191, 179); 515.12021 (353); 515.12048 (353) i

515.12035 (353), które pochodziły od kwasu chlorogenowego, kwasu 3,5-dikawoilochinowego, kwasu dikawoilochinowego i kwasu 4,5-dikawoilochinowego).

Spośród czterech ekstraktów, które przygotowałem stosując rozpuszczalniki różniące się polarnością, to ekstrakt 75% metanolowy najsilniej hamował enzym (mTYR = 274,37 µg/mL, bHYAL = 104,13 µg/mL). Następnie ekstrakt ten poddano frakcjonowaniu stosując ekstrakcję typu ciecz-ciecz. Analizując aktywność uzyskanych frakcji wobec tyrozynazy grzybowej oraz hialuronidazy ludzkiej, stwierdziłem, że najwyższą aktywność miała frakcja octanowa (mTYR = 65,5 µg/mL, bHYAL = 27,5 µg/mL).

Na podstawie analizy uzyskanych wyników badań, wykazałem, że frakcja octanu etylu hamowała hialuronidazę obecną w surowicy krwi pacjentów onkologicznych przed leczeniem (płeć: męska, wiek: 3, 4, 4, 5, 17 lat, zdiagnozowana ostra białaczka limfoblastyczna) na poziomie 55,82% (wartość średnia dla grupy) w porównaniu do kontroli, escyny 63,8% (wartość średnia dla grupy). W analizowanym modelu enzymatycznym podjąłem również próbę określenia wpływu tej frakcji na aktywność tyrozynazy w surowicy krwi dzieci, nie odnotowałem inhibicji. Równolegle określiłem cytotoksyczny wpływ frakcji na 3 linie komórek czerniaka (UACC – 647, A375, SK-MEL-30), nie odnotowałem cytotoksycznego wpływu na te linie. W kolejnym etapie sprawdziłem, czy frakcja octanu etylu może działać synergistycznie w kombinacji frakcja octanu etylu – doksorubicyna. Zauważyliśmy niezwykle ciekawą interakcję, otóż frakcja zmniejszała działanie doksorubicyny, co może wskazywać na jej protekcyjny efekt na komórki czerniaka. Obniżenie skuteczności działania doksorubicyny w obecności frakcji octanu etylu zauważono na trzech badanych liniach czerniaka. Jest to bardzo ważne odkrycie, które częściowo przeczy, wbrew przyjętym ustaleniom, że surowce adaptogenne/immunostymulacyjne powinno się stosować w trakcie leczenia onkologicznego.

Uzyskane wyniki potwierdzają skuteczność stosowania *E. divaricatus* w tradycyjnej medycynie chińskiej (TCM) w leczeniu chorób zapalnych i immunologicznych. Niemniej jednak, w świetle dotychczasowych badań, należy zachować ostrożność przy jednoczesnym stosowaniu tej rośliny i chemioterapii. Zaleca się stosowanie *E. divaricatus* przed rozpoczęciem lub po zakończeniu leczenia onkologicznego, aby wzmocnić organizm i wspomóc jego regenerację. Wyniki te są również zgodne z założeniami radzieckich naukowców z lat 70. i 80. XX wieku, którzy ostrzegali przed łączeniem adaptogenów z grupy *Eleutherococcus* z leczeniem onkologicznym. Podkreślali oni, że takie połączenie może wpływać na skuteczność terapii przeciwnowotworowej. Jednakże, aby w pełni zrozumieć

mechanizmy działania *E. divaricatus* i jego interakcje z chemioterapią, konieczne są bardziej szczegółowe badania z wykorzystaniem modeli zwierzęcych. Takie badania pozwolą na dokładniejsze określenie bezpieczeństwa i skuteczności stosowania tej rośliny w kontekście leczenia onkologicznego.

10. Publikacje będące przedmiotem rozprawy doktorskiej

10.1. Praca przeglądowa 1

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REVIEW ARTICLE

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Paving the way towards effective plant-based inhibitors of hyaluronidase and tyrosinase: a critical review on a structure–activity relationship

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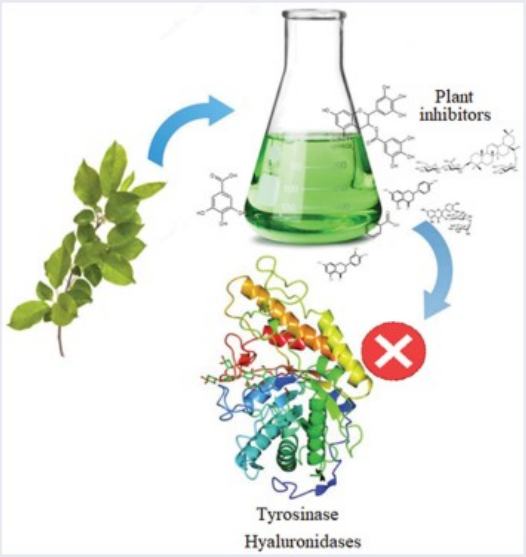
ABSTRACT

Human has used plants to treat many civilisation diseases for thousands of years. Examples include reserpine (hypertension therapy), digoxin (myocardial diseases), vinblastine and vincristine (cancers), and opioids (palliative treatment). Plants are a rich source of natural metabolites with multiple biological activities, and the use of modern approaches and tools allowed finally for more effective bioprospecting. The new phytochemicals are hyaluronidase (Hyal) inhibitors, which could serve as anti-cancer drugs, male contraceptives, and an antidote against venoms. In turn, tyrosinase inhibitors can be used in cosmetics/pharmaceuticals as whitening agents and to treat skin pigmentation disorders. However, the activity of these inhibitors is *stricte* dependent on their structure and the presence of the chemical groups, e.g. carbonyl or hydroxyl. This review aims to provide comprehensive and in-depth evidence related to the anti-tyrosinase and anti-Hyal activity of phytochemicals as well as confirming their efficiency and future perspectives.

GRAPHICAL ABSTRACT



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Hyaluronidase; tyrosinase plant-based inhibitors; structure–activity relationship; polyphenols

1. Introduction

Before the advancement of modern science, herbal medicines were widely used in ethnomedicine. Modern medicine primarily uses plant active ingredients that can be divided into primary and secondary metabolites. Secondary metabolites are defined as substances that are not directly necessary for the organism's growth and development. It is believed that they play an important role

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in adapting plants to changing external conditions. Secondary metabolites are formed in various metabolic pathways, including amino acids and sugars intermediates, therefore, plants can synthesise many structurally diverse metabolites. It is estimated that plants produce at least 250,000 natural products, many of which have not yet been identified and characterised in structure and biological activity[1]. The enormous diversity in the plant world (250,000–300,000 species) has provided many substances currently used in treatment[2,3]. The isolation of morphine and codeine from *Papaver somniferum* L. ensured an effective pain treatment[4]. Obtaining digoxin from *Digitalis purpurea* L. allowed for an effective treatment of atrial fibrillation and heart failure[5]. Salicylic acid, which is found, among others, in *Salix alba* L., after esterification forms acetylsalicylic acid, used in pain and thrombotic diseases[6]. The isolated artimizin from *Artemisia annua* L. is used in the treatment of a resistant form of malaria[7]. Other compounds used in the treatment are capsaicin from *Capsicum annuum* L.[8], quinine from *Cinchona* L.[9], and inulin[10]. Additionally, some compounds, after structure modification, have contributed to a new group of drugs. A good example is a phlorizin, which has served as the host structure for SGLT2 sodium-glucose co-transporter inhibitors, e.g. dapagliflozin[11]. The development of new branches of science, such as genetics, biotechnology, and molecular biology, allowed for a better understanding of diseases' pathophysiology[12,13]. Hyals and tyrosinases are an underestimated group of enzymes commonly found in the world of organisms. Hyal, due to its participation in many biological processes, such as fertilisation, diffusion of toxins and microorganisms, inflammatory and allergic reactions, and cancer development, is perceived as a potential therapeutic target[14]. Besides, the inhibitors of tyrosinases and Hyals can be used in cosmetology to develop cosmetics or drugs used in dermatological diseases, such as eczema, acne, discolouration, and photoaging[15–17].

This study aims to systematise knowledge about the Hyal and tyrosinase inhibitors. We have hypothesised that there are the plant-based compounds and their chemically modified derivatives which inhibit the Hyal and tyrosinase.

2. Methods

To confirm our hypothesis we have searched for the different available databases (ScienceDirect, PubMed, Scopus, Web of Science, Google Scholar, and ClinicalTrials) in the regard to the relationship between the structure and activity of inhibitors, their action's mechanism, and the prospects for their use in treatment. Search terms included "natural substances", "plant substances", "polyphenols", "phenolic acids", "chalcones", "stilbenes", "lignans", "terpenes", "alkaloids", "glycosides", "tyrosinase", "tyrosinase inhibitors", "hyaluronidase" (Hyal), "hyaluronidase inhibitors", and "bacterial lyase". The search equation was defined according to the formula [tyrosinase inhibitor OR tyrosinase OR Hyal inhibitor OR Hyal OR bacterial lyase inhibitor OR bacterial lyase] AND [natural substances OR plant substances OR polyphenols OR phenolic acids OR chalcones OR stilbenes OR lignans OR terpenes OR alkaloids OR glycosides]. Publications were searched from January 1990 to December 2021.

3. Types of inhibition

Inhibition is the process that slows down or completely stops a chemical reaction by a substance called an inhibitor. Depending on the way of binding of the inhibitor with the enzyme, one can distinguish reversible and irreversible inhibition (Table 1).

Table 1. Effect of the inhibitor on K_m and V_{max}.

Types of inhibitor	K_m	V_{max}
Competitive inhibition	↑	Unchanged
Non-competitive inhibition	Unchanged	↓
Uncompetitive inhibition	↓	↓
Mixed inhibition	↑	↓
Irreversible inhibition	Unchanged	↓

3.1. Irreversible inhibition

An irreversible inhibitor is a compound with a structure similar to the substrate or product that forms a covalent bond with a group present in the active centre. In the case of irreversible inhibition, it is not necessary to maintain the inhibitor concentration at a sufficient level to ensure enzyme-substrate interaction. The complex formed does not dissociate, so the enzyme is inactive even when the inhibitor is absent. In contrast to irreversible inhibition, reversible inhibition is characterised by dissociating the enzyme-inhibitor complex. There are the following types of reversible inhibition: competent, incompetent, acompetent, and mixed[18].

3.1.1. Competent inhibition

The inhibitor shows a structural similarity to the substrate with which it competes for access to the enzyme's active centre. The enzyme-inhibitor complex converts to an enzyme-substrate complex, and the inhibitor is displaced from the enzyme's active site. The degree of inhibition of the enzyme depends on the concentration of the substrate. The higher substrate concentration relative to the inhibitor, the fewer enzyme molecules will bind to the inhibitor. Therefore, the inhibition of the reaction caused by a competent inhibitor can be reversed by increasing the substrate concentration.

3.1.2. Noncompetent inhibition

A noncompetent inhibitor usually bears no resemblance to the structure of the substrate. The inhibitor binds to the enzyme at a different site than the substrate. The inhibitor can attach to the free enzyme or the enzyme-substrate complex. Unlike competent inhibition, incompetent inhibition does not depend on the concentration of the substrate.

3.1.3. Accompetent inhibition

The inhibitor binds to the enzyme-substrate complex, forming an enzyme-inhibitor-substrate complex.

3.1.4. Mixed inhibition

It is a case of partially competent and incompetent inhibition. The inhibitor can bind to both the free enzyme and the ES complex, reducing the maximum rate[19].

4. Inhibitors of hyaluronidase

4.1. Hyaluronic acid and hyaluronidase

Hyal is responsible for the hydrolysis of hyaluronic acid (HA). HA is a linear, high molecular weight unsulfated polysaccharide composed of alternating N-acetyl D-glucosamine and D-glucuronic acid residues linked by glycosidic bonds. That compound is present both in prokaryotes and eukaryotes, commonly found in many tissues and body fluids, such as muscles, joints, synovial

fluid, skin, and vitreous body. In addition to its structural functions, it is involved in wound healing, inflammation, and tumour development. Hyals are a group of enzymes commonly found in nature, e.g. they are an important component of the venom of bees, spiders, and snakes[20–22]. Based on the structure and mechanism of action, there are three classes of Hyals (Figure 1). The first class includes endo-β-N-acetylhexosaminidases (EC 3.2.1.35) present in mammals and in snake and hymenoptera venom. They hydrolyse β-1,4-glycosidic bonds in HA. The second class is endo-β-D-glucuronidases (EC 3.2.1.36), which hydrolyse the β-1,3-glycosidic linkages in the HA and are present in invertebrates. As a result of the activity of both enzymes, tetra, and hexasaccharide of HA are formed. The third class includes endo-β-N-acetylhexosaminidases (EC 4.2.2.1), present only in prokaryotes. Bacterial enzymes break β-1,4-glycosidic bonds in HA using a β-elimination reaction. As a result of their action, unsaturated di-, tetra-, and hexasaccharide are formed. There are five types of Hyals found in humans: HYAL-1, HYAL-2, HYAL-3, HYAL-4, and HYAL-5. HYAL-1 and HYAL-2 are found in most tissues and are involved in the circulation of HA[23]. HYAL-2 degrades long-chain hyaluronic acid (HMWHA) found in the extracellular matrix to short-chain hyaluronic acid (LMWHA), which after binding to the CD44 receptor, is transported into the cell. HYAL-1 is found inside the cell and is responsible for the degradation of LMWHA into tetra and hexasaccharide[24].

Hyals are involved in the regulation of important physiological processes, such as fertilisation and skin ageing. Present in sperm, HYAL-5 plays a key role in fertilisation in mammals as it enables the sperm to connect to the egg cell by breaking down HA in the granule layer. The overactivity of collagenases, Hyal, and elastase leads to the formation of wrinkles[25].

Hyals are an important virulence factor for many bacterial species, such as *Staphylococcus spp.*, *Streptococcus spp.*, and *Streptomyces spp.* The decomposition of HA increases the viscosity

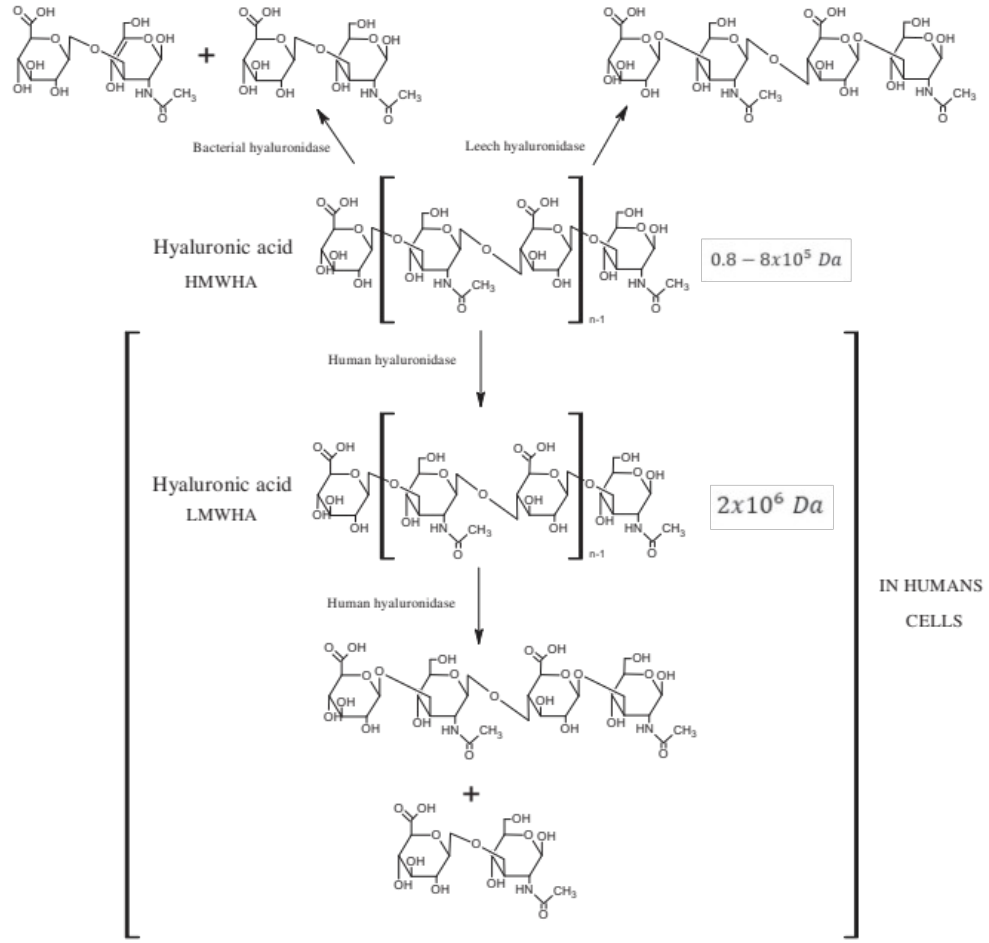


Figure 1. Classification of hyaluronidases (HMWHA: long-chain hyaluronic acid; LMWHA: short-chain hyaluronic acid).

of body fluids and reduces tissues' integrity, facilitating the penetration of microorganisms and toxins into the skin[26].

Hyals are an important component of the venom of Hymenoptera, spiders, and snakes. Hyal, present in the venom, helps to distribute the toxins throughout the body. Several studies have shown Hyal to be involved in tumour growth, metastasis, and angiogenesis[27]. However, the conducted studies do not provide unambiguous results showing that Hyals can function both as a suppressor and a promoter of carcinogenesis. HA promotes tumour metastasis, so the enzyme that breaks down hyaluronan (Hyal) inhibits their growth. On the other hand, low molecular weight HA stimulates angiogenesis, promoting tumour growth and metastasis formation[28].

4.2. Polyphenols as inhibitors of hyaluronidase: structure–activity relationships (SARS)

4.2.1. Flavonoids

Plant-based metabolites represent the chemically-different groups of compounds, of which many have been isolated for the first time from the plants used in the ethnomedicine of indigenous tribes. Those steps have allowed for their identification, biotechnological modification of their structure or to develop a synthesis path involving bacteria or fungi.

Phenolic compounds include a very numerous and important group of compounds commonly found in the world of plants. Polyphenols are plant secondary metabolites with very diverse chemical structures, containing at least two hydroxyl groups attached to an aromatic ring. Due to the number and way of connecting aromatic rings, we can distinguish phenolic acids, flavonoids, stilbenes, and lignans[29,30]. The presence of multiple hydroxyl groups gives phenolic compounds antioxidant properties[31]. Phenolic compounds also show antitumor, anti-inflammatory, antiviral, antibacterial, antifungal, hepatoprotective, antiallergic, anticoagulant, and blood vessel sealing properties[32]. Besides, many plant-derived polyphenols affect Hyal and other enzymes that regulate the metabolism of the extracellular matrix[33–36].

Inhibition of Hyal activity by polyphenolic compounds is related to, among others, the presence of hydroxyl groups (Figure 2). Hertel et al. investigated the effect of flavonoids with a different number of hydroxyl groups on Hyals' activity [flavones (apigenin and luteolin) and flavonols (kaempferol and quercetin) in concentration 0.1 mM]. In the case of bovine testicular Hyal (4 U/mL), the activity of the tested compounds was low (less than 20% for apigenin and luteolin). The inhibition of Hyal was only slightly enhanced by hydroxyl groups (quercetin or myricetin). Phenolic compounds with an additional hydroxyl group in the 3-position (quercetin ca. 90% inhibition or myricetin – ca. 70% inhibition) more strongly inhibited *Streptococcus agalactiae* hyaluronan lyase. Additionally, the introduction of a sugar moiety significantly reduces the test compounds' activity (e.g. rutin). Aglycones were more potent inhibitors than their corresponding glycosides, what may probably result from the inhibitor's difficult access to the Hyal active site in case of glycosides[37].

Another study[38,39] determined the effect of luteolin, apigenin, kaempferol, myricetin, quercetin, and morine on bovine testicular hyaluronidase (BTH). Flavonoids containing a double bond between the 2 and 3 carbon atoms showed a greater potency than flavonoids without the double bond. Additionally, a ketone group in the 4-position or hydroxyl group at 5,7, and 4′ positions may increase the inhibition of Hyal. In turn, methoxylation of the 4′-OH group in hesperitin and diosmetin reduces their activity. On the other hand, the presence of the 3-OH group did not affect the potency of Hyal inhibition. In addition, the presence of a catechol system in the B ring (3′4′-OH) may show a beneficial effect on the inhibitory activity of flavonoids. As in other studies, glycoside substituent presence completely reverses the inhibitory effect of flavonoids on BTH. The compounds possessing the malonyl group in the C-6 position of the sugar moiety, such as apigenin-7-O-(6″-O-malonyl)glucoside (IC_{50} = 360 μM) or luteolin-7-O-(6″-O-malonyl)glucoside (IC_{50} = 324 μM) show stronger inhibitory properties than compounds lacking this group, i.e. apigenin-7-O-rutinoside (IC_{50} > 1000 μM), naringenin7-O-rutinoside (IC_{50} > 1000 μM) and luteolin-7-O-glucoside (IC_{50} = 695 μM)[40]. In another study, a relationship between the position of the sugar moiety and an activity against Hyal was found [apigenin 7-O-(3″-O-acetyl)-glucuronide (200 μM, inhibition 10.0%) and apigenin 5-O-(3″-O-acetyl)-glucuronide (200 μM, inhibition 13.9%)][41].

Compounds containing a sugar residue in the C-3 position weakly block Hyal [kaempferol-3-O-α-L-rhamno pyranoside (18.26%), quercetin-3-O-α-L-rhamno pyranoside (6.88%), and quercetin-3-O-α-L-arabino pyranoside (13.81%)] (Table 2)[42].

Another possible mechanism involved in an inhibition of Hyals' activity is based on the ability to associate compounds of low molecular weight. The acid function (hydroxyl, carboxyl, phosphate, or sulphate) is necessary to form an aggregate with multiple negative charges. More research is needed to determine the possibility of aggregating by low molecular weight flavonoids that can act as effective inhibitory units.

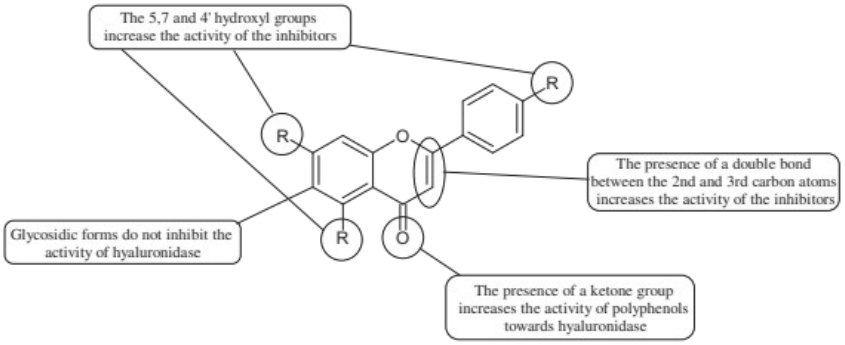


Figure 2. Chemical groups of flavonoids involved in the inhibition of hyaluronidase.

Bralley et al. determined antioxidants' influence (phenolic acids, flavonoids, and condensed tannins) in Sorghum on Hyal activity. Amongst tested compounds, i.e. condensed tannin, apigenin, luteolin, kaempferol, quercetin, and rutin, only the four first were effective. Probably condensed tannins not only denature the enzyme but also interact with the hydrophobic channel[43]. Tatemoto et al. investigated the effect of tannin, apigenin, and quercetin on Hyal activity and the fertilisation process *in vitro*. Tannins showed the greatest inhibitory properties at concentrations of 2–10 μg/mL. These data suggest that an adequate concentration of tannic acid prevents polyspermia by inhibiting sperm Hyal activity during IVF of porcine oocytes. However, the presence of apigenin or quercetin in the same concentrations as tannic acid could not prevent polyspermy[44].

The study by Zeng et al. determined how apigenin, luteolin, kempferol, quercetin, morin, naringenin, daidzein, and genistein bind to HAase. It was shown that those compounds interacted with the active centre of the enzyme through electrostatic forces, hydrophobic interactions, and hydrogen bonds. The binding of flavonoids caused changes in the active centre structure, resulting in inhibition of HAase (Table 3)[45].

4.2.2. Phenolic acids

Phenolic acids have demonstrated potent anti-Hyal properties. The impact of rosmarinic acid (IC_{50} = 24.3 μg/mL), protocatechuic acid (IC_{50} = 107.6 μg/mL), ferulic acid (IC_{50} = 396.1 μg/mL), and chlorogenic acid (IC_{50} = 162.4 μg/mL) on the activity of Hyal was noted.

Iwanaga et al. investigated the composition and effects of aqueous-acetone extracts from the aerial parts of *Cimicifuga simplex* and *Cimicifuga japonica* on HAase. The newly isolated fukiic acid derivatives (IC_{50} of compound 1. 255 μM; 2. 102 μM; 3. 173 μM; 4. 120 μM) inhibited Hyal more potently than rosmarinic acid (IC_{50} = 545 μM), caffeic acid (IC_{50} > 2000 μM), ferulic acid (IC_{50} > 2000 μM) and isoferulic acid (IC_{50} > 2000 μM). Based on the structure of compounds nr 2 and 4, the methoxy groups at the C-3′′ and C-4′′ positions may participate in the Hyal inhibition[46].

Oligomers composed of caffeic acid exhibited interesting anti-Hyal properties. Caffeic acid trimer isolated from *Dracocephalum foetidum* inhibited Hyal more strongly than disodium cromoglycate (IC_{50} = 220 μM and IC_{50} = 650 μM). Similar results were obtained by Aoshima's team studying coffee acid oligomers isolated from *Clinopodium gracile*. It was appeared that coffee acid oligomers, such as clinopodic acid M, showed more potent inhibitory activity than rosmarinic acid (IC_{50} = 19 and 226 μM, respectively). The most active compounds possessed 3–(3,4-dihydroxyphenyl)-2-hydroxypropionic acid – danshensu grouping. The activity of other compounds with the structure of danshensu amounted to: clinopodic acid E (IC_{50} = 40 μM), clinopodic acid I (IC_{50} = 112 μM), clinopodic acid K(IC_{50} = 63 μM), clinopodic acid L(IC_{50} = 26 μM), clinopodic acid N(IC_{50} = 161 μM), clinopodic acid O(IC_{50} = 66 μM), clinopodic acid P(IC_{50} = 25 μM), clinopodic acid Q(IC_{50} = 165 μM), lithospermic acid (IC_{50} = 36 μM), salvianolic acid B (IC_{50} = 107 μM), and salvianolic acid A (IC_{50} = 206 μM). The IC_{50} values for compounds without the danshensu structure were equal 206 and 653 μM for clinopodic acid J and 8-epiblechnic acid, respectively. An interesting structure for further studies may be clinopodic acid E, which has instead of the structure of 2,3-dihydrobenzofuran, 1,4-benzodioxane. This compound is four times more potent than the analogue having the structure of 2,3-dihydrobenzofuran (clinopodic acid N). Additionally, as in other studies, a beneficial effect of the increase in oligomer mass on the inhibition of Hyal activity is seen[40,47].

The structure of 1,4-benzodioxane is present in clinopodic acid C (IC_{50} = 80.1 μM) and clinopodic acid E (IC_{50} = 82.8 μM), isolated from the herbal drug takuran, which is produced from *Lycopus lucidas*. These compounds inhibited Hyal more strongly than rosmarinic acid (IC_{50} = 309 μM). It was found that an esterification of the carboxyl group associated with the structure of 1,4-benzodioxane decreased the activity of the compounds (lycopic acid A; IC_{50} = 134 μM; lycopic acid B; IC_{50} = 141 μM). Rosmarinic acid oligomers (trimer IC_{50} = 275 μM; tetramer IC_{50} = 183 μM) blocked Hyal more potent than rosmarinic acid (IC_{50} = 309 μM). Activity against hyalurinidase is also shown by seric acid A (IC_{50} = 119 μM), F (IC_{50} = 1330 μM), and G (IC_{50} = 1270 μM) isolated from the *Oenanthe javanica* root (Figures 3 and 4) (Table 4)[48].

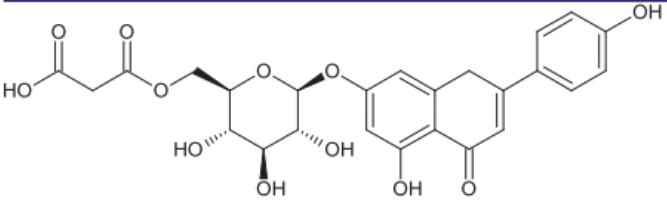
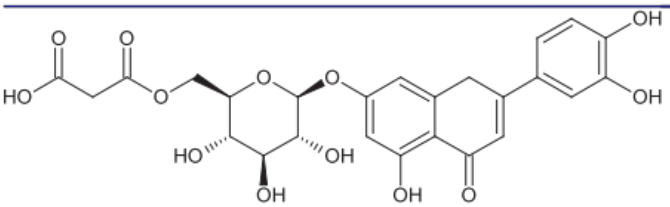
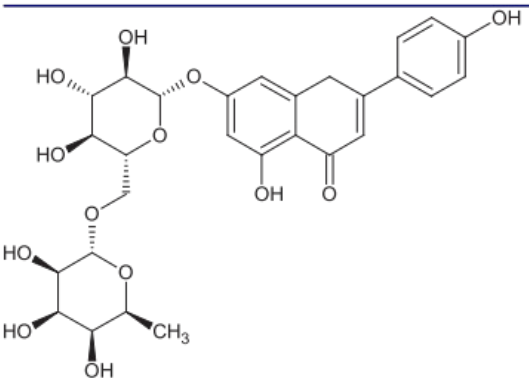
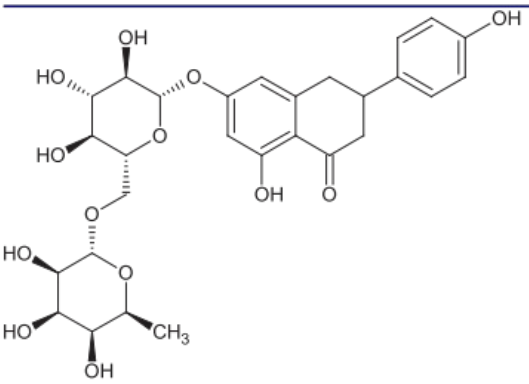
4.2.3. Tannins

Tannins are nitrogen-free plant substances of high molecular weight (500 – 3000), having numerous hydroxyl groups. Due to their chemical structure, they are divided into two groups: hydrolysing and non-hydrolysing (condensed) (Figure 5). The first group is divided into galotannins (ester combinations of gallic acid and its derivatives) or elagotannins (ester combinations of ellagic acid). The second group is formed by the condensation of catechins (flavan-3-ol products). Tannins show the ability to form complexes with proteins, resulting in an astringent effect on the skin and mucous membranes. Tannins have been shown to have many properties, such as antibacterial, antiviral, anticancer, antioxidant, anti-inflammatory, and anti-hemorrhagic[49–51].

Sugimoto et al. investigated the effect of hydrolysing tannins isolated from an ethanolic extract of *Eucalyptus globulus* Labill. on Hyal activity (400 units/mL; from bovine testis Type IV-S). The following tannins were used in the study: pedunculagin (IC_{50}= 1.51 mM), tellimagrandin I (IC_{50}= 0.9 mM), tellimagrandin II (IC_{50}= 0.58 mM), heterophylliin A (IC_{50}= 0.89 mM), 1,3-di-O-galloyl-4,6-hexahydroxydiphenoyl-β-D-glucose (IC_{50}= 0.74 mM), 1,2,4-tri-O-galloyl-β-D-glucose (IC_{50}= 1.57 mM), 1,2,3,6-tetra-O-galloyl-β-D-glucose (IC_{50}= 0.35 mM), 1,2,4,6-tetra-O-galloyl-β-D-glucose (IC_{50}= 0.68 mM), 1,2,3,4,6-penta-O-galloyl-β-D-glucose (IC_{50}= 0.55 mM), ellagic acid (IC_{50}= 4.66), gallic acid (IC_{50}= 5.0 mM), and disodium cromoglycate (IC_{50}= 0.45 mM) as a control. The activity of the tested compounds increases with the number of gallic acid residues attached to the sugar grouping (1,2,4-tri-O-galloyl-β-D-glucose – IC_{50}= 1.57 mM, 1,2,4,6-tetra-O-galloyl-β-D-glucose – IC_{50}= 0.68 mM, 1,2,3,4,6-penta-O-galloyl-β-D-glucose – IC_{50}= 0.55 mM). Also, the localisation of gallic acid residues affects the inhibitory activity (1,2,3,6-tetra-O-galloyl-β-D-glucose – IC_{50}= 0.35 mM *vs.* 1,2,4,6-tetra-O-galloyl-β-D-glucose – IC_{50}= 0.68 mM). The inhibition level was similar for both ellagotannins and gallotannins. Another gallotannin, agrimoniin (IC_{50}= 2.65 μM), also showed strong inhibition of Hyal activity[52].

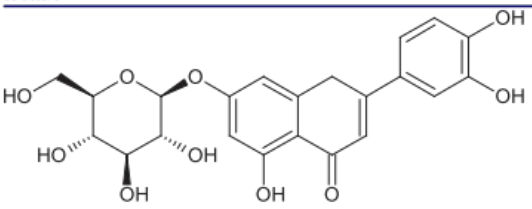
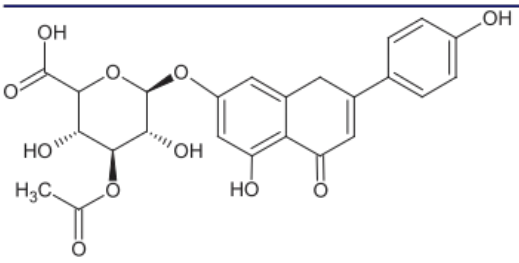
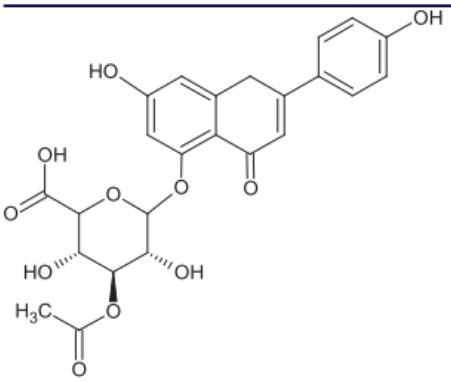
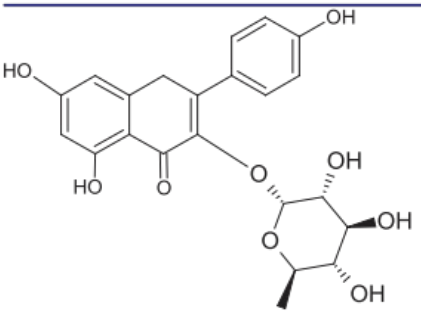
In another study[53], gallic acid esters with different n-alkanol chain lengths (from C-1 to C-12) were examined to determine their inhibitory activity against Hyal. With an increase of the alkyl chain length, the inhibitory activity was increased. Cromoglycan disodium (IC_{50}= 450 μM) was used as a positive control. Hexyl (IC_{50}= 253 μM), heptyl (IC_{50}= 112 μM), octyl (IC_{50}= 106 μM), nonyl (IC_{50}= 167 μM), and decyl (IC_{50}= 580 μM) gallates inhibited Hyal. Next, the impact of hydroxyl groups in octyl gallate on an activity was checked. Octyl 3-hydroxybenzoate and octyl 4-hydroxybenzoate did not inhibit Hyal. Octyl 3,4-dihydroxybenzoate (IC_{50}= 902 μM) blocked the enzyme to a small extent. The strongest

Table 2. Structure of flavonoid glycosides with an anti-hyaluronidase activity.

Structure	Name	IC_{50} (μM)	References
	Apigenin-7-O-(6″-O-malonyl)glucoside	360	Aoshima et al.[40]
	Luteolin-7-O-(6″-O-malonyl)glucoside	324	Aoshima et al.[40]
	Apigenin-7-O-rutinoside	>1000	Aoshima et al.[40]
	Naringenin7-O-rutinoside	>1000	Aoshima et al.[40]

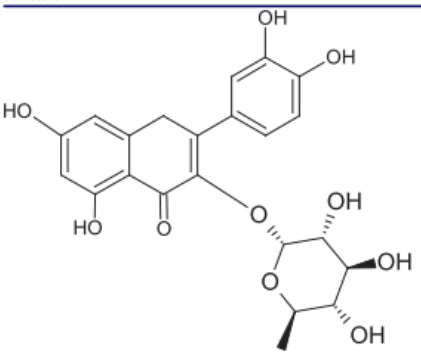
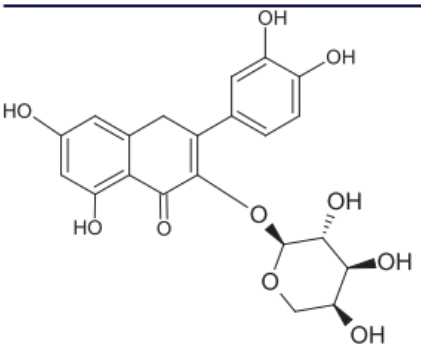
(*continued*)

Table 2. Continued.

Structure	Name	IC_{50} (μM)	References
	Luteolin-7-O-glucoside	695	Aoshima et al.[40]
	Apigenin 7-O-(3′′-O-acetyl)-glucuronide	–	Kubínová et al.[41]
	Apigenin 5-O-(3′′-O-acetyl)-glucuronide	–	Kubínová et al.[41]
	Kaempferol-3-O-α-L-rhamno pyranoside	–	Karakaya et al.[42]

(*continued*)

Table 2. Continued.

Structure	Name	IC_{50} (μM)	References
	Quercetin-3-O-α-L-rhamno pyranoside	–	Karakaya et al.[42]
	Quercetin-3-O-α-L-arabino pyranoside	–	Karakaya et al.[42]

inhibitor was octyl 3,5-dihydroxybenzoate (IC_{50}= 113 μM). This shows the significance of the 3,5-OH grouping in gallic acid (Table 5). The type of inhibition was determined only for octyl gallate, which inhibited the enzyme in a competitive manner. The K_i value was estimated to be 45 μM (Figure 6).

Tokeshi et al. examined the effect of three tannins (tannic acid TA, gallic acid GA, and ellagic acid EA) on boar sperm Hyal. TA and EA strongly inhibited Hyal in the concentration range of 2–10 μM[54]. The phlorotannins present in *Eisenia bicyclis* and *Ecklonia kurome* inhibited Hyal activity more strongly than standard substances such as disodium cromoglycate (IC_{50} = 270 μM), catechin (IC_{50} = 620 μM), and epigallocatechin gallate (IC_{50} = 190 μM). The IC_{50} values for phloroglucinol, phloroglucinol tetramer, eckol (trimer), phlorofucofuroeckol A (pentamer), dieckol, and 8,8′-bieckol (hexamers) were at the level of 280, 650, >800, 140, 120, and 40 μM, respectively. In the case of phlorofucofuroeckol A, dieckol, and 8,8′-bieckol an inhibition type (competitive inhibition) and the inhibition constant (K_i) values (130, 115, and 35 μM, respectively) were also established. Additionally, it was confirmed that the higher molecular weight of inhibitor the stronger inhibition is observed. This is probably associated with a stronger effect on the dimensional structure of the enzyme[55].

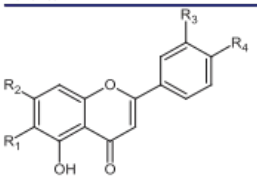
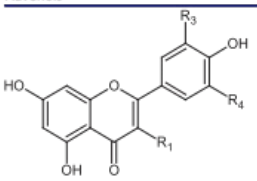
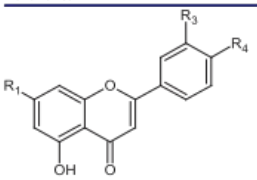
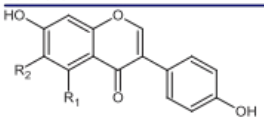
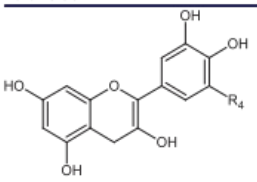
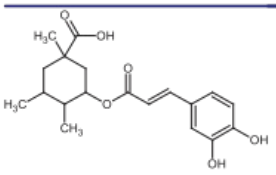
Procyanidin B1, procyanidin B3, epicatechin, and catechin exhibited a comparable inhibitory activity to disodium cromoglycate (51.1%) at the concentration of 250 μM (50.1, 28.9, 45.9, and 29.9%, respectively). At the concentration of 125 μM, epicatechin (30.3%), and procyanidin B1 (33.5%) showed a higher activity than DSCG (22.9%).

4.3. Non-polyphenols as inhibitors of hyaluronidase: structure–activity relationships (SARS)

4.3.1. Alkaloids

Natural alkaline nitrogen compounds are synthesised by plants, fungi, bacteria, and animals. Alkaloids, in minimal doses, have a strong physiological effect, especially on the central nervous system. Moreover, these compounds have antitumor, anaesthetic, antifungal, antibacterial, anti-inflammatory, and analgesic properties. Due to the heterocyclic ring system, we distinguish derivatives: pyridine and piperidine, tropane, quinoline, quinoline, indole, ergot, and purine[56]. A study by Girish et al. determined the effect of aristolochic acid on the activity of purified Indian cobra venom hyaluronidase (NNH1) and the activity of whole venom Hyal. The tested compound inhibited NNH1 non-competitively. Besides, the venom's administration with aristolochic acid to mice showed more than a twofold increase in survival time compared to mice injected with the venom alone. Lower survival was obtained by splitting the application of the inhibitor over time (10 min). Aristolochic acid did not bind to the enzyme's

Table 3. Structures of the active compounds.

		Substituent			
Flavones	Compounds	R_1	R_2	R_3	R_4
	Apigenin	H	OH	H	OH
	Luteolin	H	OH	OH	OH
	Baicalein	OH	OH	H	H
	Baicalin	OH	Glucuronide	H	H
Flavonols					
	Kaempferol	OH		H	H
	Quercetin	OH		OH	H
	Myricetin	OH		OH	OH
	Rutin	Rutinose		H	OH
Flavanones					
	Naringenin	OH		H	OH
	Hesperetin	OH		OH	OCH_3
	Naringin	Neohesperidose		H	OH
	Hesperidin	Rutinoside		OH	OCH_3
Isoflavone					
	Genistein	OH	H		
Flavan-3-ols					
	Catechin				H
	Chlorogenic acid				

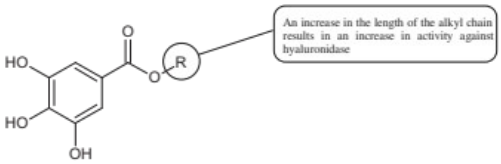


Figure 3. Effect of alkyl chain length in phenolic acids on activity against hyaluronidase.

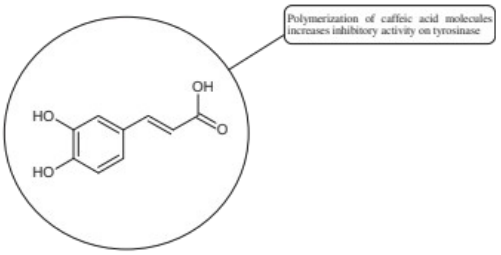


Figure 4. Potential groups engaged in an interaction oligomers phenolic acids-hyaluronidase.

active site but interacted with exposed tyrosine and tryptophan HAase residues. Aristolochic acid (50, 100, and 200 μM) inhibited NNH1 at the level of 100% for each concentration. Other alkaloids, ajmaline, and reserpine inhibited Hyal weaker than aristolochic acid (ajmaline 11, 26, 40%; reserpine 9, 23, 31%, respectively) (Table 3)[57].

Another study[58] determined the effect of alkaloids isolated from the methanolic extract of *Nelumbo nucifera* Gaertn. flowers harvested at different stages of bloom (beginning of bloom, one-third in bloom, half in bloom, three-quarters in bloom, and full bloom). Samples flowering at half (52.69 mg per dried flower) had the highest alkaloid content (Figure 7). Among the alkaloids, nornuciferin (IC_{50}= 22.5 μM), asymilobin (IC_{50}= 11.7 μM), norarmepavin (IC_{50} = 26.4 μM), coclaurin (IC_{50}= 11.4 μM), and norjuzyfin (IC_{50}= 24.3 μM) inhibited Hyal. The activity of alkaloids was more potent than that of the anti-allergic drug disodium cromoglycate (IC_{50}= 64.8 μM). The N-methyl group reduces the ability of alkaloids to inhibit Hyal, e.g. nuciferine IC_{50} > 100 μM < nornuciferine IC_{50}= 22. μM or asimilobine IC_{50}= 11.7 μM > N-methylasimilobine. On the other hand, demethylation of the hydroxyl groups increases their activity (asimilobine IC_{50}= 11.7 μM > nornuciferine IC_{50}= 22.5 μM). The observed structural relationships apply to both benzylisoquinoline alkaloids and apomorphine alkaloids (Tables 6 and 7).

4.3.2. L-ascorbic acid

Ascorbic acid (vitamin C – AA) is a compound commonly found in the world of plants and animals. Human is incapable of synthesising vitamin C, therefore, it must be supplied in the diet (parsley, red pepper, black currant, and Brussels sprouts). The ability of vitamin C to create an oxidative system (AA <=> AA <=> dehydroascorbic acid) determines its antioxidant properties. AA is the most important antioxidant of extracellular fluids in the human body. It is present in high concentrations in the eyeball and lymphocytes, protecting cells against reactive forms of oxygen and nitrogen. Besides its antioxidant activity, AA is involved in the absorption of non-heme iron; in the metabolism of fats, cholesterol, and bile; regeneration of vitamin E in the cell membrane; in the synthesis of collagen, accelerating the wound healing process. Additionally, the presence of AA in the skin may constitute a defense mechanism against an invasion of pathogenic bacteria. Despite the fact that AA is one of the most known biologically-active compound its anti-Hyal activity and SAR are still unknown in details. Several studies involving AA and AA derivatives have found to be able to inhibit Hyals[59–61].

In 2001, Li et al. first described a competitive type of AA inhibition on Hyal isolated from *Streptococcus pneumoniae* LHyal (hyaluronan lyase). The activity of AA towards Hyal is due to the structural similarity of vitamin C to glucuronic acid, being one of the basic building blocks of hyaluronan (HA) (β-1,4-glucuron-β-1,3-glucosamine). It was found that one AA molecule may bind to the enzyme's active site. The AA carboxyl group provides a negative charge that directs the molecule to the positively charged enzyme gap. In the active centre of the enzyme, AA interacts with amino acids through hydrophobic (Trp-292), ionic (Arg-243, Arg-462, and Arg-466), and hydrogen (Tyr-408, Asn-290, and Asn-580) bonds[62].

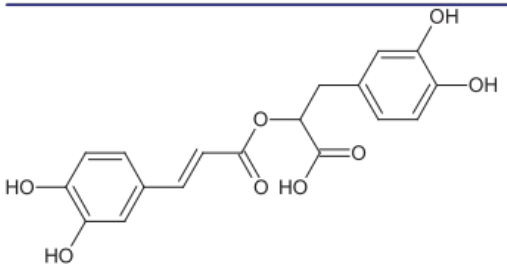
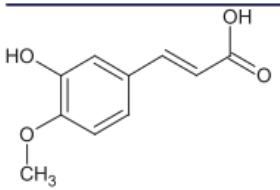
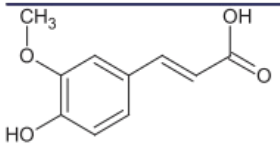
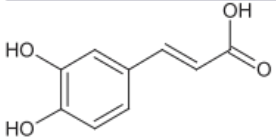
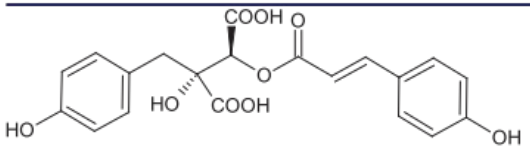
In 2003, Okorukwu et al. confirmed the inhibitory effect of AA and AA analogs on the activity of bovine testicular Hyal (BTH – final concentration 3.5 Units/mL) and LHyal (*Streptococcus zooepidemicus* – final concentration 2.5 Units/mL). Gel permeation chromatography (GPC) was used in this study to evaluate the inhibitory activity. The AA and AA derivatives (D-iso-ascorbic acid and dehydroascorbic acid) blocked the hyaluronan lyase more strongly than BTH. D-Saccharic-1,4-lactone and saccharic acid inhibited LHyal without affecting the enzymatic activity of testicular Hyal. The introduction of a carboxyl group that gives the molecule a negative charge positively affects the inhibitory effect of AA derivatives. Hydrogenation of the double bond between the 2nd and 3rd carbon atoms decreases the activity of the compounds. Saccharic acid can be used to develop selective inhibitors of bacterial hyaluronan lyase (Table 8)[63] .

In a study conducted by Botzki et al., a positive correlation was confirmed between the inhibition of Hyal activity and the increased hydrophobic interactions. L-Ascorbyl palmitate, through an increase in hydrophobic interactions with Phe343, His399, and Thr400 in the active centre, led to increased inhibition of hyaluronan lyase (competitive inhibition). A similar effect was achieved with BTH. The long alkyl chain interacts with a hydrophobic channel formed primarily by the amino acids Ala-84, Leu-91, Tyr-93, Tyr-220, and Leu-344[64].

The new LHyal inhibitors should have a larger ring system to favourably influence the hydrophobic bonding to the Trp-292 indole group and contain at least one negative charge group (carboxyl group), which brings the inhibitor to the cleft region (rich in positively charged arginine).

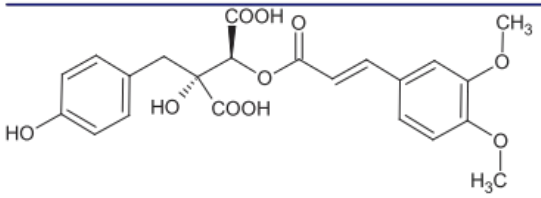
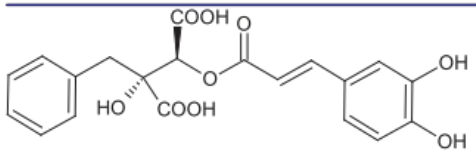
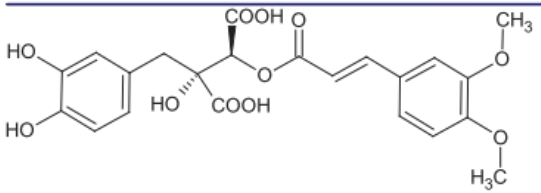
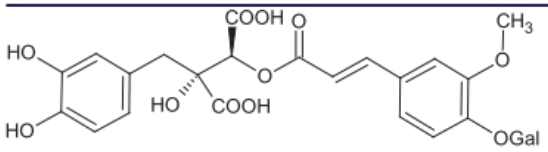
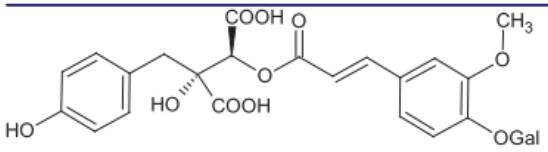
Spickenreither et al. examined the effect of 6-O-acylated AA derivatives on the Hyal activity of BTH and *Streptococcus agalactiae* strain 4755 (Sag Hyal 4755). All compounds showed more potent activity against bacterial lyase. Methylation of the endiol system reduces the activity of vitamin C analogs. On the other hand, 2 and 3 dibenzylated derivatives showed more potent inhibitory properties than AA. The increase in potency is due to additional hydrophobic interactions between the rings and the active centre. An increase in the length of the 6-O-acyl residue (13 b-j) results in increased inhibitory activity. The IC_{50} for octadecanoate was 0.9 and 39 μM for BTH and Sag Hyal 4755, respectively. Shortening of the aliphatic chain and adding phenyl, p-phenylene, or p-biphenyl groups leads to compounds with comparable inhibitory properties. Additionally, ether bonds in the synthesis of new inhibitors positively influence their activity. This

Table 4. Structure and activity of phenolic acids against hyaluronidase.

Structure	Name	IC_{50}(μM)	References
	Rosmarinic acid	545	Iwanaga et al.[46]
	Isoferulic acid	>2000	Iwanaga et al.[46]
	Ferulic acid	>2000	Iwanaga et al.[46]
	Caffeic acid	>2000	Iwanaga et al.[46]
	Cimicifugic acid K	255	Iwanaga et al.[46]

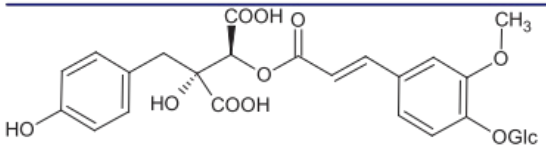
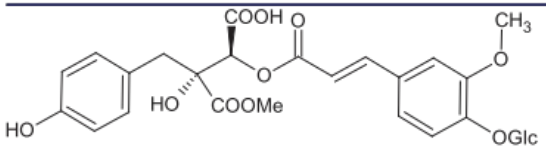
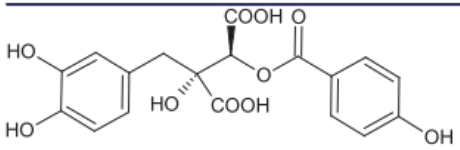
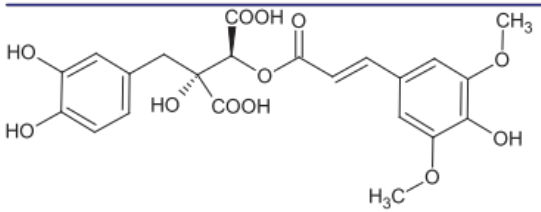
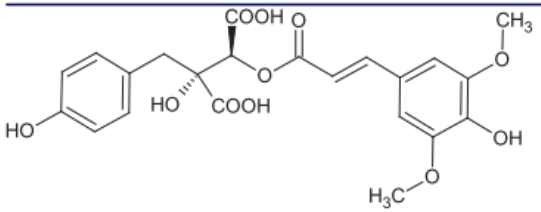
(*continued*)

Table 4. Continued.

Structure	Name	IC_{50}(μM)	References
	Cimicifugic acid L	102	Iwanaga et al.[46]
	Cimicifugic acid M	173	Iwanaga et al.[46]
	Cimicifugic acid N	120	Iwanaga et al.[46]
	Shomaside A	573	Iwanaga et al.[47]
	Shomaside B	430	Iwanaga et al.[47]

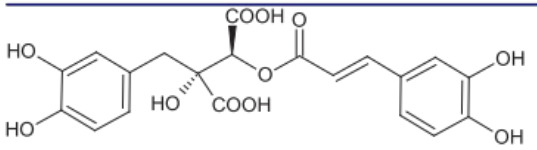
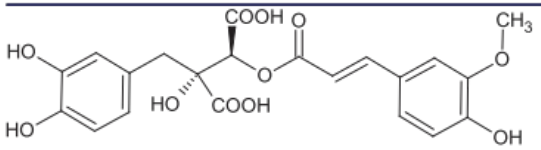
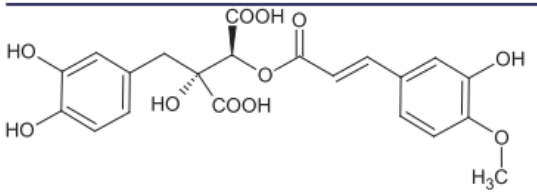
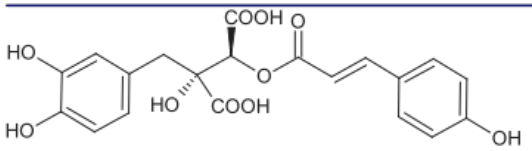
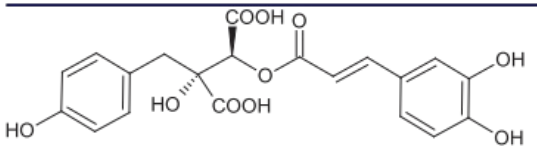
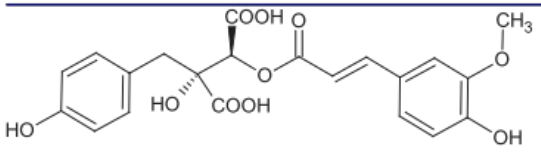
(continued)

Table 4. Continued.

Structure	Name	IC_{50}(μM)	References
	Shomaside C	663	Iwanaga et al.[47]
	Shomaside D	>658	Iwanaga et al.[47]
	Cimicifugic acid H	525	Iwanaga et al.[47]
	Cimicifugic acid I	143	Iwanaga et al.[47]
	Cimicifugic acid J	193	Iwanaga et al.[47]

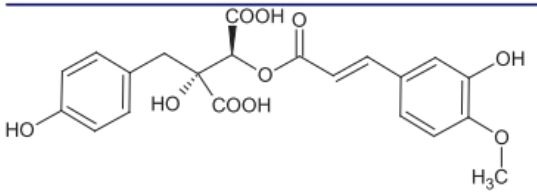
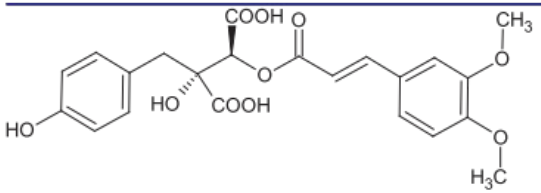
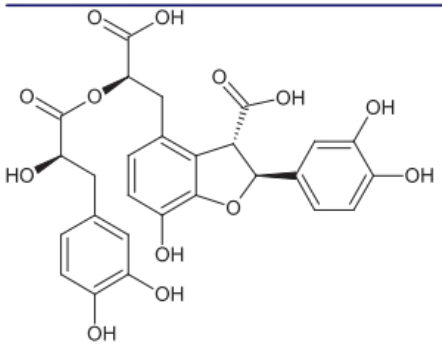
(*continued*)

Table 4. Continued.

Structure	Name	IC_{50}(μM)	References
	Fukinolic acid	144	Iwanaga et al.[47]
	Cimicifugic acid A	112	Iwanaga et al.[47]
	Cimicifugic acid B	82	Iwanaga et al.[47]
	Cimicifugic acid C	251	Iwanaga et al.[47]
	Cimicifugic acid D	153	Iwanaga et al.[47]
	Cimicifugic acid E	120	Iwanaga et al.[47]

(continued)

Table 4. Continued.

Structure	Name	IC_{50}(μM)	References
	Cimicifugic acid F	92	Iwanaga et al.[47]
	Cimicifugic acid G	138	Iwanaga et al.[47]
	Clinopodic J	206	Aoshima et al.[40]

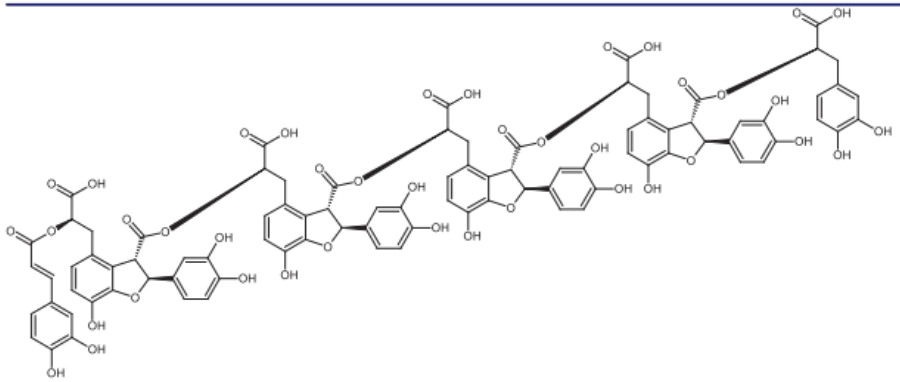
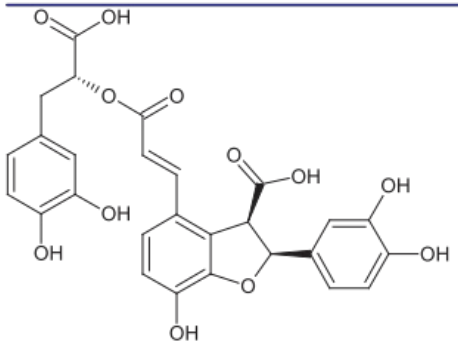
(*continued*)

Table 4. Continued.

Structure	Name	IC_{50}(μM)	References
	Clinopodic K	63	Aoshima et al.[40]
	Clinopodic L	26	Aoshima et al.[40]

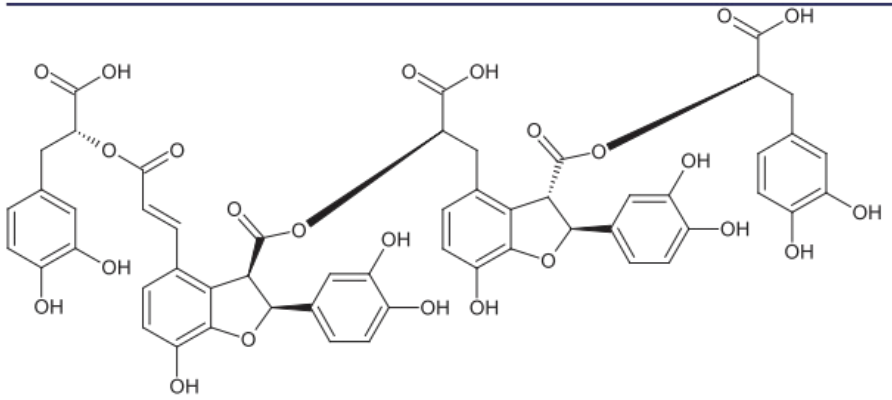
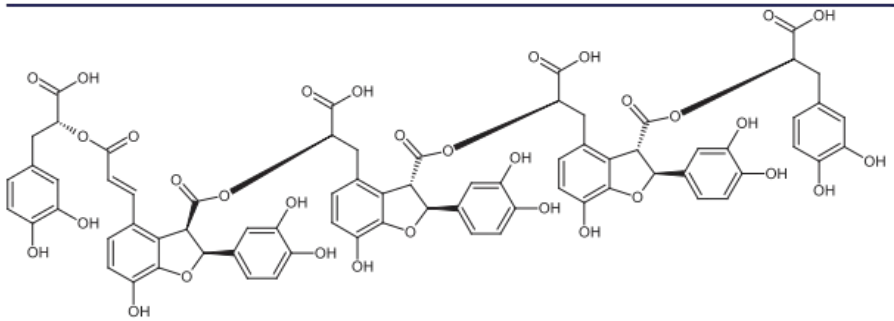
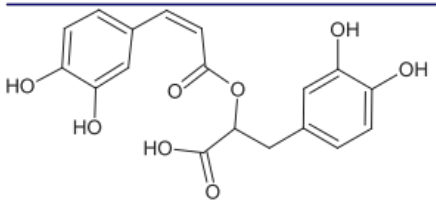
(*continued*)

Table 4. Continued.

Structure	Name	IC_{50}(μM)	References
	Clinopodic M	19	Aoshima et al.[40]
	Clinopodic N	161	Aoshima et al.[40]

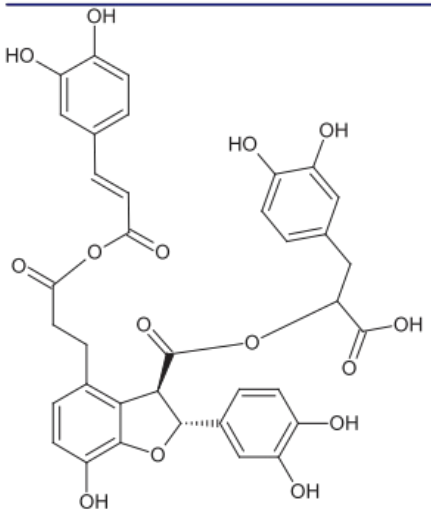
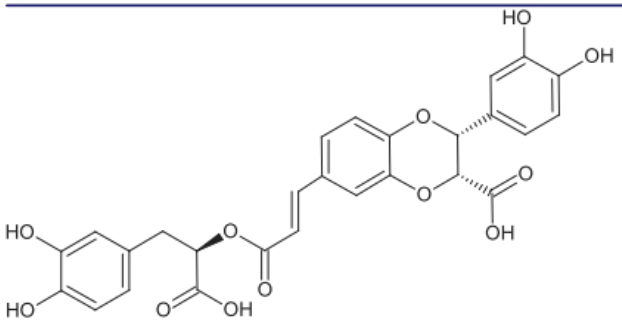
(*continued*)

Table 4. Continued.

Structure	Name	IC_{50}(μM)	References
	Clinopodic O	66	Aoshima et al.[40]
	Clinopodic P	25	Aoshima et al.[40]
	Rosmarinic acid		Aoshima et al.[40]

(*continued*)

Table 4. Continued.

Structure	Name	IC_{50}(μM)	References
	Clinopodic acid I	112	Aoshima et al.[40]
	Clinopodic acid E	40	Aoshima et al.[40]

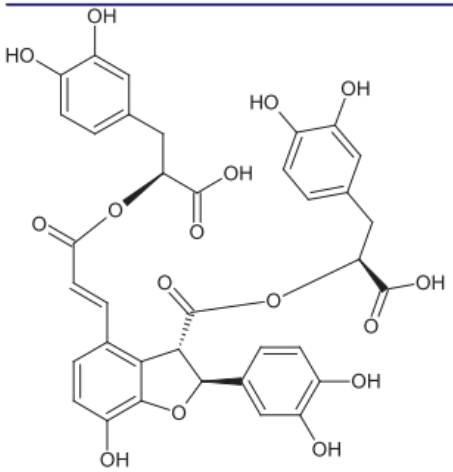
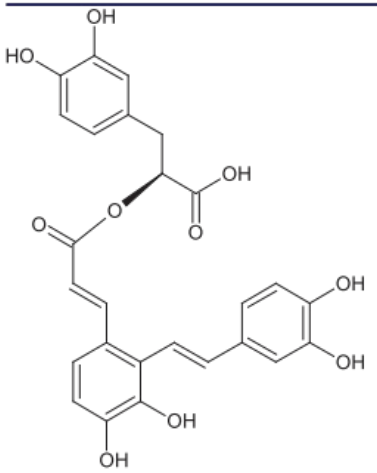
(*continued*)

Table 4. Continued.

Structure	Name	IC_{50}(μM)	References
	8-Epiblechnic acid	653	Aoshima et al.[40]
	Lithospermic acid	36	Aoshima et al.[40]

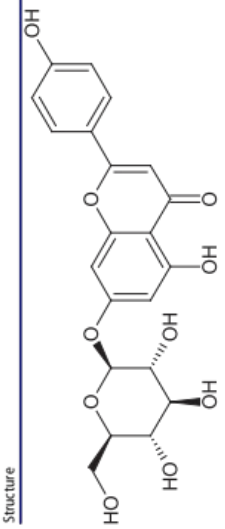
(continued)

Table 4. Continued.

Structure	Name	IC_{50}(μM)	References
	Salvianolic acid B	107	Aoshima et al.[40]
	Salvianolic acid A	206	Aoshima et al.[40]

(*continued*)

Table 4. Continued.

Structure	Name	IC_{50}(μM)	References
	Cosmosiin	>1000	Aoshima et al.[47]

is associated with the formation of additional hydrogen bonds in the active centre (Figure 8; Table 9)[65].

4.3.3. Glycosides

Glycosides are a group of the organic compounds consisting of sugar and an aglycone part. The bond between the sugar and the aglycone is called a glycosidic bond. The chemical nature of aglycones is very different, they can be alcohols, lactones, phenolic acids, thiols, etc. The sugar portion may consist of 1–12 monosaccharide, disaccharide, or oligosaccharide molecules. The glycosides widely occur in the plant world, especially in higher plants. Because of the chemical diversity of glycoside, the plant-based sources of glycosides are important in phytotherapy[66].

4.3.3.1. Cyanogenic glycosides. Cyanogenic glycosides are one of the glycosides groups that have an inhibitory effect on Hyal. In a study by Tanyildizi et al., it was checked how different doses of linamarine and amygdalin affect Hyal activity, motility, and morphology of bull sperm obtained from the Holstein bulls aged 2–3 years (Table 10). The samples were divided into 5 equal parts and mixed with linamarine at doses of 0.5, 0.75, 1, and 2 μM and with amygdalin at doses of 0.4, 0.8, 1, and 2 μM. Incubation of compounds with sperm resulted in a significant reduction (dose-dependent) of sperm motility and Hyal activity compared to the control group (isotonic saline solution). Both linamarine and amygdalin did not change sperm morphology. The authors concluded that the fertilisation ability of bull sperm could be inhibited by the over-consumption of plants rich in cyanogenic glycosides[67].

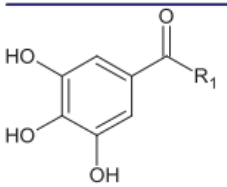
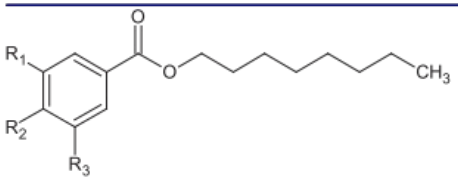
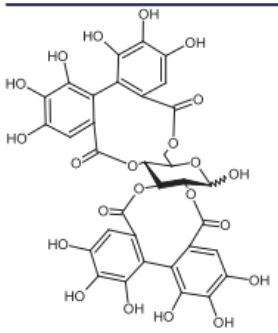
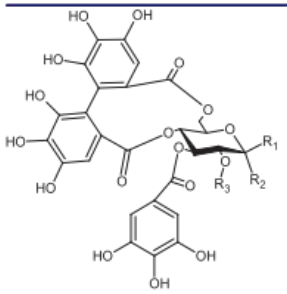
4.3.4. Terpene/terpenoids

Terpenes are composed of a varying number of isoprene units (Figure 9). They are commonly found in the plant world in hydrocarbons or oxidised forms (with hydroxyl, carbonyl, or carboxyl groups). Depending on the number of isoprene residues, we distinguish monoterpenes (C10), diterpenes (C-20), sesquiterpenes (C-15), triterpenes (C-20), and tetraterpenes (C-40). Due to their highly diverse chemical structure, terpenes exhibit a variety of biological activities, such as antibacterial, antiviral, anticancer, anti-inflammatory, and sedative effects[68].

4.3.4.1. Monoterpene/monoterpenoids. Morikawa et al. investigated the effect of methanolic extract of rhizome of *Picrorhiza kurroa* Royle ex Benth. on Hyal activity (Type IV-S from bovine testes). Seven new acylated iridoid glycosides (picrorhizaosides A–G) and six known iridoid glycosides were isolated from the extract. Among the isolates, picrorhizaosides D (IC_{50}= 43.4 μM), picrorhizaosides E (IC_{50}= 35.8 μM), picrosides I (IC_{50}= 60.7 μM), picrosides II (IC_{50}= 22.3 μM), picrosides IV (IC_{50}= 59.2 μM), and minecoside (IC_{50}= 57.2 μM), showed similar or stronger Hyal inhibitory effects than the anti-allergic drugs disodium cromoglycate (IC_{50}= 64.8 μM), ketotifen fumarate (IC_{50}= 76.5 μM), and tranilast (IC_{50}= 227 μM), but weaker than the alkaloids isolated from Nelumbo nucifera Gaertn., such as asimilobin (IC_{50}= 11.7 μM) and coclaurin (IC_{50}= 11.4 μM)[69].

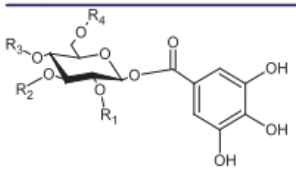
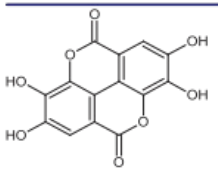
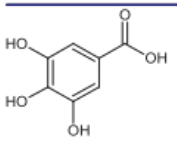
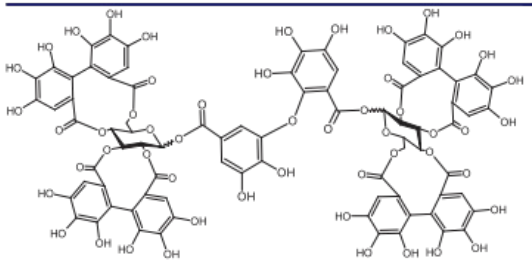
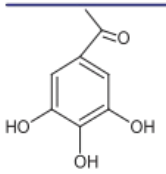
4.3.4.2. Saponin. Saponins belong to the glycosides group composed of aglycone – sapogenin (sapogenol) and glycone-sugar. Depending on the type of sapogenin we distinguish triterpene saponins, steroidal saponins, and steroidal alkaloids (Figure 9). These compounds reduce the surface tension of water solutions.

Table 5. Structures and activity of tannins and their esters with n-alkanol chain lengths against hyaluronidase.

Structure	Compound	R_1	R_2	R_3	R_4	IC_{50} (μM)	References
	Gallic acid	H				>1000	Barla et al.[53]
	Methyl gallate	OCH_3				>1000	
	Ethyl gallate	OCH_2CH_3				>1000	
	Propyl gallate	$O(CH_2)_2CH_3$				>1000	
	Butyl gallate	$O(CH_2)_3CH_3$				>1000	
	Hexyl gallate	$O(CH_2)_5CH_3$				253	
	Heptyl gallate	$O(CH_2)_6CH_3$				112	
	Octyl gallate	$O(CH_2)_7CH_3$				106	
	Nonyl gallate	$O(CH_2)_8CH_3$				167	
	Decyl gallate	$O(CH_2)_9CH_3$				580	
	Dodecyl gallate	$O(CH_2)_{11}CH_3$				>1000	
	Octyl 3-hydroxybenzoate	OH	H	H		>1000	
	Octyl 4-hydroxybenzoate	H	OH	H		>1000	
	Octyl 3,4-dihydroxybenzoate	OH	OH	H		902	
	Octyl 3,5-dihydroxybenzoate	OH	H	OH		113	
	Pedunculagin					1.51	Tokeshi et al.[54]
	Tellimagrandin I	H	OH	G		0.9	Sugimoto et al.[52]
	Tellimagrandin II	OG	H	G		0.58	
	Heterophylliin A	H	OG	H		0.89	
	1,3-Di-O-galloyl-4,6-hexahydroxydiphenoyl-β-D-glucose	OG	H	H		0.74	

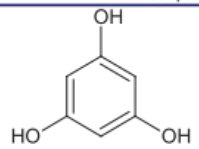
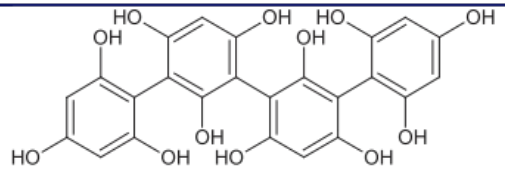
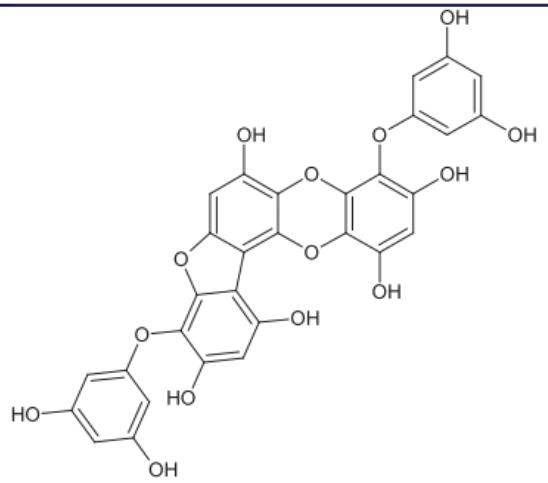
(*continued*)

Table 5. Continued.

Structure	Compound	R_1	R_2	R_3	R_4	IC_{50} (μM)	References
	1,2,4-Tri-O-galloyl-β-D-glucose	G	H	G	H	1.57	
	1,2,3,6-Tetra-O-galloyl-β-D-glucose	G	G	H	G	0.35	
	1,2,4,6-Tetra-O-galloyl-β-D-glucose	G	H	G	G	0.68	
	1,2,3,4,6-Penta-O-galloyl-β-D-glucose	G	G	G	G	0.55	
	Ellagic acid					4.66	
	Gallic acid					5.00	
	Agrimoniin					2.65	
	Galloyl group (G)						

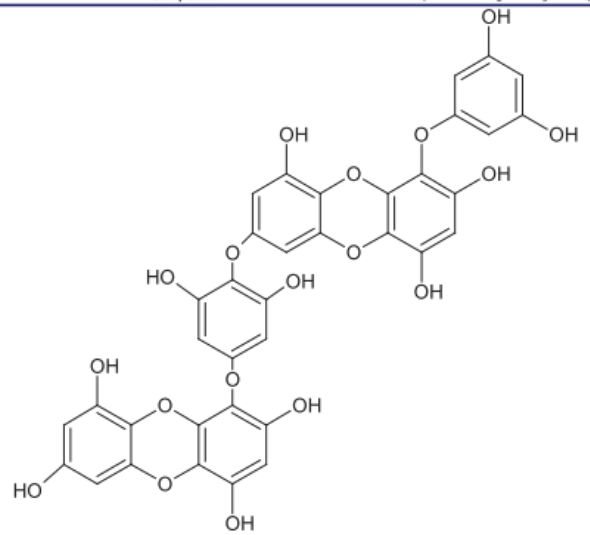
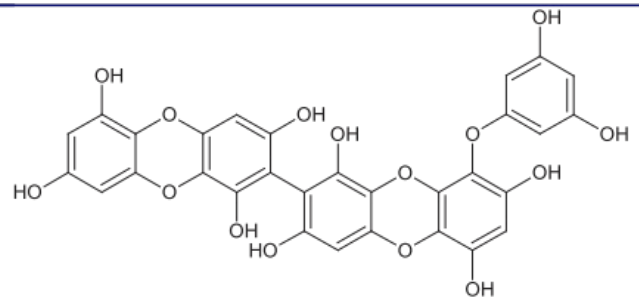
(*continued*)

Table 5. Continued.

Structure	Compound	R_1	R_2	R_3	R_4	IC_{50} (μM)	References
Phloroglucinol							
Phloroglucinol tetramer							
Phlorofucofuroeckol A							

(*continued*)

Table 5. Continued.

Structure	Compound	R_1	R_2	R_3	R_4	IC_{50} (μM)	References
Dieckol							
8,8′-Bieckol							

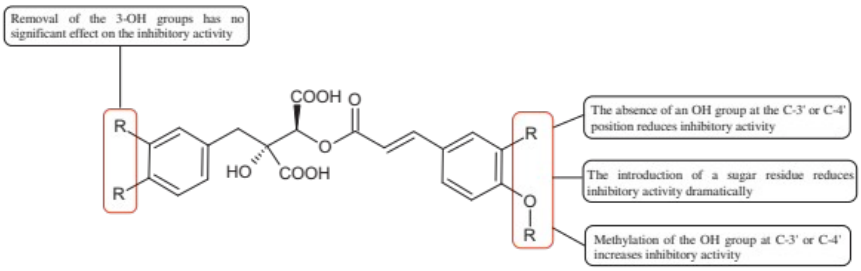


Figure 5. Potential groups engaged in an interaction fukiic acid derivative-hyaluronidase.

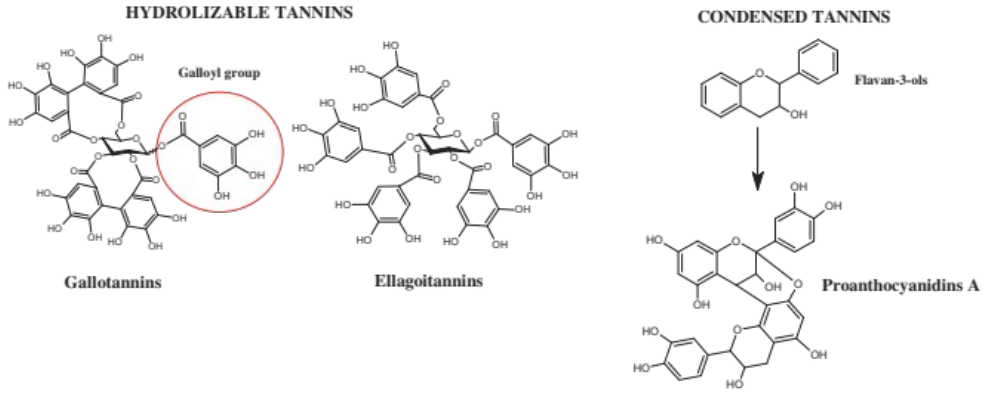


Figure 6. Chemical classification of tannins.

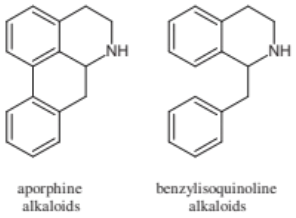


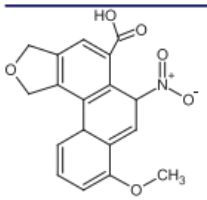
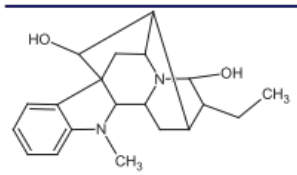
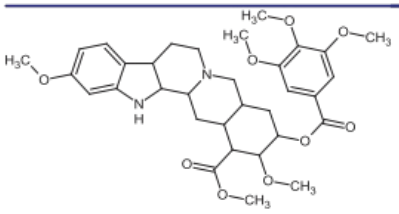
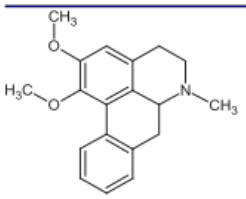
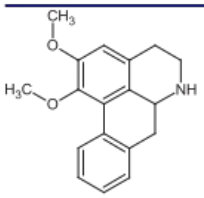
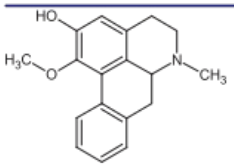
Figure 7. Structure of benzylisoquinoline alkaloids and apomorphine alkaloids.

They show anti-inflammatory, antibacterial, protozoal, antifungal, and antiviral activity, stimulate secretion of gastric juice, bile, and intestinal juice. They can affect cholesterol level[70].

In a study by Zhou et al., they determined the effects of esculeoside A and its aglycone esculeogenin A on Hyal activity *in vitro* and in mice dermatitis model. Esculeoside A, a spirosolate-type glycoside, is identified as a significant component of ripe tomato fruit. The IC_{50} for esculeogenin A and esculeoside A was about 2 and 9 μM, respectively. Administration of esculeoside A at a dose of 10 mg/kg for four weeks to mice with dermatitis significantly reduced diseases symptoms[71]. Esculeoside A is a competitive inhibitor of hyalurnidase (K_i = 11.0 μM)[72]. Also, the other steroid alkaloids present in tomato juice showed beneficial inhibitory effects against Hyal. Administration of 10 mg/kg esculeoside B to mice with dermatitis for four weeks significantly reduced skin inflammation. In addition, it was found that esculeoside B administration significantly inhibited T-lymphocyte proliferation and decreased IL-4 production[73].

More information about the effect of saponin structure on Hyal activity was provided by QSAR studies of ursolic and oleanolic acids, the results of which exhibited the higher activity of ursolic acid than oleanolic acid. In that experiment, the effect of the position of methyl groups at 29 and 30 carbon atoms was checked. Both geminal and vicinal positions had no significant impact on an inhibitor activity. For oleanolic acid, the activity increased when the methyl group was introduced at C-17 or C-16 and decreased when the methoxyl group was introduced at C-23. The 3-OH acetylation reduced the activity of the compounds. Carboxylation of C-30 increased the activity of the compounds, while esterification of the same carbon (C-30). In addition, the introduction of a sugar moiety into the 3-OH decreased their activity. In the case of ursolic acid, the activity decreased when the hydroxyl group was modified at C-3 (3-oxo, 3-hydroxyimino, and 3-acetylate derivatives) and at C-28. Replacement of the methyl group at C-23 with a carboxyl or hydroxy methylene group decreased the activity of the compounds. As in the case of oleanolic acid, the introduction of a sugar group caused a decrease in the inhibitory activity (Figure 10; Table 11)[74].

Table 6. Structures of the active alkaloids towards hyaluronidase.

Structure	Compound
	Aristolochic acid
	Ajmaline
	Rezerpine
	Nuciferine
	Nornuciferine
	N-methylasimilobine

(*continued*)

Table 6. Continued.

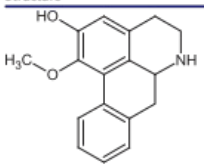
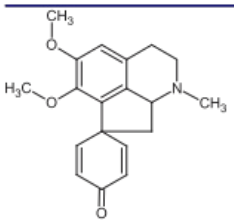
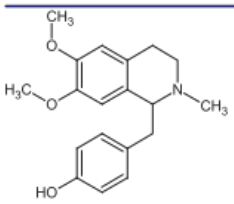
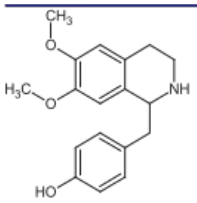
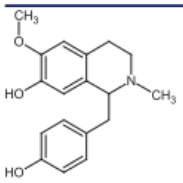
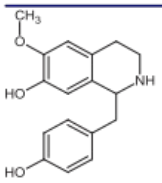
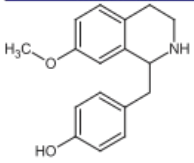
Structure	Compound
	Asimilobine
	Pronuciferine
	Armepavine
	Norarmepavine
	N-methylcoclaurine
	Coclaurine
	Norjuziphine

Table 7. Activity of benzylisoquinoline alkaloids and apomorphine alkaloids against hyaluronidase.

Compound	The half-maximal inhibitory concentration IC_{50} (μM)	Type of enzyme	Unit of enzyme activity (unit/mL)	References
Nuciferine	>100 μM	Type IV-S from bovine testes	340	Morikawa et al.[58]
Nornuciferine	22.5			
N-methylasimilobine	–			
Asimilobine	11.7			
Pronuciferine	–			
Armepavine	>100 μM			
Norarmepavine	26.4			
N-methylcoclaurine	>100 μM			
Coclaurine	11.4			
Norjuziphine	24.3			

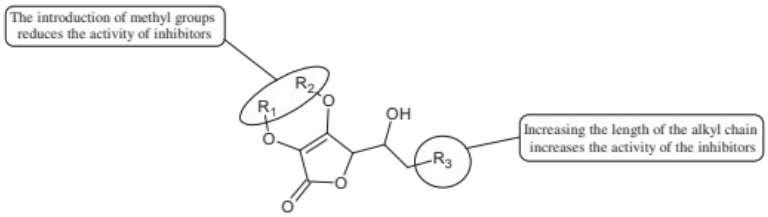


Figure 8. Chemical groups of L-ascorbic acid involved in the inhibition of hyaluronidase.

Similar results were obtained in another experiment aimed at an investigation of the impact of the oleanic acid structure modification on anti-Hyal activity. Oxidation of the 3-OH group in oleanic acid led to a decrease of the activity about 4-fold (59.3–15.9%). A similar effect was obtained by esterification of the COOH group (12.7% inhibition). The activity of the compounds was evaluated at a concentration of 40 μg/mL[75].

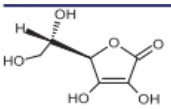
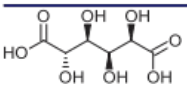
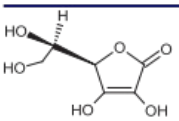
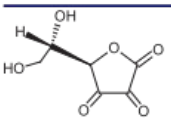
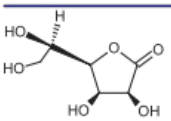
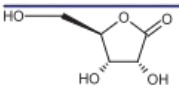
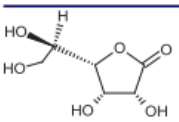
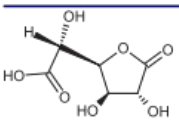
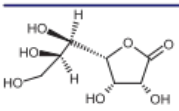
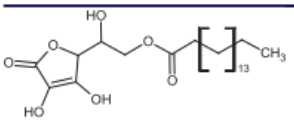
In another study[76], oleanane-type saponins, isoflavonoids, oxazoles, and glycosides (36 compounds) were isolated from the aerial part of *Oxytropis lanata* (Pall.) DC. The effect of saponins against Hyal (IC_{50}= 0.15–0.22 mM) was more potent than sodium cromoglycate, which was used as a positive control (IC_{50}= 0.37 mM). On the basis of the structure analysis, it was appeared that all isolated saponins contain a 3-O-β-D-glucuronopyranoside grouping in the sugar moiety, which shows a potent inhibition of Hyal, as well as the compounds with a ketone group at C-22 more strongly blocked the enzyme (Figure 11). Similar results were obtained when testing triterpene saponosides from the 3-O-β-D-glucuronopyranoside group, which blocked Hyal more strongly (2; IC_{50}= 1.25 mM, 3: IC_{50}= 0.68 mM, and 9: IC_{50}= 0.82 mM) than rosmarinic acid used as a control (IC_{50}= 1.36 mM)[77]. The significance of the 3-O-β-D-glucuronopyranoside grouping was also noted by examining the triterpene saponosides camelliagenin A (IPS-1 and IPS-2) isolated from the methanolic root extract of Impatiens parviflora DC. A very interesting result has been obtained, because IPS-2 (IC_{50}= 286.7 μg/mL) inhibited BTH Hyal activity more strongly than escin (IC_{50}= 303.93 μg/mL). What is interesting, escin is recommended to be used as an anti-Hyal reference compound. The higher IC_{50} value was obtained for IPS-1; 368.1 μg/mL. Considering the structure of these compounds, a clear difference may be noticed significantly since the tested saponosides differed in their acetylation at C-16. The 16-O-acetylcameliagenin A derivative showed less activity. Therefore, the free OH group at position 16 may have a beneficial effect on Hyal inhibition, e.g. *via* participation in ions chelating in the reaction's medium[78].

Myose et al. demonstrated the effect of triterpene saponins isolated from methanolic extract of *Camellia sinensis* (L.) Kuntze seeds on Hyal activity. The isolated new saponin (Teaseedsaponin A-L) blocked Hyal more potently (IC_{50}= 19.3–55.6 μM) than rosmarinic acid (IC_{50}= 240.1 μM)[79].

Another study examined how sugar moiety in saponins affects the activity of Hyals isolated from different bacterial species (*Streptococcus agalactiae* – Hyal B, *Streptomyces hyalurolyticus* – Hyal S, *Streptococcus equisimilis* – Hyal C) and bovine testes (BTH). Glycyrrhizin (1) and its aglycone glycyrrhetinic acid (2) were used as inhibitors. The tested compounds most potently blocked the activity of Hyal B [(1); IC_{50}= 0.440 mM, (2); IC_{50}= 0.060 mM]. For other Hyals, the action was weak [Hyal S – (1); 1.020 mM, (2); 0.260 mM; Hyal C – (1); NA, (2); NA; BTH – (1); 1.300 mM, (2); 0.090 mM). Considering the results obtained, glycyrrhetinic acid inhibited the activity of tested enzymes weaker, which may prove the significance of the sugar moiety in the inhibition of Hyal activity[25].

Facino et al. investigated the inhibitory influence of saponins and sapogenins isolated from seeds of *Aesculus hippocastanum* L. (escin and escinol), leaves of *Hedera helix* L. (α-hederin, hederacoside C, oleanolic acid, and hederagenin), and rhizome of *Ruscus aculeatus* L. (ruscogenin). Of the *Hedera helix* L. components, only sapogenins inhibited Hyal in a dose-dependent manner. Hederagenin (IC_{50}= 280.4 μM) inhibited the enzyme at 100 μM (12.5%); 20.3% at 150 μM, 31% at 200 μM, 56.5% at 300 μM, and 74.2% at 500 μM (plateau). Oleanolic acid (IC_{50}= 300.2 μM), 29.1% at 200 μM; 48.5% at 300 μM, and 67% (plateau) at 400 μM. Glycyrrhizic acid (positive control) blocked BTH Hyal significantly less (IC_{50}= 550.2 μM). Hederacoside C and α-hederin showed no activity against Hyal. For Aesculus hippocastanum L., escin had the highest activity (IC_{50}= 149.9 μM), inhibiting Hyal starting at 50 μM (4.2%); at higher concentrations of 100 (27.4%), 150 (52.0%), 200 (79.2%), and 300 μM (93.6%). Escinol was much less active (IC_{50}= 1.65 mM), and ruscogenin was utterly ineffective (Table 12)[80].

Table 8. Structures of L-ascorbic acid and its derivatives with an anti-hyaluronidase activity.

Name	Structure
L-Ascorbic acid	
Saccharic acid	
D-isoascorbic acid	
Dehydroascorbic acid	
L-gulonic-γ-lactone	
D-ribonic-γ-lactone	
D-gulonic-γ-lactone	
D-Saccharici-1,4-lactone	
α-D-glucoheptonic-γ-lactone	
L-Ascorbyl palmitate	

The olean-type saponins, spinasaponin A (lack of activity), spinasaponin A 28-O-glucoside (IC_{50} = 620 μM), udosaponin B (IC_{50} = 750 μM), and sandrosaponin IX (IC_{50} = 370 μM), isolated from the roots of *Oenanthe javanica*, showed moderate ability to inhibit Hyal. Esterification of the 28-COOH group with β-D-glucopranosyl increased the activity of the compounds[81].

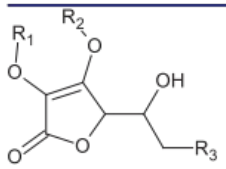
5. Inhibitors of tyrosinase

5.1. Tyrosine and tyrosinase

Tyrosinase is a key enzyme involved in melanogenesis. This enzyme belongs to the class of oxidoreductases (EC 1.14.18.1). It is responsible for the catalysis of tyrosine hydroxylation to L-DOPA and the oxidative conversion of L-DOPA to dopaquinone (Figure 12). The active centre of tyrosinase consists of two copper atoms linked by a coordination bond with three histidine residues. Three different types of tyrosinase are involved in melanin production: oxy-tyrosinase, met-tyrosinase, and deoxy-tyrosinase. Oxy-tyrosinase and met-tyrosinase have Cu (II) copper atoms in their active centre, and deoxy-tyrosinase has two Cu (I) atoms. Deoxy-tyrosinase does not perform a catalytic function, but it is easily converted into the oxy form, which is the only form of the enzyme capable of transforming both monophenol and diphenol substrates. On the other hand, met-tyrosinase is formed during the reaction catalysed by the oxy form and is responsible only for reactions with diphenolic substrates (Figure 13)[81–84].

Tyrosinase is an enzyme widely distributed in nature, including in fungi and to a lesser extent in bacteria and algae. This enzyme is also found in plants and animals. In higher vertebrates, melatonin is produced in melanocytes present in the epidermis, hair follicles, the uveal membrane of the eye (choroid, ciliary body, and iris), the inner ear (cochlea), and the central nervous system (the arachnoid and internal filum terminale). Melanins are macromolecular polymeric pigments resulting from the oxidation and polymerisation of phenolic compounds (Figure 14). Melanin synthesis takes place in vesicles called melanosomes and it is considered to be one of the most common pigments in nature. In mammals, melanosomal tyrosinase is involved in the formation of black-brown eumelanin and yellow-reddish phaeomelanin. Eumelanin has photoprotective properties, resulting from the ability to absorb ultraviolet radiation (UV) and neutralise free radicals and reactive oxygen species (ROS). On the other hand, pheomelanin has photosensitising properties and, under the influence of UV radiation, can participate in the generation of ROS. In mammals, melanin pigmentation performs many critical physiological tasks, such as adaptive colouration, protection of essential tissues against UV radiation, thermal control of the organism, regulation of vitamin D 3 biosynthesis. Abnormal tyrosinase activity is responsible for skin abnormalities such as vitiligo or freckles. Also, tyrosinase may play a role in carcinogenesis and neurodegenerative diseases such as Parkinson's disease. Tyrosinase also contributes to the formation of brown colour in fruits and vegetables due to the reaction of dopaquinone with amino acids and proteins present in these foods. In most studies on the inhibition of tyrosinase activity, fungal tyrosinase was used due to its widespread availability. The enzyme isolated from the mushroom *A. bisporus* is very similar in structure to tyrosinase occurring in mammals, making it a suitable model for studying the process of melanogenesis. Since tyrosinase is a reasonably significant target in agriculture, food, medicine, and cosmetology, much attention has been paid to the development and screening of tyrosinase inhibitors[85–87].

Table 9. Structures and activity of the vitamin C derivatives against hyaluronidase.[65]



Compound	Substituent R_1	R_2	R_3	IC_{50} (μM) or % inhibitiona, pH 5.0 SagHyal4755	BTH
1	H	H	H	6100	Inactive
6	Me	Me	H	Inactive	Inactive
7	Bn	Bn	H	355	Inactive
8	CH_2CH_2		H	24% (2000)	Inactive
9	Me	Me	$CO(CH_2)_{14}CH_3$	5% (160)	Inactive
10	Bn	Bn	$CO(CH_2)_{14}CH_3$	Inactive	Inactive
11	CH_2CH_2		$CO(CH_2)_{14}CH_3$	32% (190)	Inactive
13a	H	H	$COC(CH_3)_3$	43% (1100)	Inactive
13b	H	H	$CO(CH_2)_4CH_3$	475	Inactive
13c	H	H	$CO(CH_2)_6CH_3$	772	Inactive
13d	H	H	$CO(CH_2)_8CH_3$	102	1380
13e	H	H	$CO(CH_2)_9CH_3$	72	580
13f	H	H	$CO(CH_2)_{10}CH_3$	47	208
13g	H	H	$CO(CH_2)_{11}CH_3$	14.3	96
13h	H	H	$CO(CH_2)_{12}CH_3$	8.4	71
13i	H	H	$CO(CH_2)_{14}CH_3$	4.2	57
13j	H	H	$CO(CH_2)_{16}CH_3$	0.9	39
13k	H	H	$CO-Ph$	132	33% (1430)
13l	H	H	$COCH_2-pC_6H_4-Ph$	358	2006
13m	H	H	$CO(CH_2)_5O-Ph$	717	Inactive
13n	H	H	$CO(CH_2)_5OCH_2Ph$	437	Inactive
13o	H	H	$CO(CH_2)_5O-p-C_6H_4-Ph$	61	188
13p	H	H	$CO(CH_2)_5OCH_2-pC_6H_4-Ph$	102	543
13q	H	H	$CO(CH_2)_5O-pC_6H_4-C_2H_5$	280	Inactive
13r	H	H	$CO(CH_2)_5O-pC_6H_4-OCH_2-Ph$	76	210
13s	H	H	$CO(CH_2)_{10}O-Ph$	31	105
13t	H	H	$CO(CH_2)_{10}pC_6H_4-Ph$	7.5	37

Bn: benzyl group.

5.2. Polyphenols as inhibitors of tyrosinase: a structure–activity relationship

The positive effect of polyphenols on human health is mainly related to their antioxidant properties. The antioxidant activity of individual polyphenols depends on the number of hydroxyl groups and their location. It has been shown that the more hydroxyl groups in a molecule, the more potent antioxidant activity. Compounds with redox properties effectively prevent melanin biosynthesis due to their multidirectional mechanism of action. Tyrosinase inhibition by polyphenols is based on free radical scavenging properties and the ability to chelate copper in the tyrosinase active site.

5.2.1. Phenolic acids

Phenolic acids are one of the most common groups of organic compounds present in plants. They are made of a phenolic ring and a carboxylic acid residue. There are two subclasses of phenolic acids: derivatives of benzoic acid and cinnamic acid. Several studies have shown their inhibitory effect on tyrosinase activity and that activity was related to the number and position of hydroxyl groups[88]. Considering the structure of phenolic acids, three mechanisms of tyrosinase inhibition can be distinguished. The first is related to the chelation of copper ions in the active centre. The second mechanism is associated with the disturbance of the enzyme's tertiary structure through hydrogen bond formation. The third mechanism involves constructing hydrogen bonds between the hydroxyl groups of phenolic acids and the carbonyl oxygen of the Tyr98 ORF378 protein, preventing the interaction between tyrosinase and ORF378. Consequently, ORF378 cannot serve as a Cu (II) ion transporter to the enzyme's active site[89,90].

5.2.1.1. Hydroxybenzoic acids. Kubo et al. investigated the effect of anisic acid and its derivatives on L-DOPA oxidation by tyrosinase. As the concentration of anisic acid increased, the enzymatic activity decreased sharply but was not completely inhibited (IC_{50} = 0.60 mM). The inhibition of fungal tyrosinase by anisic acid is a reversible reaction in which the tested acid is a non-competitive inhibitor (K_i =0.603 mM). The modification of the alkyl chain in anisic acid influenced the activity and type of inhibition of the tested compounds. P-ethoxybenzoic acid showed a kind of incompetent inhibition, p-propoxybenzoic acid was of mixed, and p-butoxybenzoic acid was of competitive. With the increase in the alkyl chain's length, the inhibitory activity of the tested compounds decreased[91,92].

Chen et al. obtained similar results when testing p-alkoxybenzoic acid derivatives. The tested compounds behave as reversible tyrosinase inhibitors in the presence of L-DOPA substrate (λ 475 nm; spectrophotometric method, 6680 U/mg). Among them, *p*-hydroxybenzoic acid (IC_{50}= 1.3 mM; K_i = 0.73 mM) is a competitive inhibitor, *p*-methoxybenzoic acid (IC_{50}=0.42 mM; K_i = 0.43/0.43 mM) is non-competitive, *p*-ethoxybenzoic acid (IC_{50}=1.1 mM; K_i = 1.46/0.84 mM) is of the mixed type, and the others show a type of non-competitive inhibition (*p*-propoxybenzoic acid, *p*-butoxybenzoic acid, *p*-pentoxybenzoic acid, and *p*-hexyloxybenzoic acid). Additionally, it was appeared that increasing the chain length above two carbon atoms changed the type of braking from competitive to non-competitive[93]. (22)

Another study[94] determined the effect of methoxylation of hydroxyl groups on the tested acids' activity and their esters. Protocatechuic acid methyl ester, protocatechuic acid, vanillic acid methyl ester, vanillic acid, isovanillic acid methyl ester, isovanillic acid, veratric acid methyl ester, and veratric acid were used in the study. Only protocatechuic acid and its methyl ester inhibited the enzyme (60.1 and 75.4% inhibition; ID_{50} =0.42 μmol/mL and 0.28 μmol/mL). The hydroxyl group at the para and meta position

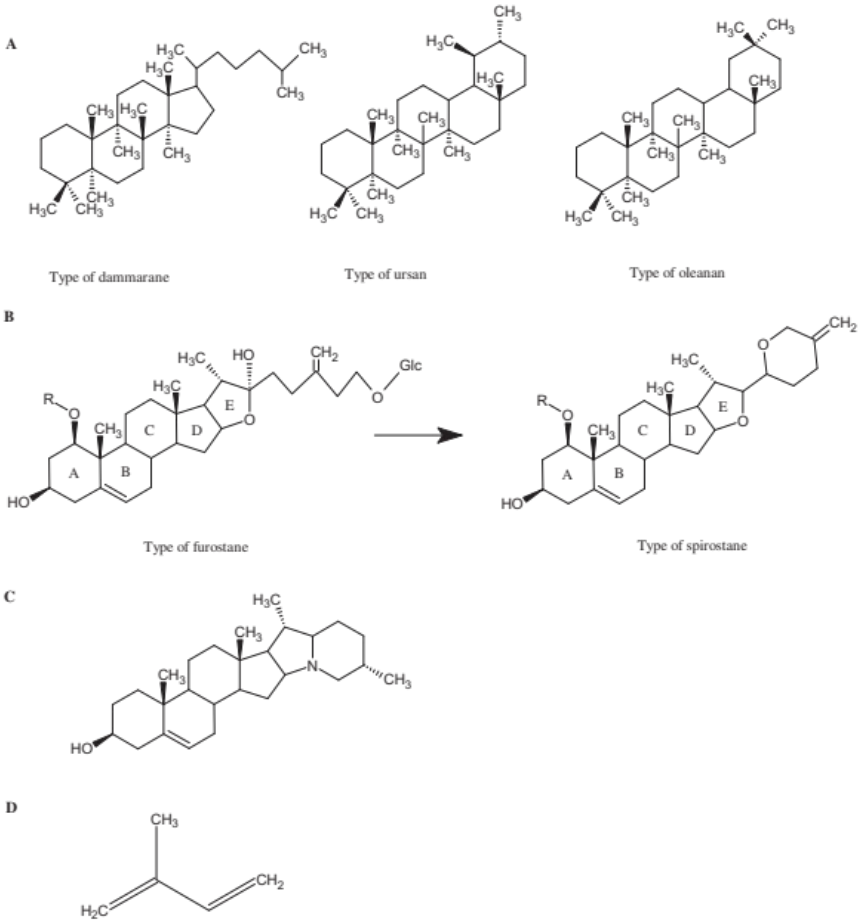
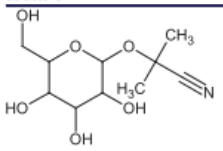
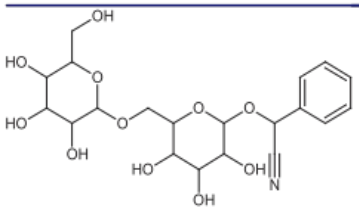


Figure 9. Chemical structures of saponins. A – triterpene saponins, B – steroid saponins, C – steroid alkaloids, and D – isoprene.

Table 10. Structures of the active cyanogenic glycosides towards hyaluronidase.

Structure	Compound
	Linamarine
	Amygdalin

is an important part of the structure of inhibitors. Protocatechuic acid methyl ester inhibited the enzyme activity more strongly than protocatechuic acid, which may result from the esterification of the carboxyl group.

Kubo et al. provided more information about the impact of an esterification on the activity of gallic acid. It was appeared that, apart from gallic acid (4.5 mM), the IC_{50} of all esters was almost comparable (<0.5 mM). Based on the above observation, it can be concluded that gallic acid esters with an increase in the number of carbon atoms of the alkyl chain (>C10) may be more challenging to incorporate into the protein pocket to reduce the rate of oxidation by the enzyme. Hence, gallates with a longer alkyl chain (>C-10) become inhibitors but not substrates, which indicates that gallates with a longer alkyl group (>C-10) can be expected as more suitable inhibitors. However, tetradecanoyl gallate (C-14) and hexadecanyl gallate (C-16) are sparingly soluble in water (Figure 12; Tables 1S and Table 13)[95].

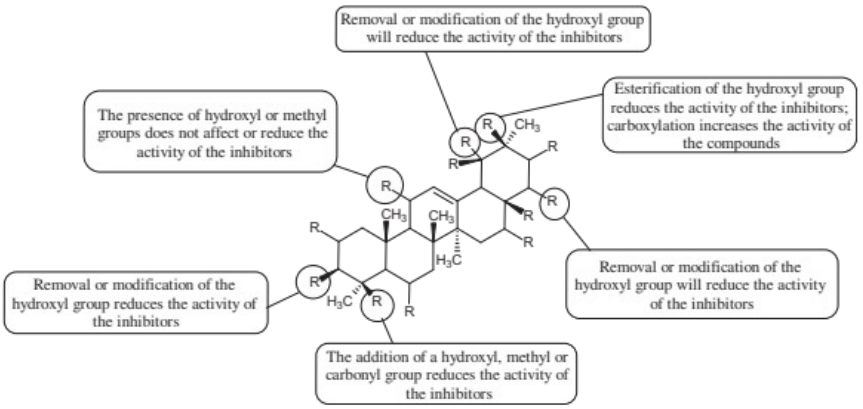


Figure 10. Chemical groups of triterpenic acids involved in the inhibition of hyaluronidase.

5.2.1.2. Hydroxycinnaminic acids. Shi et al. investigated the effect of cinnamic acid and its derivatives on the activity of fungal tyrosinase. Cinnamic acid (IC_{50}=2.10 mM), 4-methoxycinnamic acid (IC_{50}=0.42 mM), and 4-hydroxycinnamic acid (IC_{50} =0.52 mM) strongly inhibited the conversion of diphenol to dichinone. Cinnamic acid and 4-methoxycinnamic acid showed a non-competitive type of inhibition (Ki = 1.994 and 0.458 mM), interacting with a different site of the enzyme than the active site. In contrast, 4-hydroxycinnamic acid competitively inhibited the enzyme (Ki = 0.244), which may result from the similar structure of the tested compound to the tyrosinase substrate. One of the tested compounds, i.e. 2-hydroxycinnamic acid did not inhibit the enzyme activity, probably because of the presence of the 2-OH group causing spherical hindrances[96].

Another study[97] assessed the influence of methoxylation of cinnamic acid and its derivatives (cinnamic acid, 2-hydroxycinnamic acid, 3-hydroxycinnamic acid, 4-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid, 2-methoxycinnamic acid, 3-methoxycinnamic acid, and 4-methoxycinnamic acid; concentrations from 0.1 to 3 mmol/L) on the action of tyrosinase (3130 U/mg) in the presence of L-tyrosine or L-DOPA as a substrate. Cinnamic acid (IC_{50}=2.1 mM), 2-hydroxycinnamic acid (IC_{50}=0.5 mM), and the O-methyl (IC_{50}=0.42 mM) forms exhibited inhibitory properties compared to 3-hydroxycinnamic acid, 4-hydroxycinnamic acid, and 3,4-dihydroxycinnamic acid, which turned out to be substrates of tyrosinase. All inhibitors showed competitive inhibition. The inhibition constants are about the same for the oxidation of L-tyrosine and L-DOPA, indicating that the inhibitors bind to the same form of the enzyme. In the next study[98], the inhibitory effect of caffeic acid and ferulic acid on tyrosinase activity isolated from murine B16 melanoma cells (90 U) was analysed. Both ferulic acid (27.4% non-toxic conc.) and caffeic acid (24.4% non-toxic conc.) effectively inhibited melanin production in B16 melanoma cells. Ferulic acid was reducing tyrosinase activity by binding directly to the enzyme, whereas no binding was observed between caffeic acid and tyrosinase.

One of the important structural elements regulating the enzyme's activity is a type of bonds present in the inhibitor's structure. Some compounds, such as p-coumaric acid (IC_{50}=115.6 μM; % inhibition 74.4) and isoferulic acid (IC_{50}=114.9 μM; % inhibition 77.8) lose their tyrosinase inhibitory properties after saturation of the double bond (dihydro-p-coumaric acid IC_{50}=1000 μM; 74.4% inhibition and dihydroisoferulic acid IC_{50}=195.7 μM; 60.6% inhibition). None of the compounds tested were more effective than kojic acid (IC_{50}=51.6 μM) or arbutin (IC_{50}=210.5 μM). All compounds tested show a non-competitive type of inhibition. The results indicate that the reduction of double bonds weakens the inhibitory activity of the test compounds. The C = C binding is necessary for the proper interaction of the inhibitor with the active site[99].

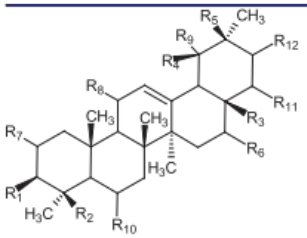
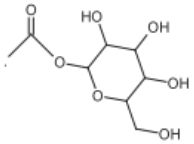
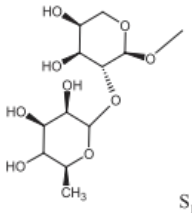
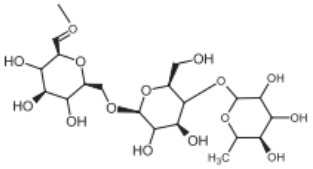
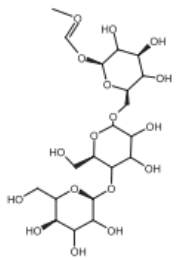
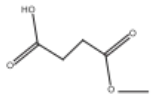
In another study, the type of inhibition and the effect of caffeic acid, *p*-coumaric acid, and rosmarinic acid on monophenolase and diphenalase activities were determined. The most active compound was *p*-coumaric acid (L-tyr, IC_{50} =0.3 μM; L-DOPA, IC_{50} =0.62 μM), which inhibited the enzyme noncompetitively (L-tyr, K_i=0.033 μM; L-DOPA, K_i=2.2 μM) followed by caffeic acid (L-tyr, IC_{50} =1.50 μM; L-DOPA, IC_{50} =2.30 μM) and rosmarinic acid (L-tyr, IC_{50} =4.14 μM; L-DOPA, IC_{50} =8.59 μM). These compounds blocked the enzyme more potently than kojic acid (L-tyr, IC_{50} =33.45 μM; L-DOPA, IC_{50} =38.98 μM) (Figure 15; Tables 2S and Table 13)[100].

5.2.2. Flavonoids

Flavonoids belong to a group of compounds commonly found in the plant world. These compounds differ from each other by the presence of a double bond between the second and third carbon atom, a ketone group at position 4, and the position of the B ring. Additionally, individual flavonoids differ from hydroxyl, methyl, isoprenoid, and methoxy groups arranged in different rings. Many flavonoids have tyrosinase inhibitory activity[101].

5.2.2.1. Hydroxyl groups. The distribution and number of hydroxyl groups in flavonoid molecules significantly affect their activity. Chrysin (flavone), having no hydroxyl groups in the B-ring, does not block the activity of tyrosinase. The presence of the hydroxyl group in the C-4′ position of apigenin (IC_{50}=c. 40 μM) significantly increased its activity compared to chrysin. The additional hydroxyl group in the C-3′ position of luteolin (IC_{50}=c. 186 μM) decreased its activity compared to apigenin. Galagin (IC_{50}=c. 10 μM) without hydroxyl groups on the B-ring blocks tyrosinase activity more strongly than chrysin. Addition of the hydroxyl group at the C-4′ or C-3′, C-4′ position reduces the activity of kaempferol (IC_{50}=c. 73 μM) and quercetin (IC_{50}=c. 30 μM) compared to galagin.

Table 11. Structures and an anti-hyaluronidase activity of oleanane-type saponins[73].

		Substituent											
	Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	R_{11}	R_{12}	IC_{50} (μM)
	1	OH	CH_3	COOH	CH_3	H	H	H	H	H	H	H	H	103.18
	2	OH	CH_2OH	COOH	CH_3	H	H	H	H	OH	H	H	H	286.95
	3	OAc	CH_3	COOH	H	CH_3	H	H	H	H	H	H	H	1466.5
	4	=O	CH_3	COOH	CH_3	H	H	H	H	H	H	H	H	162.83
	5	NOH	CH_3	COOH	CH_3	H	H	H	H	H	H	H	H	190.94
	6	OAc	CH_3	COOH	CH_3	H	H	H	H	H	H	H	H	136.92
	7	=O	CH_3	$COOCH_3$	CH_3	H	H	H	H	H	H	H	H	1184.15
	8	NOH	CH_3	$COOCH_3$	CH_3	H	H	H	H	H	H	H	H	275.68
	9	OH	CH_3	$COOCH_3$	CH_3	H	H	H	H	H	H	H	H	182.51
	10	OAc	CH_3	$COOCH_3$	CH_3	H	H	H	H	H	H	H	H	812.93
S_5														
	11	OH	CH_3	CH_3	H	H	H	H	=O	H	H	H	H	1750.91
	12	OH	CH_2OH	CH_3	CH_3	H	H	H	H	H	H	H	H	227.97
	13	OH	CH_3	CH_2OH	H	CH_3	H	H	H	H	H	H	H	206.21
	14	OH	CH_3	CH_3	CH_3	H	H	H	H	H	H	H	H	211.44
	15	OH	CH_3	COOH	H	CH_3	OH	H	H	H	H	H	H	140.91
	16	OH	CH_3	$COOCH_3$	H	CH_3	H	H	H	H	H	H	H	84.52
S_1														
	17	S_1	CH_2OH	COOH	H	CH_3	H	H	H	H	H	H	H	842.54
	18	OH	CH_3	CH_3	H	CH_3	H	H	H	H	H	H	H	215.66
	19	OH	CH_2OH	COOH	CH_3	H	H	OH	H	H	H	H	H	115.96
	20	OH	CH_3	COOH	H	CH_3	H	H	H	H	H	H	H	227.97
S_3														
	21	OH	CH_3	CH_3	H	COOH	H	H	=O	H	H	H	H	146.18
	22	S_2	CH_3	CH_3	H	COOH	H	H	=O	H	H	H	H	56.33
	23	OH	COOH	CH_3	H	CH_3	H	H	H	H	H	H	H	1482.56
	24	OH	CH_2OH	COOH	H	CH_3	H	H	H	H	H	H	H	230.00
	25	O-glucoside	CH_3	COOH	H	CH_3	OH	H	H	H	H	H	H	NA
	26	OH	COOH	CH_3	CH_3	H	H	H	H	H	H	H	H	NA
S_4														
	27	OH	S_3	CH_2OH	CH_3	H	H	OH	H	H	H	H	H	NA
	28	OH	CH_2OH	CH_2OH	H	CH_3	OH	H	H	H	H	OH	OH	NA
	29	OH	CH_2OH	$COOS_4$	CH_3	H	H	OH	H	H	OH	H	H	NA
S_2														

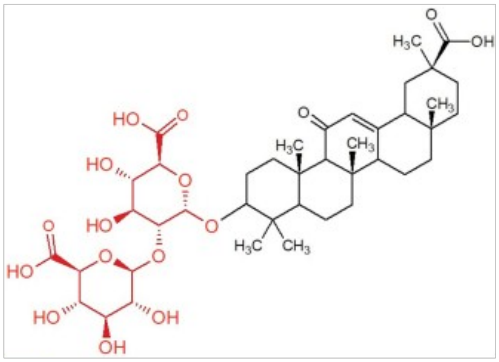


Figure 11. 3-O-β-D-glucuronopyranoside group.

Flavonols, due to the additional hydroxyl group at the C-3 position, block tyrosinase much more strongly than flavones[102].

Kim et al. provided more information regarding the influence of the position and number of hydroxyl groups on their inhibitory activity. Natural and synthetic flavones and flavonols were used in the study. L-tyrosine was used as a substrate for fungal tyrosinase. It appears that the hydroxyl groups at the C-7 (ring A) and C-4′ (ring B) positions may increase the inhibitory activity of flavonoids. In this study, 3′,4′,7,8-tetrahydroxy flavone showed the strongest inhibitory properties (IC_{50}=0.07 μM). Two catechol groups in ring A and B are probably responsible for the inhibition. Lack of the fourth hydroxyl group in 4′′,7,8-trihydroxy flavone (IC_{50}=12.95 μM) and 3′′,4′′,7-trihydroxy flavone (IC_{50}=24.1 μM) weakens their activity. The 5-OH group also has a significant influence on the activity of the inhibitors, e.g. 2′,5,7-trihydroxyflavone (IC_{50}=12.95 μM) and 3′,4′,5,7-tetrahydroxy flavone (IC_{50}=12.95 μM) block tyrosinase more strongly than 2′,7-dihydroxy flavone, and 3′,4′,7-trihydroxy flavone. Also, the hydroxyl group in the C-3 position, which divides flavonoids into flavones and flavonols, influences on the activity with a weaker inhibition for flavones. Additionally, an increase in the number of hydroxyl groups in flavonoids reduces their inhibitory activity against tyrosinase (3′,4′,5,6,7-pentahydroxy flavonol IC_{50}=314.23 μM). The location of the hydroxyl groups plays a more important role than their number. Summarising the increase in the number of hydroxyl groups on the other side of the flavonoids (C-7, C-8, C-2′, C-3′, and C-4′) increases compounds' activity[103]. In contrast, an increase in the number of hydroxyl groups on the ketone side (C-5, C-6, C-5′, C-6′) reduces the activity of inhibitors, which is related to the disturbance of the interaction with tyrosinase. Isoflavonoids are characterised by a linked ring B at the third carbon atom. The location and number of hydroxyl groups in the A-ring of isoflavonoids can strongly affect both the inhibitory power and inhibition type. 6,7,4′-trihydroxyisoflavone, daidzein, glycitin, daidzin and genistin showed strong monophenolase inhibitory activity but weak diphenolase inhibitory activity. 4′,6,7-trihydroxyisoflavone (IC_{50}=9 μM) shows the strongest properties. Presence of a single hydroxyl group in the C-7 position (4′,7-trihydroxyisoflavone IC_{50}=203 μM; 6-methoxy-7,4′-dihydroxyisoflavone IC_{50}=218 μM) or no hydroxyl groups (4′-hydroxyisoflavone-7-O-glucoside IC_{50}=267 μM) in ring A significantly reduces the activity of the compounds. The presence of the hydroxyl groups in the C-7 and C-8 position can influence the type of inhibition, shifting from reversible to irreversible type[104]. Similar results were obtained in case of anthocyanins and an assessment of the impact of the number and distribution of hydroxyl groups in the B ring on tyrosinase activity. The following compounds were investigated: pelargonidin (IC_{50}=66 μM), cyanidin (IC_{50}=27.1 μM), and delphinidin (IC_{50}=57.4 μM) in the presence of kojic acid (IC_{50}=34.8 μM) as a positive control. The substrate for the reaction was L-DOPA. These results indicate that the structure with two hydroxyl groups in ring B has the greatest inhibitory effect[105].

Another study examined the effect of isoflavonoids isolated from the roots of *Pueraria lobata*, such as daidzein and formononetin. These compounds have appeared to be of weak inhibitors with the IC_{50} values for daidzein L-tyr. 350 μM; L-DOPA 350 μM and for formononetin L-tyr. IC_{50} >350 μM; L-DOPA IC_{50} >350 μM. A significant increase in an activity was obtained by introducing OH groups into the B ring at the C-3′ position and methylation of the 4′-OH group - calycosin (L-tyr. IC_{50} =7.02 μM; L-DOPA IC_{50} =1.45 μM). This compound is more active than kojic acid (L-tyr. IC_{50} =12.10 μM; L-DOPA IC_{50} =9.14 μM). It is seen that the presence of a hydroxyl group at the C-3′ position and a methoxyl group at the C-4′ position of the isoflavone backbone plays a major role in the anti-tyrosine activity[106]. The inhibitory activity of calycosin against tyrosinase (monophenolase) was also confirmed by Kim et al. with the IC_{50} equal of 38.4 μM. In turn, the IC_{50} value for kojic acid and arbutin was 51.5 and 120.9 μM, respectively[107]. However, calycosin was more toxic than standards (LD_{50} =120 μM *vs.* LD_{50} >200 μM for kojic acid and arbutin). The results of another study conducted by Kim et al. have also shown an inhibitory activity of calycosin with the IC_{50} =30.8 μM and for kojic acid IC_{50} =50.1 μM[108].

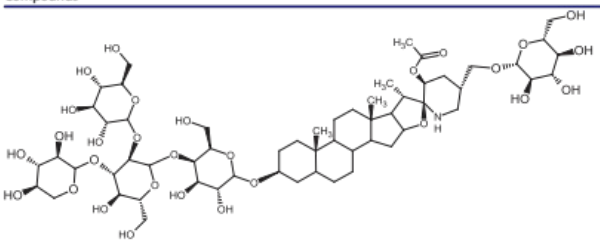
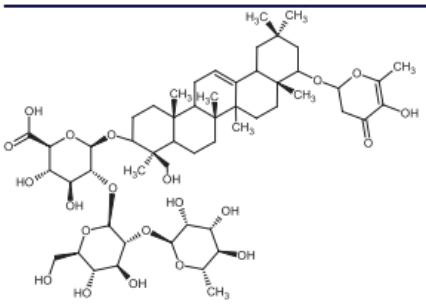
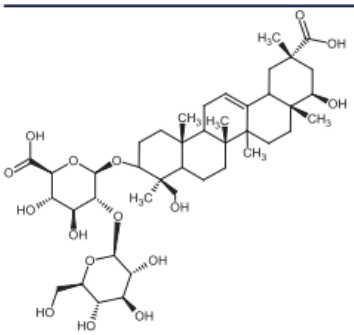
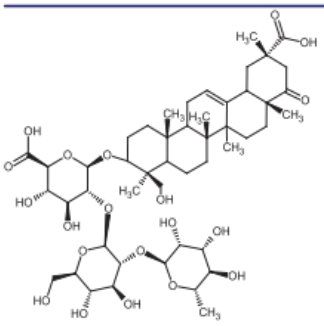
The isoflavonoids isolated from the stem of *Maackia fouriei* have also exhibited their anti-tyrosinase activity. Methylation of the 4′-OH group in formononetin, texasin, and odoratin, resulted in impaired anti-tyrosinase properties. The presence of 5-OH group in genistein (IC_{50}=33 μM) and tectorigenin (IC_{50}=20 μM) favourably affects the activity of isoflavonoids in comparison to daidzein (IC_{50}=41 μM) without 5-OH group. An interesting compound isolated from *M. fouriei* is the bishomoflavonoid derivative, mircoin (IC_{50}=5 μM). This compound inhibited the enzyme competently. Due to its strong inhibition, further structure-activity studies are needed for this compound. In this study, kojic acid (IC_{50}=45 μM) was used as a positive control[109].

In another study, the effect of OH groups at the C-6, C-7 and C-4′ positions on isoflavonoid activity was investigated. 6,7,4′-Trihydroxyisoflavone inhibited tyrosinase competently (IC_{50}=9 μM; K_I value of 5.72–6.24 μM). Methylation of the 6-OH group in glycitein (IC_{50}=264 μM), the absence of the 6-OH group in daidzein (IC_{50}=237 μM), or the presence of an OH group at the 5-OH position in genistein (IC_{50}=822 μM), decreased the anti-tyrosinase activity (Table 14)[110].

Similar effects of isoflavonoids on tyrosinase were noted by studying extracts of *Otholobium pubescens* (Pior.) J.W. Grimes. In this study, L-tyrosine as substrate and β-arbutin (IC50 = 1830 μM) as a control were used. Daidzein and its aglycone, genistein showed no effect (daidzein, IC_{50} =1580 μM; genistein, IC_{50} =7660 μM)[111].

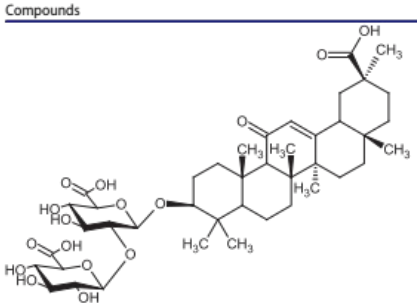
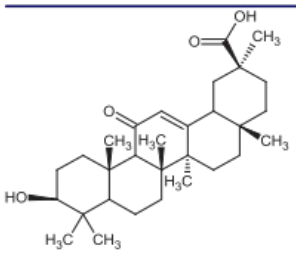
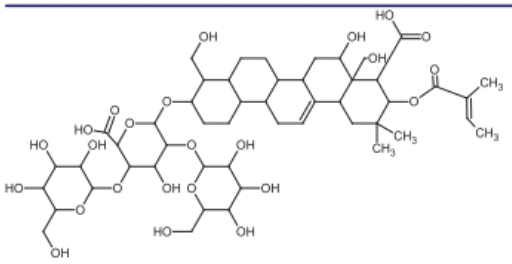
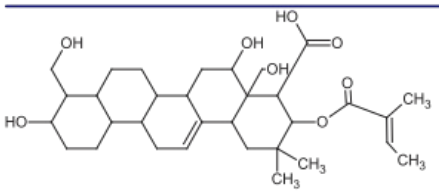
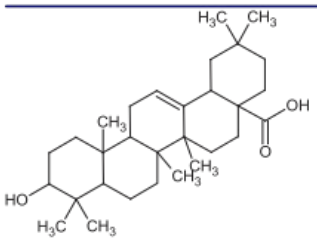
The discrepancies in the study are due to the variation in experimental conditions such as temperature, pH, and concentrations of enzyme and substrates used. Flavonols are the most potent tyrosinase inhibitors among flavonoids. This is due to the similarity of flavonols to the structure of kojic acid – 3-hydroxy-4-keto moiety (Figures 16 and 17).

Table 12. Structures and an anti-hyaluronidase activity of the chosen saponins.

Compounds	Name	IC_{50} mM	References
	Esculeoside A	2	Zhou et al.[72]
	Compound 1	0.15	Buyankhishig et al.[75]
	Compound 2	0.21	Buyankhishig et al.[75]
	Compound 3	0.22	Buyankhishig et al.[75]

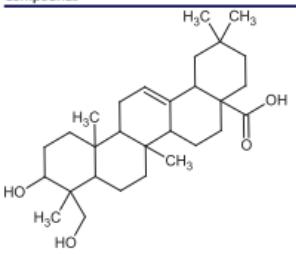
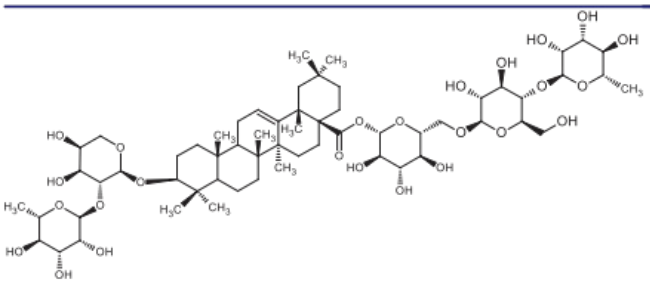
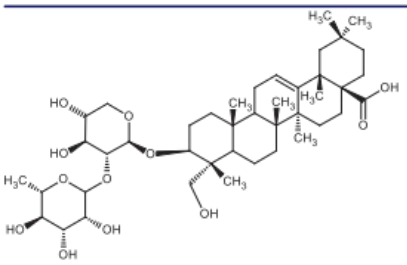
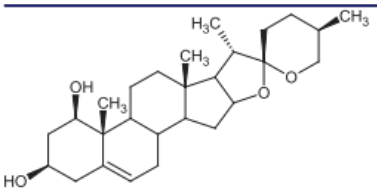
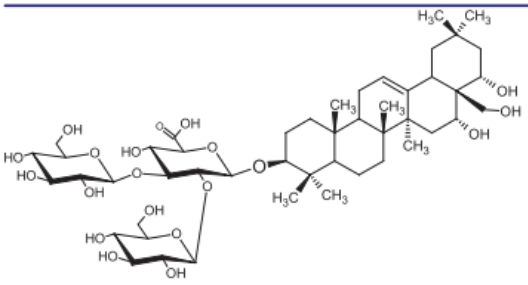
(*continued*)

Table 12. Continued.

Compounds	Name	IC_{50} mM	References
	Glycyrrhizin	0.44	Hertel et al. [37]
	Glycyrrhetinic acid	0.06	Hertel et al. [37]
	Escin	0.15	Facino et al.[79]
	Escinol	1.65	Facino et al.[79]
	Oleanolic acid	0.300	Facino et al.[79]

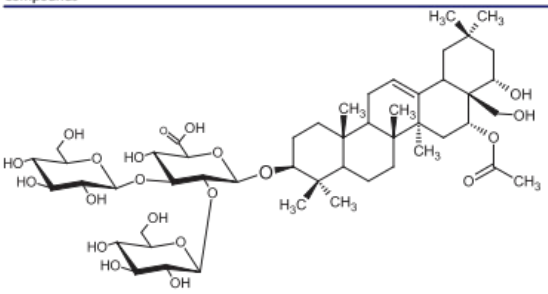
(*continued*)

Table 12. Continued.

Compounds	Name	IC_{50} mM	References
	Hederagenin	0.280	Facino et al.[79]
	Hederacoside C	Non effect	Facino et al.[79]
	α-Hederin	Non effect	Facino et al.[79]
	Ruscogenin	Non effect	Facino et al.[79]
	Glycoside camelliagenin A	303.93 µg/mL	Grabowska et al.[77]

(*continued*)

Table 12. Continued.

Compounds	Name	IC_{50} mM	References
	Glycoside 16-O-acetylcamelliagenin A	286.7 μg/mL	Grabowska et al.[77]

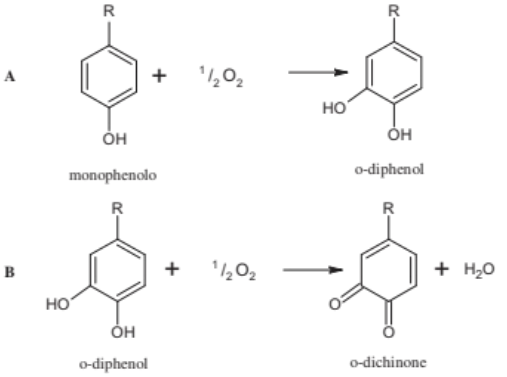


Figure 12. A – reaction hydroxylation of monophenols to *o*-diphenols; reaction B – oxidation of *o*-diphenols to *o*-quinones.

5.2.2.2. Methoxylation. Methoxylation of the hydroxyl groups in the flavonoid molecules reduces their activity. Methoxylated flavones such as 5,6,7,4′-tetramethylscutellarein (5.21% inhibition), 5,7,4′-trimethylscutellarein (3.23% inhibition), and ladanein (4.24% inhibition) showed a ten-fold decrease in the inhibitory activity than kojic acid (% inhibition 80.02). It was proved that methoxylation of isoflavonoid also decreases the inhibition e.g. 2′-hydroxygenistein (37.3% inhibition) inhibited tyrosinase stronger than its methylated forms, such as 5-O-methyl-2′-hydroxygenistein (25.8% inhibition) and 7-O-methyl-2′-hydroxygenistein (31.2% inhibition). The weaker activity of 5-O-methyl-2′-hydroxygenistein may be related to the disturbance of a hydrogen bond formation between 5-OH and carbonyl oxygen (C-4). Kojic acid (IC_{50}=11.3 μM) was used as a control and L-DOPA as a substrate[112].

More information about the effect of hydroxyl groups and methylation on flavonoid activity was provided by studying derivatives having a methoxy group at position 3, a hydroxy group at position 5, and oxidised aromatic carbons at C4′ and C7 (Table 15). The most potent inhibitors 1 (IC_{50} =6.71 μM), 2 (IC_{50} =13.20 μM), and 3 (IC_{50} =17.66 μM) have three hydroxy groups at the C-3′, C-4′ and C-5′ positions in the B ring. Compound 1, which contains an additional methoxy group at C6 and a hydroxy group at C7, was the most active. Comparing the IC_{50} of 1 *vs.* 2 and 5 (IC_{50} =73.03 μM) *vs.* 6 (IC_{50} =103.56 μM), it seems that compounds containing a methoxy group at position C6 are more active than those that are unsubstituted at this position (Table 15)[113].

5.2.2.3. Double bond. The double bond between the second and third carbon atoms is preferred for flavonoids to maintain a flat molecular structure. Naringenin (IC_{50}=c. 555 μM) showed less inhibitory activity than apigenin (IC_{50}=c. 40 μM). Dihydromyricetin (IC_{50}=c. 37 μM) showed greater inhibitory activity than myricitin (IC_{50} =c. 85 μM), while taxifolin (IC_{50}=c. 800 μM) showed less inhibitory activity than quercetin (IC_{50}=c. 30 μM). These results suggested that C2 = C3 binding affects the inhibitory properties of the flavonoids[102].

5.2.2.4. Glycosides. Flavonoids occur mainly in the form of 3- and 7-glycosides. Some studies have revealed that a sugar moiety can modify flavonoids' activity, e.g. 3-O-glycosides, hyperin (IC_{50} not detected) and rutin (IC_{50}=c. 4571 μM) show weaker tyrosinase inhibition than aglycone – quercetin (IC_{50}=c. 30 μM). Similarly, 7-O-glycosides, baicalin (IC_{50}=c. 215 μM) and naringin (IC_{50}=c. 1900 μM) also inhibited tyrosinase weaker. A clear evidence was provided when monoglycosides, diglycosides and acylated monoglycosides towards tyrosinase inhibition were tested. Monoglycosides such as luteolin-7-O-glucoside (27.35% inhibition, IC_{50}=74 μM), kaempferol-3-O-glucoside (24.2% inhibition, IC_{50}=74 μM), and isorhamnetin-3-O-glucoside (24.22% inhibition, IC_{50}=70 μM) showed stronger inhibition tyrosinases in a comparison to diglycosides such as kaempferol-3-O-rutinoside (16.05% inhibition, IC_{50}=56 μM), isorhamnetin-3-O-rutinoside (13.13% inhibition, IC_{50}=53 μM), and rutin (12.65% inhibition, IC_{50}=55 μM). It is suggested that a presence of acyl groups on sugar residues of monoglycosides kaempferol-3-O-(6″-pCm)-glucoside (14.69% inhibition, IC_{50}=11 μM), quercetin-3-O-(6″-pCm) -glucoside (21.86% inhibition, IC_{50}=55 μM), isorhamnetin-3-O-(6″-OAc)-glucoside (23.31% inhibition, IC_{50}=64 μM), isorhamnetin-7-O-(6″-pCm)-glucoside (21.10% inhibition, IC_{50}=53 μM), apigenin-7-O-(6″-pCm)-glucoside (17.66% inhibition, IC_{50}=58 μM), apigenin-7-O-(3″,6″-di-pCm)-glucoside (20.69% inhibition, IC_{50}=46 μM), chrysoeriol-7-O-(3″,6″-di-pCm)-glucoside (15.59% inhibition, IC_{50}=44 μM) promotes an inhibitory effect compared to mono and diglycosides. The increase in the size of the flavonoids may prevent the flavonoids from entering the active site of tyrosinase (Tables 3S and Table 16)[102].

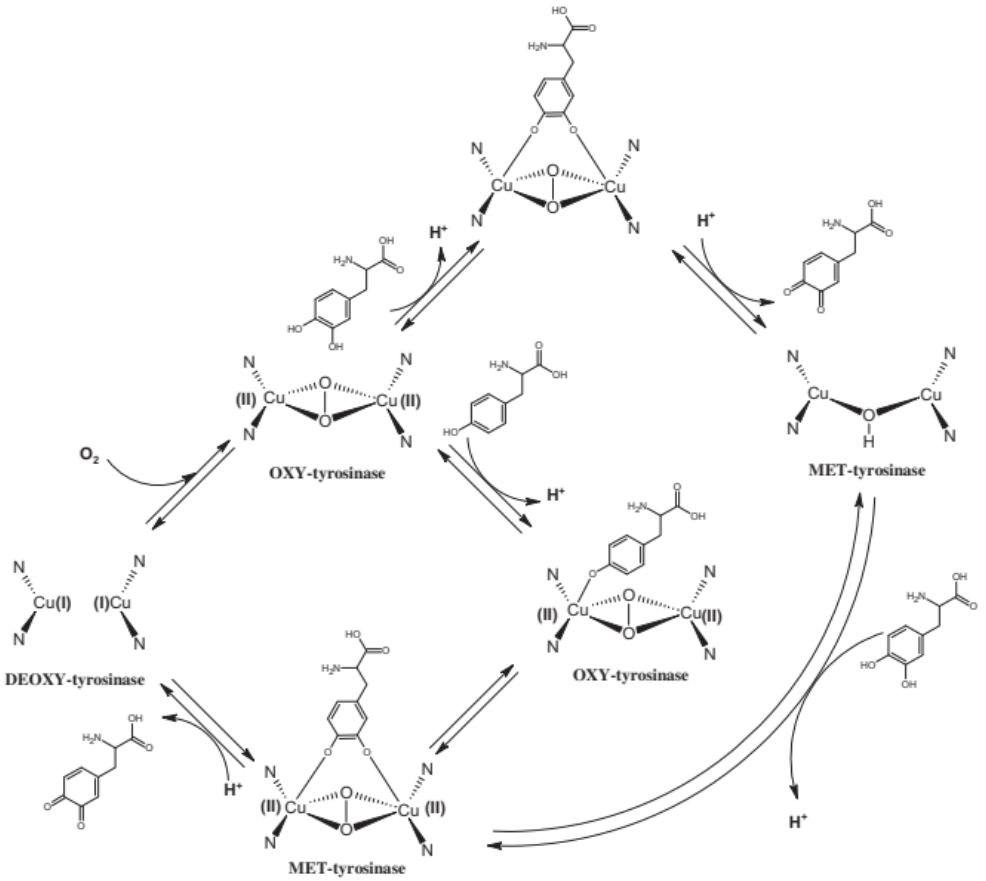


Figure 13. Mechanism of the tyrosinase action as monophenolase and diphenolase.

Comparing baicalein (IC_{50} =290 μM) with chrysin (no activity), the other hydroxyl group at the C-6 position of baicalein results in more potent tyrosinase inhibition. Comparing the inhibitory potency of baicalein (aglycone) with its glycosides, oroxin B (no activity) and oroxin A (IC_{50} =500 μM), a decrease in an inhibitory activity was noted. Glycosylation of the hydroxyl group at C7 was negatively correlated with the inhibitory activity of flavonoids. In addition, the activity of glycosides was influenced by the type of sugar moiety. The presence of the β-D-gentiobiosyl group reduced the inhibitory activity stronger than β-D-glucopyranosyl. This effect is due to spherical collapses[114].

5.2.3. Lignans

Lignans are phenylpropanoid dimers belonging to the group of plant phytoestrogens (Figure 18). These compounds are widespread in seeds (lentils), vegetables (garlic and asparagus), and fruits (pears and plums), however, the richest source is linseed and whole cereal grains. They are part of the cell wall and can be released by intestinal bacteria. Due to the similar structure to oestrogens, lignans compete for oestrogen receptors. In oestrogen deficiency, lignans gently complement their action, and when there is an excess of them, they reduce their activity because they have a much weaker oestrogenic effect. As a result, they help maintain the hormonal balance in the body and reduce the risk of various hormone-dependent diseases. Besides, these compounds protect against osteoporosis, lower LDL cholesterol, inhibit bacteria and fungi' growth, and lower blood glucose levels. The most important compounds in this group are sesamine, sesaminol, sesamoline, pinoresinol, secoisolariciresinol, matairesinol, schizandrin, and schizandrol[115–120].

Eight lignans were isolated from the methanolic extract of *Vitex negundo* L., i.e. negundin A, negundin B, 6-hydroxy-4-(4-hydroxy-3-methoxy)-3-hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthalodehydrate, vitrofolal E, (+)-lyoniresinol, (+)-lyoniresinol-3α-O-β-D-glucoside, (+)-(-)-pinoresinol, and (+)-diasyringaresinol. The lactam ring present in negundin A caused moderately strong

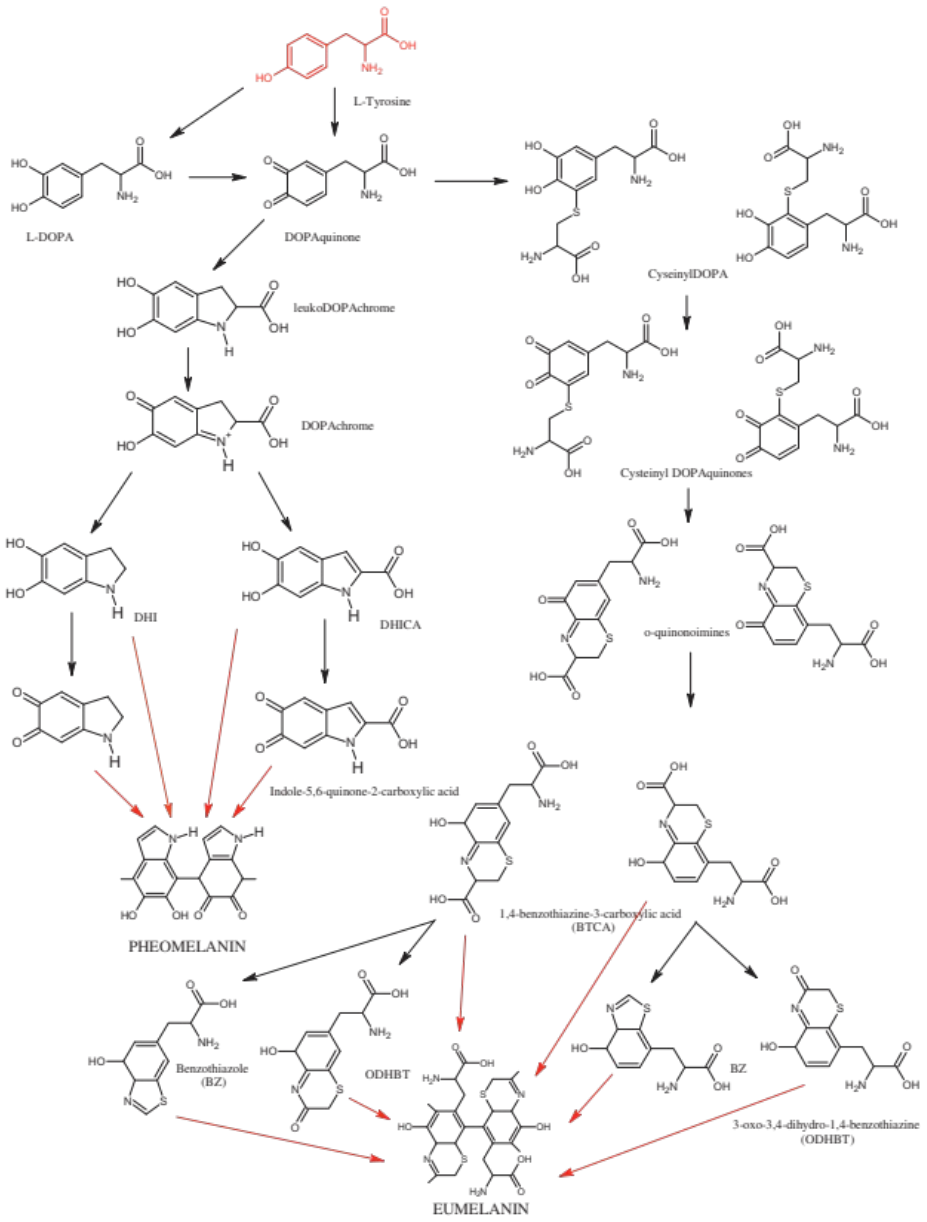


Figure 14. The pathway of melanin synthesis.

(IC_{50}=10.06 μM) inhibition of tyrosinase as compared to kojic acid (IC_{50}=16.67 μM). Negundin B, with the -CH2OH group in the C-2 position and the C=C bond between C-1 and C-2, showed stronger (IC_{50}=6.72 μM) inhibition of tyrosinase compared to kojic acid (IC_{50}=16.67 μM). Compound 3, in which the CH2OH group in the C-2 position was replaced with an aldehyde group, blocked tyrosinase to a lesser extent (IC_{50}=7.81 μM) than negundin B. Removal of the CH2OH group in the C-3 position and introduction of the C=C bond between C3 and C-4 reduced vitrofolal E's strength (IC_{50}=9.76 μM). The strongest inhibitor was

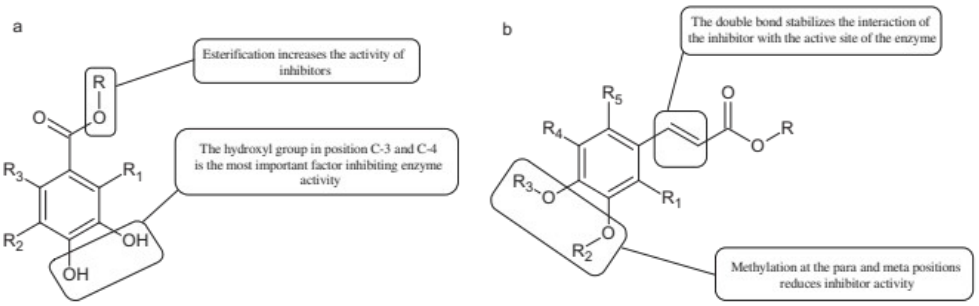


Figure 15. a) Potential groups engaged in an interaction hydroxybenzoic acid-tyrosinase and b) Potential groups engaged in an interaction hydroxycinnamic acid-tyrosinase.

(+)-lyoniresinol, in which both positions C-2 and C-3 contain the CH2OH group (IC_{50}=3.21 μM). Glycosylation of (+)-lyoniresinol at position C-3 rendered inactive. The presence of a sugar residue hinders the interaction between the enzyme's active site and the inhibitor. (+)-(-)-Pinoresinol showed moderate inhibition (IC_{50}=15.13 μM). The introduction of -OCH3 groups in the 5′ and 3′ positions in (+)-(-)-pinoresinol, lead to the formation (+)-diasyringaresinol which strongly inhibits tyrosinase (IC_{50}=5.61 μM). The compound (+)-lyoniresinol could be used as a potential lead molecule in bioprospecting[121]. Also, other lignans exhibited an anti-tyrosinase activity, e.g. 5,5-dimethoxylaryresinol-4-O-β-d-glucopyranoside and eleutheroside E_1 showed significant inhibition with the IC_{50} value of 42.1 and 28 μM, respectively[122].

Lignan glycosides showed a moderate inhibitory effect on tyrosinase (4–5 times less than kojic acid) in the presence of L-DOPA as a substrate (4-O-lariciresinol-glucoside – 17.74% inhibition and 4′-O-lariciresinol-glucoside – 12.27% inhibition). When compared to lignan diglucoside (11.06% inhibition), they showed a lower activity, probably due to the complete absence of free hydroxyl groups (Table 17)[112].

5.2.4. Flavonolignans

Phytochemicals composed of part flavonoid and part phenylpropanoid, which are commonly found in nature. The richest source of these compounds is *Silybum marianum* from the *Asteraceae* family. They show hepatoprotective, anticancer, and anti-inflammatory effects[123].

For example, isosilybin A (IC_{50} =2.1 μM) was more effective than its three mother compounds 3′-O-methyltaxifolin (IC_{50} =51.2 μM), dihydrokaempferol (IC_{50} =73.6 μM), and taxifolin (IC_{50} =23.0 μM). Analysing the structure of the mother compounds, deletion or methylation of the 3′-OH group 2.5–3 times reduces the activity of the compounds. Silychristin A (IC_{50} =3.2 μM; IC_{50} =28.8 μM) and silychristin B (IC_{50} =4.5 μM; IC_{50} =44.9 μM) having a double bond between C-2 and C-3 more potently inhibited tyrosinase activity than 2,3-dihydrosilychristin (IC_{50} =7.6 μM; IC_{50} =35.9 μM) having no double bond. The isolated compounds showed a mixed type of inhibition (Ki: L-tyr, 0.7–4.7 μM; L-DOPA, 8.5–36.7 μM). The mother compounds inhibited the enzyme in a competent manner (Table 18)[124].

5.2.5. Stilbenes

These compounds belong to phytoalexins, low molecular weight cell components with antibacterial and antifungal properties. Besides, they show other biological properties such as antioxidant, anti-inflammatory, and antiproliferative effects. A characteristic feature of their structure is the presence of a 1,2-diphenylethylene core. More than 400 natural stilbenes have been discovered, but due to the low abundance of the critical enzyme stilbene synthase, they are not widely distributed in nature. The primary source of stilbenes in the human diet are grapes, red wine, and peanuts. The most famous representative of this group is resveratrol[125].

5.2.5.1. Hydroxyl groups. Many naturally occurring stilbenes exhibit tyrosinase inhibitory activity, that is related to the characteristic elements of their structure. The inhibitory properties are due to the number and distribution of oxygen atoms attached to the aromatic rings. Dioxyl stilbene, pinosylvin, showed weak inhibitory properties (IC_{50} =46 μM), while resveratrol, a stilbene representative with three hydroxyl groups, inhibited tyrosinase even more strongly than kojic acid. However, when compared to oxyresveratrol, representative of tetroxyl stilbenes, that compound showed a nine-fold increase in the inhibition than resveratrol (IC_{50}=1.5 *vs.* 14.4 μM)[126]. To better understand, the structure–activity relationship in a model hydroxystilbene-tyrosinase new derivatives of trans-stilbene were synthesised[127]. Monohydroxy trans stilbenes showed no inhibitory effect on tyrosinase, only after attachment of another hydroxyl group to the aromatic ring resulted in an increase of inhibition. The braking force depended on the position of the hydroxyl groups to each other, e.g. 3,3′-dihydroxy-transstilbene (26.3% inhibition IC_{50} >200 μM) has a more substantial inhibitory effect than 2,3-dihydroxy-trans-stilbene (4.4% inhibition IC_{50}>200 μM) and 3,4-dihydroxy-trans-stilbene (9.5% inhibition IC_{50}>200 μM) or 3,5-dihydroxy-trans-stilbene (18.6% inhibition IC_{50} >200 μM). The 3,3′,4-trihydroxy-trans-stilbene (87.7% inhibition IC_{50} =74.3 μM) and 3,3′,4,4′-tetrahydroxy-trans-stilbene (98.3% inhibition IC_{50} =29.1 μM) showed more potent activity against the enzyme than 3,3′-dihydroxy-trans-stilbene. 3,3,4,4′-tetrahydroxy-trans-stilbene (IC_{50} =29.1 μM) inhibited the tyrosinase activity almost completely. It is seen that an increase of the inhibitory power of the hydroxystilbenes is correlated with an increase of the number of hydroxyl groups. O-methylation decreased the action of the stilbenes.

5.2.5.2. Stilbene glycosides. Other studies have examined the difference in an action between stilbene glycosides and their aglycones. Several hydroxystilbenes were isolated from the methanolic extract of *Veratrum patulum* L. (IC_{50}=100 μM), including piceid,

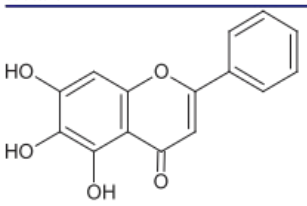
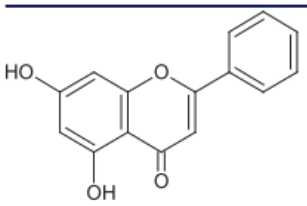
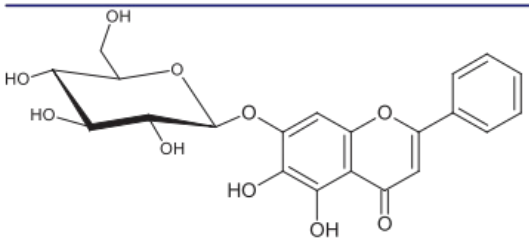
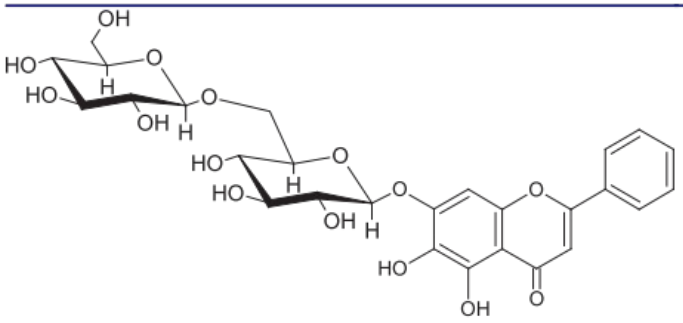
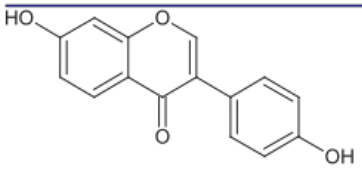
Table 13. An anti-tyrosinase activity of hydroxybenzoic and hydroxycinnaminic acids and their derivatives (NR-not reported; a-L-DOPA; b-L-tyrosin).

Substances	Substrate	Positive control (inhibitor)	Type of inhibition	Inhibition constant K_i (mM)	Source of tyrosinase	The half-maximal inhibitory concentration IC_{50}(mM)	References
Hydroxybenzoic acids							
Anisic acid (p-methoxybenzoic acid)	L- DOPA	NR	Non-competitive	0.603	Mushroom	0.6	Kubo et al.[90]
p-Hydroxybenzoic acid	L- DOPA	NR	Competitive	0.73	Mushroom	1.3	Kubo et al.[90]
p-Methoxybenzoic acid	L- DOPA	NR	Non-competitive	0.43/0.43	Mushroom	0.42	Chen et al.[92]
p-Ethoxybenzoic acid	L- DOPA	NR	Non-competitive	NR	Mushroom	NR	Kubo et al.[90]
	L- DOPA	NR	Mixed	1.46/0.84	Mushroom	1.1	Chen et al.[92]
p-Propoxybenzoic acid	L- DOPA	NR	Mixed	NR	Mushroom	NR	Kubo et al.[90]
	L- DOPA	NR	Non-competitive	0.84	Mushroom	1.85	Kubo et al.[90]
p-Butoxybenzoic acid	L- DOPA	NR	Competitive	NR	Mushroom	NR	Kubo et al.[90]
	L- DOPA	NR	Non-competitive	0.71	Mushroom	1.65	Chen et al.[92]
p-Pentyloxybenzoate acid	L- DOPA	NR	Non-competitive	0.6	Mushroom	1.4	Kubo et al.[90]
p-Hexyloxybenzoic acid	L- DOPA	NR	Non-competitive	0.49	Mushroom	1.15	Chen et al.[92]
Gallic acid	L- DOPA	NR	NR	NR	Mushroom	4.5	Kubo et al. [94]
Methyl gallate	L- DOPA	NR	NR	NR	Mushroom	0.35	Kubo et al. [94]
Propyl gallate	L- DOPA	NR	NR	NR	Mushroom	0.31	Kubo et al. [94]
Hexyl gallate	L- DOPA	NR	NR	NR	Mushroom	0.21	Kubo et al. [94]
Octyl gallate	L- DOPA	NR	NR	NR	Mushroom	0.33	Kubo et al. [94]
Decyl gallate	L- DOPA	NR	NR	NR	Mushroom	0.28	Kubo et al. [94]
Dodecyl gallate	L- DOPA	NR	Mixed	NR	Mushroom	0.49	Kubo et al. [94]
Hydroxycinnaminic acids							
Cinnamic acid	L- DOPA	NR	Non-competitive	1.99	Mushroom	2.1	Shi et al.[95]
2-Hydroxycinnamic acid	L- DOPA	NR	Not effective	Not effective	Mushroom	Not effective	Shi et al.[95]
4-Hydroxycinnamic acid	L- DOPA	NR	Competitive	0.244	Mushroom	0.5	Shi et al.[95]
4-Methoxycinnamic acid	L- DOPA	NR	Non-competitive	0.458	Mushroom	0.42	Shi et al.[95]
3,4-Dihydroxycinnamic acid	L- DOPA	NR	Non-competitive	NR	Mushroom	0.33	Garcia-Jimenez et al.[96]
4-Hydroxy-3-methoxycinnamic acid	L- DOPA	NR	Non-competitive	NR	Mushroom	0.33	Garcia-Jimenez et al.[96]
2-Methoxycinnamic acid	L- DOPA L-Tyrosine	NR	Competitive	0.51^a/0.5^b	Mushroom	NR	Garcia-Jimenez et al.[96]
3-Methoxycinnamic acid	L- DOPA L-Tyrosine	NR	Competitive	0.68^a/0.69^b	Mushroom	NR	Garcia-Jimenez et al.[96]
4-Methoxycinnamic acid	L- DOPA L-Tyrosine	NR	Competitive	1.47^a/1.54^b	Mushroom	NR	Garcia-Jimenez et al.[96]
Protocatechuic acid methyl ester	L-Tyrosine	NR	NR	NR	Mushroom	NR	Miyazawa et al.[93]
Protocatechuic acid	L-Tyrosine	NR	NR	NR	Mushroom	NR	Miyazawa et al.[93]
Vanillic acid	L-Tyrosine	NR	NR	NR	Mushroom	NR	Miyazawa et al.[93]
Vanillic acid methyl ester	L-Tyrosine	NR	NR	NR	Mushroom	NR	Miyazawa et al.[93]
Isovanillic acid	L-Tyrosine	NR	NR	NR	Mushroom	NR	Miyazawa et al.[93]
Isovanillic acid methyl ester	L-Tyrosine	NR	NR	NR	Mushroom	NR	Miyazawa et al.[93]
Veratric acid	L-Tyrosine	NR	NR	NR	Mushroom	NR	Miyazawa et al.[93]
Veratric acid methyl ester	L-Tyrosine	NR	NR	NR	Mushroom	NR	Miyazawa et al.[93]
Ferulic acid	L-Tyrosine	Hydroquinone	NR	NR	Mushroom	NR	Maruyama et al.[97]
Caffeic acid	L-Tyrosine	Hydroquinone	NR	NR	Mushroom	NR	Maruyama et al.[97]
p-Coumaric acid	L-Tyrosine	Kojic acid Arbutin	NR	NR	Mushroom	0.115	Maruyama et al.[97]

the aglycone of which is resveratrol. The inhibitory activity of piceid was 6.9 and 8.2 (L-DOPA and L-tyrosine) times lower than that of resveratrol (phenylthiourea was used as a positive control). The tested compounds showed a more significant effect on the monophenolase activity than on the diphenolase activity[128]. Kim et al. who studied the impact of mulberroside A (isolated from the ethanolic *Morus alba* L. root extract) enzymatic biotransformation to oxyresveratrol and their anti-tyrosinase activity. The inhibitory activity of oxyresveratrol was approximately 110-fold higher than that of mulberroside A (IC_{50} = 0.49 and 53.6 μM, respectively). Kojic acid and arbutin were selected as controls in the study. Inhibition of tyrosinase activity by oxyresveratrol (L-tyrosine) was 43-fold and 1503-fold higher than that of kojic acid and arbutin (IC_{50} =21.1 and 736.5 μM, respectively). Tyrosinase was almost completely inhibited by oxyresveratrol, mulberroside A, and kojic at concentrations of 2.5, 500, and 250 μM, respectively. Arbutin weakly inhibited tyrosinase (about 85%, 3000 μM). On the basis of the kinetic parameters, it has been shown that mulberroside A is a competitive inhibitor of fungal tyrosinase with L-tyrosine and L-DOPA as a substrate, oxyresveratrol showed mixed inhibition and non-competitive inhibition to L-tyrosine and L-DOPA as the substrate, respectively. Tyrosinase catalyses two different reactions: the hydroxylation of monophenols to o-diphenols (monophenolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase activity). Considering the IC_{50}(L-tyrosine 0.49; L-DOPA 11.9) and Ki (L-Tyrosine 1.093, 0.521; L-DOPA 1.272) values, oxyresveratrol had a more significant impact on the activity of monophenolase than on the diphenolase activity[129].

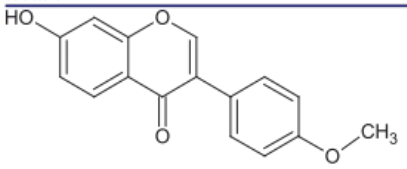
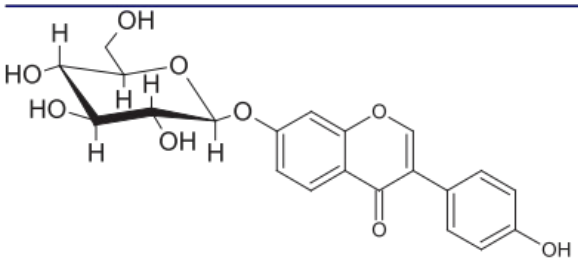
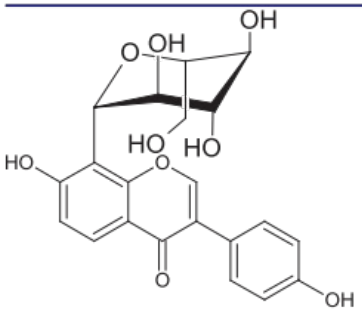
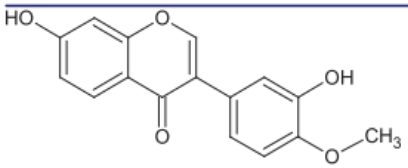
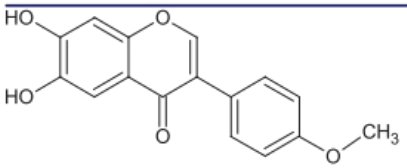
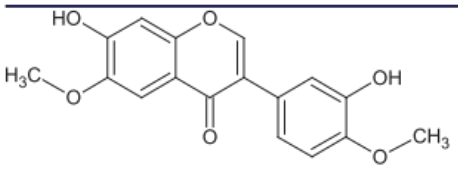
Isolated from the water-methanol extract of the rhizome of *Rheum officinale* Baill galloyl glucosides of resveratrol, e.g. 3,4′, 5-trihydroxystilbene-4′-O-β-D- (2′′-O-galloyl) glucopyranoside (A) and 3,4′, 5-trihydroxystilbene-4′-O-β-D- (6′′ -O-Galloyl) glucopyranoside (B) inhibit the activity of tyrosinase. These compounds showed a competitive type of inhibition and blocked the enzyme stronger than kojic acid. The compounds inhibited the conversion of L-tyrosine to L-DOPA more strongly than L-DOPA to DOPA quinone.

Table 14. Structure and activity of flavonoids with an anti-tyrosinase activity.

Structure	Name	IC_{50} (μM)	References
	Baicalein	290	Yin et al.[113]
	Chrysin	–	Yin et al.[113]
	Oroxin A	500	Yin et al.[113]
	Oroxin B	–	Yin et al.[113]
	Daidzein	>350[a,b]; 41[c]	Wagle et al.[105]; Kim et al.[107]

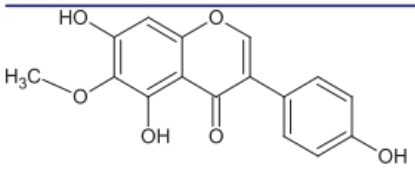
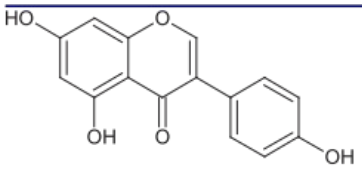
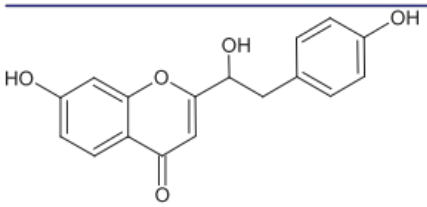
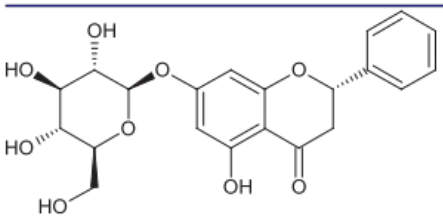
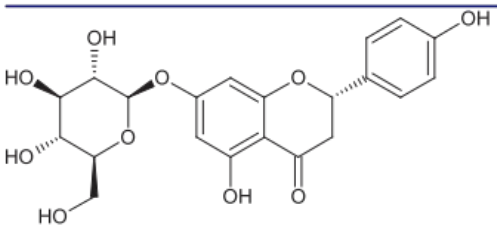
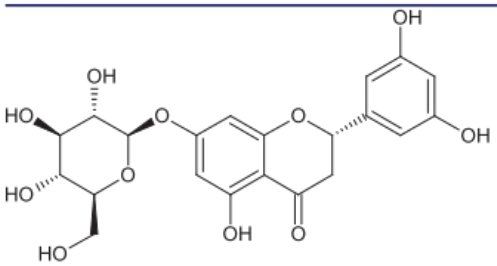
(*continued*)

Table 14. Continued.

Structure	Name	IC_{50} (μM)	References
	Formononetin	>350[a,b]; –[c]	Wagle et al.[105]; Kim et al.[107]
	Daidzin	310.67[a], >350[b]	Wagle et al.[105]
	Puerarin	438.13[a], >350[b]	Wagle et al.[105]
	Calycosin	7.02[a], 1.45[b]/38.4[d]	Wagle et al.[105]; Kim et al.[106]
	Texasin	–[c]	Kim et al.[107]
	Odoratin	–[c]	Kim et al.[107]

(*continued*)

Table 14. Continued.

Structure	Name	IC_{50} (μM)	References
	Tectorigenin	20[c]	Kim et al.[107]
	Genistein	362.54[a], >350[b]; 33[c]	Wagle et al.[105]; Kim et al.[107]
	Mirkoin	5[c]	Wagle et al.[105]; Kim et al.[107]
	(2*S*)-pinocembrin-7-*O*-*β*-D-glycoside	115.35[a], 122.34[b]	Yang et al.[152]
	(2*S*)-naringenin-7-*O*-*β*-D-glycoside	27.49[a], 39.26[b]	Yang et al.[152]
	(2*S*)-5,7,3′,5′-tetrahydroxy-flavanone 7-*O*-*β*-D-glucopyranoside	16.49[a], 20.38[b]	Yang et al.[152]

[a]L-tyrosine.[b]L-DOPA.[c]L-tyrosine for Kim et al.[108].[d]L-tyrosine for Kim et al.[107].

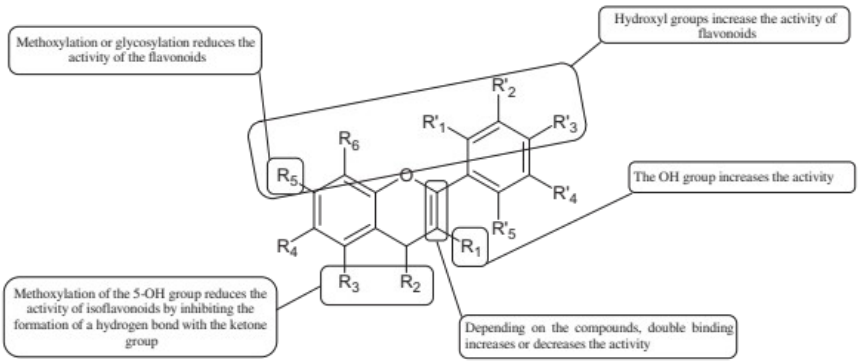


Figure 16. Potential groups engaged in an interaction flavonoid-tyrosinase.

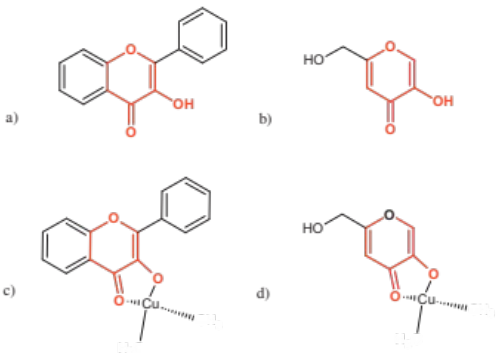


Figure 17. Structure relationship between flavanol (a) and kojic acid (b) and mode of copper chelation by 3-hydroxy-4-keto group in flavanol (c), and kojic acid (d).

Inhibitory effect for compounds A (IC_{50} =6.71 μM) and B (IC_{50} =14.7 μM) was higher than for kojic acid (IC_{50} =28.9 μM), when L-tyrosine was used as a substrate. Inhibitory effect of compound B (IC_{50} =82.3 μM) was significantly less than that of kojic acid (IC_{50} =23 μM),when L-DOPA was used as a substrate. In the case of compound A the IC_{50} value was comparable to that of kojic acid (IC_{50} =24.6 μM)[130].

The obtained results indicate that the deglycosylation of stilbenes has a positive influence on their activity and indicates that the aglycones are more active. This is probably related to particle size because as larger compounds, glycosides have restricted an access to the active site of the enzyme.

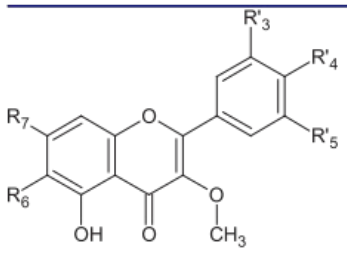
5.2.5.3. Isoprenyl chain. The inhibition of tyrosinase may also be stimulated by the compounds with isoprenyl chain in their structure, e.g. 4-[(2″E)-7″-hydroxy-3″,7″-dimethyloct-2″-enyl] – 2′,3,4′,5-tetrahydroxy-trans-stilbene (compound A) and chlorophorin, both isolated from *Chlorophora excels* (Welw.) Benth. core, showed a different inhibitory activity dependent on the presence of the isoprenyl chain. The IC_{50} values for compound A and chlorophorin were equal 96 and 1.3 μM, respectively (kojic acid, IC_{50} =20 μM). Attaching a water molecule to the geranyl chain in compound A reduced its tyrosinase activity. This is probably due to reducing the chain's interaction and the hydrophobic protein pocket close to the active site[131]. The other investigations have shown that the presence of a prenyl chain in a compound having a 4-substituted resorcinol backbone increased the inhibitory activity compared to that of oxyresveratrol (IC_{50}=0.66 and 0.98 μM, respectively).

In another study, the impact of chain length, functional groups (polarity), and cyclisation of isoprenyl chains on the anti-tyrosinase activity was investigated. An increase in chain length from one isoprene unit to two resulted in a 4-fold increase in activity (IC50 = 15.87 μM, IC50 = 60.14 μM). In turn, the introduction of hydroxyl groups or cyclisation of the isoprenyl chain caused a drastic decrease in anti-tyrosinase activity. Similar results of isoprenyl chain influence were observed in stilbene derivatives isolated from *Angelica keiskei* roots.

5.2.5.4. Double bond. Another compound inhibiting tyrosinase is gnetol, a tetrahydroxystilbene isolated from *Gnetum gnemon* L. It was appeared that gnetol is approximately 30 times more potent than kojic acid. Additionally, gnetol inhibited tyrosinase much more than dihydrognetol (100% and 20%, respectively). The double bond is crucial for the activity. The double bond's role in the stilbene backbone in inhibiting tyrosinase examined the cis-olefin structure. The cis isomer of 3,3′-dihydroxystilbene (% inhibition c. 1) compared to the trans isomer (26% inhibition) shows no inhibitory effect. The saturation of the double bond in the oxyresveratrol (IC_{50} =0.98 μM) significantly reduces the activity (IC_{50} =58 μM). However, dihydrooxyresveratrol showed eight times more inhibitory effect on the activity of fungal tyrosinase than oxyresveratrol (IC_{50} =1.6 and 12.7 μM, respectively). The higher activity of dihydrooxyresveratrol, compared to oxyresveratrol, was probably due to its dibenzyl structure, which provided greater flexibility, and thus allowed for a more effective interaction of phenolic groups with the enzyme (Figure 19)[132].

Similar results of the effect of double bond saturation were obtained by studying dihydrostilbene derivatives isolated from the 80% ethanolic extract of the *Dendrobium loddigesii* Rolfe stem. It was appeared that 3,4,5-trihydroxy-3′,4′-dihyroxyhydrostilbene; 3,5-dihydroxy-3′,4′-dihyroxyhydrostilbene and their methoxy derivatives did not inhibit tyrosinase activity. The exception to this rule is 3,5-dihydroxy-3′-dihyroxyhydrostilbene (IC_{50} = 37.90 μM). Attachment of dioxolane (aphyllals C) to the B ring of 3,5-dihyroxyhydrostilbene results in a marked increase in an activity (IC_{50} =152.56 μM). Methylation of the 3-OH group in aphyllals C abolishes the compound's activity. The above examples indicate

Table 15. Effect of the position and number of hydroxyl groups on the activity of flavonoids towards tyrosinase.



Name	R_6	R_7	R'_3	R'_4	R'_5	IC_{50} (μM)
1	OCH_3	OH	OCH_3	OCH_3	OH	6.71
2	H	OH	OCH_3	OCH_3	OH	13.20
3	H	OCH_3	OCH_3	OCH_3	OH	17.66
4	OCH_3	OH	OCH_3	OCH_3	OCH_3	62.68
5	OCH_3	OH	H	OH	H	73.03
6	H	OH	H	OH	H	103.56

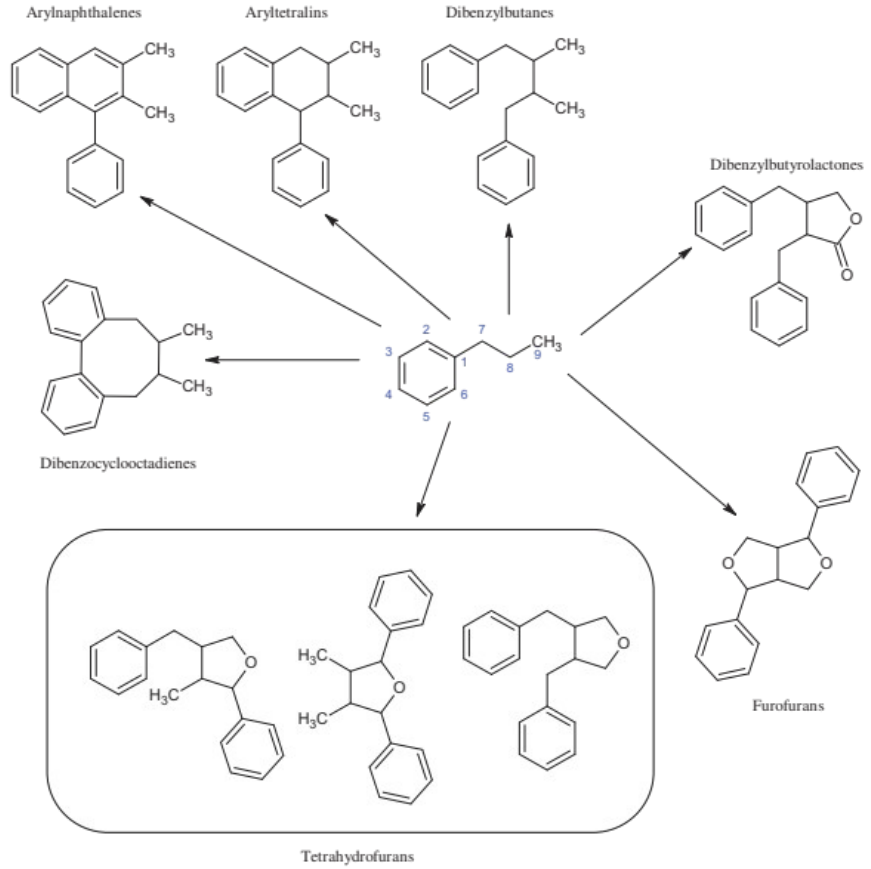


Figure 18. Chemical classification of lignans.

that methylation of the 3-OH group inactivates the compound. Kojic acid was used as a positive control (IC_{50} =8.02 μM). A noteworthy compound for (Q)SAR studies is 1,3-benzodioxol derivative (benzene ring linked to dioxolane). Due to the ambiguity of the results, further studies on the effect of a double bond in stilbenes are needed.

Table 16. An anti-tyrosinase activity of flavonoids (UE-unable to establish; c-with respect to L-tyrosine; d-with respect to both L-tyrosine and L-DOPA; NR-not reported; NT-not tested).

Substances	Substrate	Positive control (inhibitor)	Type of inhibition	Inhibition constant K_i	Source of tyrosinase	The half-maximal inhibitory concentration IC_{50} (μM)	References
FLavonols							
Galangin,	L-DOPA	Koijc acid	Competitive	NR	Mushroom	10	Fan et al.[101]
Kaempferol	L-DOPA	Koijc acid	Competitive	NR	Mushroom	73	Fan et al.[101]
Isorhamnetin	L-DOPA	Koijc acid	Mixed	NR	Mushroom	303	Fan et al.[101]
Rutin	L-DOPA	Koijc acid	Competitive	NR	Mushroom	4571	Fan et al.[101]
Myricetin	L-DOPA	Koijc acid	Mixed	NR	Mushroom	85	Fan et al.[101]
Quercetin	L-DOPA	Koijc acid	Competitive	NR	Mushroom	30	Fan et al.[101]
Morin	L-DOPA	Koijc acid	Competitive	NR	Mushroom	85	Fan et al.[101]
Diosmetin	L-DOPA	Koijc acid	Competitive	NR	Mushroom	417	Fan et al.[101]
Hyperin	L-DOPA	Koijc acid	NT	NR	Mushroom	NT	Fan et al.[101]
DIHYDROFLAVONES							
Naringenin	L-DOPA	Koijc acid	Non-competitive	NR	Mushroom	555	Fan et al.[101]
Naringin	L-DOPA	Koijc acid	Competitive	NR	Mushroom	1900	Fan et al.[101]
FLAVONES							
Chrysin	L-DOPA	Koijc acid	NR	NR	Mushroom	Non effect	Fan et al.[101]
Chrysoeriol	L-DOPA	Koijc acid	NR	NR	Mushroom	83	Fan et al.[101]
Ladanein	L-DOPA	Koijc acid	NR	NR	Mushroom	53	Fan et al.[101]
5,6,7,8,4′-Pentahydroxyflavone	L-DOPA	Koijc acid	NR	NR	Mushroom	55	Fan et al.[101]
5,6,7,4′-Tetramethylscutellarein	L-DOPA	Koijc acid	NR	NR	Mushroom	49	Fan et al.[101]
5,7,4′-Trimethylscutellarein	L-DOPA	Koijc acid	NR	NR	Mushroom	50	Fan et al.[101]
Luteolin	L-DOPA	Koijc acid	Non-competitive		Mushroom	186	Fan et al.[101]
Luteolin-7-O-glukozyd	L-DOPA	Koijc acid	NR	NR	Mushroom	74	Karioti et al.[111]
Kaempferol-3-O-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	74	Karioti et al.[111]
Isorhamnetin-3-O-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	70	Karioti et al.[111]
Kaempferol-3-O-rutinoside	L-DOPA	Koijc acid	NR	NR	Mushroom	56	Karioti et al.[111]
Isorhamnetin-3-O-rutinoside	L-DOPA	Koijc acid	NR	NR	Mushroom	53	Karioti et al.[111]
6-OH-kaempferol-3-O-rutinoside	L-DOPA	Koijc acid	NR	NR	Mushroom	55	Karioti et al.[111]
Kaempferol-3-O-(6′′-pCm)-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	11	Karioti et al.[111]
Quercetin-3-O-(6′′-pCm)-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	55	Karioti et al.[111]
Isorhamnetin-3-O-(6′′-OAc)-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	64	Karioti et al.[111]
Isorhamnetin-7-O-(6′′-pCm)-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	53	Karioti et al.[111]
Apigenin-7-O-(6′′-pCm)-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	58	Karioti et al.[111]
Apigenin-7-O-(3′′,6′′-di-pCm)-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	46	Karioti et al.[111]
Chrysoeriol-7-O-(3′′,6′′-di-pCm)-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	44	Karioti et al.[111]
Apigenin	L-DOPA	Koijc acid	Mixed	NR	Mushroom	40	Fan et al.[101]
	L-DOPA	Koijc acid	NR	NR	Mushroom	93	Karioti et al.[111]
Baicalein	L-DOPA	Koijc acid	Non-competitive	NR	Mushroom	138	Fan et al.[101]
Baicalin	L-DOPA	Koijc acid	Mixed	NR	Mushroom	215	Fan et al.[101]
DIHYDROFLAVOLS							
Dihydromyricetin	L-DOPA	Koijc acid	Mixed	NR	Mushroom	37	Fan et al.[101]
Taxifolin	L-DOPA	Koijc acid	Competitive	NR	Mushroom	800	Fan et al.[101]
ISOFLAVONOIDS							
Daidzein	L-tyrosin L-DOPA	Koijc acid	Reversibly competitive[c]	19.4	Mushroom	203/UE	Chang et al.[109]
Genistein	L-DOPA	Koijc acid	Competitive	NR	Mushroom	25	Chang et al.[109]
6,7,4′-Trihydroxyisoflavone	L-tyrosin L-DOPA	Koijc acid	Reversibly competitive[c]	1.93	Mushroom	9/UE	Chang et al.[109]
7,8,4′-Trihydroxyisoflavone	L-tyrosin L-DOPA	Koijc acid	Irreversible[d]	UE	Mushroom	191/184	Chang et al.[109]
5,7,8,4′-Tetrahydroxyisoflavone	L-tyrosin L-DOPA	Koijc acid	Irreversible[d]	UE	Mushroom	181/212	Chang et al.[109]
6-Methoxy-7,4′-dihydroxyisoflavone (glycitein)	L-tyrosin L-DOPA	Koijc acid	Reversibly competitive[c]	50.6	Mushroom	218/UE	Chang et al.[109]
4′-Hydroxyisoflavone-7-O-glucoside (daidzin)	L-tyrosin L-DOPA	Koijc acid	Reversibly competitive[c]	15.1	Mushroom	267/UE	Chang et al.[109]
5,4′-Dihydroxyisoflavone-7-O-glucoside (genistin)	L-tyrosin L-DOPA	Koijc acid	Reversibly competitive[c]	17.6	Mushroom	343/UE	Chang et al.[109]
ANTHOCYANIDINS							
Pelargonidin	L-DOPA	Koijc acid	NR	NR	Mushroom	66	Tsuda and Osawa[104]
Pelargonidln 3-O-β-D-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	61.2	Tsuda and Osawa[104]
Cyanidin	L-DOPA	Koijc acid	NR	NR	Mushroom	27.1	Tsuda and Osawa[104]
Cyanldin 3-O-β-D-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	40.3	Tsuda and Osawa[104]
Delphinldin	L-DOPA	Koijc acid	NR	NR	Mushroom	57.4	Tsuda and Osawa[104]
Delphinldin 3-O-β-D-glucoside	L-DOPA	Koijc acid	NR	NR	Mushroom	46.2	Tsuda and Osawa[104]

Table 17. An anti-tyrosinase activity of lignans.

Substances	Source of compounds	Substrate	Positive control (inhibitor)	Type of inhibition	Inhibition constant K_i (mM)	Source of tyrosinase	The half-maximal inhibitory concentration IC_{50}(μM)	References
Negundin A	*Vitex negundo*	L-DOPA	Kojic acid	NR	NR	Mushroom	10.06	Malik et al.[120]
Negundin B		L-DOPA	L-mimosine	NR	NR	Mushroom	6.72	Malik et al.[120]
6-Hydroxy-4-(4-hydroxy-3-methoxyl)-3-hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaledehyde		L-DOPA		NR	NR	Mushroom	7.81	Malik et al.[120]
Vitrofolal E		L-DOPA		NR	NR	Mushroom	9.76	Malik et al.[120]
(+)-Lyoniresinol		L-DOPA		NR	NR	Mushroom	3.21	Malik et al.[120]
(+)-Lyoniresinol-3α-O-β-D-glucoside		L-DOPA		NR	NR	Mushroom	NA	Malik et al.[120]
(+)-(−)-Pinoresinol		L-DOPA		NR	NR	Mushroom	15.13	Malik et al.[120]
(+)-Diasyringaresinol		L-DOPA		NR	NR	Mushroom	5.61	Malik et al.[120]
Eleuterozyd E_1	*Opilia amentacea*	L-DOPA	Kojic acid	NR	NR	Mushroom	28	Magid et al.[121]
4-O-Laricicresinol-glucoside	*Marrubium velutinum and*	L-DOPA	Kojic acid	NR	NR	Mushroom	64	Myose et al.[78]
4′-O-Laricitesinol-glucoside	*Marrubium cylleneum*	L-DOPA	Kojic acid	NR	NR	Mushroom	64	Myose et al.[78]
4,4′-O-Laricitesinol-bis-glucoside		L-DOPA	Kojic acid	NR	NR	Mushroom	49	Myose et al.[78]

NR: not reported; NA: not active

5.2.5.5. Stilbene oligomers. The next group with an anti-tyrosinase activity is oligomers of stilbenes. The following resveratrol oligomers: ε-viniferin as a dimer; vaticanol A, vaticanol G, and α-viniferin as trimers; vaticanol B, vaticanol C and (-)-hopeaphenol as tetramers were tested towards tyrosinase from murine B16 melanoma cells and L-DOPA as the substrate and kojic acid as a control (IC_{50} =119.7 μM; c. 49.3% inhibition). Resveratrol (IC_{50} =10.8 μM) at a concentration of 100 μM inhibited the activity of tyrosinase at the level of 98%. However, the oligomers have appeared to be weak inhibitors, e.g. dimer-ε-viniferin at a concentration of 100 μM showed approx. 24.6% inhibition, the trimmers, and tetrameters showed an inhibition level of less than 8%. It may be suggested that the inhibitory potency of the resveratrol oligomers decreases with increasing molecular weight (Figure 20; Tables 4S and Table 19)[133].

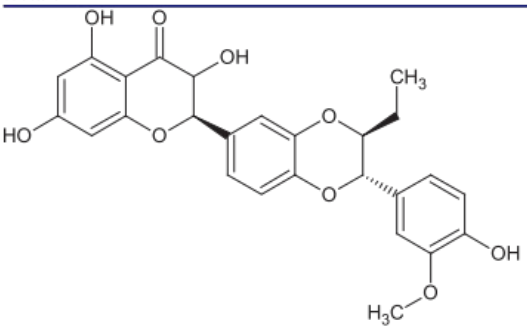
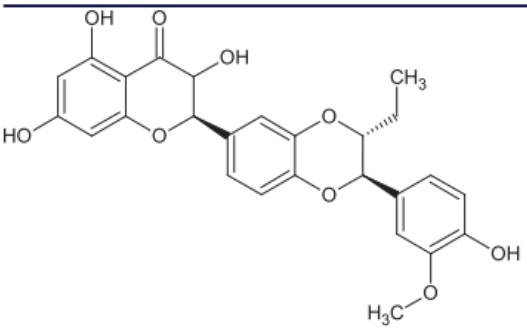
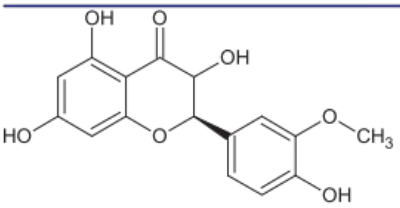
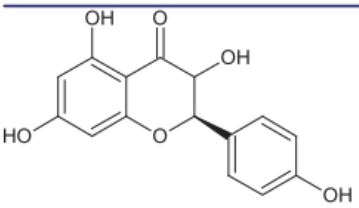
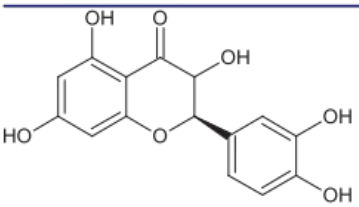
5.2.6. Chalcones

Chalcones belong to the group of unsaturated aromatic ketones and are thought to be the precursors for the synthesis of flavonoids. The core of chalcones consists of two aromatic rings linked by a three-carbon α, β-unsaturated carbonyl system (1,3-diphenyl-2-propen-1-one) (Figure 17). Chalcones can exist in trans (E) and cis (Z) forms, but cis isomers are unstable due to spherical effects. Chalcones are characterised by a broad spectrum of biological properties, including anticancer, antioxidant, antidiabetic, anti-inflammatory, antibacterial, and antiviral activities[133].

Chalcones have shown an anti-tyrosinase activity dependent on their structure and concentration. Khatib et al. studied the effect of catechol and resorcinol groupings in the A and B rings of chalcones. Two substrates for tyrosinase, L-tyrosine (first step – L-tyrosine to L-DOPA) and L-DOPA (second step – L-DOPA to o-quinone), were used. All of the compounds tested showed greater activity in blocking the first step than the second step. 3,4,2′,4′-Hydroxychalcone, with a resorcinol moiety (at the 2′ and 4′ positions) in the A ring and catechol in the B ring inhibited the first step more potently (IC_{50}=29.3 μM) than the second step (IC_{50}>100 μM). 2,4,3′,4′- Hydroxychalcone, with the opposite structure to 3,4,2′,4′- hydroxychalcone, inhibited tyrosinase about 146.5-fold more potently (IC_{50}=0.2 μM – stage 1, and IC_{50} =7.5 μM stage 2). The compound 3,5,2′,4′- hydroxychalcone, in which the OH groups of the B ring are located at positions 3 and 5 while maintaining the identical position of the catechol group as in 3,4,2′,4′- hydroxychalcone, blocked the enzyme weaker (IC_{50}=31.7 μM for step 1, IC_{50} >1000 μM for step 2). Compound 2,4,2′,4′- hydroxychalcone, made up of two resorcinol groups in rings A and B, blocked tyrosinase activity most strongly (IC_{50}=0.02 μM for stage 1 and up to 90 μM for Stage 2). Additionally, 2,4,3′,4′- hydroxychalcone is 7.5 times more active than trans-stilbene, with the same catechol and resorcinol arrangement. Moreover, the position of OH groups in the A and B ring affects the mechanism of chalcone inhibition. Compounds with a catechol group showed the ability to chelate copper ions, while 2,4,2′,4′- hydroxychalcone (resorcinol structure) did not chelate copper ions. The catechol group in ring A acted as a chelator of copper ions, while the catechol in ring B is oxidised to o-quinone. However, catechol groups in the A or B ring had no significant impact on tyrosinase inhibition. The compound with two resorcinol moieties has the most potent effect on tyrosinase activity[134].

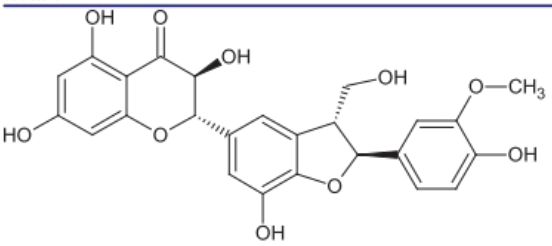
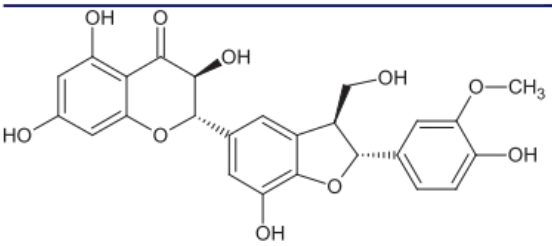
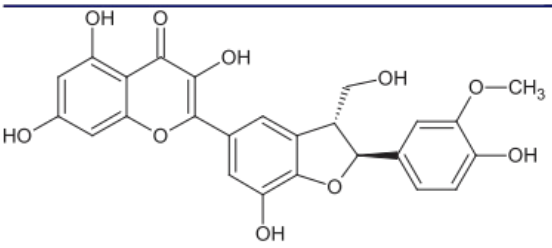
A promising group of tyrosinase inhibitors may be 2′,4′,6′-trihydroxychalcone derivatives[135]. It was confirmed, that the absence of OH groups at the 4′ or 6′ position in 2′,4′,6′-trihydroxychalcones

Table 18. An anti-tyrosinase activity of flavonolignans.

Structure	Name	IC_{50} (μM)	Ref.
	Isosilybin A	2.1[a]; 16.7[b]	Kim et al.[123]
	Isosilybin B	4.9[a]; 19.8[b]	Kim et al.[123]
	3′-O-Methyltaxifolin	51.2[a]; 150.0[b]	Kim et al.[123]
	Dihydrokaempferol	73.6[a]; >200[b]	Kim et al.[123]
	Taxifolin	23.0[a]; 27.0[b]	Kim et al.[123]

(*continued*)

Table 18. Continued.

Structure	Name	IC_{50} (μM)	Ref.
	Silychristin A	3.2[a]; 28.8[b]	Kim et al.[123]
	Silychristin B	4.5[a]; 44.9[b]	Kim et al.[123]
	Dihydrosilychristin	7.6[a]; 35.9[b]	Kim et al.[123]

[a]L-tyrosine; [b]L-DOPA.

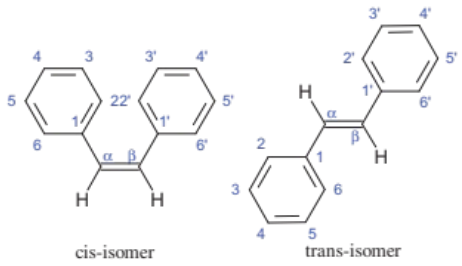


Figure 19. Chemical structure of *cis*- and *trans*-isomer of stilbenes.

results in loss of an inhibitory activity (less than 20% inhibition at 400 μM concentration). Comparing the 2′,4′,6′-trihydroxychalcone activity to kojic acid, chalcone has exhibited a 10-fold weaker inhibition (IC_{50}=120 and 12 μM, respectively). Methoxylation of hydroxyl groups at the 4′ and 6′ position of compounds: 2,2′,3,4′,6′-trihydroxychalcones, 2′,3,4,4′,5,6′-trihydroxychalcones, and 2′,3,4,4′,6′-trihydroxychalcones also cause loss of their activity. 2′,2,4,4′,6′-Trihydroxychalcones (IC_{50}=1 μM) were found to exhibit the highest activity, even better than 2,2′,4,4′-tetrahydroxychalcone (IC_{50} =5 μM) and kojic acid (IC_{50}=12 μM). In contrast, methoxylation of the 6′-OH group in 2′,2,4,4′,6′-trihydroxychalcones impairs activity (IC_{50}=3.1 μM).

Nguyen et al. provided more information about the influence of the position and number of OH groups on the activity of chalcones. It was appeared that a presence one or two hydroxyl groups in the A ring do not strength the inhibition, e.g. inhibition for 4-hydroxychalcone, 2-hydroxychalcone, and 2,4-dihydroxychalcone was about 14% at 50 μM, respectively. However, the changes had been observed after the introduction of the 4′-OH group in the B ring, which resulted in the activity's increase. The inhibition at 50 μM for 4′-hydroxychalcone, 2,4,4′-trihydroxychalcone was 71% and 67%, respectively. Additionally, the presence of the 2-OH group in the A ring drastically reduces the activity of 2,4′-dihydroxychalcone (10% inhibition at 50 μM concentration). The weakening effect of the 4′-OH group on tyrosinase activity is due to conformational changes. These changes are due to the formation of hydrogen bonds between 2-OH and the carbonyl group. The importance of the arrangement of hydroxyl groups is related to the structure of chalcones. Ring A is associated with a carbonyl carbon atom, while ring B is related to a vinyl carbon atom. This

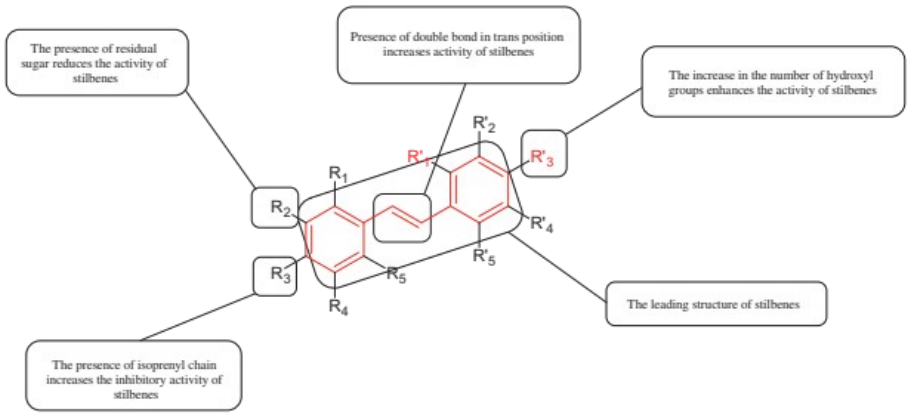


Figure 20. Potential groups engaged in an interaction stilbene-tyrosinase.

is in contrast to stilbenes, in which the rings are bound to identical carbon atoms (Figure 21)[136].

The anti-tyrosinase properties have also been confirmed in the group of the chalcone glycosides, e.g. licuraside, isoliquiritin, and the aglycone licochalcone A isolated from two species of *Glycyrrhiza* (*Glycyrrhiza uralensis* Fisch and *Glycyrrhiza inflate* Bat., respectively). The IC_{50} for licuraside, isoliquiritin, and licochalcone A, in the presence of L-tyrosine, were of 72, 38, and 25.8 μM, respectively. The compounds inhibited enzyme competitive (L-tyrosine). The inhibitory effect of chalcones on diphenolase activity (L-DOPA as substrate) was much lower. The difference in inhibition between monophenolase and diphenolase activity is related to the structure of the chalcones, those ones which inhibit monophenolase more strongly show a similarity to L-tyrosine (Figure 22)[134].

The 4-OH group in the B ring of chalcones affects the potency of the inhibitor (similarity to the tyrosine backbone). Licochalcone A is a more potent inhibitor than licurad and isoliquiritin because it has a free 4-OH group in the B ring and has less steric hindrance. The sugar residue at the 4′-OH position hinders access to the enzyme's active centre, resulting in reduced inhibitory activity. In addition, the 3,3- dimethylpropylene group at position 5 (ring B) of licochalcone A disrupts the quaternary structure of tyrosinase, inhibiting the enzyme[134].

In the case of diphenolase activity, for which the substrate is L-DOPA, the presence of both 3′-OH and 4′-OH groups in the B ring is necessary in the inhibitor's structure in order to resemble L-DOPA. The absence of the 3′-OH group in the B ring of chalcones resulted in the lack of diphenolase inhibitory activity of tyrosinase[137]. The tyrosinase activity may be also regulated *via* the introduction of alkyl chains into chalcone molecules. It has been proved that prenylated chalcone, curaridine, blocked the enzyme activity very strongly (IC_{50}=0.6 μM) compared to kojic acid (IC_{50}=20.5 μM). The important elements of the studied compound are the 2′-OH and 4′-OH groups in the B ring, and 4-OH, and the prenyl chain at C-5 (lavandulyl) in the A ring[138]. On the other hand, the addition of two isoprenyl groups at the C-5 and C-3′ positions to 4,4′,6-trihydroxychalcone abolishes tyrosinase inhibition. This is probably due to steric hindrance (Figure 23; Tables 5S and Table 20)[134].

5.2.7. Phenylpropanoid sucrose esters (PSEs)

Phenylpropanoid sucrose esters (PSEs) are composed of a sucrose core linked to one or more phenylpropanoid residues (Ph-CH = CH-CO-) *via* an ester bond. PSEs include substituted/unsubstituted caffeic, coumaric, ferulic, cinnamic, and sinapic acids. PSEs have been isolated from various species of medicinal plants in the families *Arecaceae*, *Boraginaceae*, *Brassicaceae*, *Caryophyllaceae*, *Liliaceae*, *Melanthiaceae*, *Polygonaceae*, *Poaceae*, *Polygalaceae*, *Rutaceae*, and *Rosaceae*. These compounds exhibit anti-inflammatory, antioxidant, hypoglycaemic, and anticancer activities (Figure 24)[139–141].

The PSEs isolated from *Persicaria orientalis* (L.) Spach showed low to medium tyrosinase inhibitory abilities [hydropiperoside (L-tyr, IC_{50} =27.1 μM; L-DOPA, IC_{50} =166.15 μM), vanicoside A (L-tyr, IC_{50} =37.29 μM; L-DOPA, IC_{50} =135.91 μM), vanicoside B (L-tyr, IC_{50} =62. 0 μM; L-DOPA, IC_{50} =113.13 μM), vanicoside C (L-tyr, IC_{50} =39.0 μM; L-DOPA, IC_{50} =91.38 μM), and vanicoside E (L-tyr, IC_{50} =45.23 μM; L-DOPA, IC_{50} =189.96 μM)]. In this study, kojic acid (L-tyr, IC_{50} =14.15 μM; L-DOPA, IC_{50} =181.40 μM) was used as a positive control[142]. Cho et al. investigated the inhibitory effect of PSEs isolated from the *Oryza sativa* roots on tyrosinase activity. The most active compounds were 3,6-diferuloyl-3′,6′-diacetylsucrose (IC_{50} =47.33 μM) and smilaside A (IC_{50} =45.13 μM). The IC_{50} for 3-feruloyl-4′,6′-diacetyl sucrose, 3-feruloyl-6′-acetylsucrose 3,6-diferuloylsucrose was >400 μM. None of the isolated compounds inhibited the enzyme more strongly than the positive control, kojic acid (IC_{50} =28.60 μM)[143].

Summarising results of the above studies, they indicate the significance of the presence of a feruloyl group at C-6, an acetyl group at C-6′, and another acetyl group at C-3′/C-4′ in inhibiting of the enzyme. Additionally, it seems that the introduction of additional feruloyl groups in the fructose moiety increases the activity of the compounds (Table 21).

5.3. Coumarin

Coumarins are derivatives of α-pyrone, condensed with benzene. Benzo-α-pyrone is usually substituted at C-7, less often at C-5, C-6, and C-8 positions with a hydroxyl group to which methyl groups

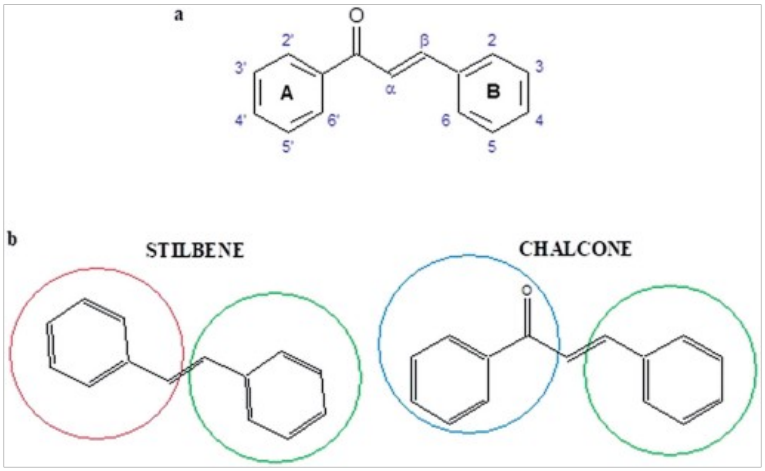


Figure 21. a) A basic structure of chalcones (1,3-diphenyl-2-propen-1-one); b) The difference in structure between stilbenes and chalcones.

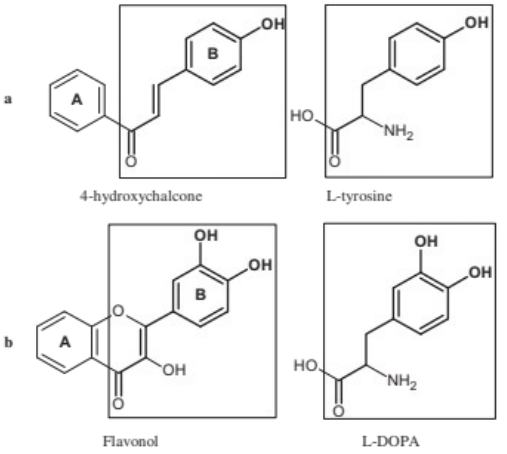


Figure 22. a) Similarity in the structure of L-tyrosine and 4-hydroxychalcones; b) Similarity in structure of L-DOPA and flavonol.

or sugar moieties may be attached. A furan or pyran ring may be condensed with the benzo-α-pyrone structure (Figure 25).

Coumarins isolated from *Euphorbia lathyris* seeds showed weak inhibitory properties against tyrosinase. The exception was esculetin, whose IC_{50} was 43 μM (kojic acid; IC_{50} =10 μM) I[144]. Different results were obtained for the constituents present in the leaf extract from *M. alba* L. Scopoletin showed the strongest anti-tyrosinase properties (IC_{50} =0.2 μM), while esculetin and scopoline appeared to be less potent with the IC_{50} equal 6.9 and 15.9 μM, respectively. All inhibitors blocked the enzyme competently[145].

Coumarin glycosides present in the *Morus nigra* roots showed weak inhibitory properties against tyrosinase, probably the presence of a sugar residue reduced the inhibitory activity[146].

Some coumarins present in *Rhododendron collettianum* inhibited tyrosinase more strongly than kojic acid (IC_{50} =16.67 μM). 8′-Epi-cleomiscosin A (IC_{50} =1.33 μM) blocked the enzyme activity most strongly. Cleomiscosin A (IC_{50} =18.69 μM) differs from 8′-epi-cleomiscosin A by the position of the proton at position 8. Due to the change in stereochemistry of the single proton, the inhibitory activity of the compounds changes drastically. This may be due to stereochemically favourable binding conditions at the enzyme active site. Aquillochin (IC_{50} =15.69 μM) and 5,6,7-trimethoxycoumarin (IC_{50} =8.65 μM) also inhibited the enzyme more strongly than caffeic acid. This study also showed a negative impact of the sugar residue on an anti-tyrosinase activity (8-O-β-D-glucopyranosyl-6-hydroxy-2-methyl-4H-1-benzopyrane-4-one; IC_{50} =256.97 μM)[147].

More information on the structure–activity relationship was provided by examining semi-synthetic/synthetic coumarin derivatives. In a study conducted by Matos et al., the effect of 3-phenylcoumarin and 3-thiophenylcoumarin derivatives on tyrosinase activity was investigated. L-DOPA was used as a substrate for the enzyme. The results showed that some synthesised derivatives exhibited an inhibitory activity against mashroom tyrosinase. The two most active compounds (5,7-dihydroxy-3–(3-thiophenyl)coumarin and 3–(4′-bromophenyl)-5,7-dihydroxycoumarin) showed tyrosinase inhibitory activity (IC_{50}=0.19 and 1.05 μM, respectively), higher than kojic acid (IC_{50}=17.90 μM). The presence of two hydroxyl groups at the C-5 and C-7 positions of the coumarin scaffold improved the inhibitory activity. The presence of the resorcinol grouping enhanced the ability to chelate copper ions[148].

In subsequent studies, the effect of the position of hydroxyl, methoxyl, ethoxyl, and bromine groups in 3-phenylcoumarin derivatives on the activity against tyrosinase was examined. 3-Phenyl-6-hydroxy-8-bromocoumarin (IC_{50} =215 μM) inhibited tyrosinase more strongly than kojic acid (IC_{50} =420 μM). In addition, the introduction of more hydroxyl groups in the coumarin grouping increased the inhibitory activity. Compared to 6-hydroxy-8-bromocoumarin (IC_{50} =302 μM), one more hydroxyl group was introduced in the 3-phenyl-6-hydroxy-8-bromocoumarin, which

Table 19. An anti-tyrosinase activity of stilbenes.

Substances	Source of compounds	Substrate	Positive control (inhibitor)	Type of inhibition	Inhibition constant K_i	Source of tyrosinase	The half-maximal inhibitory concentration IC_{50} (μM)	References
Pinosylvin	Gnetum cleistostachyum	L-tyrosine	Kojic acid	Competitive[ab]	NR	Mushroom	46.0	Likhitwitayawuid[125]
Resveratrol-4′-O-(6′′-galloyl)glucoside	Rheum officinale	L-tyrosine L-DOPA	Kojic acid	Competitive[ab]	NR	Mushroom	6.71[a] 24.6[b]	Iida et al.[129]
Resveratrol-4-O-(6′′-O-galloyl)glucoside		L-tyrosine L-DOPA	Kojic acid	Competitive[ab]	NR	Mushroom	14.7[a] 82.3[b]	Iida et al.[129]
Mulberroside A	Morus alba	L-tyrosine L-DOPA	Kojic acid, arbutin	Competitive[ab]	49.82[a] 124.764[b]	Mushroom	53.6[a]	Kim et al.[128]
Oxyresveratrol		L-tyrosine L-DOPA	Kojic acid, arbutin	Competitive – non-competitive[a] Non-competitive[b]	1.093[a] 0.521[b]	Mushroom	0.49[a] 11.9[b]	Kim et al.[128]
Resveratrol	Veratrum patulum	L-tyrosine L-DOPA	Kojic acid	NR	NR	Mushroom	43.5 123.3	Kim et al.[127]
Resveratrol	Dipterocarpaceae plants	L-DOPA	Kojic acid	NR	NR	Murine	10.8	Ohguchi et al.[131]
Piceid	Veratrum patulum	L-tyrosine L-DOPA	Phenylthiourea	NR	NR	Mushroom	>100[a] > 500[b]	Kim et al.[127]
Piceid	Dipterocarpaceae plants		Kojic acid	NR	NR	Murine	71.3	Ohguchi et al.[131]
Pinostilben (3,5-Dihydroxy-4′-methoxystilbene)	Veratrum patulum	L-tyrosine L-DOPA	Kojic acid	NR	NR	Mushroom	86.8[a]187.9[b]	Kim et al.[127]
Pterostilbene (3,4′-Dimethoxy-5-hydroxystilbene)		L-tyrosine L-DOPA	Kojic acid	NR	NR	Mushroom	>100[a] 398.3[b]	Kim et al.[127]
Rhaponticin		L-tyrosine L-DOPA	Kojic acid	NR	NR	Mushroom	>100[a] >500[b]	Kim et al.[127]
Chlorophorin	Chlorophora excelsa	L-DOPA	Kojic acid	Competitive	NR	Mushroom	1.3	Shimizu et al.[130]
Chlorophorin	Artocarpus incisus	L-tyrosine	Kojic acid	Competitive[b]	>13.4[b]	Mushroom	0.26	Shimizu et al.[130]
4-Prenyloxyresveratrol		L-tyrosine	Kojic acid	Competitive[b]	8.7[b]	Mushroom	0.66	Shimizu et al.[130]
4-[(2″E)-7″-Hydroxy-3″,7″-dimethyloct-2″-enyl]-2′,3,4′,5-tetrahydroxy-trans-stilbene	Chlorophora excelsa	L-DOPA	Kojic acid	NR	NR	Mushroom	96.0	Shimizu et al.[130]
Gnetol	Gnetum gnemon	L-DOPA	Kojic acid	NR	NR	Murine		Ohguchi et al.[126]
Dihydrooxyresveratrol		L-tyrosine	Kojic acid	Competitive		Mushroom	58.0	Shimizu et al.[130]
Vaticanol A	Dipterocarpaceae plants	L-DOPA	Kojic acid	NR	NR	Murine	NR	Ohguchi et al.[131]
Vaticanol G		L-DOPA	Kojic acid	NR	NR	Murine	NR	Ohguchi et al.[131]
α-Viniferin		L-DOPA	Kojic acid	NR	NR	Murine	NR	Ohguchi et al.[131]
Vaticanol B		L-DOPA	Kojic acid	NR	NR	Murine	NR	Ohguchi et al.[131]
Vaticanol C		L-DOPA	Kojic acid	NR	NR	Murine	NR	Ohguchi et al.[131]
(−)-Hopeaphenol		L-DOPA	Kojic acid	NR	NR	Murine	NR	Ohguchi et al.[131]

[a]L-tyrosine; [b]L-DOPA; NR: not reported.

Table 20. An anti-tyrosinase activity of chalcones.

Substances	Substrate	Positive control (inhibitor)	Type of inhibition	Inhibition constant K_i	Source of tyrosinase	The half-maximal inhibitory concentration IC_{50} (μM)	References
3,4,2′,4′-Hydroxychalcone	L-tyrosine	Kojic acid	NR	NR	Mushroom	29.3[a]/>100[b]	Khatib et al.[133]
2,4,3′,4′-Hydroxychalcone	L-DOPA	Kojic acid	NR	NR	Mushroom	0.2[a]/>7.5[b]	Khatib et al.[133]
3,4,2′,4′-Hydroxychalcone		Kojic acid	NR	NR	Mushroom	31.68[a]/>1000[b]	Khatib et al.[133]
2,4,2′,4′-Hydroxychalcone		Kojic acid	NR	NR	Mushroom	0.02[a]/90[b]	Khatib et al.[133]
2′,4′,6′-Trihydroxychalcone	L-tyrosine	Kojic acid	NR	NR	Mushroom	120	Jun et al.[134]
2′,4′-Dihydroxychalcone		Kojic acid	NR	NR	Mushroom	NA	Jun et al.[134]
2′,6′-Dihydroxychalcone		Kojic acid	NR	NR	Mushroom	NA	Jun et al.[134]
2′,3,4,4′,6′-Pentahydroxychalcone		Kojic acid	NR	NR	Mushroom	193	Jun et al.[134]
2′,3,4,4′,5,6′-Hexahydroxychalcone		Kojic acid	NR	NR	Mushroom	200	Jun et al.[134]
2′,3,4,4′,6′-Pentahydroxychalcone		Kojic acid	NR	NR	Mushroom	NA	Jun et al.[134]
3,3′,4,4′-Tetrahydroxychalcone		Kojic acid	NR	NR	Mushroom	NA	Jun et al.[134]
2′,3,4-Trihydroxychalcone		Kojic acid	NR	NR	Mushroom	NA	Jun et al.[134]
2′,4′,6′-Trihydroxy-3,4-dimethoxychalcone		Kojic acid	NR	NR	Mushroom	150	Jun et al.[134]
2,2′,3-Trihydroxy-4′,6′-dimethoxychalcone		Kojic acid	NR	NR	Mushroom	NA	Jun et al.[134]
2′,3,4,5,-Tetrahydroxy-4′,6′-dimethoxychalcone		Kojic acid	NR	NR	Mushroom	NA	Jun et al.[134]
2′,3,4-Trihydroxy-4′,6′-dimethoxychalcone		Kojic acid	NR	NR	Mushroom	NA	Jun et al.[134]
2,2′,4,4′-Tetrahydroxychalcone		Kojic acid	NR	NR	Mushroom	5	Jun et al.[134]
2,2′,4,4′-Tetrahydroxy-6′-methoxychalcone		Kojic acid	NR	NR	Mushroom	3.1	Jun et al.[134]
2,2′,4,4′,6′-Pentahydroxychalcone		Kojic acid	Competitive	3.1	Mushroom	1	Jun et al.[134]
Licochalcone A	L-DOPA	Kojic acid	Competitive	NR	Mushroom	25.8	Fu et al.[136]
Licuraside		Kojic acid	Competitive	NR	Mushroom	72	Fu et al.[136]
Isoliquiritin		Kojic acid	Competitive	NR	Mushroom	38	Fu et al.[136]

[a]L-tyrosine; [b]L-DOPA; NR: non reported; NA: non active

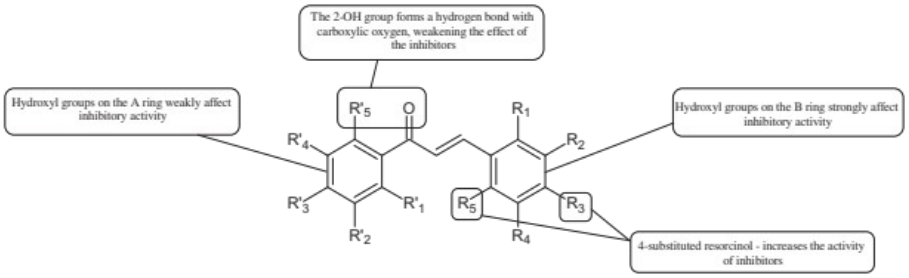


Figure 23. Potential groups engaged in an interaction chalcone-tyrosinase.

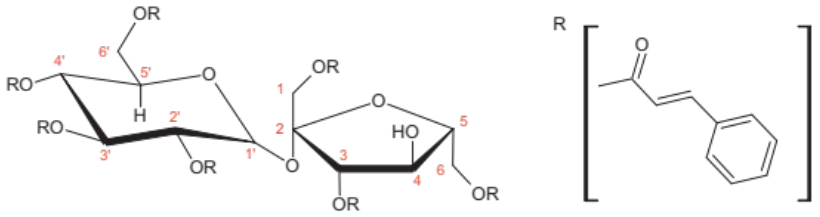


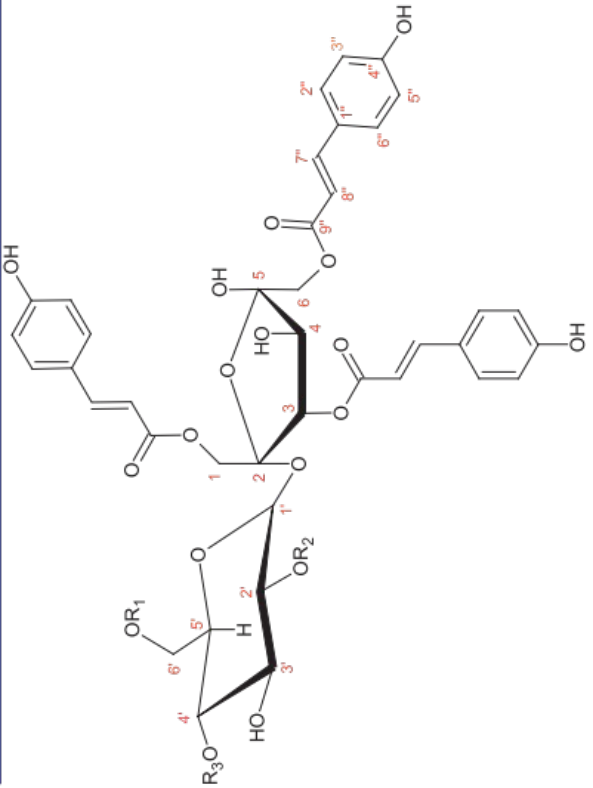
Figure 24. Structure of Phenlpropanoid Sucrose Esters – PSEs (R: phenylpropanoid residues).

improved the inhibitory activity about 1.5 times. A bromine substituent and a C-4′ hydroxyl group at the C-6 positions increase the inhibitory activity. In turn, methoxy and ethoxy derivatives weakly inhibited the enzyme. It seems that brominated hydroxycoumarin derivatives may be promising inhibitors of tyrosinase[149].

In the study of Asthan et al., the effect of the position of the hydroxyl group in benzo-α-pyrone on tyrosinase activity was checked. The position of the hydroxyl group at C-6 and C-7 causes the molecule to behave as a weak substrate for the enzyme. This is related to the interaction of the hydroxyl group with the copper ion in the enzyme's active centre, which means that the compounds with the OH group in the pyrone ring cannot be substrates for tyrosinase. Among investigated compounds, only 3-hydroxycoumarin inhibited the enzyme activity. The studies indicate the possibility of application of 3-hydroxycoumarin structure as a new class of tyrosinase inhibitors[150].

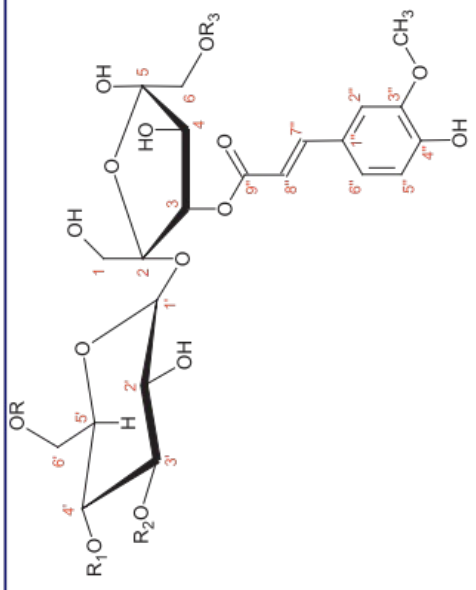
In another study, thiosemicarbothioamide derivatives, such as 2–(1-(coumarin-3-yl)ethylidene)hydrazinecarbothioamide inhibited

Table 21. Structure and an anti-tyrosinase activity of phenylpropanoid sucrose esters.

Structure	Name	R_1	R_2	R_3	IC_{50} (μM)	Ref.
	Hydropiperoside	H	H	H	27.1[a]/166.15[b]	Masum et al.[141]
	Vanicoside A	feruloyl	Ac	H	37.29[a]/135.91[b]	Masum et al.[141]
	Vanicoside B	feruloyl	H	H	62.0[a]/113.13[b]	Masum et al.[141]
	Vanicoside C	H	Ac	H	39.0[a]/91.38[b]	Masum et al.[141]
	Vanicoside E	feruloyl	Ac	Ac	45.23[a]/181.40[b]	Masum et al.[143]

(continued)

Table 21. Continued.

Structure	Name	R_1	R_2	R_3	IC_{50} (μM)	Ref.
	3-Feruloyl-4′,6′-diacetyl sucrose	Ac	Ac	H	>400	Cho et al.[142]
	3,6-Diferuloyl-3′,6′′-diacetylsucrose	Ac	H	Ac	feruloyl	47.33	Cho et al.[142]
	Smilaside A	Ac	Ac	H	feruloyl	45.13	Cho et al.[142]
	3,6-Diferuloyl-6′-acetylsucrose	Ac	H	H	feruloyl	372.6	Cho et al.[142]
	3-Feruloyl-6′-acetylsucrose	Ac	H	H	H	>400	Cho et al.[142]
	3,6-Diferuloylsucrose	H	H	H	feruloyl	>400	Cho et al.[142]

[a]L-tyrosine; [b]L-DOPA.

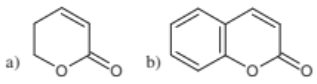


Figure 25. Structure of α-piron (a); benzo-α-piron (b).

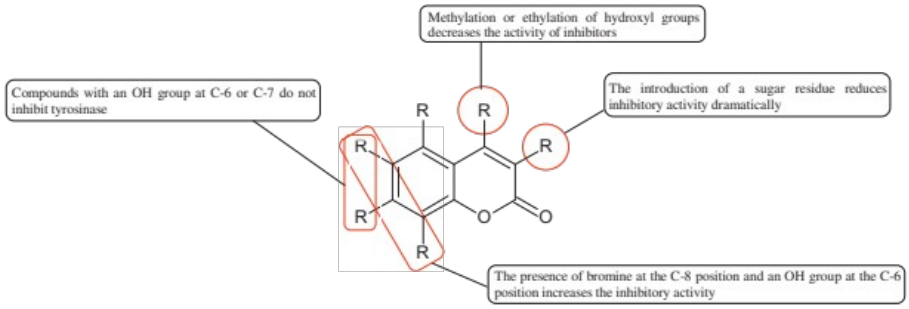
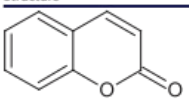
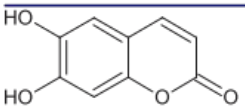
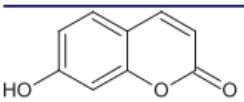
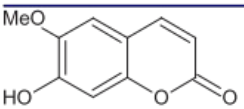
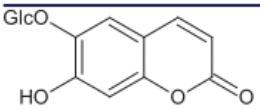
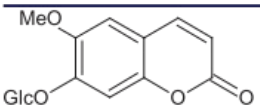


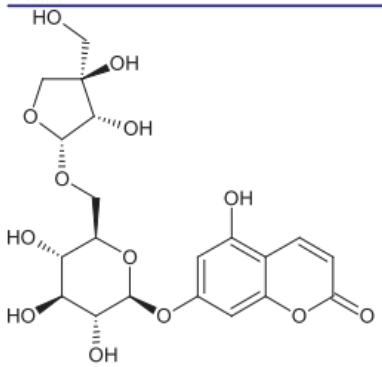
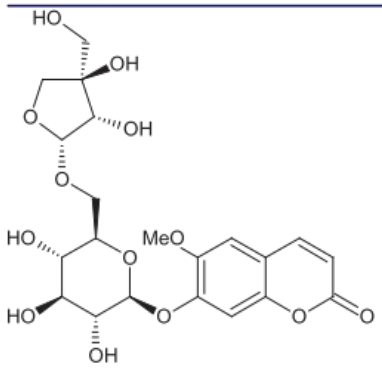
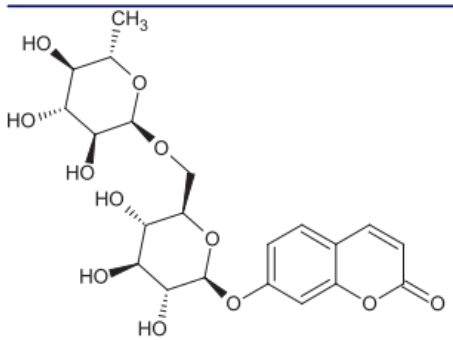
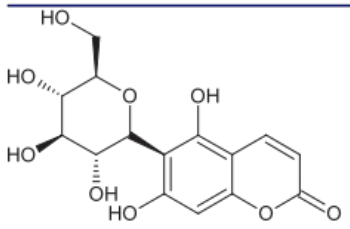
Figure 26. Potential groups engaged in an interaction coumarin-tyrosinase.

Table 22. Structure and activity of coumarin against tyrosinase.

Structure	Name	IC_{50} (μM)	Ref.
	Coumarin	8100	Masamoto et al.[143]
	Esculetin	43/6.9	Masamoto et al.[143]; Li et al.[144]
	Umbelliferone	420	Masamoto et al.[143]
	Scopoletin	2600/0.2	Masamoto et al.[143]; Li et al.[144]
	Esculin	>14000	Masamoto et al.[143]
	Scopoline	15.9	Li et al.[144]

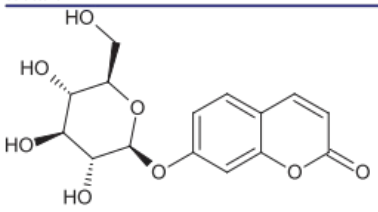
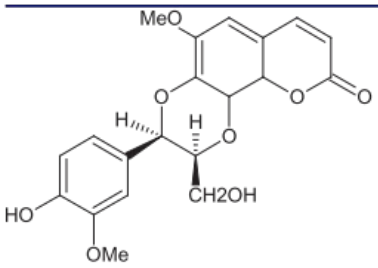
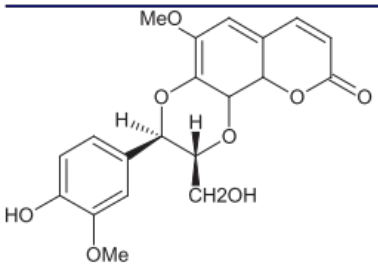
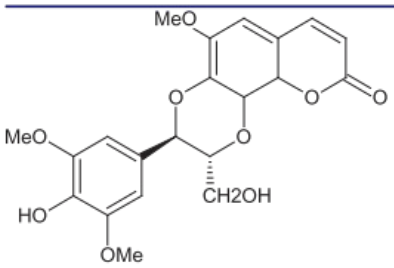
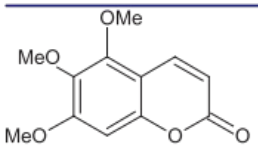
(*continued*)

Table 22. Continued.

Structure	Name	IC_{50} (μM)	Ref.
	5,7-dihydroxycoumarin-7-(6-O-β-D-apiofuranosyl-β-Dglucopyranoside)	>400	Zheng et al.[145]
	xeroboside	>400	Zheng et al.[145]
	7-[[6-O-(6-deoxy-R-L-mannopyranosyl)-β-Dglucopyranosyl]oxy]-2H-1-benzopyran-2-one	>400	Zheng et al.[145]
	mulberroside B	>500	Zheng et al.[145]

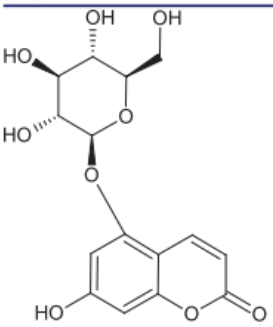
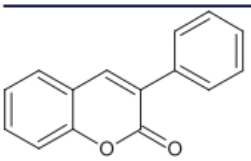
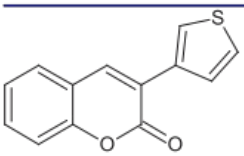
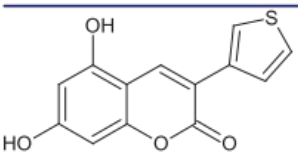
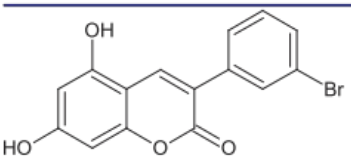
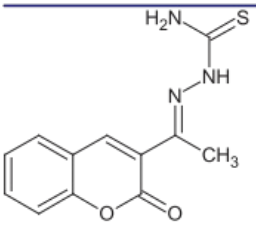
(*continued*)

Table 22. Continued.

Structure	Name	IC_{50} (μM)	Ref.
	5,7-dihydroxycoumarin-7-O-β-D-glucopyranoside	>400	Zheng et al.[145]
	8′-epi-cleomiscosin A	1.33	Ahmad et al.[146]
	Cleomiscosin A	18.69	Ahmad et al.[146]
	aquillochin	15.69	Ahmad et al.[146]
	5,6,7-trimethoxycoumarin	8.65	Ahmad et al.[146]

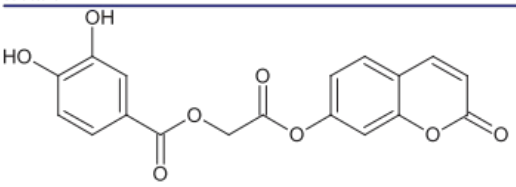
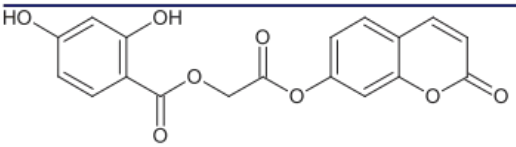
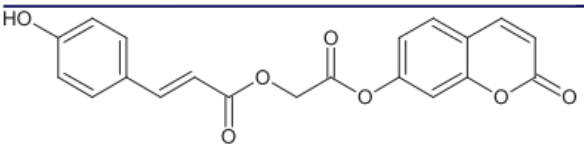
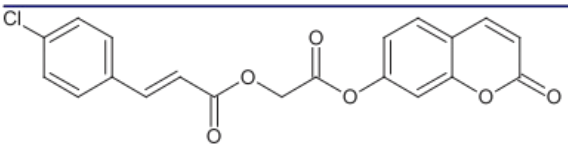
(*continued*)

Table 22. Continued.

Structure	Name	IC_{50} (μM)	Ref.
	8-O-b-Dglucopyranosyl-6-hydroxy-2-methyl-4H-1-benzopyrane-4- one	256.97	Ahmad et al.[146]
	3-Phenylcoumarin	>1000	Matos et al.[147]
	3-Thiophenylcoumarin	>1000	Matos et al.[147]
	5,7-Dihydroxy-3-(3-thiophenyl)coumarin	0.19	Matos et al.[147]
	3-(4′-Bromophenyl)-5,7-dihydroxycoumarin	1.05	Matos et al.[147]
	2-(1-(Coumarin-3-yl)ethylidene) hydrazinecarbothioamide	3.44	Liu et al.[150]

(*continued*)

Table 22. Continued.

Structure	Name	IC_{50} (μM)	Ref.
	4c	118.48	Ashraf et al.[151]
	4e	8.96	Ashraf et al.[151]
	6a	123.77	Ashraf et al.[151]
	6b	80.20	Ashraf et al.[151]

tyrosinase stronger than kojic acid with the IC_{50} values 3.44 and 23.0 μM, respectively. The compound blocked the enzyme irreversibly[151].

Ashraf et al. synthesised several umbelliferone derivatives and studied their effects on tyrosinase. Compounds 4e and 4c, having 2,4-dihydroxy and 3,4-dihydroxyphenyl group, inhibited fungal tyrosinase most potently with the IC_{50} values 8.96 and 118.48 μM, respectively. The other umbelliferone derivatives showed weak activity against mashroom tyrosinase compared to kojic acid (IC_{50} = 16.69 μM). On the other hand, umbelliferone derivatives obtained in this study inhibited tyrosinase more potently than umbelliferone (IC_{50} = 420 μM) (Figure 26; Table 22)[152].

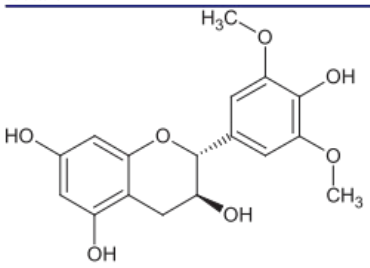
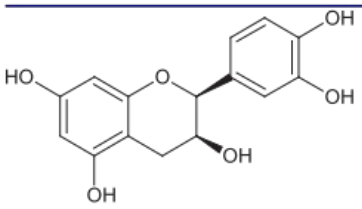
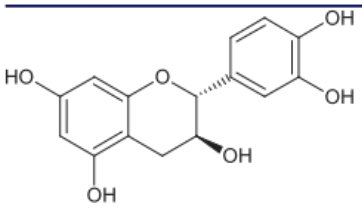
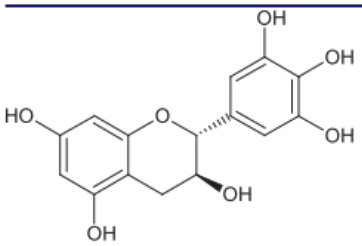
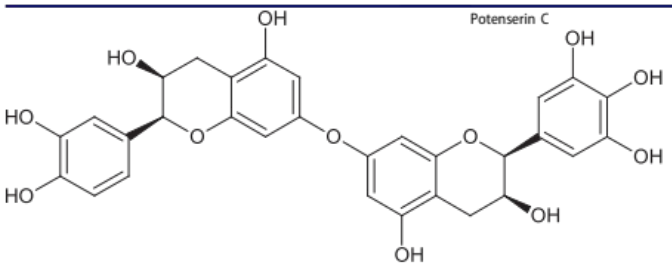
5.4. Tannins

Another group of natural compounds with tyrosinase inhibitory potential is tannins. The flavan-3-ol derivatives isolated from *Potrntilla anserina* L. rhizome showed interesting inhibitory properties against tyrosinase. In both cases, the compounds inhibited diphenolases more strongly than monophenolases. Flavan-3-ol dimers (potenserin C – L-tyr IC_{50} = 16.53 μM, L-DOPA IC_{50} = 2.63 μM; gallocatechin-(4′→O → 7)-epigallocatechin-L-tyr IC_{50} = 18. 55 μM, L-DOPA IC_{50} = 6.62 μM; bis-6,8′-catechinylmethane – L-tyr IC_{50} = 30.56 μM, L-DOPA IC_{50} = 12.26 μM; bis-8,8′-catechinylmethane – L-tyr IC_{50} = 33. 89 μM, L-DOPA IC_{50} = 15.11 μM; catechin (4α → 8) catechin – L-tyr IC_{50} = 39.09 μM, L-DOPA IC_{50} = 25.49 μM; catechin (4α → 8) epicatechin – L-tyr IC_{50} = 37.22 μM, L-DOPA IC_{50} = 21.76 μM) showed similar or stronger inhibitory properties than kojic acid (L-tyr IC_{50} = 48.55 μM, L-DOPA IC_{50} = 21.00 μM). The presence of an additional OH group at the C-3′ position of catechins (L-tyr IC_{50} = 65.26 μM, L-DOPA IC_{50} = 42.71 μM) enhanced the anti-tyrosinase properties (gallocatechin – L-tyr IC_{50} = 41.96 μM, L-DOPA IC_{50} = 30.11 μM). In turn, methylation of the 3′-OH and 5′-OH groups in (2 R, 3S)- 3′, 5′-dimethoxy gallocatechin decreases the inhibitor activity[153]. A similar result was obtained by testing proanthocyanidin oligomers (OPC) from red wine (*Vitis vinifera*) for tyrosinase activity at a concentration of 1 mM. Procyanidin dimers as procyanidin B-3 (69.6% inhibition) and B-4 (69.4% inhibition), which possess (+)-catechin, more strongly blocked the enzyme than procyanidin B-1 (16.7% inhibition) and B-2 (26.0% inhibition). The IC_{50} values for PB3 and PB4 were 545 and 726 μM, respectively. The trimeric OPCs showed an inhibition rate of 25.3–31.1% at 1 mM, meaning no significant differences between the trimeric proanthocyanidins[154].

The procyanidin epicatechin-(4β→8, 2β→O → 7)-epicatechin-(4β→8)-epicatechin isolated from *Guioa villosa* and procyanidin B1 have shown a minimal effect on tyrosinase activity[155,156].

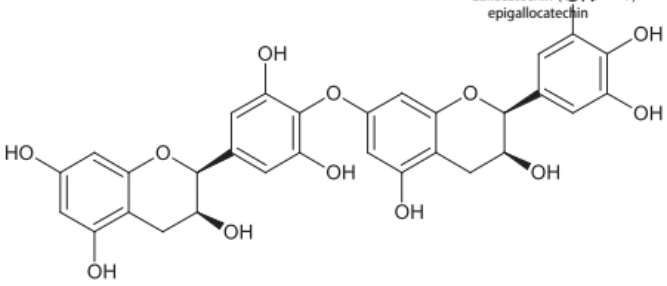
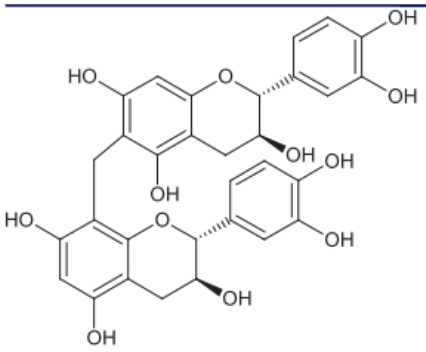
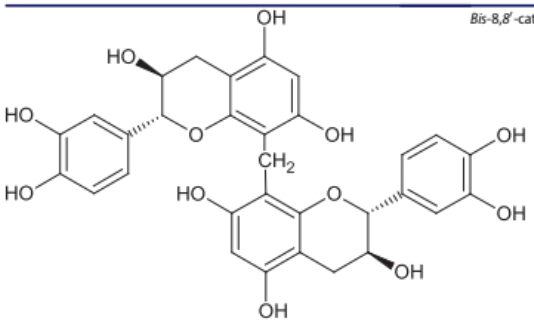
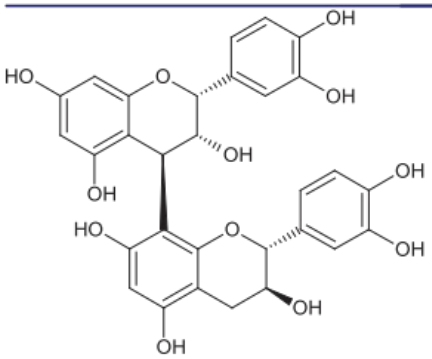
Another study examined the effect of tannins isolated from the methanolic extract of *Ecklonia stolonifera*. The extract contained five phlorotannins: phloroglucinol, eckstolonol, eckol, phlorofucofuroeckol A, and dieckol. Particularly noteworthy was dieckol (IC_{50} = 2.16 μg/mL), which blocked the enzyme more strongly than kojic acid (IC_{50} = 6.32 μg/mL) and arbutin (IC_{50} = 112.0 μg/mL). Phloroglucinol and eckstolonol showed the competent type of inhibition. Eckol, phlorofucofuroeckol A, and dieckol inhibited the enzyme incompetently. The isolated phlorotannin derivatives owe

Table 23. Structure and activity of tannin against tyrosinase.

Structure	Name	IC_{50} (μM)	Ref.
	(2R, 3S)- 3′, 5′-Dimethoxy gallocatechin	47.33[a], 35.79[b]	Yang et al.[152]
	Epicatechin	73.88[a], 48.98[b]	Yang et al.[152]
	Catechin	65.26[a], 42.71[b]	Yang et al.[152]
	Gallocatechin	41.96[a], 30.11[b]	Yang et al.[152]
	Potenserin C	16.53[a], 2.63[b]	Yang et al.[152]

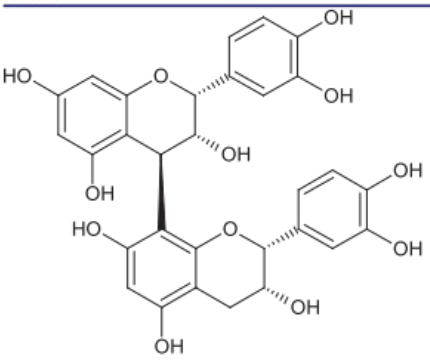
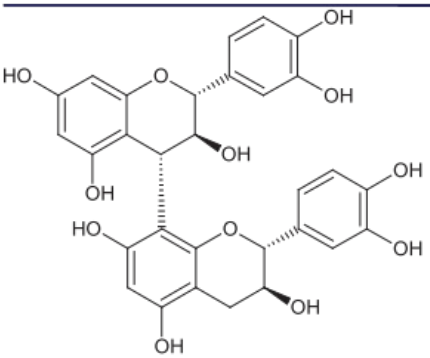
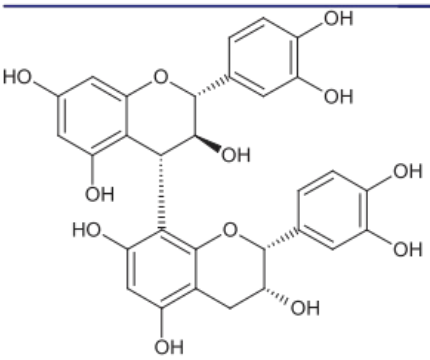
(*continued*)

Table 23. Continued.

Structure	Name	IC_{50} (μM)	Ref.
	Gallocatechin-(4β → 7)-epigallocatechin	18.55[a], 6.62[b]	Yang et al.[152]
	*Bis*-6,8′-catechinylmethane	30.56[a], 12.26[b]	Yang et al.[152]
	*Bis*-8,8′-catechinylmethane	33.88[a], 15.11[b]	Yang et al.[152]
	Epicatechin-(4β→8)-catechin - Procyanidin B-1	>346[a,b]	Momtaz et al.[154]

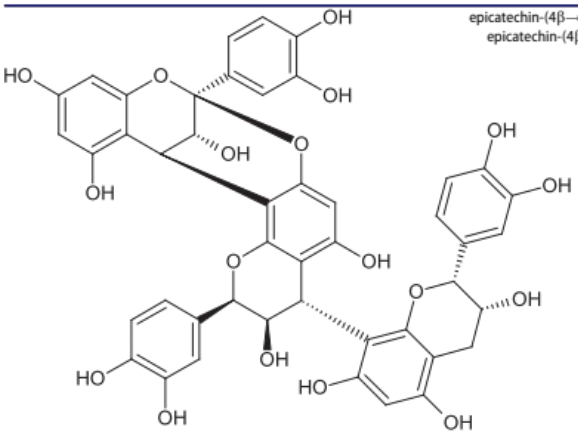
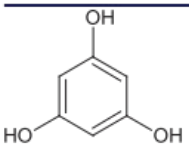
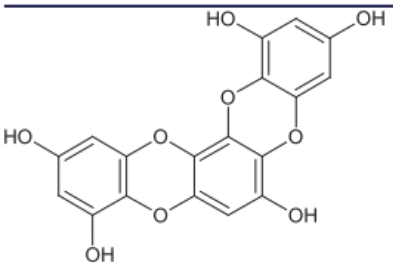
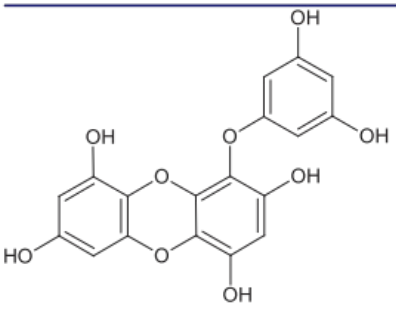
(*continued*)

Table 23. Continued.

Structure	Name	IC_{50} (μM)	Ref.
	(−)-Epicatechin-(4β→8)-(−)-epicatechin Procyanidin B-2	–	Fujimaki et al.[153]
	Catechin (4α → 8) catechin - procyanidin B-3	39.09[a], 25.49[b]	Yang et al.[152]
	Catechin (4α → 8) epicatechin - procyanidin B-4	37.22[a], 21.76[b]	Yang et al.[152]

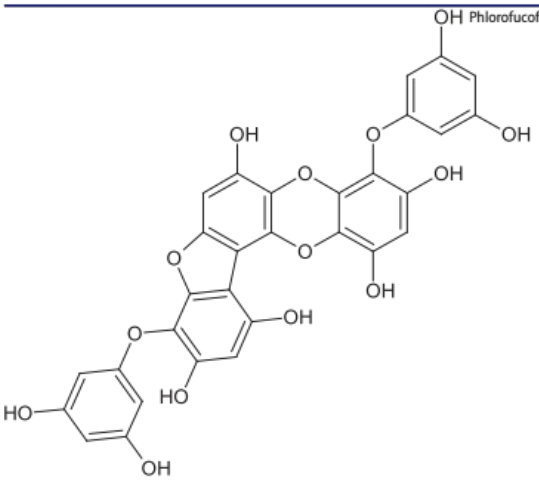
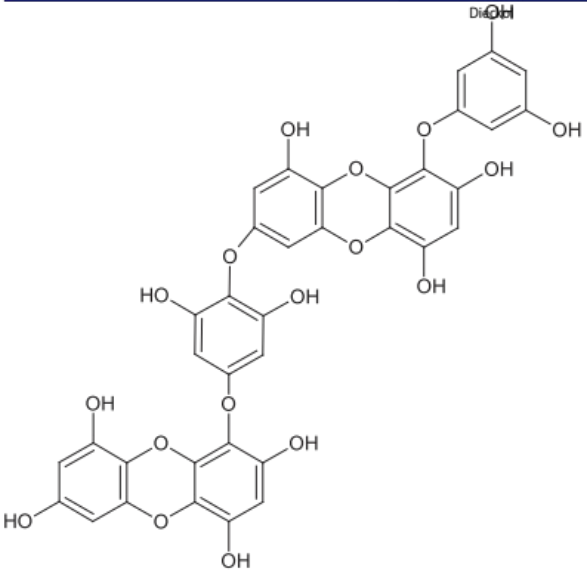
(*continued*)

Table 23. Continued.

Structure	Name	IC_{50} (μM)	Ref.
	epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatechin	nd	Fujimaki et al.[153]
	Phloroglucinol	92.8 μg/mL	Kang et al.[156]
	Eckstolonol	126.0 μg/mL	Kang et al.[156]
	Eckol	33.2 μg/mL	Kang et al.[156]

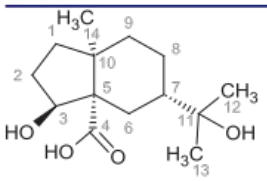
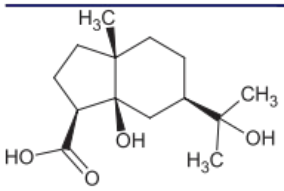
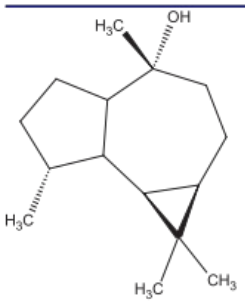
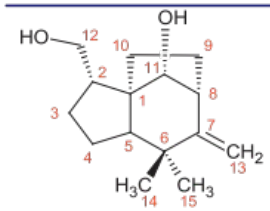
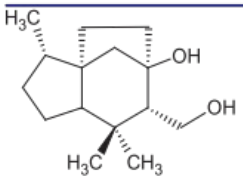
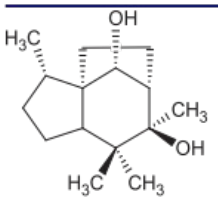
(*continued*)

Table 23. Continued.

Structure	Name	IC_{50} (μM)	Ref.
	Phlorofucofuroeckol A	177.0 μg/mL	Kang et al.[156]
	Dieckol	2.16 μg/mL	Kang et al.[156]

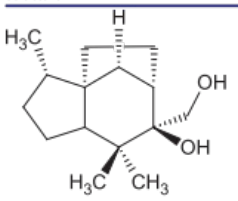
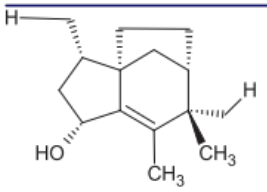
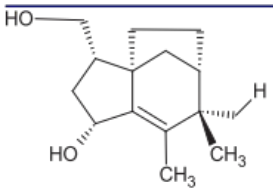
[a]L-tyrosine.[b]L-DOPA.

Table 24. Structure and an anti-tyrosinase activity of terpenes.

Structure	Name	IC_{50} (μM)	Ref.
	3-β-11-dihydroxyisoiphion-4-one	14.17	Lin et al.[158]
	5-β-11-dihydroxy-iphionan-4-one	10.08	Lin et al.[158]
	(-)-globulol	9.79	Lin et al.[158]
	Agarozizanol A	–	Yang et al.[159]
	Agarozizanol B	–	Yang et al.[159]
	Agarozizanol C	–	Yang et al.[159]

(*continued*)

Table 24. Continued.

Structure	Name	IC_{50} (μM)	Ref.
	Agarozizanol D	–	Yang et al.[159]
	Agarozizanol E	–	Yang et al.[159]
	Agarozizanol F	–	Yang et al.[159]

their properties to the presence of a resorcinol group, which can chelate copper in the active tyrosinase center[157].

Shoji et al. examined the effect of procyanidin polymerisation on tyrosinase activity. All oligomer fractions (1mer to 7mer) strongly inhibited tyrosinase activity, as did kojic acid. The IC_{50} values were similar for all groups: monomer, 74 μM; dimer, 235 μM; trimmer, 140 μM; tetramer, 149 μM; pentamer, 184 μM; hexamer, 127 μM; and heptamer 103 μM. No correlation was observed between the degree of procyanidin polymerisation and tyrosinase inhibition. Nevertheless, these observations suggest that procyanidins are effective inhibitors of tyrosinase (Table 23)[158].

5.5. Terpenes

A very promising group of tyrosinase inhibitors are terpenes, for which that activity was proved by Lin et al. who examined the effect of terpenes isolated from the *Eucalyptus globulus* Labill leaves. Among few tested terpenes, the sesquiterpene, (-)-globulol (IC_{50} = 9.79 μM) showed the strongest inhibition, followed by the isoiphionane sesquiterpene derivatives, 5-β-11-dihydroxy-iphionan-4-one (IC_{50} = 10.08 μM) and 3-β-11-dihydroxyisoiphion-4-one (IC_{50} = 14.17 μM). They have appeared to be more effective inhibitors than kojic acid (IC_{50} = 17.32 μM)[159]. Isolated from the Aquilaria genus prezizane-type sesquiterpenes, agarosanol A–F weakly inhibited tyrosinase at a concentration of 100 μM (less than 30%) (Table 24)[160].

6. Conclusion and future perspectives

Despite the fact that the plant-derived substances have always been a mainstay in medical treatment, the use of plants as a source of new drugs is still poorly studied. Of the 250,000–500,000 plant species, only a small number have been adequately studied. Besides the possibility of a direct use of plant materials in medicine, isolated substances can be used as a source of the structures for the synthesis of new drugs.

Commercially available Hyal and tyrosinase inhibitors are characterised by a range of adverse properties. Tyrosinase overactivity can be reduced by inhibitors such as hydroquinone, arbutin, vitamin C, azelaic acid, kojic acid, and ellagic acid. However, those inhibitors have many side effects, e.g. hydroquinone, the most commonly used inhibitor, shows mutagenic and irritating effects (dermatitis and irritation), while arbutin, a prodrug of hydroquinone, is a chemically unstable compound. Kojic acid is carcinogenic, L-AA is quickly degraded, ellagic acid, due to its poor solubility, shows a low bioavailability. Therefore, it is necessary to search for new safe inhibitors[161–165].

One of the Hyal inhibitors available for treatment is escin (aescin). Unfortunately, due to its physicochemical properties, the compound is absorbed orally only to a small extent[166].

From this review, it is clear that currently used tyrosinase and Hyal inhibitors are not without their drawbacks, therefore, there is a great need to search for the new inhibitors with more valuable pharmacological properties. In the further bioprospecting, the following criteria should be considered:

1. Examine the effect of extracts and newly isolated compounds on cell cultures to determine cytotoxicity. Some compounds, e.g. alkaloids or terpenes, may exhibit toxic effects. Therefore, early determination of their toxicity is necessary. In addition, the study of new inhibitors using cell cultures will allow us to initially determine the metabolic pathways and mode of absorption (active or passive transport) of the tested compounds.
2. To better understand the utility of new substances in inhibiting Hyal or tyrosinase, more studies using animal models should be planned to determine the safety and efficacy of the tested inhibitors.
3. Most of the work was done with tyrosinase isolated from the fungus *Agaricus bisporus*. Human tyrosinase is membrane-bound, while fungal tyrosinase is a cytosolic enzyme. Furthermore, the human enzyme is a monomer that undergoes an intensive glycation process, unlike the fungal tyrosinase, which is a tetramer. Knowledge about the effects of tyrosinase inhibitors on the human enzyme is limited, thus, it is necessary to determine the suitability of fungal tyrosinase inhibitors relative to the human enzyme.
4. The search for selective Hyal inhibitors is also essential. The availability of selective inhibitors that target one type of Hyal without affecting another Hyal is important because different isoforms of Hyal regulate physiological processes. Additionally, the search for selective Hyal inhibitors will allow for a better understanding of the role of Hyals in both physiological and pathological processes.
5. A significant problem when comparing the results of studies is the deficiencies in analysing enzymatic reaction kinetic. Information on Km, Vmax, and Cmax of the enzymatic reaction is often missing in studies.
6. Many studies, especially on Hyal, focus only on determining the properties of anti-Hyal extracts. A more careful analysis of the active ingredients is needed.

Disclosure statement

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 article.

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10.2. Praca oryginalna 2

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Article

Eleutherococcus divaricatus Fruits Decrease Hyaluronidase Activity in Blood Serum and Protect from Oxidative Damages in In Vitro Model

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Abstract: Fruits are very important dietary components and a source of biologically active compounds used in nutritional pharmacology. Particularly due to the presence of polyphenolic compounds, fruits play an important role in the prevention of diseases of civilization. Therefore, it is important to study the phytochemicals and biological activity of fruits, especially those with a long-standing use in ethnomedicine. In this study, we determined the chemical profile and biological activity of a methanolic extract of the *Eleutherococcus divaricatus* fruits. Amongst nine polyphenols studied, only chlorogenic acid, protocatechuic acid, and eleutheroside E have been detected. The extract showed a weak anti-hyaluronidase activity from bovine testicular in a range of 9.06–37.70% and quite high for human serum hyaluronidase from children diagnosed with acute leukemia in a range of 76–86%. A weak anti-tyrosinase activity was obtained in a range of 2.94–12.46%. Moreover, the extract showed antioxidant properties against DPPH radical, ABTS radical, and $O_2^{\bullet -}$. In addition, the antioxidant activity of the extract was evaluated by FRAP assay and Fe^{2+} ion chelation assay. These preliminary studies partially justify the traditional use of the plant in inflammatory- and immune-related diseases, in which hyaluronidase and free radicals can participate. A difference in human serum hyaluronidase inhibition may result from the inter-patient variability. Regardless of that, the results mean that polyphenolic compounds may stimulate activity of hyaluronidase, as well as to protect cells from the oxidative damages. However, further studies in ex vivo and in vivo models are needed, including blood isolated from a larger number of patients.

Keywords: antioxidants; anti-tyrosinase; anti-hyaluronidase; adaptogenic plants; phytochemicals; *Eleutherococcus*; leukemia

1. Introduction

Plants are a crucial source of medicines for treating human diseases. For centuries, humans have used them for various disease entities without knowledge of the compounds that determine their biological activity [1–3]. The beginning of the "conscious" use of plants was the isolation of morphine by German apothecary Friedrich Sertürner in 1804 [4]. This research marked the beginning of a new era of work on the medicinal properties of plants, making it possible to treat diseases such as hypertension (reserpine), gout (colchicine), or cancer (paclitaxel, vincristine, and vinblastine) [5]. After a temporary decline in interest in plants due to the development of combinatorial chemistry in the search for drugs, the

21st century has seen a "renaissance" of drugs of natural origin [6]. This is related to two aspects: (1) plants are rich in secondary metabolites with a wide variety of chemical structures, which generate many pharmacophores with unique spatial structures, and (2) compounds of natural origin, unlike synthetic molecules, due to their biochemical functions in the plant, offer a good chance of potential interaction with proteins and the ability of intercellular permeation.

Leukemia is a blood-related malignancy characterized by transformed hematopoietic progenitors and diffuse infiltration of bone marrow. Globally, in 2020, leukemia accounted for approx. 2.5% and 3.1% of all new cancer incidence and mortality. As mentioned above, plants have had significant therapeutic potential for preventing or treating human diseases for thousands of years. Current advances in leukemia therapy promote the use of natural products to prevent the onset of cancer; as well, they are a source of new anticancer drugs. It is estimated that almost half of the drugs used currently in treating cancer are plant-based compounds and their derivatives. Vincristine and vinblastine are the first plant-derived anticancer drugs used to treat leukemia or other types of cancers. On the other hand, plant compounds are also utilized as chemo-preventive and supporting a standard treatment. However, in many cases, their use has not been scientifically proven and patients take a risk when they connect administration of such compounds with standard treatments. Additionally, some of them are ingredients of so-called nutriproducts, which are very popular products with multidirectional functions [7–9].

When looking for new plant-based drugs, we should follow the ethnopharmacological knowledge of our ancestors, who could treat people with great success. The *Araliaceae* family is an interesting group of plants which have been used in traditional healing systems to treat immune-related diseases [10,11]. This family includes, among others, the most important in traditional Chinese medicine (TCM), used for 4000 years, a "panacea" for many diseases, *Panax ginseng* C.A. Meyer. A well-known representative of this family, used as a substitute for *P. ginseng*, is *Eleutherococcus senticosus* (Rupr. and Maxim.) Maxim [12]. In both scenarios, the root serves as a valuable medicinal ingredient. However, the substantial expense involved in obtaining this raw material necessitates the exploration of alternative sources [13–15]. In our laboratory, research is under way on the fruits of *E. senticosus*, which, unlike the root, do not require a long maturation period (about five years). A little-known representative of the *Eleutherococcus* is *E. divaricatus* (Siebold and Zucc.) S. Y. Hu. *E. divaricatus*, also known as five fingers, a plant species that belongs to the *Araliaceae* family. It is native to Northeast Asia, including China, Korea, and Japan. This plant is known for its medicinal properties; it is believed to improve one's immune system and overall health [16]. The roots of *E. divaricatus* are commonly used in traditional Chinese medicine as a tonic for the spleen and kidneys. It is also used to treat rheumatism, hypertension, and diabetes [17]. In addition, the plant's leaves and berries can be used to make a tea which is said to have a calming effect on the body and mind [18]. The plant is also used extensively in the cosmetic industry due to its antioxidant and anti-inflammatory properties. It is believed to promote healthy skin and reduce the signs of aging [19–22]. However, the details on the mechanism of action are still uncompleted. Moreover, there is not much information about that species cultivated in Poland. For these reasons, we have hypothesized that the fruits contain phytochemicals that may be responsible for their anti-enzymatic and antioxidant activities. We chose hyaluronidase and tyrosinase as enzymes participating in many diseases, including leukemia and skin cancers. To obtain more reliable results, we studied both enzymes commercially available (hyaluronidase and tyrosinase) as well as serum hyaluronidase from children diagnosed with acute leukemia. In order to prove our hypothesis, the phytochemical techniques of in vitro and ex vivo biological tests were applied.

2. Results and Discussion

Fruits play a crucial role in the diet of both humans and animals. They are a vital source of vitamins, mineral salts, and fiber. It is interesting to note that nearly 3/4 of the

food consumed by humans is estimated to be the nutrients of fruits and seeds on a dry weight basis. In addition to primary metabolites, fruits also contain an important group of compounds known as secondary metabolites, which are responsible for a wide range of health-promoting effects [23]. Among these, polyphenolic compounds present in fruits exhibit a broad spectrum of activity. These insights highlight the immense nutritional and health benefits offered by fruits.

Table 1 presents the quantitative results for eleutherosides and phenolic acids. Figure 1 presents a chromatogram for eleutherosides and phenolic acids.

Table 1. Antioxidant activity of *E. divaricatus* fruits. The results are presented as IC_{50} (mg/mL ± SD).

	ABTS *	DPPH **	CA **	$O_2^{\bullet-}$ **
Extract	0.28 ± 0.01	1.30 ± 0.01	1.45 ± 0.11	1.51 ± 0.11
BHA	0.0025 ± 0.00	0.40 ± 0.01	-	-
AA	-	-	-	0.05 ± 0.00
EDTA	-	-	0.19 ± 0.00	-

BHA—butylhydroxyanisole, AA—ascorbic acid, EDTA—ethylenediaminetetraacetic acid, and CA—ion chelation. * $p < 0.05$ and ** $p < 0.01$, in the table column, indicate statistically significant differences (Kruskal–Wallis test).

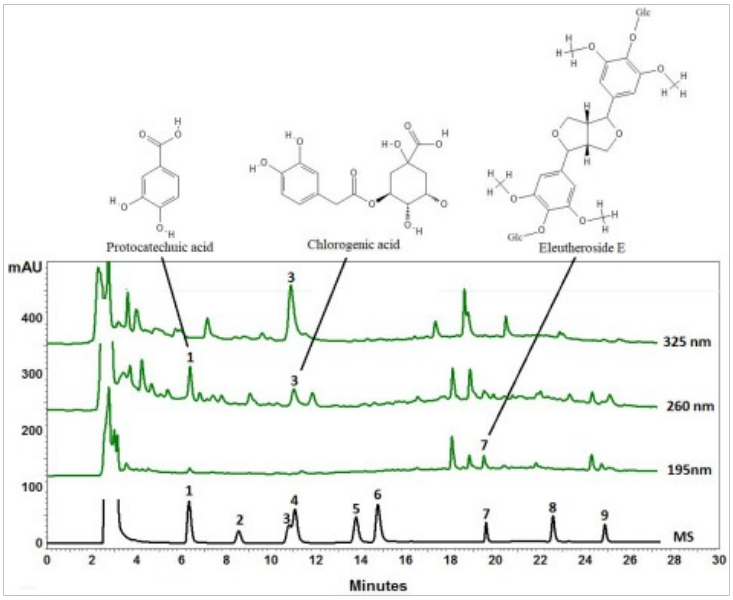

Figure 1. An exemplary HPLC chromatogram of *Eleutherococcus divaricatus* fruits extract (green line) and reference compounds (black line): 1-protocatechuic acid, 2-eleutheroside B, 3-chlorogenic acid, 4-*p*-hydroxybenzoic acid, 5-vanillic acid, 6-caffeic acid, 7-eleutheroside E, 8-ferulic acid, and 9-eleutheroside E1. Conditions: RP18 reversed-phase column Kinetex at 25 °C, a mixture of acetonitrile (solvent A) and water (solvent B), both acidified with 0.025% of trifluoroacetic acid, were used as the mobile phase. The compounds were separated by gradient elution with program: 0.0–8.0 min A 10%, B 90%; 8.1–18.0 min A 10–20%, B 90–80%; 18.1–28.0 min A 20%, B 80%; 28.1–35.0 min A 20–25%, B 80–75%; and 35.1–40.0 min A 25%, B 75%. Flow rate was 1.0 mL/min.

The extraction of the fruits resulted in 22.5% dry extract yield and the total polyphenol content was found to be at a level of 14.61 mg GAE/g. However, using the spectrophotometric methods, flavonoids, phenolic acids, and tannins were not detected.

The literature provides limited information on the chemical composition of *E. divaricatus* fruits. The low polyphenol content could be attributed to the solvent and extraction method used. Załuski et al. highlighted the impact of the solvent on the total polyphenol content of extracts prepared from *E. divaricatus* fruits, reporting 52.03 ± 0.5 mg GAE/g for 75% EtOH (accelerated solvent extraction) and 41.1 ± 0.5 mg GAE/g for infusion (95 °C distilled water). Additionally, the fruits were collected in 2016 and the differences may result from a seasonal change [24]. In a previous study, the contents of polyphenols, flavonoids, and phenolic acids in the intractum made from other *Eleutherococcus* species, i.e., *E. senticosus* fruits, were found to be 1.02 ± 0.04 mg GAE/g DW, 0.1 ± 0.05 mg QE/g DW, and 0.30 ± 0.07 mg CAE/g DW, respectively [25]. An investigation of 75% ethanol extracts made from fresh and dried fruits of *E. senticosus* and *E. henryi* revealed no statistically significant differences between the polyphenol levels (for *E. senticosus*—fresh dried 4110 mg GAE/100 g, storage 3850 mg GAE/100 g; and for *E. henryi*—fresh dried 4350 mg GAE/100 g, storage 4140 mg GAE/100 g) [26]. Conversely, the methanolic extract made from *E. senticosus* fruits (1:10 dry weight material to MeOH) contained 229.83 ± 9.34 mg GAE/g of polyphenols [27]. Similar results were obtained in a study examining polyphenol levels from fresh fruits of *E. divaricatus*. The total polyphenol content (TPC) for other species (*E. senticosus*, *E. gracilistylus*, *E. sessiliflorus*, *E. henryi*, and *E. setchuensis*) ranged from 6.9 ± 0.01 to 19.7 ± 0.01 mg/g [28]. In those cases, it should be noted that the content of phytochemicals in plants is dependent on a few factors, including, among others, the seasonal and weather conditions. Therefore, a quality control is a needed process at every step of plant raw material processing.

In the next step, the contents of phenolic acids (protocatechuic acid, chlorogenic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, and ferulic acid) and eleutherosides (B, E, and E1) were examined. Out of all tested compounds, only chlorogenic acid (0.13 ± 0.01 mg/g extract ± SD), protocatechuic acid (1.47 ± 0.10 mg/g extract ± SD), and eleutheroside E (0.23 ± 0.01 mg/g extract ± SD) have been detected. Eleutheroside B and E have also been detected in the fruits of this species by Kim et al., in the amounts of 1.06 and 7.08 µg/mg, respectively [29]. Comparing these results with the results obtained for the fruits of *E. senticosus*, used very often as a model species for that genus, Bączek discovered eleutheroside B (0.356 mg/g) and E (0.298 mg/g) in an ethanol extract of *E. senticosus* fruits [30]. Our previous investigation also confirmed their presence in *E. senticosus* (0.66 and 0.74 mg/gDW, respectively) [31]. Taking into consideration the phenolic acids, an extract from *E. senticosus* fruits contained protocatechuic acid, 4-OH-benzoic acid, vanillic acid, caffeic acid, ferulic acid, and rosmarinic acid [32]. The extract from *E. senticosus* fruits contained 4.1 mg/g of chlorogenic acid and 0.84 mg/g of rosmarinic acid. These compounds were present in four-year-old raw materials, while two-year-old and three-year-old raw materials did not contain these substances [30]. The study by Załuski et al. identified protocatechuic acid (0.45 mg/gDW), 4-OH-benzoic acid (2.0 mg/gDW), vanillic acid (4.2 mg/gDW), *trans*-caffeic acid (41.2 mg/gDW), and *trans*-ferulic acid (3.6 mg/gDW) in the *intractum* from the *E. senticosus* fruits [33]. The varying content of polyphenolic compounds can be attributed to different extraction methods, the solvents used in the study, and the location where the raw material was harvested.

The antioxidant capacity of a methanolic extract from *E. divaricatus* fruits was evaluated using several methods. The application of multiple methods enabled a more precise estimation of the extracts' antioxidant properties. The extract's activity was assessed against $DPPH^{\bullet}$, $ABTS^{+}$, and $O_2^{\bullet -}$. This marks the first time, to our knowledge, that the $O_2^{\bullet -}$ scavenging capacity of an extract made from fruits of this species has been evaluated. The antioxidant activity was also assessed using the Fe^{2+} ion chlorination test and the FRAP method. The results, presented in Table 1, showed that the extract exhibited moderate antioxidant properties. In the DPPH and ABTS assays, the IC_{50} values were 1.36 and 0.28 mg/mL, respectively. The activity against superoxide radical was 1.51 mg/mL. The IC_{50} for chelating Fe^{2+} ions were 1.45 mg/mL. In the FRAP test at concentrations of

1 mg/mL and 0.1 mg/mL, the values were 6.01 and 5.02 mg Trolox/g, respectively, in comparison to BHA, 28.91 and 12.83 mg Trolox/g.

The antioxidant properties of the fresh *E. divaricatus* fruits, expressed as the EC_{50} value, were evaluated in a study involving five *Eleutherococcus* species. The fruits of *E. divaricatus* showed the highest activity (2.7 mg/mL) in the linolenic acid oxidation inhibition test. The activity of other species (*E. setchuensis*, *E. senticosus*, *E. gracilistylus*, and *E. henryi*) ranged from 3.4–4.9 mg/mL. In this study the chelating capacity of Fe^{2+} ion was also evaluated, with the EC_{50} value obtained for all extracts equal 0.4 mg/mL. The EC_{50} of *E. divericatus* fruit extract against the DPPH radical was 86.2 ± 2.0 mg/mL. The range of activity for other species was from 4.5 ± 0.2 to 63.4 ± 0.5 [28]. Załuski et al. reported on an anti-DPPH activity of the *E. senticosus* and *E. henryi* ethanol fruits extracts with the IC_{50} in a range of 0.1–0.29 mg/mL [26]. The acidified 80% MeOH extract of *E. senticosus* fruits exhibited stronger antioxidant properties against DPPH, ABTS, and hydroxyl radicals (IC_{50} values of 11.2, 4.3, 14.5 µg/mL, respectively) when compared to cyanidin-3-O-(2″-O-xylosyl)-glucoside isolated from this extract (IC_{50} values of 85.2, 43.7, and 126.6 µg/mL, respectively) [34]. Kim et al. determined the antioxidant activity of aqueous extracts made from *E. senticosus* and *E. koreanum* fruits. *E. koreanum* showed strong antioxidant properties in DPPH, ABTS, FRAP, and ORAC tests compared to extracts made from the roots, leaves, and stem of this plant. In contrast, for *E. senticosus*, the antioxidant activity in these tests was significantly weaker [29].

For centuries, plants have been used as medicines to treat human diseases. The use of plants as therapeutic agents was made possible by the presence of a variety of chemical compounds characterized by a diverse biological activity. In the above work, we tested how an extract made from the fruits of *E. divaricatus* affects human serum hyaluronidase from children diagnosed with acute leukemia (AL), bovine hyaluronidase, and fungal tyrosinase. The results are shown in Tables 2 and 3. It is well known that the activity of hyaluronidase increases in many diseases, e.g., in leukemia, skin conditions, or GI tract diseases. Simultaneously, in this case, it should be noted that the overactivity plays both positive and negative roles, with the overweighting of the negative one. The elevated amount in serum has been detected in the case of hepatitis C and pancreaticocutaneous fistula. In Crohn's disease, the elevated hyaluronidase amount was detected in colonic fibroblasts. The latest reports provide information about the subcutaneous injection of rituximab and hyaluronidase for the treatment of adults with follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), or chronic lymphocytic leukemia (CLL). In this case, hyaluronidase serves as a spreading factor and is inactivated in skin shortly after injection [35].

Table 2. Inhibition of *E. divaricatus* fruits towards serum human hyaluronidase from children diagnosed with acute leukemia (AL) before starting treatment (%). The results are presented for an extract concentration of 100 µg and expressed as %.

Patient's Age	Serum Hyal (U/mL)	*E. divaricatus* (%)	Escin (%)
6	130.29	84.94 ± 10.28	56.17 ± 18.95
8	38.60	86.13 ± 9.29	95.62 ± 24.07
7	136.55	76.46 ± 0.18	61.35 ± 11.74
Means	101.81	82.51	71.04

The Kruskal–Wallis test revealed statistically significant differences between the effects of the extract and escin ($p < 0.05$), regardless of the patient. However, subsequent post hoc tests did not show statistically significant differences between the extract and escin within individual patients.

Table 3. Inhibition of *E. divaricatus* fruits towards bovine hyaluronidase and tyrosinase. The results are presented as % of inhibition and IC_{50} (mg/mL ± SD).

		Hyal *		Tyr *	
	Concentration (µg/300 µL)	**(%)**	**(IC_{50})**	**(%)**	**(IC_{50})**
Extract	100	37.70 ± 3.09	0.45 ± 0.04	12.46 ± 2.30	2.67 ± 0.05
	10	9.06 ± 1.54		6.02 ± 3.02	
	1	0.00 ± 0.00		2.94 ± 2.41	
Escin	100	58.96 ± 1.69	0.28 ± 0.01		
	10	9.88 ± 2.05			
	1	4.15 ± 1.62			
Kojic acid	100			99.39 ± 0.33	0.027 ± 0.00
	10			34.04 ± 1.60	
	1			14.32 ± 1.90	

Hyal—hyaluronidase, Tyr—tyrosinase. * $p < 0.01$, in the table column, indicates statistically significant differences (Kruskal–Wallis test).

To assess whether the fruits might regulate the hyaluronidase activity, we used serum from patients diagnosed with acute leukemia (AL) before starting treatment. Firstly, the activity of hyaluronidase was established; next, the extract in a dose 100 µg was added to each serum sample. The dose 100 µg has been previously selected in research on the immunostimulative activity of the fruits of *Eleutherococcus senticosus* and eleutherosides, another species with the *E. divaricatus*-like activity and which is used as a model species in our long-standing research. Table 3 presents the findings of the study on the effectiveness of the extract against human serum hyaluronidase. The extract exhibited an activity range of 76.46% to 86.13% when it is compared to escin (56.17% to 95.62%, respectively). In the case of bovine hyaluronidase, the methanolic extract showed moderate activity (9.06–37.70%); similar results were obtained for escin 4.15–58.96%. Moreover, a weak anti-tyrosinase activity was obtained in a range of 2.94–12.46% and strong for kojic acid 14.32–99.39%. To our knowledge, this is the first report on the impact of *Eleutherococcus* fruits extract on human serum hyaluronidase.

The IC_{50} for extracts made from the fruits of *E. senticosus* and *E. divaricatus* against bovine hyaluronidase (hyal) ranged from 0.58 to 0.87 mg/mL [30]. In a test of the activity of *E. senticosus* fruit *intractum* against hyal, the IC_{50} was 217.44 ± 10.72 µg/mL, against tyrosinase was equal 586.83 ± 2.36 µg/mL [29]. The activity of methanol extracts made from the roots of *E. gracilistylus*, *E. divaricatus*, *E. senticosus*, *E. henryi*, and *E. sessiliflorus* was 19.6–32% against hyal [36]. In the other study, autumn leaves inhibited hyal stronger when compared to spring leaves (74.3 and 33%, respectively) [37].

An inhibition of hyaluronidase and tyrosinase have been described by many researchers who tested both the extracts and isolated compounds [38–40]. There are many factors the inhibition is reliant on. It makes the comparison of results, sometimes, impossible. Additionally, there is a lack of standardized units of enzyme activity and in different investigations different units are used. In many publications, no units' activity are provided, even for those enzymes commercially available. Another problem is a lack of an absorbance assay for the extracts alone. The extracts and some isolated plant-based compounds contain dyes or are dyes themselves; finally, they can strengthen the absorbance, giving a false positive result. In the case of oxidoreductases, some compounds might act also as substrates and inhibitors or just like substrates, especially when studied in an isolated form [41]. In this case, a series of other tests are needed, like NMR titration, and also establishing what are the most reliable tests from a pharmacological point of view, including cells or animal model tests.

3. Materials and Methods

3.1. Chemicals and Reagents

Nitrotetrazolium blue chloride (NBT), xanthine, xanthine oxidase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), potassium persulfate, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (ferrozine), iron (II) chloride tetrahydrate ($FeCl_2 \times 4H_2O$, 1,3,5-Tri(2-pyridyl)-2,4,6-triazine (TPTZ), iron (III) chloride ($FeCl_3$), aluminum chloride ($AlCl_3$), potassium acetate, Folin−Ciocalteu reagent, sodium nitrite, sodium molybdate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ascorbic acid, 2(3)-t-Butylhydroquinone monomethyl ether (BHA), 2(3)-t-Butyl-4-hydroxyanisole, hyaluronic acid (IV), escin, hyaluronidase from bovine testes, hexadecyltrimethylammonium bromide (CTAB), L-tyrosine, koji acid, and tyrosine from mushrooms were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The standards of eleutheroside B ≥98.0% (HPLC), eleutheroside E ≥98.0% (HPLC), eleutheroside E1 ≥98.0% (HPLC), protocatechuic acid ≥97%, p-hydroxybenzoic acid 99%, vanillic acid ≥97%, caffeic acid ≥98%, and ferulic acid ≥99% were also purchased from Sigma-Aldrich. Solvents used for extraction were purchased from Avantor Performance Materials (Gliwice, Poland).

3.2. Preparation of Extract

The fruits were collected from the Arboretum SGGW in Rogów, Poland, in 2021 and authenticated by Prof. D. Załuski. The fresh fruits were air-dried at room temperature and macerated with a 75% methanol solution (15 g/150 mL). The extract was then subjected to ultrasound treatment for 15 min and repeated three times. The resulting extract was evaporated and stored in a refrigerator at 2 °C. The extraction yield was calculated based on the dry weight of the extract (%).

3.3. Phytochemical Panel

Spectrophotometric and chromatographic methods were used to determine the quantitative and qualitative composition of the extract. The methods used are described in more detail in a previous paper.

3.3.1. Chemical Composition

Determination of Total Phenolic Content (TPC)

A modified version of the Folin–Ciocalteu method was used to determine the total phenolic content [42]. The extract (1 mg/mL in MeOH) was mixed with Folin−Ciocalteu reagent (diluted in pure water, 1:3) at a ratio of 1:1 each. The mixture was then incubated for 5 min after adding distilled water. Sodium carbonate (10%) solution was added and the mixture was incubated in the dark at room temperature for an hour. The absorbance was measured at 750 nm. The TPC results were expressed in milligrams of gallic acid (GA) equivalents (GAE) per gram of the sample (mg GAE/g sample).

Determination of Total Phenolic Acid Content (TTC)

To determine total tannin content, we used the Zhu method with slight modification [43]. The polyvinylpolypyrrolidone (PVPP) was used to precipitate the tannins. Quickly, to 1 mL of extract in methanol, we added 1 mL of PVPP (0.5%). The samples underwent a thorough mixing process using a vortex. Following this, they were subjected to a low-temperature incubation at 4 °C for a duration of 10 min. The final step in the procedure involved centrifugation at a speed of 5000 rpm, lasting for 5 min. The supernatant was used to determine the total phenolic content by the methods described above. The TTC results were expressed in milligrams of tannic acid (TA) equivalents (TAE) per gram of the sample (mg TAE/g sample).

Determination of Total Flavonoids Content (TFC)

The total flavonoid content was determined using a method that involved the reaction between $AlCl_3$ and flavonoids [44]. In short, extract (1 mg/mL in MeOH) and EtOH was mixed and, then, aluminum chloride (10%) and potassium acetate (1 M) were added. The mixture was incubated for 30 min after adding distilled water. The absorbance was measured at 510 nm and the results of TFC were expressed in milligrams of quercetin equivalents (QE) per gram of the sample (mg QE/g sample).

Determination of Total Phenolic Acid Content (TPAC)

The method outlined in Polish Pharmacopeia VI was followed to determine the total phenolic acid content [45]. The extract (1 mg/mL in MeOH) was mixed with distilled water, HCl (0.5 M), and Arnov's reagent (10.0 g of sodium molybdate and 10.0 g of sodium nitrite in 100 mL distilled water). Solution of NaOH (1M) was added and the mixture was immediately measured at 492 nm. The TPAC results were expressed as milligrams of caffeic acid (CA) equivalents (CAE) per gram of the sample (mg CAE/g sample).

3.3.2. HPLC-PDA-Based Metabolomic Profiling of Phenolic Compounds in the Extract

The EliteLaChrom chromatograph with PDA detector and EZChrom Elite software 3.2.0 (Merck, Darmstadt, Germany) was used to perform the analyses. The gradient chromatographic system used in the experiment consisted of an RP18 reversed-phase column Kinetex (Phenomenex, Torrance, CA, USA) measuring 25 cm × 4.6 mm i.d., with a particle size of 5 µm, maintained at a temperature of 25 °C. A mobile phase comprising acetonitrile (solvent A) and water (solvent B) was used, containing 0.025% trifluoroacetic acid. The compounds were separated by gradient elution using a program that included various percentages of solvents A and B over a period. The flow rate was 1.0 mL/min and data were collected between 190 and 400 nm. The identity of compounds was established by comparing their retention times and UV spectra with corresponding standards. Quantitative analysis was performed at specific wavelengths for each compound (260 nm for protocatechuic acid, 325 nm for chlorogenic acid, and 195 nm for eleutheroside E).

3.4. Enzymatic Panel

3.4.1. Bovine Hyaluronidase Inhibition Assay

Bovine hyaluronidase inhibitor assays were performed in 96-well plates using a modified method described by Di Ferrante [46] and Studzińska-Sroka [47]. The precipitation of the undigested hyaluronic acid with cetyltrimethylammonium bromide (CTAB) was determined as the activity of the compounds/extracts. Three concentrations of extracts 0.1, 1.0, and 10 mg/mL (final concentration in well: 1.0, 10, and 100 µg/300 µL), acetate buffer (pH = 5.35), incubation buffer (pH = 5.35, 0.01% BSA, and 0.45% NaCl) and enzyme (30 U/mL in incubation buffer) were mixed. The mixture was incubated at 37 °C for 10 min. Then, hyaluronic acid solution (0.3 mg/mL in acetate buffer pH = 5.35) was added. The plates were further incubated for 45 min at 37 °C. After incubation, undigested HA was precipitated by adding 2.5% of CTAB. The plates were kept at 25 °C for 10 min. The intensity of complex formation was measured at 600 nm. The presence of inhibition was determined by measuring the absorbance of the solution without inhibitor (A_C) and enzyme (A_T). All samples were tested in triplicate. The hyaluronidase inhibition was calculated using the following equation and escin was used as a standard:

$$\%_{INH} = \left(\frac{A_S - A_C}{A_T - A_C}\right) \times 100\%$$

A_S—absorbance of the HA + sample + enzyme
A_C—absorbance of the HA + enzyme
A_T—absorbance of the HA + sample.

3.4.2. Human Serum Hyaluronidase

Three children with a median age of 7 years (range, 6–8 years), diagnosed with acute leukemia (AL) before starting treatment, were included in the study. The patients were diagnosed at the Department of Pediatric Hematology and Oncology (Jurasz University Hospital, Bydgoszcz, Poland) in 2019–2020. Venous blood was collected from each child under fasting conditions and placed into serum tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Blood samples were allowed to clot for 30 min at room temperature and then were centrifuged for 20 min at 2000× *g* at room temperature. They were collected and stored at −80 °C until analyses. The local bioethics committee approved the study (608/2019). It was carried out in accordance with the Declaration of Helsinki.

Level of Human Serum Hyaluronidase

The commercially available kit (LS-F6310 Human Hyaluronidase (Sandwich ELISA) ELISA Kit) was utilized to measure the concentration of human hyaluronidase in serum. This kit operates on the sandwich assay principle and is capable of detecting hyaluronidase levels down to 0.115 nanograms per milliliter.

Human Serum Hyaluronidase Inhibition Assay

The inhibition of human serum hyaluronidase was evaluated using modified methods [25]. The activity of the compounds/extracts was determined by precipitating the undigested hyaluronic acid with cetyltrimethylammonium bromide (CTAB). Briefly, 10 µL of extract 10 mg/mL (final concentration in well 100 µg/300 µL) and 50 µL of serum was incubated at 37 °C for 15 min. Subsequently, a 40 µL of solution of hyaluronic acid (0.3 mg/mL in acetate buffer with pH = 5.35) was added. The plates were incubated for an additional 45 min at 37 °C. After incubation, undigested HA was precipitated by adding 2.5% CTAB. The plates were shaken out at 25 °C for 10 min. The intensity of complex formation was measured at a wavelength of 600 nm. All samples were tested in triplicate. The inhibition of hyaluronidase was calculated using a specific equation, with escin used as a standard.

$$\%_{INH} = \left(\frac{A_S - A_C}{A_T - A_C}\right) \times 100\%$$

A_S—absorbance of the HA + sample + enzyme
A_C—absorbance of the HA + enzyme
A_T—absorbance of the HA + sample.

3.4.3. Tyrosinase Inhibitor Assays

Tyrosinase inhibitor assays were performed in 96-well plates according to a modified method [25,48]. Conversion of L-tyrosine to L-DOPA and L-DOPA to DOPA-quinone, accompanied by the browning of the solution, is catalyzed by tyrosinase enzyme. Briefly, 10 µL of three concentrations of extracts 0.1, 1.0, and 10 mg/mL sample (final concentrations in well: 1.0, 10, and 100 µg/200 µL) and 150 µL of phosphoric buffer with mushroom tyrosinase (pH = 6.88, 100 U/mL) were mixed and incubated for 10 min at room temperature. In addition, a control without inhibitor was prepared (A_C). After incubation, L-tyrosine (0.3 mg/mL) was added to each well and the absorbance was measured at 492 nm (kinetic model, every 5 min). Next, two time points (t_1 and t_2) were selected in the linear range of the graph. All samples were tested in triplicate. The tyrosinase inhibition was calculated using the following equation and kojic acid was used as a standard:

$$\%_{INH} = \left(\frac{A_S - A_C}{A_C}\right) \times 100\%$$

A_S—the difference in absorbance between times t_2 and t_1 for sample,
A_C—the difference in absorbance between times t_2 and t_1 for positive control.
All analyses were performed in triplicate.

3.5. Antioxidant Panel

3.5.1. ABTS Free Radical Scavenging Activity

The method described by Wu et al. was followed to test for ABTS free radical scavenging [49]. A working solution of ABTS+ was prepared by mixing 10 mL of ABTS (7 mM in H_2O) with 10 mL of potassium persulfate (2.45 mM in H_2O), which was left to incubate in the dark for 12 h. The $ABTS^+$ solution was diluted with water to achieve an absorbance of 0.700 ± 0.03 at 405 nm. Next, extracts at concentrations of 0.1 mg/mL, 1 mg/mL, and 10 mg/mL (final concentration in well: 1, 10, and 100 µg/200 µL) were mixed with 190 µL of the $ABTS^+$ solution and incubated for 30 min. After incubation, the absorbance at 405 nm was measured. BHA was used as control. The antioxidant activity was calculated using the provided equation.

$$\%_{INH} = \left(\frac{A_S - A_C}{A_C}\right) \times 100\%$$

A_S—the absorbance for sample + ABTS
A_C—the absorbance without sample + ABTS.

3.5.2. DPPH Free Radical Scavenging Activity

The method for testing DPPH free radical scavenging was followed as outlined by Naseer et al. [50]. A working $DPPH^•$ solution was created by dissolving 24 mg of DPPH in 100 mL of distilled water. The solution was then diluted with methanol until an absorbance of 0.900 ± 0.03 at 515 nm was reached. Next, extracts at concentrations of 0.1 mg/mL, 1 mg/mL, and 10 mg/mL (final concentration in well: 1, 10, and 100 µg/200 µL) were mixed with 190 µL of the $DPPH^•$ solution and incubated for 60 min. The absorbance at 515 nm was measured after incubation. BHA was used as control. The antioxidant activity was then calculated using the given equation.

$$\%_{INH} = \left(\frac{A_S - A_C}{A_C}\right) \times 100\%$$

A_S—the absorbance for sample + DPPH
A_C—the absorbance without sample + DPPH.

3.5.3. Ferric-Ion-Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was conducted by mixing extracts of 0.1 and 1.0 mg/mL (at final concentrations of 1 and 10 µg/300 µL) with 290 µL of a working solution consisting of acetate buffer (15 mL), TPTZ solution (1.5 mL), and $FeCl_3 \times 4H_2O$ (1.5 mL). The mixture was then incubated for 30 min before measuring the absorbance at 593 nm. Trolox and BHA were used as control. The results of the FRAP assay were expressed in milligrams of Trolox per gram of the sample (mg Trolox/g sample) [51].

3.5.4. Iron (II) Ion Chelation Assay

Li et al. method was employed to determine the ion chelation assay [52]. Firstly, extracts of 0.1 and 1.0 mg/mL (with final concentrations of 10 and 100 µg/260 µL in the well) were mixed with MeOH and $FeCl_2$ (2 mM). Then, ferrozine (5 mM) was added. After incubation, the absorbance at 510 nm was measured. The chelation was calculated using the following equation, with EDTA being used as a positive control.

$$\%_{chel.} = \left(1 - \frac{A_S}{A_C}\right) \times 100\%$$

A_S—the absorbance for sample + ferrozine + $FeCl_2$
A_C—the absorbance without sample + ferrozine + $FeCl_2$.

3.5.5. $O_2^{\bullet-}$ Scavenging Capacity Assay

Scavenging of the superoxide anion was examined by using a xanthine–xanthine oxidase system with the nitro blue tetrazolium chloride (NBT) described by Choi et al. [53]. Briefly, 50 µL of extract at concentration 10, 1, and 0.1 mg/mL (500, 50, and 5 µg/200 µL), 100 µL of a solution of xanthine with NBT (1:1 (*v*/*v*); 0.4 mM and 0.24 mM, respectively) and 50 µL of a mixture of xanthine oxidase (10 mU). After 20 min of incubation at 37 °C, the absorbance was determined at 560 nm. All reagents were dissolved in PBS. As a positive control, ascorbic acid was used.

3.6. Statistical Analysis

Statistically significant differences between the extracts and control substances were calculated based on the obtained percentage values of anti-enzymatic or antioxidant activity. None of the samples exhibited a normal distribution; therefore, non-parametric Kruskal–Wallis tests were used.

4. Conclusions

It is clearly seen that there are compounds in the extract which have a significant anti-hyaluronidase activity with the mean value of 82.51% by a patient's group. We can suggest that chlorogenic acid, because of its already confirmed anti-hyaluronidase activity, may represent these compounds [54–56].

Overexpression of hyaluronidases causes increased cell proliferation; therefore, their inhibition is a new strategy for the treatment of several diseases, as well as neoplastic inflammatory-related diseases [57–61]. Considering our results, research on synergism and antagonism with drugs used in the treatment of the above-mentioned diseases are needed, including blood samples isolated from a larger number of patients.

Based on these findings, it can be concluded that *E. divaricatus* fruits could strengthen a general vitality of the body. And the results meet criteria about plant-based compounds, which should act rather gently and be used for a longer time to be effective.

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10.3. Praca oryginalna 3

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Ethyl Acetate Fraction from *Eleutherococcus divaricatus* Root Extract as a Promising Source of Compounds with Anti-Hyaluronidase, Anti-Tyrosinase, and Antioxidant Activity but Not Anti-Melanoma Activity

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Abstract: *Eleutherococcus divaricatus* (Siebold and Zucc.) S. Y. Hu. has been used in Traditional Chinese Medicine (TCM) due to its anticancer, immunostimulant, and anti-inflammatory activities. However, its mechanism of action and chemical composition are still insufficiently understood and require more advanced research, especially for cases in which anti-inflammatory properties are beneficial. The aim of this study was to evaluate the impact of *E. divaricatus* root extracts and fractions on proinflammatory serum hyaluronidase and tyrosinase in children diagnosed with acute lymphoblastic leukemia. Antioxidant and anti-melanoma activities were also examined and correlated with metabolomic data. For the first time, we discovered that the ethyl acetate fraction significantly inhibits hyaluronidase activity, with mean group values of 55.82% and 63.8% for aescin used as a control. However, interestingly, the fraction showed no activity against human tyrosinase, and in A375 melanoma cells treated with a doxorubicin fraction, doxorubicin activity decreased. This fraction exhibited the most potent antioxidant activity, which can be attributed to high contents of polyphenols, especially caffeic acid (24 mg/g). The findings suggest an important role of the ethyl acetate fraction in hyaluronidase inhibition, which may additionally indicate its anti-inflammatory property. The results suggest that this fraction can be used in inflammatory-related diseases, although with precautions in cases of patients undergoing chemotherapy.

Keywords: *Eleutherococcus divaricatus*; human hyaluronidase; human tyrosinase; metabolites

1. Introduction

Enzymes such as hyaluronidase and tyrosinase, which are naturally present in the human body, play crucial roles in a variety of physiological processes [1–6]. This includes the facilitation of fertilization and the production of melanin. However, these enzymes are also implicated in several pathogenic processes, including the formation of cancerous metastases and the development of age spots [7]. The discovery and subsequent characterization of inhibitors of hyaluronidase could potentially pave the way for the creation of novel anticancer treatments, effective contraceptives, and antidotes for various venoms

and toxins [8–12]. Conversely, inhibitors of tyrosinase have potential applications in the cosmetic and pharmaceutical industries as skin-lightening agents or in the treatment of pigmentation-related skin conditions [13–17].

The Araliaceae family encompasses trees, shrubs, and climbers. This family boasts numerous species that are widely utilized as ornamental plants, including *Schefflera arboricola* L. and *Fatsia japonica* (Thunb.) Decne. & Planch [18]. Notably, the Araliaceae family also comprises plants of significant medicinal value such as ginseng (*Panax* spp.) and ivy (*Hedera* spp.) [19–21]. The representative of this family is also *Eleutherococcus divaricatus* (Siebold and Zucc.) S. Y. Hu., a plant that has been used in Far Eastern traditions for centuries [22].

E. divaricatus root contains many compounds, including eleutherosides, flavonoids, triterpenoids, and phenolic acids. The main metabolites vary considerably and are called the eleutherosides, with eleutherosides B (syringin 4-β-D-glucoside) and E ((−)-siringaresinol 4,4″-*O*-β-D-diglucoside) accounting for the majority. *E. divaricatus* is a plant that has been used for medicinal purposes in traditional medicine systems for many years. It has various pharmacological properties, such as anti-inflammatory, anti-cancer, anti-depressant, antidiabetic, anti-fatigue, neuroprotective, hepatoprotective and immunostimulative activities [23–28]. Despite its long history of use in ethnopharmacology to treat the above-mentioned diseases, the mechanism of its action remains largely unexplored. Załuski's et al. previous research indicated the presence of MMP-1 and MMP-9 inhibitors in chloroform extracts [29].

We hypothesized that the roots of *E. divaricatus* contain compounds with anti-hyaluronidase (an enzyme related to tissue degradation) and anti-tyrosinase (an enzyme involved in melanin production) activity. To prove our hypothesis, HPLC-PDA, UHPLC-DAD/ESI-TOF-MS, and biological techniques were used. To obtain more reliable results, we studied both commercially available enzymes (hyaluronidase and tyrosinase), as well as serum hyaluronidase from children diagnosed with acute lymphoblastic leukemia. Additionally, antioxidant and anti-melanoma activities were tested.

2. Results and Discussion

2.1. Chemical Panel

Phytochemicals, which are very often characteristic of only a small group of plants, are responsible for their pharmacological effects. The *Eleutherococcus* genus is rich in large numbers of compounds, which makes it is a potential source of plant-based medicines. However, the activity of traditional medicines is usually caused by the combination of compounds, which means that no single active compound can be isolated. In some cases, a fractionation process is the most reasonable approach, which leads to the attainment of active fractions. The first step of this study was to determine the most effective solvent for extraction, expressed as the lowest IC_{50} value for enzyme inhibition. On the basis of these results, 75% methanol extract was chosen for phytochemical analysis as a promising source of inhibitors of hyaluronidase and tyrosinase. Subsequently, using liquid–liquid extraction, 75% methanol extract was fractionated for four fractions. Fractionation resulted in 2.02, 1.02, 5.11, and 9.26 g of n-hexane, ethyl acetate, n-butanol, and water mass fraction, respectively.

The total contents of phenolic compounds (TPC), flavonoids (TFC), and phenolic acids (TPAC) are shown in Table 1. Our study revealed that the ethyl acetate fraction had the highest concentrations of polyphenols (110.89 ± 6.32 mg/g), flavonoids (27.95 ± 4.11 mg/g), and phenolic acids (2.81 ± 0.48 mg/g). Literature data do not provide much information about polyphenols in *Eleutherococcus* spp., especially with respect to fractions. Załuski et al. studied ethanolic extract obtained from the roots of *E. divaricatus*, which contained 6.9 ± 0.4 mg GAE/g polyphenols per dry sample [30]. In turn, Adamczyk et al. reported that the polyphenol and flavonoid contents in 75% MeOH extracts were equal 9.4 ± 0.9 gGAE/g and 6.5 ± 1.1 gQE/g, respectively [31]. The contents of polyphenols, flavonoids, and phenolic acids of hydrophobic–hydrophilic extract from the roots of *E.*

senticosus enriched with naringenin were 159.27 ± 2.73 mgGAE/g, 137.47 ± 5.23 mgQE/g, and 79.99 ± 3.57 mgCAE/g, respectively [32].

Table 1. Chemical composition of the 75% methanol extract of *E. divaricatus* after its fractionation using liquid–liquid extraction [mg/g ext. ± SD] and mass of fraction [g]. Different superscript lowercase letters indicate a statistically significant difference between the fractions within the same column, with $p < 0.05$.

Fraction	TPC [mgGAE/g]	TFC [mgQE/g]	TPAC [mgCAE/g]	Mass of Fraction [g]
n-Hexane	68.16 ± 1.32 ^{ab}	21.80 ± 1.53 ^{ab}	1.03 ± 0.18 ^{ab}	2.02
Ethyl acetate (EtOAc)	110.89 ± 6.32 ^{b}	27.95 ± 4.11 ^{b}	2.81 ± 0.48 ^{b}	1.02
n-Butanol (n-BuOH)	22.03 ± 0.77 ^{ab}	0.65 ± 0.77 ^{a}	1.74 ± 0.24 ^{ab}	5.11
Water	4.02 ± 2.88 ^{a}	3.36 ± 2.17 ^{ab}	0.55 ± 0.033 ^{a}	9.26

In the next step, the phenolic composition of the fractions was characterized using mass spectrometry. Chromatographic parameters and mass spectra were compared with standards, or components were tentatively identified based on the literature. A representative chromatogram of the most abundant fraction, ethyl acetate, is shown in Figure 1. The mass data used for identification are summarized in Table 2.

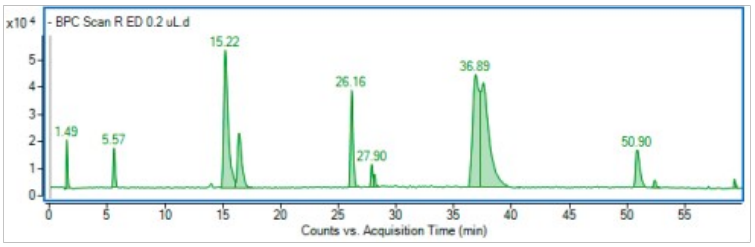

Figure 1. Base peak chromatogram (BPC) of the ethyl acetate fraction obtained in negative ionization mode.

Table 2. Phenolic composition of *E. divaricatus* fractions obtained using UHPLC-DAD/ESI-TOF-MS.

N°	Rt (min)	Observed Ion Mass [M − H]$^-$/(Fragments)	Δ ppm	Formula	Identified
1	5.57	153.01973	2.58	C7H6O4	Protocatechuic acid *
2	8.73	137.02481	2.84	C7H6O3	Hydroxybenzoic acid
3	14.00	289.07203	0.92	C15H14O6	Catechin *
4	15.20	179.03505 (135, 191)	0.38	C9H8O4	Caffeic acid *
5	16.42	353.08835 (191, 179)	1.54	C16H18O9	Chlorogenic acid *
6	26.16	389.12423 (227)	0.10	C20H22O8	Piceid (Resveratrol der.)
7	36.84	515.12021 (353)	1.38	C25H24O12	3,5-dicaffeoylquinic acid *
8	37.63	515.12048 (353)	1.90	C25H24O12	Dicaffeoylquinic acid
9	50.90	515.12035 (353)	1.65	C25H24O12	4,5-dicaffeoylquinic acid *
10	59.33	577.13521 (198, 385)	0.10	C30H26O12	Diferulic acid derivative

*—identification was confirmed by comparison with standards.

The analysis revealed the presence of 10 compounds, predominantly derivatives of cinnamic acid such as caffeic acid and its derivatives, including chlorogenic acid, 3,5-dicaffeoylquinic acid, dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid (Figure 2, Table 3). Furthermore, the fraction contains derivatives of benzoic acid, such as protocatechuic acid, hydroxybenzoic acid, and a diferulic acid derivative, along with a low amount of

catechin. None of the investigated eleutherosides (eleutherosides B and E) were found in the fractions.

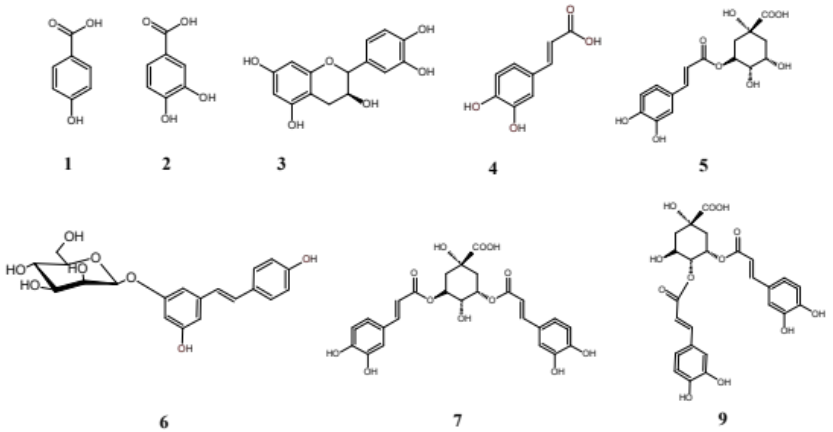

Figure 2. Formulae of the compounds present in the EtOAc fraction according to Table 2.

Table 3. The results of the quantification of the main identified components, expressed in mg per g of dried fractions.

	PA	CA	ChA	3,5-DCA	DCA	4,5-DCA
n-Hexane	0.017 ± 0.001	0.077 ± 0.006	0.593 ± 0.020	0.283 ± 0.001	0.334 ± 0.001	0.150 ± 0.009
Ethyl acetate	9.293 ± 0.105	24.018 ± 0.045	16.653 ± 0.055	126.97 ± 3.08	150.63 ± 3.65	26.615 ± 0.253
n-Butanol	0.492 ± 0.001	0.595 ± 0.003	59.198 ± 0.153	24.87 ± 0.109	29.51 ± 0.129	6.383 ± 0.061
Water	0.169 ± 0.008	0.281 ± 0.004	44.360 ± 0.102	1.294 ± 0.011	1.540 ± 0.013	0.129 ± 0.009

PA—protocatechuic acid; CA—caffeic acid; ChA—chlorogenic acid; DCA—dicaffeoylquinic acid.

The fractions were rich in dicaffeoylquinic acid and chlorogenic acid. It is very interesting that EtOAc was rich in caffeic acid as a representative of simple phenolic acids. A similar phytochemical composition was observed in methanolic extracts obtained from *E. henryi* leaves, which contained caffeoylquinic acid derivatives such as 5-caffeoylquinic acid (5-CQA—27.54 mg/g), 4-caffeoylquinic acid (4-CQA—5.91 mg/g), 3,4-dicaffeoylquinic acid (3,4-DCQA—0.66 mg/g), 3,5-dicaffeoylquinic acid (3,5-DCQA—5.91 mg/g), 1,5-dicaffeoylquinic acid (1,5-DCQA—0.853 mg/g), and 4,5-dicaffeoylquinic acid (4,5-DCQA—3.81 mg/g) [33]. A 75% methanolic extract of *E. divaricatus* was found to contain benzoic acid (salic acid and protocatechuic acid) and cinnamic acid (caffeic acid, ferulic acid, and *p*-coumaric acid) derivatives [31]. Phenolic acids such as trans-4-hydroxycinnamic acid, trans-caffeic acid, and methyl caffeate were found in the methanolic extract from the stem of *E. divaricatus*. In addition, the extract was rich in phenolic alcohols (4-(3-methoxy-1-propen-1-yl)-1,2-benzenediol, coniferyl alcohol, and 4-[(1*E*)-3-methoxy-1-propenyl]phenol) and stilbens ((þ)-pinoresinol, (þ)-medioresinol, (þ)-syringaresinol, acanthoside B, obtusifoside A, acanthoside D, (þ)-sesamin, (þ)-lariciresinol-9-O-*β*-D-glucopyranoside, (þ)-alangilignoside C, and (þ)-salvadoraside) [34].

2.2. Anti-Enzymatic Panel

2.2.1. Inhibition of Bovine Hyaluronidase (bHYAL) and Fungal Tyrosinase (mTYR) by Crude Extracts and Fractions

The *Eleutherococcus* genus, known for its diverse phytochemical composition, exhibits a wide range of biological activities. The *Eleutherococcus* species have been used in traditional

medicine for centuries, offering benefits such as adaptogenic, immunostimulant, stress-combating, anti-fatigue, antioxidant, anti-inflammatory, anti-tumor, neuroprotective, and antidiabetic properties.

Our study explored the impact of *E. divaricatus* root extract on the activity of bovine hyaluronidase (bHYAL) and fungal tyrosinase (mTYR). The initial phase of the study was dedicated to determining the most effective solvent for extraction, expressed as the lowest IC_{50} value for enzyme inhibition (Table 4). The IC_{50} values for bovine hyaluronidase ranged between 100.8 and 181.27 µg/mL, and those for tyrosinase ranged between 103.6 and 274.37 µg/mL. The most active appeared to be 75% methanol, with an IC_{50} of 100.8 µg/mL for bHYAL and 103.6 µg/mL for mTYR.

Table 4. Activity of polar and nonpolar extracts against hyaluronidase from bovine testes and mushroom tyrosinase. IC_{50} values are shown in µg/mL. Different superscript lowercase letters indicate a statistically significant difference between the fractions within the same column, with $p < 0.05$.

Type of Extract	bHYAL	mTYR
Chloroform ($CHCl_3$)	111.73 ± 0.75^{a}	188.50 ± 1.83^{ab}
Ethyl acetate	104.13 ± 2.51^{a}	274.37 ± 3.69^{b}
75% methanol	100.80 ± 0.9^{a}	103.60 ± 4.23^{a}
$CHCl_3$:MeOH:H_2O	181.27 ± 0.92^{a}	221.83 ± 2.21^{ab}

$CHCl_3$:MeOH:H_2O volume ratios, 7:3:0.4; bHYAL—bovine hyaluronidase; mTYR—mushroom tyrosinase.

In the next step, the 75% methanol extract was subjected into liquid–liquid extraction using nonpolar, medium-polar, and polar solvents (n-hexane, ethyl acetate, n-butanol, and water, respectively). It was found that ethyl acetate fraction showed the highest activity, with an IC_{50} value equal to 27.5 µg/mL for bHYAL and equal to 65.5 µg/mL for mTYR (Table 5). It should be noted that the activity of *E. divaricatus* was stronger than that of the positive control, aescin (IC_{50} = 388.8 ± 1.81 µg/mL). In the case of tyrosinase, none of the fractions showed activity exceeding the value for kojic acid (IC_{50} = 4.44 ± 0.06 µg/mL). The activities of phenolic acids and eleutherosides present in the highest amounts were further determined (Table 6). For both bHYAL and mTYR, the most active compound was caffeic acid (bHYAL—IC_{50} = 111.34 ± 3.59 µg/mL; mTYR—IC_{50} = 60.77 ± 2.37 µg/mL). Eleutherosides B, E, and E1 showed no activity.

Table 5. Activity of fractions obtained from methanolic extract of *E. divaricatus* root against bHYAL and mTYR. IC_{50} values are shown in µg/mL. Different superscript lowercase letters indicate a statistically significant difference between the fractions themselves and between the fractions and control within the same column, with $p < 0.05$.

Type of Fraction	bHYAL	mTYR
n-Hexane	94.44 ± 0.80^{ab}	207.50 ± 3.63^{b}
Ethyl acetate	27.50 ± 0.65^{a}	65.50 ± 1.35^{ab}
n-Butanol	56.10 ± 6.86^{ab}	85.40 ± 2.51^{ab}
Water	71.60 ± 3.87^{ab}	81.10 ± 5.32^{ab}
Aescin	388.8 ± 1.81^{b}	
Kojic acid		4.44 ± 0.06^{a}

bHYAL—bovine hyaluronidase; mTYR—mushroom tyrosinase.

There are many research papers investigating the effects of phenolic acids on tyrosinase and hyaluronidase. In a previous study, the fruits of *E. divaricatus* demonstrated moderate inhibitory activity against hyaluronidase and weak anti-tyrosinase activity (IC_{50} = 0.45 and IC_{50} = 2.67 mg/mL, respectively) [35]. Isolated phenolic acids (derivatives of seric acid) from *Oenanthe javanica* inhibited the activity of bovine hyaluronidase (IC_{50} = 0.19–1.33 mM) [36]. Cimicifugic acids K-N (IC_{50} = 102–255 µM) isolated from the aboveground parts of *Cimicifuga*

simplex and *C. japonica*, exhibited more potent hyaluronidase-inhibitory activities than rosmarinic acid (IC_{50} = 545 µM) [37].

Table 6. Activity of selective phenolic acid and eleutherosides against bHYAL and mTYR. IC_{50} values are shown in µg/mL. Different superscript lowercase letters indicate a statistically significant difference between the different acids within the same column, with $p < 0.05$.

Standard	bHYAL	mTYR
Eleutheroside B	NA	NA
Eleutheroside E	NA	NA
Eleutheroside E_1	NA	NA
Caffeic acid	111.34 ± 3.59 ^{a}	56.22 ± 0.67 ^{a}
Chlorogenic acid	519.14 ± 17.94 ^{ab}	107.52 ± 3.46 ^{ab}
Protocatechuic acid	920.20 ± 87.71 ^{b}	134.57 ± 3.46 ^{b}

bHYAL—bovine hyaluronidase; mTYR—mushroom tyrosinase.

2.2.2. Inhibition of Human Hyaluronidase (hHYAL) and Human Tyrosinase (hTYR) in Blood Samples from Children Diagnosed with Acute Lymphoblastic Leukemia by Ethyl Acetate Fraction

Hyaluronidase and tyrosinase contribute to the progression of many diseases, which very often have a cancerous background, and their overactivity is observed. Taking this into consideration, we decided to establish the level of these enzymes in blood samples from acute leukemia patients and to examine the influence of the ethyl acetate fraction on their activity. We chose leukemic patients because it is known that in their case, e.g., hyaluronidase levels are high [38]. Five boys with a median age of 4.5 years diagnosed with acute lymphoblastic leukemia (ALL) before starting treatment were included in the study. Serum levels of hHYAL ranged between 25.20 and 162.15 ng/mL, while hTYR levels ranged between 4.68 and 78.94 ng/mL (Table 7). The obtained results show that the ethyl acetate fraction contains inhibitors of hyaluronidase with aescin-like activity (Table 7). The EtOAc fraction inhibited hHYAL in a range of 30.43–89.85%, with mean group values of 55.82% and 63.8% with aescin used as a control. However, interestingly, the fraction showed no activity against hTYR.

Table 7. Activity of ethyl acetate fraction (EtOAc) against hyaluronidase and tyrosinase from human serum isolated from the blood children diagnosed with acute lymphoblastic leukemia (N°). Results are presented in %. Different superscript lowercase letters indicate a statistically significant difference between the samples within the same column, with $p < 0.05$.

N°	Level of hHYAL [ng/mL]	hHYAL [%] EtOAc Mean ± SD	Level of hTYR [ng/mL]	hTYR [%] EtOAc Mean ± SD
1	95.27	53.47 ± 12.37 ^{ab}	4.68	NA
2	116.90	89.85 ± 7.73 ^{b}	9.31	NA
3	162.15	66.67 ± 20.00 ^{ab}	15.26	NA
4	81.86	38.71 ± 5.59 ^{ab}	78.94	NA
5	25.20	30.43 ± 3.07 ^{a}	52.17	NA
Mean value	96.27	55.82	32.07	-

NA—Not Active.

There is a lack of studies in the literature on the activity of natural and synthetic compounds against human hyaluronidases and tyrosinases isolated directly from the blood. To the best of our knowledge, information about the activity of *E. divaricatus* roots against these enzymes was obtained for the first time in this study. In our previous studies, we proved the activity of 75% methanolic *E. divaricatus* fruit extract against hHYAL (76.46–86.13%) [35]. In another study, we evaluated the activity of the *intractum of E. senticosus* fruits. The *intractum* significantly inhibited human hyaluronidase activity in ranges of 58.80–76.32% and

20.00–47.37% with aescin used as a control. The results mean that the *intractum* inhibited hyaluronidase activity with mean group values of 60% and 40% with aescin used as a control [38].

2.3. Antioxidant Panel

Free radicals are responsible for many of pathogenic processes in the human body, resulting in the development of, e.g., inflammation-related diseases. Taking into consideration the ability to inhibit hyaluronidase (proinflammatory agent), it is important to evaluate the antioxidative activity of fractions. When evaluated for their reactivity towards the ABTS cation radical, the ethyl acetate and n-butanol fractions exhibited the most significant activity, with values of 9.69 ± 0.0035 µg/mL and 10.10 ± 0.21 µg/mL, respectively. Similarly, against the DPPH radical, these fractions demonstrated the highest potency, with values of 36.83 ± 2.43 µg/mL and 61.49 ± 1.87 µg/mL, respectively. The results are presented in Table 8.

Table 8. Antioxidants activity of *Eleutherococcus divaricatus* fractions. IC_{50} values are shown in µg/mL. The results for ferrozine are shown in %. Different superscript lowercase letters indicate a statistically significant difference between the fractions themselves and between the fractions and control within the same column, with $p < 0.05$.

Fraction	IC_{50} ABTS	IC_{50} DPPH	Ferrozine *
n-Hexane	80.20 ± 5.40 ^{b}	NA	80.15 ± 0.73
Ethyl acetate	9.69 ± 0.035 ^{ab}	36.83 ± 2.43 ^{ab}	34.44 ± 3.99
n-Butanol	10.10 ± 0.21 ^{ab}	61.49 ± 1.87 ^{ab}	23.52 ± 3.24
Water	24.99 ± 0.095 ^{ab}	106.10 ± 4.51 ^{b}	4.09 ± 2.48
BHA	2.35 ± 0.11 ^{a}	62.52 ± 4.13 ^{ab}	
AA	2.27 ± 0.07 ^{a}	24.93 ± 0.28 ^{ab}	
TROLOX	2.85 ± 0.18 ^{ab}	13.68 ± 3.53 ^{a}	
EDTA			98.9 ± 0.01

NA—not active; * results for 1 mg/mL.

There are few studies on the antioxidant properties of *E. divaricatus* roots. The activity of 75% methanolic extract of *E. divaricatus* roots (0.8 mg/mL) against the DPPH radical after 90 min was 23.00 ± 0.79% [31]. In another study, IC_{50} values of the chloroform and ethanol extracts of *E. divaricatus* roots against the DPPH radical were 50.1 ± 0.5 and 1.2 ± 0.2, respectively. For chelation, IC_{50} values for chloroform and ethanol extracts were 0.9 ± 0.51 mg/mL and 0.8 ± 0.01 mg/mL, respectively [30]. In our previous investigations of *E. divaricatus* fruits, 75% methanolic extract showed moderate activity against the ABTS radical, DPPH, and chelating properties, with IC_{50} values of 280, 1300, and 1450 µg/mL, respectively [35]. In a study by Yu et al., the most active fractions against the DPPH radical were EtOAc and BuOH methanolic extract of *E. senticosus* root. The antioxidant activities in the EtOAc and BuOH fractions were higher than or similar to those of α-tocopherol [39]. Furthermore, concentrated powder from *E. senticosus* produced by Sheng Chang Pharmaceutical inhibited DPPH generation by 58.3 ± 2.8% at 1000 µg/mL [40]. The IC_{50} values for hydrophobic–hydrophilic naringenin-enriched extract (1 mg/mL) against the DPPH radical and ABTS were 138.17 ± 4.28 µg/mL and 18.10 ± 0.20 µg/mL, respectively. The chelating capacity at a concentration of 1 mg/mL was 26.34 ± 1.14% [32]. In turn, the IC_{50} values obtained for essential oil from *E. simonii* leaves were 1125 µg/mL, 945 µg/mL, and 862 µg/mL using the DPPH radical, and the ABTS and FRAP methods, respectively [41].

2.4. Principal Component Analysis (PCA)

The PCA plot shows (Figure 3) a clear grouping of *E. divaricatus* extract fractions according to the solvents used (n-hexane, ethyl acetate, n-butanol, and water), suggesting that the solvents influence the chemical composition of the fractions, as well as their anti-enzymatic and antioxidant properties. The first principal component explains 44.85% of the variance, and the second principal component explains 41.40%. The H_2O and n-BuOH fractions, located on the left side of the diagram, are associated with higher chlorogenic acid contents and higher IC_{50} values against DPPH. The n-hexane fraction, located on the right side of the plot, is mainly associated with higher IC_{50} values against ABTS and tyrosinase compared to the other extracts. The EtOAc fraction shows correlations with the vectors for protocatechuic and caffeic acids, as well as TPAC content.

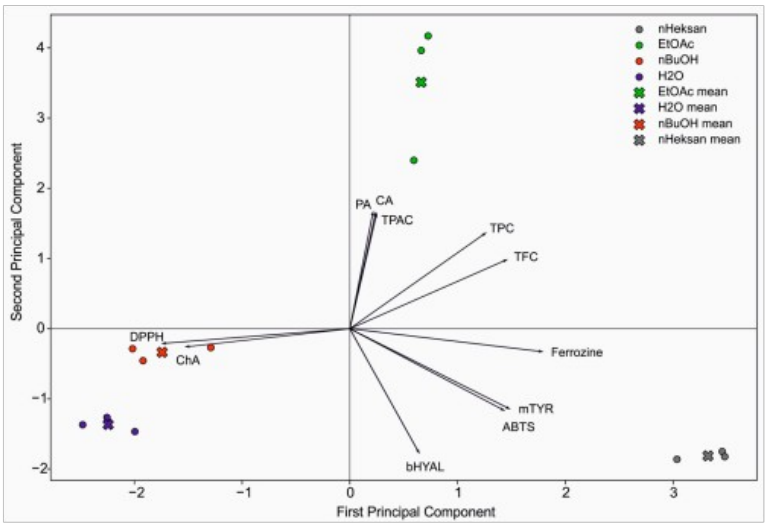

Figure 3. Principal component analysis of the studied fractions of *E. divaricatus* extract based on phenolic compound content and anti-enzymatic and antioxidant activities.

2.5. Cytotoxicity Panel

Ethyl Acetate Fraction of *E. divaricatus* Does Not Affect the Viability of Normal and Cancerous Skin Cells

Three different human melanoma cell lines, namely SK-MEL-30, UACC-647, and A375, as well as normal BJ fibroblasts, were chosen to investigate the cytotoxicity of the ethyl acetate fraction. We observed no major changes in the viability of the tested cell lines in doses of up to 200 µg/mL, which suggests that the fraction does not have any toxic effects on melanoma cells. Additionally, the fraction did not cause any changes in normal fibroblasts, which may indicate that at the doses used in this study, this fraction is safe. Doxorubicin was used as a control, for which the IC_{10}, IC_{50}, and IC_{90} values were established (Figure 4). In SK-MEL-30 cells, 10% of maximal inhibition was generated by DOX at a dose of 0.24 µM, 50% of maximal inhibition was generated at a dose of 1.52 µM, and 90% of maximal inhibition was generated at a dose of 9.63 µM. In UACC-647 cells, DOX yielded IC_{10}, IC_{50}, and IC_{90} values of 0.36, 1.16, and 3.76 µM, respectively. In the A375 cell line, the IC_{10} for DOX was found to be 0.09 µM, the IC_{50} was found to be 0.33 µM, and the IC_{90} was found to be 1.19 µM. The cell lines exhibited differences not only in IC_{50} values but also in the level of inhibition. For instance, the maximal level of inhibition was estimated to be 96.38% in A375 cells. Under the same conditions, the viability of SK-MEL-30

was observed to drop by a maximum of 81.87%. In contrast, the viability of UACC-647 cells was found to be suppressed by only 39.15%.

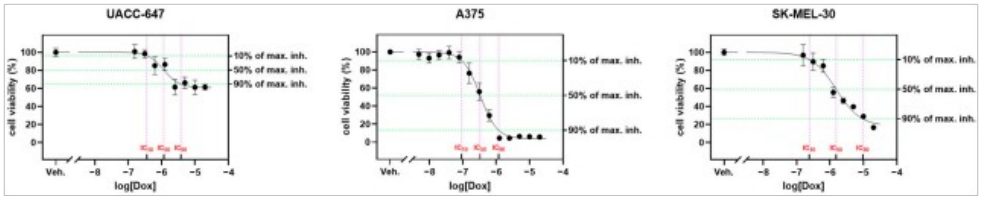

Figure 4. Effect of doxorubicin (DOX) on the viability of UACC-647, A375, and SK-MEL-30 melanoma cell lines. The 10, 50, and 90% levels of maximal viability inhibition are indicated by horizontal dotted lines. The IC_{10}, IC_{50}, and IC_{90} values are indicated by vertical dotted lines.

Natural compounds are frequently used concurrently with chemotherapeutic drugs in the treatment of different diseases. Thus, it is crucial to investigate potential interactions between these types of compounds. In the next step, a potential interaction between DOX and the ethyl acetate fraction was examined (Figure 5). Three melanoma cell lines—UACC-647, A375, and SK-MEL-30—were treated with DOX at three different doses (IC_{10}, IC_{50}, and IC_{90}, as determined independently for each cell line) simultaneously with the EtOAc fraction (200 µg/mL). In all cell lines, a protective effect of the EtOAc fraction was observed, which was concomitant with a decrease in DOX-dependent cytotoxicity. For instance, A375 cells subjected to the EtOAc fraction in combination with the IC_{90} of DOX experienced only a 37% drop in viability (Figure 5, middle panel), whereas DOX alone (at IC_{90}) inhibited the viability of A375 cells by 96%.

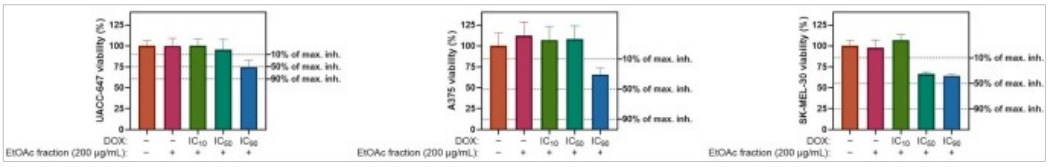

Figure 5. Effect of doxorubicin (DOX) and the EtOAc fraction on the viability of UACC-647, A375, and SK-MEL-30 melanoma cell lines. The cells were exposed to either the IC_{10}, IC_{50}, or IC_{90} of DOX in combination with the EtOAc fraction at a dose of 200 µg/mL. The 10, 50, and 90% levels of maximal viability inhibition elicited by DOX alone are indicated by horizontal dotted lines.

The literature does not provide sufficient information about the cytotoxicity of the root of *E. divaricatus*. Among the five studied species, *E. henryi* extract showed the strongest inhibition of HL-60 cell line growth, with an IC_{50} value of 270 µg/mL, while for *E. divaricatus*, the IC_{50} value was 650 µg/mL [31]. Fruit extract and hydrophobic–hydrophilic extract from the root of *E. senticosus*, which is enriched with naringenin, did not affect the viability of two cancer cell lines, namely FaDu and HepG2 [32]. The 70% ethanol extract of *E. sessiliflorus* leaves, which is rich in flavonoids, terpenoids, and xylogens, showed weak cytotoxic activity against the A549 cell line [42]. In contrast, taiwanin E, a compound isolated from a branch of *E. trifoliatus*, demonstrated strong cytotoxicity. It exhibited potent antiproliferative effects on the growth of the MCF-7 human breast adenocarcinoma cell line, with an IC_{50} value of 1.47 µM [43]. Elesesterpenes A–K, extracted from the leaves of *E. sessiliflorus* have shown significant antiproliferative activity against several human cancer cell lines, including hepatocellular carcinoma (HepG2), lung adenocarcinoma (A549), and glioblastoma multiforme (LN229), with IC_{50} values ranging from 1.05 to 8.60 µM [44].

3. Materials and Methods

3.1. Chemicals and Reagents

The standards of eleutheroside B ≥ 98.0% (HPLC) and eleutheroside E ≥ 98.0% (HPLC), protocatechuic acid ≥ 97%, *p*-hydroxybenzoic acid 99%, vanillic acid ≥ 97%, caffeic acid ≥ 98%, ferulic acid ≥ 99%, rosmarinic acid ≥ 98%, DMEM, RPMI 1649 Medium, phosphate buffered saline (PBS), gradient grade acetonitrile, and trifluoroacetic acid ≥ 99%, ascorbic acid, 2(3)-t-butylhydroquinone monomethyl ether (BHA), 2(3)-t-butyl-4-hydroxyanisole, hyaluronic acid (IV), aescin > 95%, hyaluronidase from bovine testes, hexadecyltrimethylammonium bromide (CTAB), L-tyrosine ≥ 98%, kojic acid, and muschroom's tyrosinase were obtained from Sigma-Aldrich (St. Louis, MO, USA). Additionally, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), potassium persulfate, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate 97% (ferrozine), iron(II) chloride tetrahydrate >98% ($FeCl_2 \times 4H_2O$), 1,3,5-Tri(2-pyridyl)-2,4,6-triazine (TPTZ), iron (III) chloride > 98% ($FeCl_3$), aluminum chloride ($AlCl_3$), potassium acetate, Folin−Ciocalteu reagent, sodium nitrite, sodium molybdate, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were used. All these substances were purchased from Sigma-Aldrich Corp (Saint Louis, MO, USA). The solvents used for extraction were sourced from Avantor Performance Materials (Gliwice, Poland).

3.2. Extraction and Plant Material

The roots of *E. divaricatus* were harvested from the Arboretum of the Warsaw University of Life Sciences in Rogów, Poland, in October 2020. The identification of the raw material was carried out by prof. Daniel Załuski. The extraction process began by preparing an extract from 5 g of *E. divaricatus* root. The root was dried for 4 weeks at room temperature in a dark place. Then, 50 mL of chloroform was poured over the ground root and left for 24 h. Next, the mixture was placed in an ultrasonic bath for 15 min, and the resulting solution was filtered. This process was repeated three times. The filtrate was then concentrated using a vacuum evaporator at 40 °C. After the initial chloroform extraction, the raw material underwent subsequent extractions with ethyl acetate and 75% methanol. The extraction procedure for these solvents was identical to that used for chloroform.

Next, the extracts were tested against hyaluronidase and tyrosinase, and the most active one was subjected to fractionation using liquid–liquid extraction. A 75% methanolic extract of *E. divaricatus* was prepared in a larger amount. Then, 115.109 g of root powder was extracted with 75% methanol solution for three days at room temperature. The flask was then placed in an ultrasonic bath for 15 min. The mixture was filtered, and the crude was poured over 50 mL of 75% MeOH and placed back in a water bath. The procedure was repeated until the filtrate was decolorized.

The obtained extract was concentrated in a vacuum evaporator at 40 °C, yielding 190.08 g/kg of raw material. This residue was fractionated using liquid–liquid extraction (Figure 1).

Liquid–Liquid Extraction of Polyphenols

The dried 75% methanol extract, obtained as described in Section 2.2, was suspended in 250 mL of water and extracted with 100 mL of n-hexane. The organic layer was collected, and the procedure was repeated twice using n-hexane. The aqueous solution was then extracted with 100 mL of ethyl acetate and, finally, with 100 mL of saturated n-butanol, as shown in Figure 6. The obtained fractions were concentrated in a vacuum evaporator at 40 °C. The obtained fractions were stored in a refrigerator.

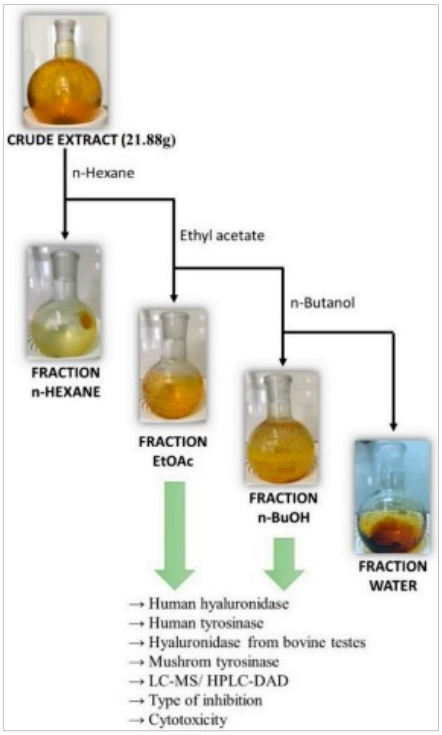

Figure 6. Scheme of liquid–liquid extraction of the 75% methanol extract.

3.3. Phytochemical Panel

3.3.1. Chemical Composition

Determination of Total Phenolic Content (TPC)

A modified Folin–Ciocalteu method was employed to quantify the total phenolic content [45]. First, 25 µL of extract (1 mg/mL in methanol) was combined with 25 µL the Folin−Ciocalteu reagent (diluted 1:3 in pure water) in a 1:1 ratio. After adding 150 µL of distilled water, the mixture was incubated for 5 min. A solution of sodium carbonate (10%) was then added, and the mixture was left to incubate in the dark at room temperature for an hour. The absorbance of the solution was measured at a wavelength of 750 nm. The total phenolic content (TPC) results are expressed in terms of milligrams of gallic acid (GA) equivalents (GAE) per gram of the sample (mg GAE/g sample).

Determination of Total Flavonoid Content (TFC)

The total flavonoid content was quantified using a method that relies on the reaction between aluminum chloride ($AlCl_3$) and flavonoids [46]. Briefly, 25 µL extract (1 mg/mL in methanol) was mixed with 75 µL ethanol. Subsequently, 10% aluminum chloride (10 µL) and 1M potassium acetate (10 µL) were added to the mixture. After adding 130 µL of distilled water, the mixture was incubated for 30 min. The absorbance of the solution was then measured at a wavelength of 510 nm. The total flavonoid content (TFC) results are expressed as milligrams of quercetin equivalents (QE) per gram of the sample (mg QE/g sample).

Determination of Total Phenolic Acid Content (TPAC)

The total phenolic acid content was determined following the method outlined in the Polish Pharmacopeia VI [47]. First, 25 µL of extract (1 mg/mL in methanol) was combined with 150 distilled water (150 µL), hydrochloric acid (25 µL, 0.5M), and Arnov's reagent (25 µL, a solution of 10.0 g of sodium molybdate and 10.0 g of sodium nitrite in 100 mL of distilled water). Then, 25 µL of solution of sodium hydroxide (1M) was added to the mixture, which was immediately measured at a wavelength of 492 nm. The total phenolic content (TPC) results are expressed in terms of milligrams of caffeic acid (CA) equivalents (CAE) per gram of the sample (mg CAE/g sample).

3.3.2. Chromatographic Analysis

All standards, formic acid, and MS-grade acetonitrile were from Sigma-Aldrich (St. Louis, MO, USA). MS data were acquired using an Infnity Series II ultra-high-performance liquid chromatograph (UHPLC) with an Agilent 6224 ESI/TOF mass detector (Agilent Technologies, Santa Clara, CA, USA). The conditions were as follows: an RP18 reversed-phase Titan column (Supelco, Sigma-Aldrich, Burlington, MA, USA) (10 cm × 2.1 mm i.d., 1.9 µm particle size), a thermostat temperature of 30 °C, and a flow rate of 0.2 mL/min. Water with 0.05% formic acid (solvent A) and acetonitrile with 0.05% formic acid (solvent B) were used as the mobile phase composition. The gradient elution program was as follows: 0–8 min from 97% A to 95% A, 8–15 min at 95% A, 15–29 min from 95% A to 85% A, 29–40 min at 85% A, 40–50 min from 85% A to 80% A, and 50–60 min from 80% A to 65%. LC–MS conditions were as follows: drying gas temperature of 325 °C, drying gas flow of 8 L min^{-1}, nebulizer pressure of 30 psi, capillary voltage of 3500 V, and a 65 V skimmer. The voltage of the fragmentator was 220 V. Ions were acquired in the range of 100 to 1200 m/z in negative ions.

Quantitative analyses were performed using an EliteLaChrom chromatograph equipped with a PDA detector and EZChrom Elite software (Version 3.3.2 SP2 build 3.3.2.1037) from Merck (Darmstadt, Germany) and an RP18 reversed-phase Kinetex column measuring 25 cm × 4.6 mm i.d. with a particle size of 5 µm (Phenomenex, Torrance, CA, USA). The elution conditions are described above. The flow rate was set at 1.0mL/min, and data were collected within the wavelength range of 190 to 400 nm. The identities of the compounds were confirmed by comparing their retention times and UV spectra with those of corresponding standards. Quantitative analysis was carried out at the following specific wavelengths for each compound: 260 nm for protocatechuic acid and 325 nm for chlorogenic acid and dicaffeoylquinic acid.

3.4. Enzymatic Panel

3.4.1. Bovine Hyaluronidase Inhibition Assay

Bovine hyaluronidase inhibitor assays were conducted in 96-well plates, following a modified method originally described by Di Ferrante [48] and Studzińska-Sroka [49]. The activity of the compounds or extracts was determined based on the precipitation of non-hydrolyzed hyaluronic acid using cetyltrimethylammonium bromide (CTAB). A 10% water solution of DMSO was used to dissolve the extract. Then, 15 µL of extracts at concentrations of 10.0, 1.0, and 0.1 mg/mL in the well were mixed with an acetate buffer (15 µL, pH = 5.35), an incubation buffer (25 µL, pH = 5.35, 0.01% BSA, 0.45% NaCl), and an enzyme solution (25 µL, 30 U/mL in the incubation buffer). This mixture was incubated at 37 °C for 10 min. Following this, a hyaluronic acid solution (25 µL, 0.3 mg/mL in an acetate buffer with a pH of 5.35) was added. The plates were then incubated for an additional 45 min at 37 °C. After incubation, non-hydrolyzed hyaluronic acid was precipitated by adding 2.5% CTAB (200 µL). The plates were then maintained at 25 °C for 10 min. The intensity of the complex formation was measured at a wavelength of 600 nm. The presence of inhibition was determined by measuring the absorbance of the solution without the

inhibitor (A_C) and the enzyme (A_T). All samples were tested in triplicate. The inhibition of hyaluronidase was calculated using a specific equation, with aescin used as a standard.

$$INH_{HYAL} = \frac{A_S - A_C}{A_T - A_C} * 100\%$$

A_S—absorbance of the HA + sample + enzyme;
A_C—absorbance of the HA + enzyme;
A_T—absorbance of the HA + sample.

3.4.2. Human Serum Hyaluronidase from Children Diagnosed with Acute Lymphoblastic Leukemia

Blood Samples

Five boys (3, 4, 4, 5, and 17 years old) diagnosed with acute lymphoblastic leukemia (ALL) before starting treatment were included in the study. These patients were diagnosed at the Department of Pediatric Hematology and Oncology at Jurasz University Hospital in Bydgoszcz, Poland, between 2019 and 2020. Venous blood samples were collected from each child while fasting and placed into serum tubes supplied by Becton Dickinson, located in Franklin Lakes, NJ, USA. The blood samples were left to clot at room temperature for 30 min, then centrifuged for 20 min at 2000× *g*, also at room temperature. The samples were then collected and stored at a temperature of −80 °C until the time of analysis. The study was approved by the local Bioethics Committee (approval number 608/2019) and was conducted in accordance with the Declaration of Helsinki.

Level of Human Serum Hyaluronidase

A commercially available kit, the LS-F6310 Human Hyaluronidase (Sandwich ELISA) ELISA Kit (LSBio, Lynnwood, WA, USA), was used to measure the concentration of human hyaluronidase in serum. This kit functions based on the Sandwich assay principle and can detect hyaluronidase levels down to a certain limit (0.115 nanograms per milliliter).

Human Serum Hyaluronidase Inhibition by the Ethyl Acetate Fraction

The inhibition of human serum hyaluronidase was assessed using modified methods [50]. The activity of the compounds or extracts was determined by precipitating non-hydrolyzed hyaluronic acid with cetyltrimethylammonium bromide (CTAB). In brief, 10 µL of ethyl acetate fraction (1 mg/mL) and 50 µL of serum were incubated at 37 °C for 15 min. Following this, a solution of hyaluronic acid (0.3 mg/mL in an acetate buffer with a pH of 5.35) was added in a volume of 40 µL. The plates were then incubated for an additional 45 min at 37 °C. After incubation, non-hydrolyzed hyaluronic acid was precipitated by adding 2.5% CTAB. The plates were then shaken at 25 °C for 10 min. The intensity of the complex formation was measured at a wavelength of 600 nm. All samples were tested in triplicate. The inhibition of hyaluronidase was calculated using a specific equation, with aescin used as a standard.

$$\mathrm{INH_{HYAL}} = \frac{\mathrm{A_S} - \mathrm{A_C}}{\mathrm{A_T} - \mathrm{A_C}} * 100\%$$

A_S—absorbance of the HA + sample + enzyme;
A_C—absorbance of the HA + enzyme;
A_T—absorbance of the HA + sample.

3.4.3. Tyrosinase Inhibition Assay

Tyrosinase inhibitor assays were conducted in 96-well plates using a modified method originally described by Wróbel-Biedrawa [51]. The process involves the conversion of L-tyrosinase to L-DOPA, then to DOPA-quinone, facilitated by the tyrosinase enzyme. This reaction results in the solution turning brown. In brief, 10 µL of the sample (concentrations of 10.0, 1.0, and 0.1 mg/mL in 10% DMSO) was combined with 150 µL of a phosphoric

buffer containing mushroom tyrosinase (pH = 6.88, 100 U/mL). This mixture was then incubated for 10 min at room temperature. A control (A_C) was also prepared that did not contain any inhibitor. Following incubation, L-tyrosine (0.3 mg/mL) was added to each well, and the absorbance was measured at 492 nm (using a kinetic model every 5 min). Two time points (t_1 and t_2) were selected within the linear range of the graph. All samples were tested in triplicate. The inhibition of tyrosinase was calculated using a specific equation, with kojic acid used as a standard.

$$INH_{TYR} = \frac{A_C - A_S}{A_C} * 100\%$$

A_S—the difference in absorbance between times t_2 and t_1 for the sample;
A_C—the difference in absorbance between times t_2 and t_1 for the positive control.

3.5. Antioxidant Panel

3.5.1. ABTS Free Radical Scavenging Activity

The ABTS free radical scavenging test was conducted following the method outlined by Wu et al. [52] A working solution of ABTS+ was prepared by combining 10 mL of ABTS (7 mM in H_2O) with 10 mL of potassium persulfate (2.45 mM in H_2O). This mixture was then left to incubate in the dark for 12 h. The ABTS solution was subsequently diluted with water until it reached an absorbance of 0.700 ± 0.03 at 405 nm. Then, volumes of 10 µL of extracts at concentrations of 1 mg/mL, 0.1 mg/mL, and 0.01 mg/mL (dissolved in MeOH) were combined with 190 µL of the ABTS+ solution and incubated for 30 min. The absorbance at 405 nm was measured after the incubation period. Butylated hydroxyanisole (BHA) was used as a control. The antioxidant activity was then calculated using a specific equation.

$$INH_{ABTS} = \frac{A_C - A_S}{A_C} * 100\%$$

A_S—the absorbance for sample + ABTS;
A_C_the absorbance without sample + ABTS.

3.5.2. DPPH Free Radical Scavenging Activity

The DPPH free radical scavenging test was conducted according to the procedure established by Naseer et al. [53]. A working solution of DPPH• was prepared by dissolving 24 mg of DPPH in 100 mL of distilled water. This solution was further diluted with methanol until it reached an absorbance of 0.900 ± 0.03 at 515 nm. Subsequently, volumes of 10 µL of extracts at concentrations of 1 mg/mL, 0.1 mg/mL, and 0.01 mg/mL (dissolved in MeOH) were combined with 190 µL of the DPPH• solution and allowed to incubate for 60 min. The absorbance at 515 nm was measured post incubation. Butylated hydroxyanisole (BHA) was used as a control. The antioxidant activity was then calculated using a specified equation.

$$INH_{DPPH} = \frac{A_C - A_S}{A_C} * 100\%$$

A_S—the absorbance for sample + DPPH;
A_C—the absorbance without sample + DPPH.

3.5.3. Iron (II) Ion Chelation Assay

The ion chelation assay was conducted using the method proposed by Li et al. [54]. Initially, 100 µL of extract at a concentration of 1.0 mg/mL was combined with methanol (150 µL, MeOH) and iron(II) chloride (5 µL, $FeCl_2$) at a concentration of 2 mM. Subsequently, 5 µL of ferrozine was added at a concentration of 5 mM. Following a period of incubation, the absorbance was measured at a wavelength of 510 nm. The degree of chelation was then

calculated using a specific equation, with ethylenediaminetetraacetic acid (EDTA) serving as a positive control.

$$INH_{Fe^{2+}} = \frac{A_C - A_S}{A_C} * 100\%$$

A_S—the absorbance for sample + ferrozine + $FeCl_2$;
A_C—the absorbance without sample + ferrozine + $FeCl_2$.

3.6. Cytotoxicity Panel

Cell Culture and Cytotoxicity Assessment

The following human melanoma cell lines were used in this study: A375 (ATCC:CRL-1619), UACC-647 (CVCL_4049), and SK-MEL-30 (DSMZ:ACC 151). A375 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM). UACC-647 and SK-MEL-30 were cultured in RPMI-1640. All basal media were supplemented with fetal bovine serum (FBS) at a final concentration of 10%. The control used in the study was BJ foreskin fibroblasts (ATCC:CRL-2522). To facilitate the growth of BJ cells, Eagle's Minimum Essential Medium (EMEM) with 10% FBS was utilized. Tissue culture-treated Petri dishes were used to support all cell lines. A temperature of 37 °C and 5% CO_2 were maintained routinely. The cells were expanded for a few passages, then seeded onto 96-well plates, where they were left to attach overnight. The following day, the cells were exposed to *E. divaricatus* fractionated extracts at descending concentrations, starting from 200 µg/mL, or to a vehicle (DMSO, 0.1%). Alternatively, the cells were treated with doxorubicin (DOX) to independently determine the inhibitory concentrations causing the 10, 50, and 90% levels of maximal inhibition (IC_{10}, IC_{50}, and IC_{90}, respectively) of cellular viability for each melanoma cell line. Then, the melanoma cells were treated with the DOX at IC_{10}, IC_{50}, or IC_{90}, followed by the addition of the fractions of the extracts from *E. divaricatus* at a dose of 200 µg/mL. Upon 24 h of incubation, the viability of the cells was assessed by MTT assay.

3.7. Statistics

Dose–response curves were obtained using GraphPad Prism 8.4.3 software running on a personal computer. Experimental datapoints were fitted to the sigmoidal equation, and the IC_{50} value was calculated. IC_{10} and IC_{90} values were computed using a web tool available at https://www.graphpad.com/ accessed on 20 May 2023.

Statistically significant differences were assessed using Friedman's ANOVA. The Dunn–Bonferroni–Holm test was utilized as a post hoc test. Principal component analysis (PCA) was employed to demonstrate differences between the studied fractions. Analyses were conducted in PQStat software 1.8.6.

4. Conclusions

Advances in phytochemical analysis and phytopharmacology make it possible to identify biologically active substances and to assess their mechanisms of molecular activity. Most knowledge about plant-based compounds comes from ethnopharmacology, in combination with modern concepts in that field, allowing for the development of effective, safe, and standardized plant extracts.

The obtained results confirm the effectiveness of using *E. divaricatus* in TCM to treat inflammation- and immune-related diseases. Simultaneously, in light of what has already been published we should be careful when this plant is administered together with chemotherapy, which is a popular approach to strengthen the body. From this study, it is clear that the EtAOc fraction may decrease the activity of doxorubicin, while it does not inhibit human tyrosinase isolated from the leukemic children. Therefore, it is questionable whether adaptogenic or/and immunostimulant compounds should be use during chemotherapy. Keeping these questions in mind, further research using in vivo models is needed.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board Bioethics Committee of Nicolaus Copernicus University in Toruń at the Ludwik Rydygier Collegium Medicum in Bydgoszcz, Poland, with assigned approval number 608/2019 for studies involving humans.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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

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

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11. Wnioski

Gatunki z rodzaju *Eleutherococcus* ze względu na zróżnicowany skład chemiczny, charakteryzują się szerokim spektrum aktywności farmakologicznej. Zasadność ich wielowiekowego stosowania w etnomedycynie Chin i Rosji jest obecnie potwierdzana za pomocą współczesnych narzędzi analitycznych. Jednym z mniej znanych gatunków tego rodzaju, zwłaszcza w Europie, jest *E. divaricatus*. Na podstawie uzyskanych wyników badań można sformułować następujące wnioski:

1. analizowane substancje roślinne (korzenie i owoce) zawierają związki polifenolowe (kwas kawowy i jego estry z kwasem chinowym), których ugrupowania mają zdolność inhibicji hialuronidazy i w mniejszym stopniu tyrozynazy. Bogatym źródłem tych pochodnych jest frakcja octanu etylu.
2. związki fenolowe obecne w korzeniu oraz owocach *E. divaricatus* wykazują działanie anty-hialuronidazowe, szczególnie w kontekście hialuronidazy obecnej w surowicy krwi dzieci ze zdiagnozowaną białaczką. Uzasadnia to potencjalną skuteczność korzenia oraz owoców w leczeniu chorób zapalnych, w przebiegu których aktywność hialuronidaz wzrasta.
3. frakcja octanu etylu zmniejsza aktywność doksorubicyny w modelu *in vitro*. Wyniki te mogą wskazywać na niekorzystne działanie adaptogenów lub preparatów immunostymulujących/immunomodulujących stosowanych w trakcie chemioterapii doksorubicyną. Jest to niezwykle ważne odkrycie, które częściowo potwierdza obserwacje pojawiające się w literaturze naukowej już w latach 70. i 80. na temat bezpieczeństwa łączenia chemioterapii z adaptogenami z grupy *Eleutherococcus*. Niemniej jednak konieczne jest przeprowadzenie więcej badań na poziomie molekularno-biochemicznym w modelach *in vivo*.
4. właściwości antyoksydacyjne korzenia, szczególnie frakcji octanu etylu oraz n-butanolu, uzasadniają jego stosowanie w chorobach z udziałem reaktywnych form tlenu (RFT).

12. Wkład wyników w rozwój nauki, możliwości aplikacyjne i perspektywy dalszych badań

Inhibitory hialuronidaz

- **Wspomaganie leczenia nowotworów** – wysoki poziom hialuronidaz występuje w wielu chorobach nowotworowych takich jak, rak płaskonabłonkowy głowy i szyi, rak stercza, rak pęcherza moczowego lub rak piersi. U pacjentów z czerniakiem oraz rakiem piersi hamowanie hialuronidaz zmniejszyło ryzyko przerzutów nowotworowych.
- **Wspomaganie leczenia infekcji bakteryjnych** – czynnikiem wirulencji wielu bakterii chorobotwórczych jest hialuronidaza. Enzym ten umożliwia bakteriom oraz ich toksynom przenikać przez błony biologiczne.
- **Doustna antykoncepcja u mężczyzn** – jedną z najczęściej stosowanych metod antykoncepcji jest terapia hormonalna. Stosowanie hormonów wiążę się z ryzykiem wielu działań niepożądanych. Stosowanie selektywnych inhibitorów hialuronidaz może zostać wykorzystane do uzyskania preparatów hamujących wnikanie plemnika do komórki jajowej. Ważną zaletą tej grupy leków jest brak wpływu leków na spermatogenezę oraz poziomu hormonów u mężczyzn.
- **Wspomaganie leczenia ukąszeń** – ukąszenia węży są istotną przyczyną śmiertelności w krajach tropikalnych. Podczas ukąszenia do organizmu człowieka dostaje się nie tylko toksyna, ale także hialuronidaza, która ułatwia rozprzestrzenianie się toksyny. Opracowanie inhibitorów hialuronidazy mogłoby spowolnić rozprzestrzenianie się toksyny, zwiększając tym samym szanse na przeżycie.

Inhibitory tyrozynazy

- **Leczenie chorób skóry** – nadaktywność tyrozynazy jest przyczyną wielu schorzeń skóry takich jak plamy starcze, przebarwienia posłoneczne czy piegi. Inhibitory tyrozynazy mogą zostać wykorzystane w leczeniu przebarwień skóry.
- **Poprawa wyglądu przetworów owocowych** – istotnym problemem w przemyśle spożywczym podczas produkcji soków oraz przetworów z owoców jest ich szybkie ciemnienie. Opracowanie skutecznych i bezpiecznych inhibitorów tyrozynazy pozwoli na rozwiązanie tego problemu.

- **Wspomaganie leczenia czerniaka** – wysoki poziom melaniny w komórkach czerniaka chroni je przed promieniowaniem oraz zmniejszają skuteczność radioterapii. Zdolność chelatująca melaniny dezaktywuje leki stosowane w chemioterapii. Dodatkowo wysokie stężenie melaniny może prowadzić do generowania reaktywnych form tlenu prowadząc do dalszych uszkodzeń materiału genetycznego i progresji choroby. Hamowanie produkcji melaniny w komórkach czerniaka może zwiększyć skuteczność chemio- i radioterapii.

Perspektywy dalszych badań

Moje dalsze plany badawcze związane są z analizami nad biodostępnością związków obecnych w *E. divaricatus*, zwłaszcza we frakcji octanu etylu. Pozwoli to na określenie stopnia przenikania tych związków przez skórę w modelu *post mortem* i *in vitro* (test PAMPA). Jest to niezwykle istotne pod kątem praktycznego wykorzystania tej frakcji w preparatach stosowanych na skórę. Pozwoli to na dokładne zrozumienie mechanizmów działania tych związków oraz ich potencjalnych korzyści terapeutycznych.

Wyniki tych badań mogą przyczynić się do opracowania nowych, skuteczniejszych preparatów dermatologicznych, które będą mogły być stosowane w leczeniu różnych schorzeń skórnych.

Dodatkowo, planuję współpracę z innymi ośrodkami badawczymi, aby zweryfikować wyniki moich badań oraz poszerzyć zakres analiz o dodatkowe testy *in vitro* i *in vivo*.

13. Piśmiennictwo

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14. Spis rycin i tabel

14.1. Ryciny

14.2. Tabele

15. Oświadczenia

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Oświadczam, że jestem autorem poniższych 3 publikacji wchodzących w skład pracy doktorskiej:

1. **Gębalski J.**, Graczyk F., Załuski D. Paving the way towards effective plant-based inhibitors of hyaluronidase and tyrosinase: A critical review on a structure–activity relationship. J Enzyme Inhib Med Chem, 2022; 37(1), 1120-1195.

Mój udział w przygotowaniu pracy polegał on na opracowaniu koncepcji pracy, wyszukiwaniu publikacji w bazach, krytyczna analiza danych, przygotowanie manuskryptu i oceniam go na 90%

2. **Gębalski J**., Małkowska M., Gawenda-Kempczyńska D., Słomka A., Strzemski M., Styczyński J., Załuski D. *Eleutherococcus divaricatus* Fruits decrease hyaluronidase activity in blood serum and protect from antioxidative damages in in vitro model. Int. J. Mol. Sci. 2024; 25(4):2033.

Mój udział w przygotowaniu pracy polegał on na opracowaniu koncepcji badań, metodologii, przeprowadzeniu analiz biologicznych i fitochemicznych, redagowaniu manuskryptu i oceniam go na 70%

3. **Gębalski J**., Małkowska M., Wnorowska S., Gawenda-Kempczyńska D., Strzemski M, Wójciak M., Słomka A., Styczyński J., Załuski D. Ethyl acetate fraction from *Eleutherococcus divaricatus* root extract as a promising source of compounds with anti-hyaluronidase, anti-tyrosinase, and antioxidant activity but not anti-melanoma activity. Molecules. 2024; 29(15):3640.

Mój udział w przygotowaniu pracy polegał na przygotowaniu materiału roślinnego, ekstrakcji, izolacji, analizach fitochemicznych i biologicznych, analizie uzyskanych danych, przygotowaniu manuskryptu i oceniam go na 60%.

11.03.24 .....
data i podpis autora

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Uniwersytetu Mikołaja Kopernika w Toruniu

OŚWIADCZENIE WSPÓŁAUTORA

W związku z ubieganiem się mgr Jakuba Gębalskigo o stopień doktora nauk farmaceutycznych, oświadczam, że jestem współautorem poniższych 3 publikacji wchodzących w skład pracy doktorskiej:

1. Gębalski J., Graczyk F., **Załuski D.** Paving the way towards effective plant-basedinhibitors of hyaluronidase and tyrosinase: A critical review on a structure–activity relationship. J Enzyme Inhib Med Chem, 2022; 37(1), 1120-1195.

2. Gębalski J., Małkowska M., Gawenda-Kempczyńska D., Słomka A., Strzemski M., Styczyński J., **Załuski D.** *Eleutherococcus divaricatus* fruits decrease hyaluronidase activity in blood serum and protect from antioxidative damages in in vitro model. Int. J. Mol. Sci. 2024; 25(4):2033.

3. Gębalski J., Małkowska M., Wnorowska S., Gawenda-Kempczyńska D., Strzemski M, Wójciak M., Słomka A., Styczyński J., **Załuski D.** Ethyl acetate fraction from *Eleutherococcus divaricatus* root extract as a promising source of compounds with anti-hyaluronidase, anti-tyrosinase, and antioxidant activity but not anti-melanoma activity. Molecules. 2024; 29(15):3640.

Oświadczam, że mój udział w wyżej wymienionych publikacjach związany był z opieką merytoryczną nad doktorantem i miał charakter konsultacji merytorycznych, korekty manuskryptów, a swój udział w każdym oceniam na 5%.

Kierownik
Katedry Botaniki Farmaceutycznej
i Farmakognozji
dr hab. Daniel Załuski, prof. UMK

09.09.2024

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OŚWIADCZENIE WSPÓŁAUTORA

W związku z ubieganiem się mgr Jakuba Gębalskiego o stopień doktora nauk farmaceutycznych, oświadczam, że jestem współautorem poniższych 2 publikacji wchodzących w skład pracy doktorskiej:

1. Gębalski J., Małkowska M., Gawenda-Kempczyńska D., Słomka A., Strzemski M., **Styczyński J.**, Załuski D. *Eleutherococcus divaricatus* fruits decrease hyaluronidase activity in blood serum and protect from antioxidative damages in in vitro model. Int. J. Mol. Sci. 2024; 25(4):2033.

2. Gębalski J., Małkowska M., Wnorowska S., Gawenda-Kempczyńska D., Strzemski M, Wójciak M., Słomka A., **Styczyński J.**, Załuski D. Ethyl acetate fraction from *Eleutherococcus divaricatus* root extract as a promising source of compounds with anti-hyaluronidase, anti-tyrosinase, and antioxidant activity but not anti-melanoma activity. Molecules. 2024; 29(15):3640.

Oświadczam, że mój współudział w wyżej wymienionych publikacjach polegał na zebraniu i dostarczeniu próbek surowicy od dzieci z białaczką i oceniam go na 5%.

15.09.2024 Styczyński

data i podpis autora

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OŚWIADCZENIE WSPÓŁAUTORA

W związku z ubieganiem się mgr Jakuba Gębalskigo o stopień doktora nauk farmaceutycznych, oświadczam, że jestem współautorem poniższej publikacji wchodzącej w skład pracy doktorskiej:

Gębalski J., Małkowska M., Wnorowska S., Gawenda-Kempczyńska D., Strzemski M, **Wójciak M.**, Słomka A., Styczyński J., Załuski D. Ethyl acetate fraction from *Eleutherococcus divaricatus* root extract as a promising source of compounds with anti-hyaluronidase, anti-tyrosinase, and antioxidant activity but not anti-melanoma activity. Molecules. 2024; 29(15):3640.

Mój udział w przygotowaniu pracy polegał na współprzeprowadzeniu analizy chromatograficznej i oceniam go na 5%.

15.09.2024

.....

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OŚWIADCZENIE WSPÓŁAUTORA

W związku z ubieganiem się mgr Jakuba Gębalskigo o stopień doktora nauk farmaceutycznych, oświadczam, że jestem współautorem poniższych 2 publikacji wchodzących w skład pracy doktorskiej:

1. Gębalski J., Małkowska M., Gawenda-Kempczyńska D., Słomka A., **Strzemski M.**, Styczyński J., Załuski D. *Eleutherococcus divaricatus* fruits decrease hyaluronidase activity in blood serum and protect from antioxidative damages in in vitro model. Int. J. Mol. Sci. 2024; 25(4):2033.

2. Gębalski J., Małkowska M., Wnorowska S., Gawenda-Kempczyńska D., **Strzemski M.**, Wójciak M., Słomka A., Styczyński J., Załuski D. Ethyl acetate fraction from *Eleutherococcus divaricatus* root extract as a promising source of compounds with anti-hyaluronidase, anti-tyrosinase, and antioxidant activity but not anti-melanoma activity. Molecules. 2024; 29(15):3640.

Mój udział w przygotowaniu pracy polegał na współprzeprowadzeniu analizy chromatograficznej i oceniam go na 5%.

Uniwersytet Medyczny w Lublinie
Katedra Chemii, Zakład Chemii Analitycznej
17.09.2024 Maciej Strzemski
dr hab. n. farm. Maciej Strzemski

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OŚWIADCZENIE WSPÓŁAUTORA

W związku z ubieganiem się mgr Jakuba Gębalskiego o stopień doktora nauk farmaceutycznych, oświadczam, że jestem współautorem poniższych 2 publikacji wchodzących w skład pracy doktorskiej:

1. Gębalski J., Małkowska M., Gawenda-Kempczyńska D., **Słomka A.**, Strzemski M., Styczyński J., Załuski D. *Eleutherococcus divaricatus* fruits decrease hyaluronidase activity in blood serum and protect from antioxidative damages in in vitro model. Int. J. Mol. Sci. 2024; 25(4):2033.

2. Gębalski J., Małkowska M., Wnorowska S., Gawenda-Kempczyńska D., Strzemski M, Wójciak M., **Słomka A.**, Styczyński J., Załuski D. Ethyl acetate fraction from *Eleutherococcus divaricatus* root extract as a promising source of compounds with anti-hyaluronidase, anti-tyrosinase, and antioxidant activity but not anti-melanoma activity. Molecules. 2024; 29(15):3640.

Oświadczam, że mój współudział w wyżej wymienionej publikacji polegał na redagowaniu manuskryptu i oceniam go na 5%.

17.09.2024 

.....

data i podpis autora

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OŚWIADCZENIE WSPÓŁAUTORA

W związku z ubieganiem się mgr Jakuba Gębalskiego o stopień doktora nauk farmaceutycznych, oświadczam, że jestem współautorem poniższych 2 publikacji wchodzących w skład pracy doktorskiej:

1. Gębalski J., Małkowska M., **Gawenda-Kempczyńska D.**, Słomka A., Strzemski M., Styczyński J., Załuski D. *Eleutherococcus divaricatus* fruits decrease hyaluronidase activity in blood serum and protect from antioxidative damages in in vitro model. Int. J. Mol. Sci. 2024; 25(4):2033.

2. Gębalski J., Małkowska M., Wnorowska S., **Gawenda-Kempczyńska D.**, Strzemski M, Wójciak M., Słomka A., Styczyński J., Załuski D. Ethyl acetate fraction from *Eleutherococcus divaricatus* root extract as a promising source of compounds with anti-hyaluronidase, anti-tyrosinase, and antioxidant activity but not anti-melanoma activity. Molecules. 2024; 29(15):3640.

Oświadczam, że mój współudział w wyżej wymienionej publikacji związany był z przeprowadzeniem analizy statystycznej i oceniam go na 5%.

11.09.2024 .....
data i podpis autora

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OŚWIADCZENIE WSPÓŁAUTORA

W związku z ubieganiem się mgr Jakuba Gębalskiego o stopień doktora nauk farmaceutycznych, oświadczam, że jestem współautorem poniższej publikacji wchodzącej w skład pracy doktorskiej:

1. Gębalski J., **Graczyk F.**, Załuski D. Paving the way towards effective plant-based inhibitors of hyaluronidase and tyrosinase: A critical review on a structure–activity relationship. J Enzyme Inhib Med Chem, 2022; 37(1), 1120-1195.

Oświadczam, że mój współudział w wyżej wymienionej publikacji polegał na częściowym przeglądzie i korekcie manuskryptu i oceniam go na 5%.

Filip Graczyk

.....
data i podpis autora

mgr Sylwia Wnorowska
Zakład Chemii Medycznej
Uniwersytet Medyczny w Lublinie

OŚWIADCZENIE WSPÓŁAUTORA

W związku z ubieganiem się mgr Jakuba Gębalskiego o stopień doktora nauk farmaceutycznych, oświadczam, że jestem współautorem poniższej publikacji wchodzącej w skład pracy doktorskiej:

Gębalski J., Małkowska M., **Wnorowska S.**, Gawenda-Kempczyńska D., Strzemski M, Wójciak M., Słomka A., Styczyński J., Załuski D. Ethyl Acetate Fraction from Eleutherococcus divaricatus Root Extract as a Promising Source of Compounds with Anti-Hyaluronidase, Anti-Tyrosinase, and Antioxidant Activity but Not Anti-Melanoma Activity. Molecules. 2024; 29(15):3640.

Oświadczam, że mój współudział w wyżej wymienionej publikacji polegał na wykonaniu części badań cytotoksycznych i oceniam go na 5%.

16.09.2024 Sylwia Wnorowska
data i podpis autora

mgr Milena Małkowska
Katedra Botaniki Farmaceutycznej i Farmakognozji
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytetu Mikołaja Kopernika w Toruniu

OŚWIADCZENIE WSPÓŁAUTORA

W związku z ubieganiem się mgr Jakuba Gębalskiego o stopień doktora nauk farmaceutycznych, oświadczam, że jestem współautorem poniższych 2 publikacji wchodzących w skład pracy doktorskiej:

1. Gębalski J., **Małkowska M.**, Gawenda-Kempczyńska D., Słomka A., Strzemski M., Styczyński J., Załuski D. *Eleutherococcus divaricatus* fruits decrease hyaluronidase activity in blood serum and protect from antioxidative damages in in vitro model. Int. J. Mol. Sci. 2024; 25(4):2033.

2. Gębalski J., **Małkowska M.**, Wnorowska S., Gawenda-Kempczyńska D., Strzemski M, Wójciak M., Słomka A., Styczyński J., Załuski D. Ethyl acetate fraction from *Eleutherococcus divaricatus* root extract as a promising source of compounds with anti-hyaluronidase, anti-tyrosinase, and antioxidant activity but not anti-melanoma activity. Molecules. 2024; 29(15):3640.

Oświadczam, że mój współudział w wyżej wymienionej publikacji polegał na wykonaniu części badań biologicznych i oceniam go na 5%.

11.09.24 Małkowska
.....
data i podpis autora

16. Zgoda Komisji Bioetycznej

Uniwersytet Mikołaja Kopernika w Toruniu

Collegium Medicum im L. Rydygiera w Bydgoszczy

KOMISJA BIOETYCZNA

Ul. M. Skłodowskiej-Curie 9, 85-094 Bydgoszcz, tel.(052) 585-35-63

KB 608/2019 Bydgoszcz, 28.02.2023 r.

Działając na podstawie art.29 ustawy z dnia 5 grudnia 1996 roku o zawodzie lekarza (Dz.U. z 1997 r. Nr 28 poz. 152 (wraz z późniejszymi zmianami), rozporządzenia Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych (Dz.U. Nr 47 poz.480) oraz Zarządzenia Nr 21 Rektora UMK z dnia 4 marca 2009 r. z późn. zm. w sprawie powołania oraz zasad działania Komisji Bioetycznej Uniwersytetu Mikołaja Kopernika w Toruniu przy Collegium Medicum im Ludwika Rydygiera w Bydgoszczy oraz zgodnie z zasadami zawartymi w ICH – GCP

Komisja Bioetyczna przy UMK w Toruniu, Collegium Medicum w Bydgoszczy

(której skład podano w załączeniu) na posiedzeniu w dniu **28.02.2023 r.** przeanalizowała prośbę o:

- poszerzenie panelu wykonywanych badań laboratoryjnych w uzyskanej surowicy krwi o oznaczenie stężenie tyrozynazy i hialuronidazy metodą immunoenzymatyczną;
- dołączenie do zespołu badawczego: dr hab. Daniel Załuski, prof. UMK, mgr Jakub Gębalski.

którą złożył:

prof. dr hab. n. med. Jan Styczyński
Klinika Pediatrii, Hematologii i Onkologii
Collegium Medicum w Bydgoszczy UMK w Toruniu

w sprawie badania:

„Metabolizm żelaza w świetle stanu jego przeładowania (hyperferrytynemii) u dzieci z ostrymi białaczkami lub poddawanych transplantacji komórek krwiotwórczych".

Po zapoznaniu się ze złożonym dokumentem i w wyniku przeprowadzonej dyskusji oraz głosowania jawnego Komisja przyjęła do wiadomości podane informacje i wyraża zgodę na powyższe pod warunkami określonymi w uchwale Komisji podjętej w dniu 25.06.2019 r. oraz w ewentualnych aneksach do tejże uchwały jak również pod warunkiem:

- zawarcia obowiązkowego ubezpieczenia odpowiedzialności cywilnej podmiotu przeprowadzającego eksperyment medyczny, w stosunku do noworekrutowanych uczestników.

Zgoda na kontynuowanie przedmiotowego badania obowiązuje do końca 2023 r.

Prof. dr hab. med. Karol Śliwka

Przewodniczący Komisji Bioetycznej

Otrzymuje:

prof. dr hab. n. med. Jan Styczyński
Klinika Pediatrii, Hematologii i Onkologii
Collegium Medicum w Bydgoszczy UMK w Toruniu

Lista obecności

na posiedzeniu Komisji Bioetycznej

w dniu 28.02.2023 r.

Lp.	Imię i nazwisko	Funkcja/ Specjalizacja	Podpis
1.	Prof. dr hab. med. Karol Śliwka	*medycyna sądowa*	
2.	Mgr prawa Joanna Połetek-Żygas	*prawniczka*	
3.	Prof. dr hab. med. Mieczysława Czerwionka-Szaflarska	*pediatra, alergologia i gastroenterologia dziecięca*	
4.	Prof. dr hab. med. Marek Grabiec	*położnictwo, ginekologia onkologiczna*	
5.	Prof. dr hab. n med. Maria Kłopocka	*choroby wewnętrzne, gastroenterologia*	
6.	Prof. dr hab. med. Zbigniew Włodarczyk	*chirurgia ogólna, transplantologia kliniczna*	
7.	Dr hab. n. med. Maciej Słupski, prof. UMK	*chirurgia ogólna, transplantologia kliniczna*	
8.	Dr hab. n. med. Katarzyna Sierakowska, prof. UMK	*anestezjologia i intensywna terapia*	
9.	Ks. dr hab. Wojciech Szukalski, prof. UAM	*duchowny*	
10.	Dr n. med. Radosława Staszak-Kowalska	*pediatria, choroby płuc*	
11.	Mgr prawa Patrycja Brzezicka	*prawniczka*	
12.	Mgr farm. Aleksandra Adamczyk	*farmaceutka*	
13.	Mgr Lidia Iwińska-Tarczykowska	*pielęgniarka*	