LATVIJAS BIOZINĀTŅU UN TEHNOLOĢIJU UNIVERSITĀTE
LATVIA UNIVERSITY OF LIFE SCIENCES AND TECHNOLOGIES

Mg. Chem. **ANNA ANDERSONE**

Promocijas darbs

SMILTSĒRKŠĶU LIGNOCELULOZES BIOMASAS BEZATLIKUMA IZMANTOŠANAS IZPĒTE ILGTSPĒJĪGAI MULTIFUNKCIONĀLO PRODUKTU RAŽOŠANAI

RESEARCH ON THE ZERO-WASTE USES OF SEA BUCKTHORN LIGNOCELLULOSIC BIOMASS FOR SUSTAINABLE MANUFACTURING OF MULTIFUNCTIONAL PRODUCTS

Zinātnes doktora grāda
zinātnes doktors (Ph.D.) inženierzinātnēs un tehnoloģijās iegūšanai

promocijas darba tematiski vienotu zinātnisko publikāciju kopa

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Promocijas darbs izstrādāts Latvijas Valsts Koksnes ķīmijas institūtā sadarbībā ar Latvijas Universitātes Bioloģijas fakultāti, Rīgas Stradiņš Universitāti un Latvijas Biozinātņu un tehnoloģiju universitātes Zemkopības institūtu. Doktorantūras studiju laiks Rīgas Tehniskajā Universitātē no 2010. – līdz 2023. gadam un Latvijas Biozinātņu un tehnoloģiju universitātē no 2023. līdz 2024. gadam. Pētījumu finansiālais atbalsts ir ERAF projekts Nr. 1.1.1.1/19/A/146 "Biorefinery processing of sea buckthorn non-fruit biomass using innovative techniques and comprehensive analytical investigation, for obtaining prospective high value-added products for the Latvian bioeconomy, including serotonin" un LZP projekts Nr. lzp-2022/1-0599 "Innovative anti-inflammatory and antipathogenic systems based on proanthocyanidins isolated from fruit shrubs agricultural lignocellulosic waste".

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2. *Dr.sc.ing.* Inese FIĻIPOVA, RTU Individuālo aizsardzības līdzekļu laboratorijas vecākā zinātniskās darbības eksperte;
3. *Ph.D.* Andrey PRANOVITCH, Abo Akadēmijas (Somija), vadošais pētnieks.

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ANOTĀCIJA

Smiltsērkšķis pieder pie ražīgām, diezgan senām augļu kultūrām, kuras augļi satur praktiski visus dabā esošus vitamīnus. Ikgadējā agrotehnisko pasākumu rezultātā, veicot smiltsērkšķu sezonālo atzarošanu vai ogu novākšanu, katru gadu veidojas liels apjoms ar nogrieztiem smiltsērkšķu zariem, kurus šobrīd sadedzina, neizmantojot to kā izejvielas potenciālu bioloģiski aktīvo vielu un ekoloģiski drošu produktu/līdzekļu ražošanai. Pilnvērtīga un racionālā zaru kā lignocelulozes biomasas pārstrāde produktos ar pievienoto vērtību ir viens no atjaunīgo resursu ilgtspējīgās izmantošanas nosacījumiem, veicinot tautsaimniecības izaugsmi, paaugstinot vietējo atjaunīgo resursu produktivitāti un nodrošinot to stabilu izmantošanu rūpnieciskos nolūkos, neradot kaitējumu videi un cilvēku veselībai. Augu izcelsmes produktu ieviešana tirgū un interese par tiem nepārtraukti pieaug, balstoties uz pieprasījuma pēc dabiskiem produktiem veselības aprūpē, farmācijā un pat lauksaimniecībā, kā piemēram, augu aizsardzības līdzekļu ieguvei. Smiltsērkšķu lignocelulozes biomasā ir sakoncentrētas dažādas bioloģiski aktīvas vielas, tostarp polifenolu savienojumi un serotonīns ar daudzšķautņainu ķīmisko un bioloģisko aktivitāti.

Promocijas darbā novērtēts trīs Latvijā kultivēto smiltsērkšķu šķirņu (*Maria Bruvele, Tatjana, Botaničeskaja Lubiteļskaja*) lignocelulozes biomasas kā izejvielas potenciāls bioloģiski aktīvo vielu – proantocianidīnu (PAC) un serotonīna ieguvei. Veicot lignocelulozes biomasas ekstrakciju, vispiemērotākais ekstrahents mērķsavienojumu izdalīšanai bija etanola-ūdens maisījums (1:1 w/w). Izstrādājot piemērotāko mērķsavienojumu izdalīšanas paņēmienu, rezultātā iegūti PAC ar augstu tīrības pakāpi (91-94% PAC/SM) un serotonīnu saturošā frakcija (28% serotonīna/SM). Savstarpēji salīdzinot pavasarī un rudenī ievākto zaru ekstraktu sastāvu, noskaidrots, ka rudenī ievāktā zaru biomasa ir vispiemērotākā izejviela serotonīna un PAC ieguvei. Tā kā smiltsērkšķu lapas nesaturēja PAC un serotonīna daudzums bija <0.5%, tos atdalot, mērķsavienojumu iznākums no lignocelulozes biomasas palielinājās par 8–10%. Izmantojot LC-UV-ESI-QTOF MS un LC-DAD-ESI-MS/MS analīzi, noteikts, ka PAC sastāvā ir katehīna, epikatehīna, gallokatehīna un epigallokatehīna monomēra vienības, galvenokārt A un B tipa procianidīnu maisījumi ar polimerizācijas pakāpi 2-3. Pierādītā zaru hidrofīlo ekstraktu un mērķsavienojumu augstā antioksidatīvā, antibakteriālā, fungicīdā un pretiekaisuma aktivitāte nodrošina plašas izmantošanas iespējas dažādās nozarēs: farmācijā, pārtikas un lopbarības rūpniecībā, kosmētisko līdzekļu un augu aizsardzības līdzekļu ražošanā, veselības aprūpē un profilaksē.

Veicot *in vitro* analīzes, noteikta mērķsavienojumu ietekme uz gremošanas fermentu aktivitāti. Serotonīnu saturošā frakcija uzrādīja katalizējošo iedarbību alfa-amilāzes un aizkuņģa dziedzera lipāzes aktivitātē, savukārt proantocianidīniem bija novērota pretēja iedarbība.

Smiltsērkšķu koksnes atlikums pēc ekstrakcijas kā lignocelulozes biomasa ir piemērots substrāts lopbarības piedevas ieguvei ar iespēju regulēt to sagremojamību, mainot lopbarības sastāvdaļu masas attiecību. Bagātinot atlikumu ar mikro- un makroelementiem, iegūta videi draudzīga augsnes piedeva, kas palielina dārzeņu un graudu produktivitāti, papildus uzlabojot augsnes kvalitāti.

Promocijas darba rezultāti ir atspoguļoti 8 SCI zinātniskajās publikācijās un četros recenzētos zinātnisko konferenču rakstos. Ir iesniegts 1 Latvijas Republikas patents. Darbs izpildīts LV Koksnes ķīmijas institūtā, Lignīna ķīmijas laboratorijā laika posmā no 2020. gada līdz 2024. gadam.

ABSTRACT

Sea buckthorn belongs to prolific, quite ancient fruit species, whose fruits contain practically all vitamins found in nature. As a result of annual agrotechnical measures, a large volume of cut sea buckthorn branches is formed every year during seasonal pruning of sea buckthorn or harvesting of berries, which are burned currently without using their potential as a raw material for the production of biologically active substances and ecologically safe products/ preparations. The full and rational use of branches as lignocellulosic biomass into products with added value is one of the conditions for the sustainable use of renewable resources, promoting the growth of the national economy, increasing the productivity of local renewable resources and ensuring their stable use for industrial purposes, without harming the environment and human health. The introduction of herbal products into the market and the interest in them is constantly increasing, based on the demand for natural products in healthcare, pharmaceuticals, and even agriculture, such as for the extraction of plant protection products. Sea buckthorn lignocellulosic biomass contains various biologically active substances, including polyphenolic compounds and serotonin with multifaceted chemical and biological activity.

The thesis evaluates the lignocellulosic biomass potential of three varieties of sea buckthorn cultivated in Latvia (*Maria Bruvele, Tatjana, Botaničeskaja Lubiteļskaja*) as a raw material to produce biologically active substances - proanthocyanidins and serotonin. During the extraction of lignocellulosic biomass, the most suitable extractant for the release of target compounds was ethanol-water mixture (1:1 w/w). Development of the most appropriate isolation technique for the target compounds resulted in high purity proanthocyanidins (PACs) (91-94% PACs/DM) and a fraction containing serotonin (28% serotonin/SM). By mutually comparing the composition of twigs extracts collected in spring and autumn, it was found that the biomass of twigs collected in autumn was the most suitable raw material for the production of serotonin and PACs. Since sea buckthorn leaves do not contain PACs and serotonin, separating them increases the yield of target compounds from the biomass of branches by 8–10%. Using LC-UV-ESI-QTOF MS and LC-DAD-ESI-MS/MS analyses, PACs were determined to contain monomer units of catechin, epicatechin, gallocatechin, and epigallocatechin, mainly forming a mixture of type A and type B procyanidins with a degree of polymerization of 2–3. The proven high antioxidant, antibacterial, antifungal, and anti-inflammatory activity of the hydrophilic extracts and target compounds of twigs provides vast application opportunities in various industries: pharmaceuticals, food and feed industry, production of cosmetics and plant protection products, healthcare, and prevention.

By performing *in vitro* analyses, the effect of the target compounds on the activity of digestive enzymes was determined. The fraction containing serotonin showed a catalytic effect on the activity of alpha-amylase and pancreatic lipase, while the opposite effect was observed for proanthocyanidins.

The wood residue of sea buckthorn after extraction, as lignocellulosic biomass, is a suitable substrate for the production of fodder additive with the possibility of regulating its digestibility by changing the mass ratio of fodder components. By enriching the residue with micro- and macro minerals, an environmentally friendly soil additive is obtained, which increases the yield of vegetables and grains, additionally improving the quality of the soil.

The results of the doctoral thesis are reflected in 8 SCI scientific publications and four peer-reviewed scientific conference papers. There is 1 patent of the Republic of Latvia submitted.
The research was conducted at the LV Institute of Wood Chemistry, Lignin Chemistry Laboratory in the period from 2020 to 2024.

SATURS/*CONTENTS*

PUBLIKĀCIJU SARAKSTS / *LIST OF PUBLICATIONS*

Promocijas darba rezultāti ir atspoguļoti astoņās publikācijās, uz kurām atsauces tekstā veidotas, izmantojot romiešu ciparus:
This thesis is based on eight publications, referred to by Roman numerals in the text:

I. **Andersone A.***, Janceva S.*, Lauberte L., Nikolajeva V., Zaharova N., Jurkjane V., Rieksts G., Spulle U., Telysheva G. (2024) Sea Buckthorn, Aronia, and Black Currant Pruning Biomass as a Source of Multifunctional Anti-aging Cosmetic and Pharmaceutical Creams Ingredients (**pirmo divu autoru vienāds ieguldījums publikācijas rakstīšanā, sagatavota iesniegšanai*).
II. Janceva S., **Andersone A.**, Lauberte L., Zaharova N., Telysheva G., Krasilnikova J., Rieksts G. (2024) A Comparative Assessment of Sea Buckthorn (*Hippophae rhamnoides* L.) Pruning Waste as a Potential Source of Serotonin. BioResources 19(1), 886-897. 10.15376/biores.19.1.886-897
III. **Andersone A.**, Janceva S., Lauberte L., Skadins I., Nikolajeva V., Logviss K., Zaharova N., Rieksts G., Telysheva G. (2023) A Comparative Analysis of the Proanthocyanidins from Fruit and Non-fruit Trees and Shrubs of Northern Europe: Chemical Characteristics and Biological Aactivity. Sustainable Chemistry and Pharmacy, Volume 36, 101266. doi.org/10.1016/j.scp.2023.101266
IV. **Andersone A.**, Janceva S., Lauberte L., Krasilnikova J., Zaharova N., Nikolajeva V., Rieksts G., Telysheva G. (2023) Lignocellulosic Waste Compounds for Pancreatic Lipase Inhibition: Preliminary Extraction by Freon, Obtaining of Proanthocyanidins and Testing on Lipase Activity. Metabolites, 13(8), 922. doi.org/10.3390/metabo13080922
V. **Andersone A.**, Janceva S., Lauberte L., Zaharova N., Chervenkov M., Jurkjane V., Jashina L., Rieksts G., Telysheva G. (2023) Granulated Animal Feed and Fuel Based on Sea Buckthorn Agro-Waste Biomass for Sustainable Berry Production. Sustainability, 15(14), 11152. doi.org/10.3390/su151411152
VI. **Andersone A.**, Janceva S., Lauberte L., Ramata-Stunda A., Nikolajeva V., Zaharova N., Rieksts G., Telysheva G. (2023) Anti-Inflammatory, Anti-Bacterial, and Anti-Fungal Activity of Oligomeric Proanthocyanidins and Extracts Obtained from Lignocellulosic Agricultural Waste. Molecules, 28(2), 863. doi.org/10.3390/molecules28020863
VII. **Andersone A.**, Janceva S., Svarta A., Zaharova N., Rieksts G., Telysheva G. (2023) Lignin and Lignocellulose-based Organomineral Complex for Organic Agriculture. 23rd SGEM International Multidisciplinary Scientific GeoConference. 10.5593/sgem2023/3.1/s13.30
VIII. Janceva S., **Andersone A.**, Lauberte L., Bikovens O., Nikolajeva V., Jashina L., Zaharova N., Telysheva G., Senkovs M., Rieksts G., Ramata-Stunda A., Krasilnikova J. Sea Buckthorn (*Hippophae rhamnoides*) Waste Biomass after Harvesting as a Source of Valuable Biologically Active Compounds with Nutraceutical and Antibacterial Potential. Plants 2022, 11, 642. doi.org/10.3390/plants11050642.

PĒTĪJUMU REZULTĀTU PREZENTĒŠANA KONFERENCĒS / *APPROBATION OF RESEARCH RESULTS*

Pētījuma rezultāti ir prezentēti četrās zinātniskajās konferencēs: /
Study results have been presented in three scientific conferences:

IX. **Andersone A**., Janceva S., Zaharova N., Rieksts G., Telysheva G. (2022) Granulated Animal Feed Additives on the Basis of Sea Buckthorn Biomass. Proceedings of the XIII International Scientific Agricultural Symposium “Agrosym 2022”, 1109 – 1115.

X. **Andersone A.**, Janceva S., Zaharova N., Rieksts G., Telysheva G. (2022) Bioactivity of Silylated Lignocellulosic Biomass of Sea Buckthorn. Proceedings of the XIII International Scientific Agricultural Symposium "Agrosym 2022", 796 – 802.
XI. **Andersone A.**, Janceva S., Lauberte L., Zaharova N., Senkovs M., Ramata-Stunda A., Telysheva G., Rieksts G. (2022) Comparative Analysis of the Biological Activity of Proanthocyanidins from Fruit and Non-fruit Trees and Shrubs of Northern Europe. 9th IUPAC International Conference on Green Chemistry, 432 – 433.
XII. Janceva S., **Andersone A.**, Lauberte L., Zaharova N., Nikolajeva V. Fruit shrubs' twigs as a source of valuable oligomeric polyphenolic compounds with antibacterial and antifungal potential. Proceedings of the 15th International Scientific and Practical Conference "Environment. Technology. Resources". Rezekne, Latvia, June 27 – June 28, 2024 (apstiprināts publicēšanai).

Autoru ieguldījums publikācijās / *The contribution of the authors*

Nr. p.k. No.	**Ideja / *Original idea***	**Pētījuma plāns / *Study design***	**Datu ievākšana / *Data collection***	**Datu analīze / *Data analysis***	**Manuskripta sagatavošana / *Manuscrpit preparation***	**Doktora grāda pretendenta ieguldījums, % / *The contribution of the author, %***
I	**A.A.**, S.J., G.T.	**A.A.**, S.J., G.T.	**A.A.**, S.J., L.L., V.N., N.Z., V.J., G.R.	**A.A.**, S.J., U.S.	**A.A.**, S.J., G.T., L.L., N.Z., G.R.	75
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VII	**A.A.**, S.J., G.T.	**A.A.**, S.J., A.S.	**A.A.**, S.J., A.S., N.Z., G.R.	**A.A.**, S.J., A.S., N.Z.	**A.A.**, S.J., N.Z., G.T.	80
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IX	**A.A.**, S.J., G.T.	**A.A.**, S.J., N.Z.	**A.A.**, S.J., N.Z., G.R.	**A.A.**, S.J., N.Z.	**A.A.**, S.J., N.Z.	70
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Jelena Krasilnikova, M.C. – Mihail Chervenkov, A.S. – Agrita Svarta, I.S. – Ingus Skadiņš, K.L. - Konstantins Logviss, M.S. – Māris Seņkovs, V.J. – Vilhelmīne Jurkjāne, O.B. – Oskars Bikovens, G.R. – Gints Rieksts.

Ir iesniegts Latvijas Republikas patents (patenta pieteikuma Nr. LVP2023000055, atbilstoši likumam tiks publicēts 20.12.2024.)

SAĪSINĀJUMI / *ABBREVIATIONS*

PAC – proantocianidīni

MB - Marija Bruvele

BL - Botaničeskaja Ljubiteļskaja

TAT – Tatjana

50% EtOH- etanola-ūdens šķīdums (1:1, v/v)

50% EtOH ekstrakts – ekstrakts, kas iegūts ar etanol-ūdens šķīdumu (1:1, v/v)

MB_E_H2O – ekstrakts, kas iegūts no MB zariem, izmantojot destilēto ūdeni

BL_E_H2O – ekstrakts, kas iegūts no BL zariem, izmantojot destilēto ūdeni

TAT_E_H2O – ekstrakts, kas iegūts no TAT zariem, izmantojot destilēto ūdeni

MB_E_50% EtOH – ekstrakts, kas iegūts no MB zariem, izmantojot 50% EtOH

BL_E_50% EtOH - ekstrakts, kas iegūts no BL zariem, izmantojot 50% EtOH

TAT_E_50% EtOH - ekstrakts, kas iegūts no TAT zariem, izmantojot 50% EtOH

LS – lipīdu saturs

IK – inhibēšanas koncentrācija

GC – gāzes hromatogrāfija

MS – masspektrs

GS – galluskābe

AS – askorbīnskābe

1. DARBA VISPĀRĪGS RAKSTUROJUMS

1.1. Tēmas aktualitāte

Saskaņā ar Apvienoto Nāciju Organizācijas datiem paredzams, ka pasaules iedzīvotāju skaits no pašreizējiem 7.7 miljardiem pieaugs līdz 9.7 miljardiem 2050. gadā ("World population prospects," 2017), kas palielinās atjaunīgo resursu patēriņu. Pašreizējā pasaules patēriņa apmierināšana nav ilgtspējīga, jo cilvēces resursu patēriņš pārsniedz to, ko daba spēj atjaunot. Pārvēršot bioatkritumus resursā, ne tikai tiks aizvietoti fosilie resursi, kuri tiek plaši izmantoti enerģētikā, ķīmiskajā rūpniecībā un farmācijā, bet arī tiks atrisināta izejvielu trūkuma problēma meža produktu rūpniecībā.

Eiropas Savienībā (ES) augļu koku audzēšanai kopumā atvēlēti 11 301 345 hektāru (ha) (Vanghele et al., 2022). Šo koku atzarošana kopā ar stādījumu nozāģēšanu vai izņemšanu rada milzīgu daudzumu koksnes atkritumu. Teorētiskais koksnes atkritumu aprēķins ES lēš, ka katru gadu augļu koku atzarošanas rezultātā tiek iegūti līdz 25 miljoni tonnu koksnes zaru veidā (Aliaño-González et al., 2022). Šie koksnes atkritumi parasti tiek sadedzināti vai sasmalcināti ar to turpmāko iestrādi augsnē, kas nerada tiešus ekonomiskus ieguvumus. Tādējādi ir racionāli ieviest augļkoku koksnes pārstrādi vērtīgos produktos ar augsto pievienoto vērtību. Atjaunīgo resursu efektīvā un racionālā pielietošana ir viena no Latvijas Bioekonomikas stratēģijas 2030 prioritātēm, veicinot tautsaimniecības izaugsmi, paaugstinot vietējo atjaunīgo resursu produktivitāti un nodrošinot to stabilo izmantošanu rūpnieciskos nolūkos, neradot kaitējumu videi un cilvēku veselībai ("Latvijas bioekonomikas strategija 2030," 2017).

Viens no Latvijā aktuālajiem un mazizpētītajiem atjaunīgiem resursiem ir smiltsērkšķu koksne. Smiltsērkšķis savu uzmanību izpelnījies, pateicoties augļu bagātīgam sastāvam un vērtīgām īpašībām, kuras pielieto daudzās nozarēs. Izstrādājot smiltsērkšķu atzarojumu koksnei shēmu par šīs lignocelulozes biomasas racionālo bezatlikuma pārstrādi produktos ar augstu pievienoto vērtību, tas būs noderīgs un pielietojams arī citu augļkoku vai ogulāju koksnes atkritumu pilnvērtīgai izmantošanai.

Koksni vislabāk var definēt kā biopolimēra kompozītu, kas sastāv no celulozes, hemicelulozes, lignīna un ekstraktvielām. Koksnes ekstraktvielās ir daudz visdažādāko klašu organisko savienojumu — polifenoli, terpēni, polisaharīdi, taukskābes un alkaloīdi (N'Guessan et al., 2023). Polifenoli ir visbiežāk sastopamākie bioloģiski aktīvi savienojumi. Starp šiem savienojumiem ir sastopami hidroksitirosols, resveratrols, protokatehīnskābes un proantocianidīni (Pandey and Rizvi, 2009). Šo savienojumu bioaktivitāte ir pierādīta zemākās koncentrācijās *in vitro* pētījumos. Bioaktīviem savienojumiem, it īpaši polifenoliem, piemīt antioksidantas, pretmikrobu, fungicīdas, biostimulantu, pretiekaisuma, kardioprotektīvas un pretveža īpašības (Bié et al., 2023), kas liecina, ka augļu koksnes ekstraktiem var būt vairāki pielietojumi lauksaimniecībā, medicīnā un pārtikas, farmācijas, uztura un kosmētikas rūpniecībā. Piemēram, olīvkoku koksnes ekstrakts reducē trombīna izraisītu trombocītu agregāciju *in vitro* (Zbidi et al., 2009); vīnkopības koka koksnes ekstrakts darbojas kā konservants vīnā, aizstājot SO_2 (Guerrero and Cantos-Villar, 2015); kastaņkoka koksnes ekstraktam piemīt fungicīdas īpašības *in vitro* (Prapaiwong et al., 2021; Romani et al., 2021). Turklāt koksnes izmantošana veicina virzību gan uz ilgtspējīgāku attīstību, gan uz aprites ekonomiku.

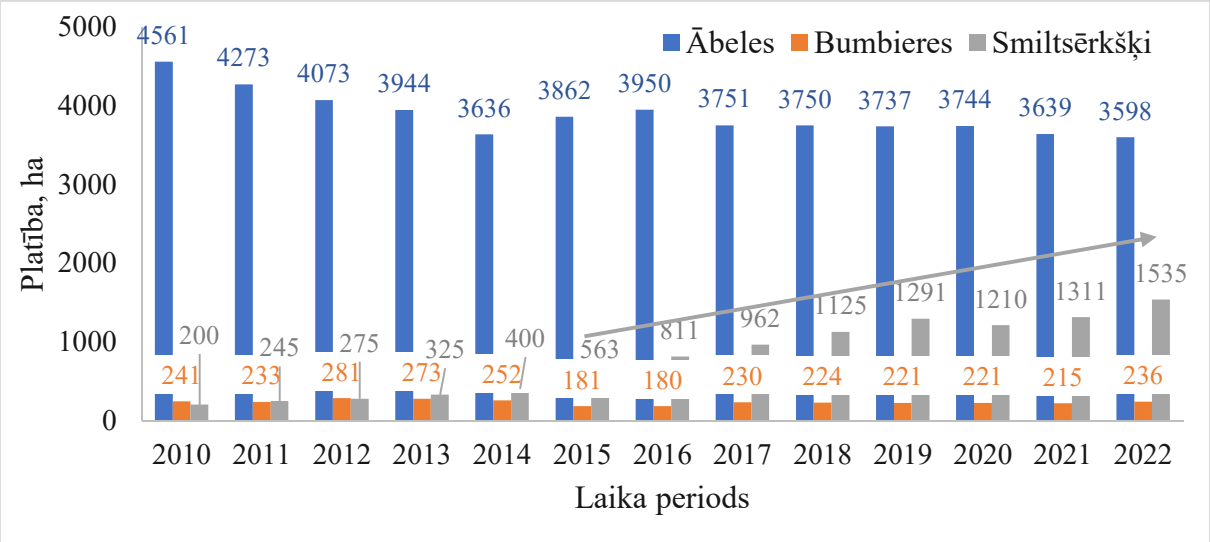
1.2. Augļu koku atkritumu valorizācija produktos

Eiropas Savienībā augļu koku audzēšanā olīvu dārzi aizņem lielāko platību ar vairāk nekā 5 miljoniem hektāru (45% no kopējās platības), kam seko vīna dārzi ar vairāk nekā 3 miljoniem ha (28%). Mandeļu koki un citi rieksti ir trešie bagātīgākie (11%) ar vairāk nekā 1 200 000 ha.

Savukārt 611 540 ha (5%) atvēlēti ābelēm, bumbierēm, kauleņaugļiem, citrusaugļiem un ogulājiem, piemēram, smiltsērkšķiem (Aliaño-González et al., 2022). Savvaļā smiltsērkšķi aug 52 valstīs, vislielākās smiltsērkšķu plantācijas Eirāzijas teritorijā var sastapt Kaukāzā, Rietumāzijā, Vidusāzijā, Mongolijā. Ķīnā smiltsērkšķu audzes aizņem 2 071 000 ha, tai skaitā 722 000 ha dabīgu audžu un 1 287 000 ha mākslīgi veidotu plantāciju ekoloģiskiem un ekonomiskiem mērķiem ("The Annual Report of International Seabuckthorn Development," 2021).

Smiltsērkšķis ir zināms kā vasarzaļš, ražīgs, *Eleagnus* dzimtas divmāju augļkoks ar ērkšķainiem zariem, kas savvaļā aug smilšainās un oļainās augsnēs ar augstu gruntsūdens līmeni. *Hippophaë rhamnoides* L. ir visizplatītākā smiltsērkšķu ģints suga. Smiltsērkšķis ir ārkārtīgi izturīgs augs gan pret sausumu, gan pret aukstumu, līdz pat –43 °C. Pateicoties smiltsērkšķu spēcīgai un veģetatīvi attīstītai sakņu sistēmai, tas tiek izmantots meliorācijā tā slāpekli piesaistošo īpašību dēļ (8 līdz 10 gadus vecs smiltsērkšķu stādījums spēj piesaistīt 180 kg slāpekļa/ha/gadā) un kultivētas savvaļas biotopu un augsnes bagātināšanas dēļ (Husain et al., 2018).

Latvijā smiltsērkšķis ir otra visvairāk izplatītā augļu koku suga pēc ābeles un viens no kultūraugiem, kuru stādījumu platība arvien pieaug. Liels pētījumu skaits un ieguldījums smiltsērkšķu šķirņu vērtēšanā, selekcijā, pavairošanā, augļu audzēšanā un ražas novākšanas tehnoloģiju izstrādē ļāva audzētājiem popularizēt šo kultūru un palielināt šīs kultūras audzētāju skaitu. Salīdzinot ar 2010. gadu, smiltsērkšķu platību skaits Latvijā palielinājās no 200 līdz 1 535 ha (skatīt 1. attēlu), līdzvērtīgi palielinoties arī smiltsērkšķu ogu ražai no 200 t/gadā līdz 814 t/gadā ("Augļu koku un ogulāju stādījumi (ieskaitot zemenes) 2000 - 2022," n.d.). Kvalitātes ziņā Latvijā audzētas smiltsērkšķu ogas ar dēvēto nosaukumu "Latvijas zelts" pārspēj Rumānijā un Vācijā audzētās ogas, bet Rumānijas ogas ir lētākas, un ogu pārstrādātāji ekonomisko apsvērumu dēļ bieži izvēlas lētākas ogas, nevis kvalitatīvākas. Savukārt liela konkurence smiltsērkšķu ogu tirgū liek aizdomāties gan par plantāciju palielināšanu, gan par videi draudzīgu, racionālu tehnoloģiju izveidi, kas ļaus pārstrādāt visas augu daļas, kas veidojas kā atlikumi augu kopšanas vai augļu novākšanas rezultātā, produktos ar augstu pievienoto vērtību.



1.1. att. **Prevalējošo augļkoku kopplatības izmaiņas Latvijā no 2010. līdz 2022. gadam** ("Augļu koku un ogulāju stādījumi (ieskaitot zemenes) 2000 - 2022," n.d.).

Smiltsērkšķu platību skaits var būt vēl lielāks, jo Latvijā smiltsērkšķus galvenokārt audzē privātos stādījumos, un iespējams, ka ne visi dārzi ir uzskaitīti oficiālajos statistikas datos. Pamatojoties uz Lauku atbalsta dienesta (LAD) datiem, 2023. gadā Latvijā bija 2 miljoni a neapstrādātas lauksaimniecības platības ("LIZ apsekošanas rezultāti, 2023. gads," 2024). Daļu

no šīm platībām ir iespējams izmantot smiltsērkšķu audzēšanai, sekmējot šo zemes platību racionālu un ilgtspējīgu izmantošanu vietējo kvalitatīvo produktu – ogu un atjaunīgo resursu – koksnes nodrošināšanai. Paplašinot smiltsērkšķu audzēšanas plantācijas, papildus tiks piesaistīts jauns darbaspēks, veidojot pozitīvu sociālās vides sekmēšanu lauku reģionos.

Augļu koku atzarošana ir svarīgs process daudzu augļu plantāciju apsaimniekošanā. Tās galvenais mērķis ir atvieglot vainagu veidošanu optimālai augļu ražošanai un efektīvai ražas novākšanai. Pareizi veidots vainags spēj ražot un nest lielu augļu ražu. Smiltsērkšķu audzēšanas tehnoloģija paredz četrus gadus vecu mazienesīgu plantācijās augošo koku nozāģēšanu un vienu līdz divus gadus vecu smiltsērkšķu dzinumu apgriešanu gan pavasarī, pirms sāk aktīvi cirkulēt sulas un plaukt pumpuri, veidojot skaistu vainaga formu un samazinot krūma sēnīšu infekciju risku gan vasarā vai agrajā rudenī – ogu novākšanas rezultātā, gan rudenī, veicot sanitāro atzarošanu, noņemot nolūzušos un sausos zarus. Vasarā un rudenī veidojās vislielākais zaru apjoms. Asi, ērkšķaini zari traucē ogu noņemšanu, un komercnolūkos audzēto ogu novākšanas procesa paātrināšanai zari tiek nogriezti kopā ar ogām. Pēc ogu atdalīšanas, iepriekš visu zaru ar ogām sasaldējot, veidojas liels zaru apjoms, kas vidēji sastāda ap 20-30% no ogoto zaru masas. Vidēji ikgadējā smiltsērkšķu atzarošana labos klimatiskajos un agronomiskajos apstākļos parasti var radīt no 0.5 līdz 2.0 tonnām/ha sausas koksnes. Šobrīd smiltsērkšķu audzētāji gandrīz visu šo zaru apjomu sadedzina, nenovērtējot šīs biomasas potenciālu, kaut gan šī koksnes daļa var būt vērtīga izejviela bioloģiski aktīvo vielu un jaunu produktu ieguvei, radot papildus ienākumus smiltsērkšķu audzētājiem. Smiltsērkšķa ogas, to izspaidas, eļļa un sēklas – ir bagātas ar vitamīniem (A, C, E, K, riboflavīns, folijskābe), karotinoīdiem (α, β, δ-karotīns, likopēns), fitosterīniem (ergosterols, stigmasterīns, lansterīns, amirīni), organiskajām skābēm (ābolskābe, skābeņskābe), polinepiesātinātajām taukskābēm un dažām neaizvietojamām aminoskābēm (Suryakumar and Gupta, 2011). Visplašāk tiek izpētīti smiltsērkšķu augļi, un sēklas, nesalīdzināmi mazāk pētījumu ir par lapām, un trūkst sistēmisko pētījumu par smiltsērkšķa zariem. Smiltsērkšķu ogas un lapas ir izmantoti tradicionālajā ķīniešu medicīnā kopš Tangu dinastijas (Olas, 2018). Šī auga ogas un eļļa ir plaši izmantota austrumu tradicionālajā medicīnas sistēmā astmas, ādas slimību, kuņģa čūlu un plaušu slimību ārstēšanai. Nesen ir ziņots par plašu smiltsērkšķu eļļas farmakoloģiskās iedarbības spektru, tostarp antioksidantu, imūnmodulējošu, anti-aterogēnu, anti-stresa, hepatoprotektīvu, radioprotektīvu un audu atjaunošu (Y. Chen et al., 2023; Wang et al., 2022).

1.2. att. **Smiltsērkšķu plantācija (A); pavasara zaru biomasa (B); rudens zaru biomasa (C)**

Neskatoties uz smiltsērkšķa ātraudzību un koksnes pozitīvām īpašībām (cieta, izturīga, blīva, un vidēja svara), tas tiek uzskatīts par mazvērtīgu koku sugu, kas nesasniedz pietiekami lielas dimensijas, lai tas būtu izmantojams celtniecībā, iepakojuma ražošanā, būvgaldniecībā vai mēbeļu ražošanā. Bieži no smiltsērkšķu stumbra koksnes izgatavo nažu rokturus, un tas ir lielisks materiāls iemutņu, spieķu, lietussargu rokturu, virtuves piederumu, trauku un rotaļlietu izgatavošanai. Smiltsērkšķu atzarojumu atlikumos lielāko daļu aizņem maza diametra zari, tādēļ to izmantošana kurināmo granulu ieguvei ir apgrūtināta augsta pelnu satura dēļ. Saskaņā ar Scopus® bāzes datiem, pēdējo desmit gadu laikā publikāciju skaits par tēmu "augļu koksnes

valorizācija" ir pieaudzis 7 reizes, piedāvājot augļkoku koksnes izmantošanu papīra ieguvei un koksnes kompozītmateriālu ražošanā.

Latvijā no augu resursiem vairāk ir izpētīti mežā augošu koku atlikumi (miza, atzarojumi). Ņemot vērā meža biomasas daudzveidīgo sastāvu lapkoku un skujkoku maisījuma veidā, ne vienmēr šie resursi ir noderīgi individuāla mērķsavienojuma vai savienojumu grupas ar sinerģisko bioloģisko aktivitāti ieguvei. Alternatīva šiem resursiem var būt plantācijās audzēto ātraudzīgo augļkoku – smiltsērkšķu – pārstrādes koksnes atlikumi, kas ietver sevī gan organisko daļu, gan makro – un mikroelementus. Augi ražo sekundāros metabolītus, kas nav tieši iesaistīti organisma augšanas, attīstības un vairošanās pamatfunkcijās, bet ir būtiski ilgtermiņa izdzīvošanai un pilda vairākas funkcijas, tostarp aizsardzību pret plēsējiem vai apputeksnētāju piesaistei. Iepriekš minētie metabolīti ir apveltīti ar daudzām bioloģiskām aktivitātēm, padarot tos arī ārkārtīgi svarīgus cilvēku veselībai. Turklāt to ķīmisko un bioloģisko īpašību dēļ, virknei augu šo metabolītu pielietojums ir atrasts arī daudzās citās jomās, kas kalpo kā pigmenti, kosmētika, antioksidanti u.c. Šobrīd salīdzinoši labi ir zināms smiltsērkšķu augļu un lapu ķīmiskais sastāvs.

1.3. Smiltsērkšķu ogu ķīmiskais raksturojums

Smiltsērkšķu ogu nozīmīgais veselības potenciāls ir analizēts un aprakstīts daudzos zinātniskos rakstos un publikācijās. Zināms, ka smiltsērkšķu ogās ir vairāk nekā 190 dažādu bioloģiski aktīvu vielu, tajā skaitā taukos šķīstošie vitamīni (K, E, D, A), ūdenī šķīstošie vitamīni (C, B - B1, B2, B6), taukskābes (t.sk. omega-3, 6, 7 un 9), organiskās skābes, ogļhidrāti, aminoskābes (no kurām astoņas ir neaizstājamas), zemmolekulārie un augstmolekulārie polifenoli (proantocianidīni) un augu sterīni, kā arī dabiskie antioksidanti (askorbīnskābe, tokoferoli, karotinoīdi (īpaši beta karotīns - provitamīns A), likopēns, zeaksantīns un vismaz 24 mikroelementi (Bal et al., 2011; A. Chen et al., 2023). Kopējais polifenola saturs smiltsērkšķu ogās (aprēķināts galluskābes ekvivalentā [GAE]) svārstās no 33 līdz 1417 mg uz 100 g sausnas (Y. Chen et al., 2023). Starp dominējošām fenolskābēm smiltsērkšķu ogās ir identificētas galluskābe, salicilskābe un kumarīnskābes. No flavonoīdiem ir identificēti: kaempferols, kvercetīns, izorhamnetīns, mirekinīts un šo pārstāvju glikozīdi. No augstmolekulāriem polifenolu savienojumiem smiltsērkšķu ogās tika atklāti 60 savienojumi ar polimerizācijas pakāpi no 2 līdz 11, kas sastāv no (epi)katehīna un/vai (epi)gallokatehīna vienībām. Augstmolekulāro polifenolu savienojumu saturs smiltsērkšķu ogās ir 390–1940 mg 100 g^{-1} sausnas (Yang et al., 2016), kas salīdzinoši vairāk nekā dzērvenēs – 133–367 mg 100 g^{-1} (Blumberg et al., 2013) un mellenēs 28 to 146 mg 100 g^{-1} (Wang et al., 2019).

1.4. Smiltsērkšķu lapu ķīmiskais raksturojums

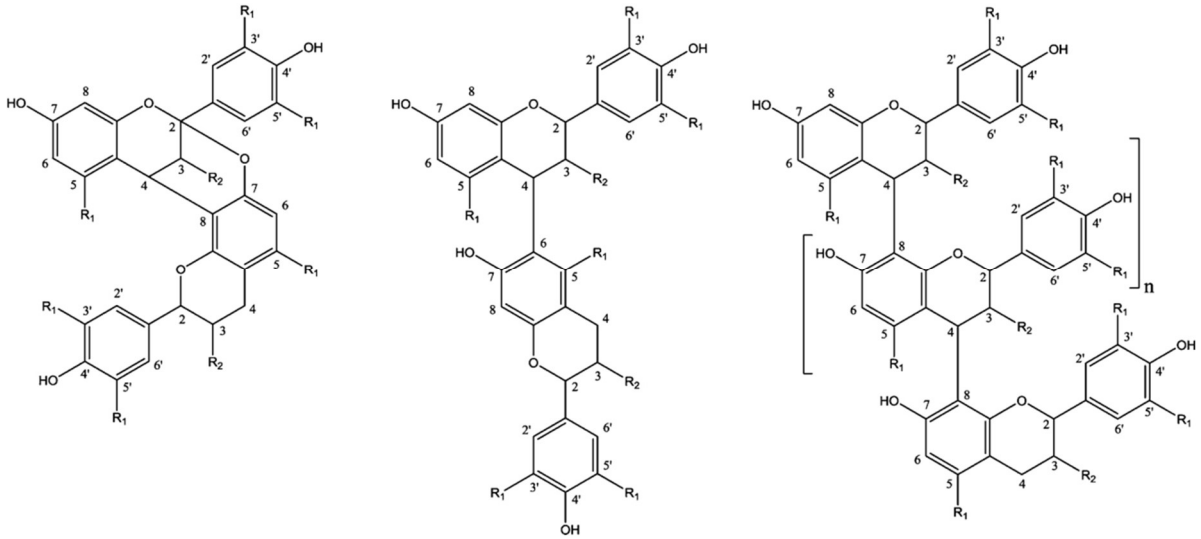
Lapu lipofīlais komplekss ir īpaši interesants kā potenciāls daļējs augļu eļļas aizstājējs, kas satur fosfolipīdus, karotīnus, tokoferolus, hlorofilu un augstākas taukskābes. Lapu hidrofilās frakcijās tiek atklāti vairāki fenola rakstura savienojumi: fenolkarbonskābes un to atvasinājumi, katehīni, leikoantocianīni un citi. Smiltsērkšķu lapu flavonoīdu sastāvs ir nedaudz daudzveidīgāks nekā augļiem. Kopējais flavonoīdu saturs ir ap 4.0%. No lapu flavonoīdiem ir identificēti kaempferola, kvercetīna un izorhamnetīna grupas flavonoli. No zemmolekulāriem polifenoliem smiltsērkšķu lapās visvairāk ir identificētas fenolskābes. No fenolskābēm ir konstatētas: 2,5-dihidroksibenzoskābe, galluskābe, pirokatehīnskābe, salicilskābe, vanilskābe, kofeīnskābe, p-kumarīnskābe, p-hidroksifenil-skābe un ferulīnskābe un rutīns (Asofiei et al., 2019). Smiltsērkšķu lapas ir bagātīgs ellagitanīnu avots, un lielākā daļa no tām tika identificētas apmēram pirms 30 gadiem (Yoshida et al., 1991). Somijas smiltsērkšķu šķirnēm UHPLC

analīze parādīja, ka ellagotanīni var veidot līdz ~ 90% no visiem lapu fenoliem, un to kopējais saturs ir ~ 55–70 mg g^{-1} no lapu svaigā svara (Tian et al., 2017).

Smiltsērkšķu lapu ekstraktu ķīmiskā sastāva un farmakoloģisko īpašību komplekss paver iespējas tās izpētīt kā izejvielu brūču dzīšanas, pret čūlu un vitamīnu preparātu ražošanai. Smiltsērkšķu lapu eļļa ir vērtīga ārstnieciska un profilaktiska piedeva ārstnieciskiem un kosmētiskiem preparātiem. Ziņots, ka lapu ekstrakti ir netoksiski devā LD_{50}>10 g kg^{-1} žurku ķermeņa svara (Saggu et al., 2007).

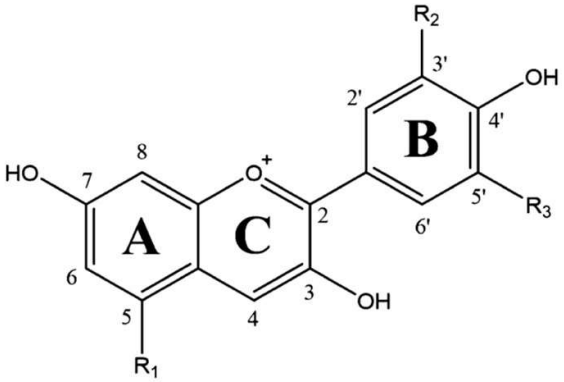
1.5. Smiltsērkšķu koksnes potenciāls bioloģiski aktīvo vielu ieguvei

Vieni no vērtīgākiem koksnes kā lignocelulozes biomasas ķīmiskiem savienojumiem ir polifenoli, kas uzkrājas augu augšanas un attīstības procesā, nodrošinot augiem svarīgas fizioloģiskās un morfoloģiskās īpašības, pasargājot augu no biotiskajiem un abiotiskajiem stresiem un nodrošinot aizsardzību pret infekcijas slimību ierosinātājiem (Kumar et al., 2023). Polifenolu koncentrācija augā un to sastāvs ir atkarīgs gan no auga veģetācijas fāzes un. no šķirnes, gan no ģeogrāfiskās augšanas vietas un fizioloģiskā brieduma. Iepriekšējie pētījumi parādīja, ka proantocianidīni ir dominējošie savienojumi oligomēro polifenolu sastāvā, kas iegūti no lapkoku un skujkoku lignocelulozes biomasām (koksne, miza) (Neto et al., 2020). Pastāv dažādi proantocianidīnu veidi, kuru pamatā ir flavan-3-ola monomēru vienības, kas saistītas ar C_4, C_8 vai C_6 atomiem (B tipa proantocianidīnu) vai papildus C_2–O–C_7 vai C_2–O–C_5 saiti (A tipa proantocianidīni, 1.3. att.) (Yang et al., 2011).



1.3. att. **A-tipa (pa kreisi) un B-tipa (pa vidu un pa labi) proantocianidīnu (PAC) struktūra (R_1 = H vai OH; R_2 = OH vai gallāti**) (Neto et al., 2020)

Augu proantocianidīnos bieži ietver procianidīnus, prodelfinidīnus un propelargonidīnus, kas sastāv attiecīgi no (epi)katehīna, (epi)gallokatehīna un (epi)afzelechīna vienībām (Chen et al., 2018; Li et al., 2016).



	R_1	R_2	R_3
Cianidīns	OH	OH	H
Delfinidīns	OH	OH	OH
Fisetinidīns	H	OH	H
Pelargonidīns	OH	H	H
Robinetinidīns	H	OH	OH
Guibortinidīns	H	H	H

1.4. att. **Antocianidīnu vispārējā molekulārā struktūra to flavīlija katjonu formā**

Proantocianidīnu sastāvā esošam monomēru skaitam vai polimerizācijas pakāpei ir galvenā loma bioaktivitātē (De La Iglesia et al., 2010), kā piemēram, zemmolekulārie proantocianidīni ir efektīvāki kā superoksīda anjonu iznīcinātāji, brīvo radikāļu iznīcinātāji un ksantīna oksidāzes inhibitori, nekā lielmolekulārie proantocianidīni. Savukārt lielmolekulārie proantocianidīni efektīvāk palēnina zarnu α-glikozes uzsūkšanos, efektīvāk inhibējot zarnu α-glikozidāzes aktivitāti (Neilson et al., 2016). Atkarībā no proantocianidīna izcelsmes avota lielākajai daļai no proantocianidīniem ir 2–6 flavan-3-ola monomēra vienību skaits (Janceva et al., 2015). Dažos zinātniskos rakstos minēts, ka kvebraho un mimozas mizas proantocianidīniem molekulārā masa, kas noteikta ar MALDI-TOF-MS palīdzību, bija 2333 Da (oktamēri) (Kusano et al., 2011; Pasch et al., 2001). Veicot pētījumus Latvijas mežos augošiem lapkokiem, tika konstatēts, ka alkšņi satur oligomērus proantocianidīnus, sastāvošus no katehīna un/vai epikatehīna vienībām (Janceva et al., 2015). Proantocianidīnu saturs meža pārstrādes atlikumos variē no 6 līdz pat 14% (*Picea mariana* miza satur 60,6 mg PAC/g SM (Diouf et al., 2009); *Pinus taeda* skujas -103 mg PAC g^{-1} SM (Booker et al., 1996); *Betula nana* lapas – 140 mg PAC g^{-1} SM). Latvijā augošo lapkoku mizā proantocianidīnu saturs variē no 2 līdz 12% (Janceva et al., 2015). Priežu mizas Pycnogenol® satur oligomērus proantocianidīnus, katehīnu, epikatehīnu, ferulskābi, kofeīnskābi un taksifolīnu (Bedekar et al., 2010). Tritikāles salmi izrādījās bagātīgs proantocianidīnu avots, kas satur 862.5 mg 100 g^{-1} SM (Hosseinian and Mazza, 2009).

Proantocianidīnu saturs un to sastāvs augļkoku lignocelulozes biomasā netika pētīts, līdz ar to šī promocijas darba ietvaros viens no uzdevumiem bija novērtēt smiltsērkšķu zaru biomasas kā izejvielas potenciālu proantocianidīnu ieguvei.

1.6. Smiltsērķšu polifenolu īpašības

Paplašināta zinātnieku interese par augu izcelsmes polifenolu savienojumiem ir saistīta ar to plaša spektra bioloģisko aktivitāti un zemo toksicitāti. Smiltsērkšķu ogu un lapu polifenoliem piemīt antioksidantas (Ji et al., 2020),(Di Mauro et al., 2017; Xu et al., 2017), pretiekaisuma (Li et al., 2017; Ren et al., 2019), kardioprotektīvas (Larmo et al., 2009; Olas et al., 2017) un pretvēža (Shilpa G. Patil, 2016) īpašības, kas saistītas ar vielmaiņas un veselības uzlabošanos, tostarp svara regulēšanu, lipīdu un glikozes profilu uzlabošanu, aizkuņģa dziedzera atjaunošanos un hipertensijas mazināšanu (Fredes et al., 2014; Wani et al., 2016).

Smiltsērkšķu ogu etanola ekstraktam ir nozīmīgas citoprotektīvas īpašības pret perifēra vazodilatatora nātrija nitroprusīda (pretspiediena medikaments) izraisītu oksidatīvo stresu limfocītos (Geetha et al., 2002). Šis ekstrakts arī mazināja nikotīna izraisīto oksidatīvo stresu žurku aknās un sirdī (Taysi et al., 2010). Turklāt smiltsērkšķu kopējie flavoni nodrošina aizsardzību pret H_2O_2 izraisītu apoptozi uz asinsvadu endotēlija šūnām, samazinot kaspāzes-3 ekspresiju (Cheng et al., 2011). Ogu etanola ekstrakts arī uzrādīja imūnmodulējošu iedarbību pret T-2 toksīna izraisītu imūndepresiju 15 dienas veciem cāļiem (Ramasamy et al., 2010).

Smiltsērkšķu ogu ekstraktam bija arī aizsargājoša iedarbība uz antioksidantu enzīmu līmeni, un tas veicināja lipīdu peroksidācijas samazināšanos, kā rezultātā samazinājās šūnu oksidācijas procesu līmenis. Turklāt Yasukawa et al. ziņoja, ka smiltsērkšķu zaru etanola ekstraktam, kas satur (+)-katehīnu, (+)-gallokatehīnu, (-)-epigallokatehīnu un ursolskābi, bija pretvēža aktivitāte (Yasukawa et al., 2009).

Flavonoīdu saturošam ekstraktam, kas satur izorhamnetīnu un kvercetīnu, ir aizsargājoša iedarbība uz miokarda išēmiju un reperfūziju, mikrocirkulāciju un vairogdziedzera funkcijas regulēšanu [2]. Polifenolu savienojumi dažādās gremošanas fāzēs labvēlīgi ietekmē resnās zarnas mikrobu daudzveidību, palielinot kopējo fenola saturu un kopējo antioksidantu aktivitāti kuņģa un tievās zarnas gremošanas laikā (Attri et al., 2018).

Papildus ir ziņojumi par smiltsērkšķu augļu un sēklu polifenolu pretiekaisuma iedarbību (Larmo et al., 2009), aizkuņģa dziedzera darbības uzlabošanu žurkām ar izraisīto cukura diabētu (Sharma, 2011), vielmaiņas uzlabošanu (Yang et al., 2017), kā arī novērots hepatoprotektīvais efekts (Gao et al., 2003) un pierādīta citoprotektīva un antioksidatīva iedarbība (Geetha et al., 2009).

Vairāku autoru ziņojumi norādīja ekstraktu drošumu devā LD_{50} > 10 g kg^{-1} ķermeņa masas, nenovērojot būtiskas izmaiņas bioķīmiskajos parametros attiecībā uz lipīdu metabolismu, kā arī nieru vai aknu darbību žurkām (Nishad et al., 2012; Saggu et al., 2007; Tulsawani, 2010). Histopatoloģisku bojājumu trūkums galvenajos orgānos jebkurā devā liecina, ka nenovērotas nelabvēlīgas ietekmes līmenis (NOAEL, no-observed-adverse-effect-level) ir augstāks par 500 mg kg^{-1} ķermeņa masas. Pat pie lielas devas no 2000 līdz 8000 mg kg^{-1} ķermeņa masas tika ziņots par toksicitātes un blakusparādību neesamību, kas apstiprina, ka smiltsērkšķu ekstrakts ir drošs produkts.

1.7. Dažādu augu proantocianidīnu bioloģiskā aktivitāte

Proantocianidīni sniedz ievērojamu labumu veselībai, kā ziņots vairākos pētījumos, izmantojot cilvēku un dzīvnieku modeļus. Tiem ir aizsargājoša iedarbība pret sirds un asinsvadu slimībām, vielmaiņas traucējumiem un onkogēniem notikumiem (Rauf et al., 2019). Ir ziņots, ka oligomēru proantocianidīnu kompleksi demonstrē antioksidantu, antibakteriālu, pretvīrusu, pretkancerogēnu, pretiekaisuma, antialerģisku un vazodilatējošu (paplašina asinsvadus) iedarbību (Nie et al., 2023).

Oligomērie dzērveņu proantocianidīni spēj inhibēt pro-iekaisuma citokīnu molekulu (interleikīna-1β, audzēja nekrozes faktora-α) veidošanos un iejaucas patogēno baktēriju rezistences mehānismos, tādējādi uzlabojot antibiotiku (īpaši ciprofloksacīna) aktivitāti (Nie et al., 2023), ar spēju saistīties ar olbaltumvielām, kas atrodas uz *Escherichia coli* un citu baktēriju virsmas, neļaujot tām piestiprināties pie urīnceļu sieniņām. Jaunākie pētījumi liecina, ka dzērveņu proantocianidīni novērš rezistences attīstību pret tetraciklīnu *Escherichia coli* un *Pseudomonas aeruginosa* baktērijām, palielina antibiotiku efektivitāti un kavē bioplēves veidošanos (Maisuria et al., 2022). *In vitro* eksperimentos uz pelēm ar lipīdu vielmaiņas traucējumiem kanēļa proantocianidīni uzlaboja aizkuņģa dziedzera β-šūnu darbību, cīnoties ar oksidatīvo stresu, uzlabojot jutību pret insulīnu un to sekrēciju vai pat ietekmējot dažu enzīmu aktivitāti vielmaiņas procesā (Sun et al., 2016). Parādīts arī vīnogu izspiedu proantocianidīnu potenciāls diabēta ārstēšanā, regulējot α-glikozidāzes un lipāzes aktivitāti, samazinot glikēmiju pēc ēšanas (Liu et al., 2017; Zhang et al., 2018; Zhu et al., 2015). Proantocianidīniem piemīt arī spēcīga tirozināzes inhibējošā aktivitāte (Takagi and Mitsunaga, 2003). *In vivo* un *in vitro* pētījumi parāda proantocianidīnu ietekmi uz metastātisku procesu kavēšanu plaušās, krūtīs, aizkuņģa dziedzerī, gremošanas traktā un aknās (Mao et al., 2019; Prasad and Katiyar, 2013; Ravindranathan et al., 2019), un tiem ir perspektīva pielietošanā vēža ārstēšanai un profilaksei (Cádiz-Gurrea et al., 2017). Proantocianidīni var novērst H_2O_2 veidošanos, proteīnu oksidāciju, lipīdu peroksidāciju un DNS bojājumus šūnās (Mantena, 2005). Proantocianidīni var tieši

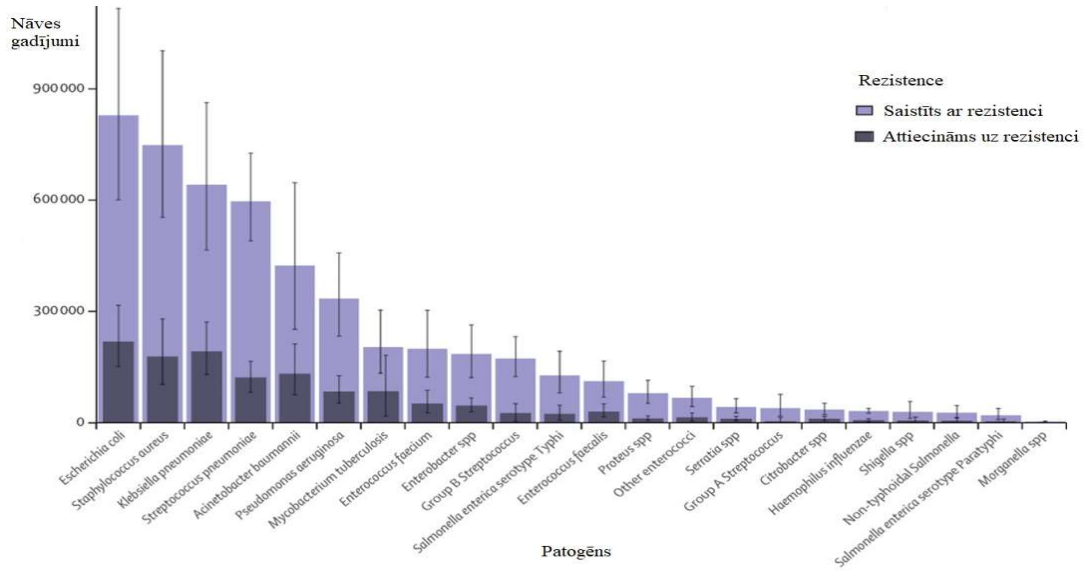
saistīties ar signālmolekulām, kas iesaistītas daudzos šūnu procesos, un regulē to darbību (Unusan, 2020). Proantocianidīniem piemīt antihipertensīvās īpašības, kas saistītas ar endotēlija disfunkcijas un novecošanas inhibēšanu (Oak et al., 2018). Proantocianidīnu zinātnisko pētījumu un atklājumu klāsts ir plašs, toties ir jāatceras par proantocianidīnu sastāvu, saturu un polimerizācijas pakāpi, kas var atšķirties, tos iegūstot no dažādiem augu valsts produktiem un izejvielām, jo tie var būtiski ietekmēt iegūto proantocianidīnu bioaktivitāti. Proantocianidīnu spēja veidot nešķīstošus kompleksus ar toksiskām vielām ļauj tos izmantot kā līdzekli perorālās saindēšanās novēršanai. No smiltsērkšķu koksnes vai mizas iegūtu proantocianidīnu bioaktivitāte nav pētīta.

1.8. Proantocianidīnu avoti un to rūpnieciskā izmantošanas veidi

Augu izcelsmes savienojumu – tā saucamo naturālo preparātu – nozīme strauji pieaug dažādās nozarēs. Ar tiem aizvietojot ķīmiski sintezētus savienojumus, rodas mazāks vides piesārņojums, tie ir arī drošāki ražošanā un pielietošanā cilvēku vai dzīvnieku organismiem. Proantocianidīnu (kondensēto tanīnu) komerciālo avotu vidū ir kvebraho (*Schinopsis lorentzii, Schinopsis balansae*) koksne un miza (Venter et al., 2012), akācijas (*Acacia mollissima, Acacia decurrens, Acacia pycnantha*) miza, vīnogu (*Vitis vinifera*) sēklas, priežu un egļu (*Picea abies, Pinus radiate*) miza, un ozola (*Quercus montana, Quercus borealis*) miza (Das et al., 2020; Kumar Das et al., 2020). Proantocianidīni tiek plaši izmantoti jēlādu miecēšanai ādas apstrādes rūpniecībā, kā arī kā adsorbenti ūdens attīrīšanai no smagiem metāliem un olbaltumvielu izgulsnēšanās nolūkos. Tos izmanto arī līmes ražošanā kokrūpniecībā un pretkorozijas līdzekļu ražošanā (Dargahi et al., 2015; Pizzi, 2006; Vorobyova et al., 2023).

1.9. Smiltsērkšķu proantocianidīnu izmantošanas potenciāls

Smiltsērkšķu proantocianidīnu vai to saturošo ekstraktu izmantošana rūpnieciskos nolūkos, piemēram, koksnes adhezīvu iegūšanai, vai to izmantošana ūdens attīrīšanai, vai viskozitātītes regulēšanai nav ekonomiski lietderīgi nepieciešamo lielo apjomu dēļ, līdz ar to šī promocijas darba ietvaros tika meklēti jauni pielietojuma virzieni ar augstāko pievienoto vērtību, un primāri ir pētīta proantocianidīniem piemītošā bioloģiskā aktivitāte. Tādējādi, smiltsērkšķa proantocianidīni no zariem varēs aizvietot ražošanā izmantotos citas izcelsmes proantocianidīnus, piemēram, no dzērvenēm, vai preparātus no smiltsērkšķa augļiem, kuru iegūšana konkurē ar pārtikas ķēdi un līdz ar to ir daudz dārgāka. Turklāt tas ne tikai ļaus izstrādāt smiltsērkšķa ilgtspējīgo pārstrādes ciklu, bet arī ļaus iegūt proantocianidīnus no vietējās izcelsmes koksnes, kas būs vērtīgs pienesums Latvijas ekonomikai. Smiltsērkšķu pielietojums medicīnas un veterinārijas nozarēs ir ļoti aktuāls, ņemot vērā dažādu infekcijas slimību izplatību un to apgrūtinātu ārstēšanu, kas joprojām ir nozīmīga sabiedrības veselības problēma. Tam par iemeslu ir infekciju izraisošie patogēni mikroorganismi, kas kļuvuši rezistenti pret antibiotikām pārmērīgas un neatbilstošas antibiotiku lietošanas dēļ ("Antibiotiku rezistences celoni," 2020). Starp šiem patogēniem mikroorganismiem ir *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aureginosa* u.c. (skatīt 1.5. att.). Rezistenti patogēnie mikroorganismi izdzīvo antibiotiku klātbūtnē un turpina vairoties, paildzinot slimību vai pat izraisot slimnieka nāvi. Jau šobrīd Eiropā no multirezistento patogēno mikroorganismu izraisītām infekcijām mirst ap 35 tūkstošiem cilvēku gadā, savukārt pasaulē šis skaits ir vēl lielāks. 2019. gadā nāves gadījumu skaits no multirezistento patogēno mikroorganismu izraisītām infekcijām bija lielāks par 1.27 miljoniem un papildus veicināja 4.95 miljonus nāves gadījumu. Jaunu antibiotiku trūkums, kas ir saistīts ar to laikietilpīgu un dārgu attīstību, saasina šo problēmu (Murray et al., 2022). Tādēļ ir nepieciešams identificēt jaunus pretmikrobu līdzekļus baktēriju un sēnīšu infekciju ārstēšanai.



1.5. att. **Nāves gadījumu skaits, kas saistīti ar patogēnu baktēriju rezistenci pret mikrobu līdzekļiem, 2019 (Murray et al., 2022)**

Augu ekstraktu izmantošanai kā jaunai stratēģijai cīņā pret patogēniem mikroorganismiem ir būtiska loma. Augu ārstnieciskās īpašības ir zināmas kopš seniem laikiem, kad tās tika plaši izmantotas vairāku patoloģiju ārstēšanā. Ir plašs pierādījumu kopums par polifenolu spēcīgo antibakteriālo un fungicīdo iedarbību. Turklāt dažiem polifenoliem ir sinerģiska iedarbība, ja tos kombinē ar antibiotikām un fungicīdiem. Polifenolus izmanto arī kā dabīgus konservantus pārtikas rūpniecībā to antioksidanto un pretmikrobu īpašību dēļ (Bouarab Chibane et al., 2019; Efenberger-Szmechtyk et al., 2021; Gutiérrez-del-Río et al., 2018; Olszewska et al., 2020; Skroza et al., 2019).

Lai samazinātu un racionalizētu antibotiku lietošanu, kas ir viena no galvenajām ES prioritātēm, un ar tiem saistītas blakusparādības, projektā tiks noteikta sakarība starp proantocianidīnu saturu ekstraktā un to antimikrobiālo un pretiekaisuma aktivitāti. Iegūtie rezultāti sniegs lietišķo pierādījumu bāzi par proantocianidīnu, kā perspektīvu alternatīvu sintētiskiem līdzekļiem, mikrobiālās rezistences attīstības ierobežošanai, kam ir būtiska nozīme cilvēku infekciju ārstēšanā un kas rada ne tikai veselības bet arī ekonomiska rakstura problēmas.

1.10. Serotonīns

Dažos zinātniskos pētījumos atzīmēts, ka smiltsērkšķu dzinumu mizā ir tā sauktais "laimes hormons" - serotonīns, turklāt daudz lielākā daudzumā, nekā tas ir, piemēram, šokolādē vai banānos. Saldie ķirši satur ievērojamu daudzumu serotonīna, tas konstatēts arī vīnogās un vīnā. Ir parādīts, ka serotonīna saturs dažādās augu daļās atšķiras un atšķiras arī attiecīgajos audos.

Serotonīna fizioloģiskās funkcijas augos vēl nav skaidras. Vairāki zinātnieki sniedz ziņojumus par tādu serotonīna iespējamo funkciju nodrošināšanu augos, kā: detoksikācija, jonu caurlaidība, antioksidatīvā iedarbība, auga aizsardzība pret plēsoņiem. Serotonīnam piemīt arī auksīniem līdzīgs efekts, nodrošinot organoģenēzi, dīgtspējas stimulēšanu, sakņu augšanu un attīstības regulēšanu.

Turklāt, ir konstatēts, ka daudzas augu sugas, kas ir neiroloģiski aktīvas cilvēkiem, satur serotonīnu, kā arī citus neirotransmiterus. Šādi dati norāda, ka serotonīns mūsu uzturā un augu izcelsmes zālēs var ietekmēt cilvēka veselību un var ietekmēt vairākas hroniskas slimības (Bell and Janzen, 1971; Kumarasamy et al., 2003). Ziņots, ka serotonīns ir izdalīts no saflora sēklām un tiem piemīt antioksidatīva, pretiekaisuma un pretveža iedarbība, kā arī ir potenciāls stresa,

depresijas un trauksmes mazināšanā (Hotta et al., 2002; Kumarasamy et al., 2003; Nagatsu et al., 2000; Yamamotová et al., 2007).

Serotonīnu ietver to pacientu ārstēšanā, kuri cieš no Parkinsona slimībai līdzīgiem simptomiem un aptaukošanās (Bell and Janzen, 1971).

Serotonīna daudzums smiltsērkšķu ogās ir ap 30 mkg g^{-1}. Lai nodrošinātu tādu serotonīna līmeni, kas nodrošina jūtamu efektu, nepieciešama aptuveni viena sauja jeb 40-50 grami smiltsērkšķu ogu. Serotonīns jeb 5-hidroksitriptamīns (5-HT) pieder pie neirotransmiteriem – vielām, kas pārraida impulsus starp nervu šūnām. Organismā tas veidojas smadzenēs un gremošanas traktā, no kurienes ar asinīm nonāk arī trombocītos. Organismā serotonīns veidojas no neaizvietojamās aminoskābes L-triptofāna, kas nāk ar pārtiku. Serotonīnam ir plašs bioloģisko efektu klāsts – veicinot psiholoģisko un fizisko labsajūtu, darbojoties kā antioksidants, veicinot imūnsistēmas darbību, regulējot apetīti, gremošanu, miegu, atmiņu un dzimumfunkciju. Savukārt tā trūkums organismā var izraisīt depresiju, emocionālu nestabilitāti, traucēt nervu sistēmas darbību, kā arī veicināt bezmiegu. Balstoties uz šīm īpašībām, promocijas darbā kā otrs mērķsavienojums izvēlēts serotonīns, novērtējot Latvijā augošo smiltsērkšķu atlikumu kā šī savienojuma avotu, to izdalot, attīrot un raksturojot, lai novērtētu papildu izmantošanas iespējas.

Lai nodrošinātu bezatlikuma elastīgo tehnoloģijas izstrādi, koksnes kā lignocelulozes biomasas atlikums pēc bioloģiski aktīvo vielu atdalīšanas var būt lietderīgi izmantots kā lopbarības piedeva vai kā augsnes piedeva, atgriežot dabas apritē no tās paņemto organisko daļu, kas nepieciešama augsnes-biotiskā kompleksa labvēlīgai darbībai.

1.11. Promocijas darba hipotēze

Analizējot literatūrā pieejamo informāciju, ir izvirzīta **promocijas darba hipotēze**: cirkulāras ekonomikas ietvaros smiltsērkšķim ir iespējams izstrādāt biorafinēšanas shēmu, pārstrādājot visas koka veģetatīvas daļas vērtīgos mērķproduktos (proantocianidīni un serotonīns), ar pretiekaisuma un antimikrobiālām īpašībām, un no pārstrādes atlikuma iegūstot lopbarības un augsnes piedevu.

1.12. Promocijas darba mērķis

Novērtēt Latvijā kultivēto augļkoku – smiltsērkšķu (*Hippophae rhamnoides* L.) – lignocelulozes biomasu kā potenciālu serotonīna un proantocianidīnu avotu, piedāvājot inovatīvu risinājumu lignocelulozes biomasas bezatlikuma kompleksai pārstrādei konkurētspējīgu, funkcionālu, videi, cilvēkiem un dzīvniekiem nekaitīgu produktu iegūšanai.

1.13. Promocijas darba uzdevumi

Promocijas darbā izvirzīti sekojoši uzdevumi:

1. novērtēt smiltsērkšķu atzarojumu koksnes potenciālu kā izejvielu bioloģiski aktīvo vielu – serotonīna un oligomēro proantocianidīnu – ieguvei un noteikt piemērotāko ekstrakcijas un attīrīšanas veidu mērķsavienojumu ieguvei;
2. noteikt antimikrobiālo, antioksidatīvo, pretiekaisuma aktivitāti un citotoksicitāti ekstraktiem, oligomēro proantocianidīnu un serotonīnu saturošām frakcijām un izvērtēt to praktiskās izmantošanas iespējas; noteikt mērķsavienojumu un ekstraktu iedarbību uz cilvēku gremošanas fermentu aktivitāti;
3. noteikt pēcekstrakcijas koksnes atlikuma potenciālu kā izejvielu granulētas lopbarības un augu augšanas veicinātāja ieguvei;

4. izstrādāt biorafinērijas shēmu bezatlikuma smiltsērkšķu atzarojumu – lignocelulozes biomasas kompleksai izmantošanai.

1.14. Promocijas darbā izvirzītās tēzes

Promocijas darbā ir izvirzītas sekojošā tēzes:

- smiltsērkšķu koksnes biomasa ir perspektīva izejviela oligomēro proantocianidīnu un serotonīnu ieguvei kā individuālu komponentu vai kompleksā veidā, radot sinerģiju bioaktivitātē. Izstrādātais tehnoloģiskais paņēmiens ir efektīvs mērķsavienojumu izdalīšanai no ekstrakta;
- pierādīta mērķsavienojumu un to saturošo ekstraktu augsta antioksidatīvā, antimikrobiālā un pretiekaisuma aktivitāte, kā arī ietekme cilvēku gremošanas fermentu aktivitātē, gan inhibējot, gan aktivējot to darbību. Iegūtie mērķsavienojumi un to saturošie ekstrakti nav toksiski darbības koncentrāciju diapazonā;
- smiltsērkšķu koksnes atlikums pēc ekstrakcijas ir piemērota izejviela lopbarības piedevas ieguvei, kas spēj kontrolēt sagremojamību un gāzes veidošanos, mainot izejvielu masas attiecību. Bagātinot smiltsērkšķu koksnes atlikumu ar mikro- un makroelementiem, iegūtā augsnes piedeva veicina dārzeņu un graudu augšanu un attīstību, papildus uzlabojot augsnes kvalitāti, paaugstinot organisko vielu saturu augsnes sastāvā.

1.15. Zinātniskā novitāte

- Pirmo reizi novērtēts Latvijā kultivēto smiltsērkšķu atlikumu potenciāls oligomēro proantocianidīnu un serotonīnu ieguvei.
- Izmantojot cilvēkiem un videi drošas ķimikālijas, t.sk. ozonam drošu freonu HFC R134a, izstrādāts piemērots mērķsavienojumu izdalīšanas paņēmiens, kas ļāva novērtēt vielu lomu ekstraktu sastāvos un bioaktivitātē, t.sk. cilvēka gremošanas fermentu (alfa-amilāze, aizkuņģa dziedzera lipāze) aktivitātē.
- Pirmo reizi noteikts piemērotākais sezonas laiks un smiltsērkšķu zaru vecums proantocianidīnu, serotonīna un to saturošo ekstraktu ieguvei.
- Pamatojoties uz asu nepieciešamību pēc dabiskiem antimikrobiālajiem līdzekļiem, mērķsavienojumi gan individuālā, gan maisījuma veidā testēti pret septiņiem patogeniem mikroorganismiem, papildus novērtējot to ietekmi bioplēves veidošanā, izmantojot triju veidu baktērijas.
- Pirmo reizi ir noteikts no smiltsērkšķu koksnes biomasas izdalīto proantocianidīnu sastāvs un to loma pretiekaisuma aktivitātē.
- Veikta smiltsērkšķu ekstraktu un mērķsavienojumu antioksidatīvās aktivitātes izpēte lipīdu saturošos produktos.
- Novērtētas smiltsērkšķu koksnes atlikuma pēc ekstrakcijas izmantošanas iespējas lopbarības piedevas ieguvei, ķīmiski to raksturojot un nosakot gremošanas efektivitāti atgremotāju kuņģa modeļu sistēmā.
- Bagātinot pēc ekstrakcijas smiltsērkšķu koksnes atlikumu ar silīciju saturošo komponentu, novērtēta iegūtas augsnes piedevas ietekme uz augu augšanu un attīstību, atgriežot dabas apritē organisko daļu, kas nepieciešama augsnes-biotiskā kompleksa labvēlīgai darbībai.

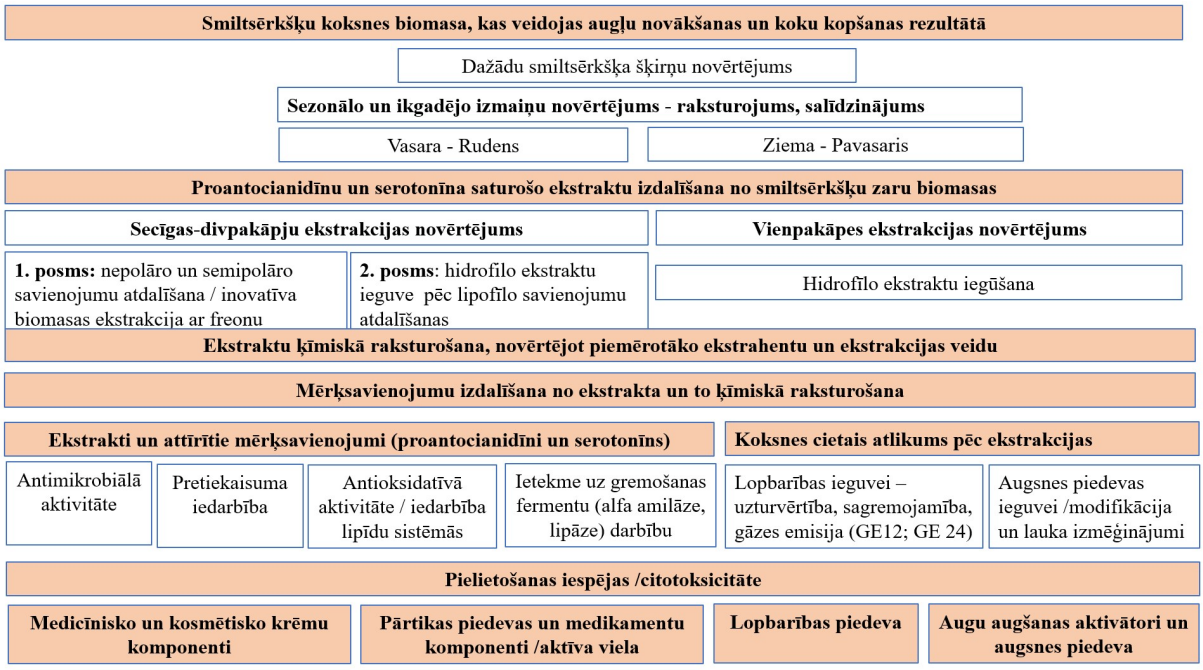
1.16. Darba tautsaimnieciskā nozīmība

- Parādīta iespēja no smiltsērkšķu zariem iegūt proantocianidīnu un serotonīnu saturošus produktus, kas dos būtisku ieguldījumu bioekonomikas attīstīšanā, kas aktuāla Latvijas tautsaimniecībai un atbilst Eiropas Savienības stratēģiskajiem uzdevumiem.
- Proantocianidīni un to saturošie ekstrakti ir potenciāli antioksidanti medicīniskiem un kosmētiskajiem krēmiem, radot alternatīvu sintētiskiem, ekoloģiski nedrošiem antioksidantiem.
- Proantocianidīni, serotonīns un to saturošie ekstrakti ir potenciāli antimikrobiālie līdzekļi, radot alternatīvu vai papildinot antibiotikas cīņai pret rezistentiem mikrobiem.
- Serotonīnu gremošanas fermentu stimulējošā un proantocianidīnu kavējošā darbība parāda mērķsavienojumu potenciālu veselības aprūpē dažādu ar gremošanu saistītu problēmu risināšanai.
- Parādīta iespēja izmantot zaru atlikumu pēc ekstrakcijas lopbarības piedevas ieguvei, un kā substrātu augsnes piedevas ieguvei.
- Izstrādātā smiltsērkšķu lignocelulozes biomasas racionālā bezatlikuma pārstrādes shēma produktos ar pievienoto vērtību būs noderīga un pielietojama arī citu augļkoku vai ogulāju atzarošanas atkritumu pilnvērtīgai izmantošanai.

1.17. Promocijas darba uzbūve

Promocijas darba struktūra ir pakārtota iepriekš minētajiem darba uzdevumiem. Darbs ir strukturēts trijās nodaļās (skatīt 1.6 att.). 3.1. nodaļā ir novērtēts smiltsērkšķu koksnes-lignocelulozes biomasas kā oligomēro proantocianidīnu un serotonīnu ieguves avota potenciāls gan ekstrakta veidā, nosakot piemērotāko ekstrakcijas veidu un ekstrahentu, gan individuālā veidā, tos attīrot no piemaisījumiem, izstrādājot piemērotāko mērķsavienojumu attīrīšanas paņēmienu. Fokusējoties uz polifenoliem, iegūtiem ekstraktiem ir noteikts ķīmiskais sastāvs, un veikta mērķsavienojumu izdalīšana. Lai novērtētu iegūto ekstraktu un mērķsavienojumu praktiskās izmantošanas iespējas, 3.2. nodaļā ir veikta to raksturošana, nosakot citotoksicitāti, antioksidatīvo, antimikrobiālo, pretiekaisuma aktivitāti, kā arī iedarbību uz gremošanas fermentu aktivitāti. 3.3. nodaļā saskaņā ar smiltsērkšķu biomasas bezatlikuma izmantošanas koncepciju izvērtēts pēc ekstrakcijas koksnes atlikuma un lapu potenciāls augsnes piedevas un lopbarības piedevas ieguvei. Nobeigumā, pamatojoties uz iegūtiem rezultātiem, izstrādāta un rekomendēta smiltsērkšķu biomasas pilnvērtīgas izmantošanas elastīga tehnoloģiskā shēma, atkarībā no patērētāju klāsta, pieprasījuma un smiltsērkšķu augšanas reģiona. 1.7 attēlā ir parādītas iespējamie mērķsavienojumu un to saturošo ekstraktu pielietošanas virzieni.

1.6. att. **Promocijas darba uzbūve**



1.7. att. **Pētījumu shēma un iespējamie mērķsavienojumu pielietojuma virzieni**

2. MATERIĀLI UN METODES

Promocijas darbā pētīti smiltsērkšķu zaru un lapu paraugi, kas bija saņemti neapstrādātā veidā no smiltsērkšķu audzētāja SIA "Bruwell". Kopumā ir veiktas 3 dažādu Latvijā audzēto šķirņu (*Maria Bruvele, Botaničeskaja Ļubiteļskaja,* un *Tatjana*) analīzes (skatīt 2.1. tabulu).

2.1. tabula.

Analizējamo paraugu saraksts

Parauga apzīmējums	**Smiltsērkšķu šķirne**	**Paraugu veids**	**Ievākšanas sezona**	**Paraugu ievākšanas veids**
Rudens koksnes paraugi				
MB/Z/2020/R	Maria Bruvele	Zari	Augusts – Septembris	Augļu novākšanas rezultātā
MB/Z/2021/R				
MB/Z/2022/R				
MB/Z/2023/R				
BL/Z/2020/R	Botaničeskaja Ļubiteļskaja (Bot. Ļub.)			Augļu novākšanas rezultātā
BL/Z/2021/R				
BL/Z/2022/R				
TAT/Z/2020/R	Tatjana			Augļu novākšanas rezultātā
TAT/Z/2021/R				
TAT/Z/2022/R				
Pavasara koksnes paraugi				
MB/1Z/2021/P	Maria Bruvele	1 gadīgie zari	Marts	Augļkoka kopšanas rezultātā
MB/2Z/2021/P		2 gadīgie zari		
MB/3Z/2021/P		3 gadīgie zari		
MB/4Z/2021/P		4 gadīgie zari		
MB/1-2Z/2022	Maria Bruvele	1 un 2 gadīgo zaru maisījums	Marts / Augusts	
BL/1-2Z/2022	Bot. Ļub.			
TAT/1-2Z/2022	Tatjana			
Miza no četrgadīgiem zariem				
MB/M/2021/R	Maria Bruvele	Miza	Augusts – Septembris	Četrgadīgo zaru atmizošanas rezultātā
BL/M/2021/R	Bot. Ļub.			
TAT/M/2021/R	Tatjana			
Lapu paraugi				
MB/L/2020/R	Maria Bruvele	Lapas	Augusts – Septembris	Augļu novākšanas rezultātā
MB/L/2021/R				
MB/L/2022/R				
BL/L/2020/R	Botaničeskaja Ļubiteļskaja			Augļu novākšanas rezultātā
BL/L/2021/R				
BL/L/2022/R				
TAT/L/2020/R	Tatjana			Augļu novākšanas rezultātā
TAT/L/2021/R				
TAT/L/2022/R				

Rudens koksnes paraugi – smiltsērkšķu zari – ievākti laika periodā 2020.-2023. gada augustā/septembrī augļu novākšanas rezultātā, atbilstoši ražas vākšanas tehnoloģijai nogriežot zarus kopā ar ogām un tos atdalot pēc sasaldēšanas. Pavasara koksnes paraugi ievākti 2021. un 2022. gada martā auga kopšanas (atzarošanas) rezultātā, iegūstot četrus pavasara koksnes paraugu veidus – viengadīgie, divgadīgie, trīsgadīgie un četrgadīgie zari. Salīdzināšanai iegūta miza četrgadīgo zaru atmizošanas rezultātā, kas bija ievākti 2021. gada rudenī. Visas trīs smiltsērkšķu šķirnes audzētas Tukuma rajonā vienā plantācijā un vienādos klimatiskajos apstākļos. Paraugu apzīmējumi un atšifrējumi ir doti 2.1. tabulā.

2.1. Smiltsērkšķu biomasas analītiskā pirolīze

Analītiskā pirolīze balstās uz ātrās pirolīzes procesu, kurā paraugs tiek uzkarsēts ar ātrumu 1000˚C/sec., ar sekojošo gaistošo produktu gāzes hromatogrāfijas analīzi. Produktu identifikācija notiek ar masu spektrometrisko detektoru. Pirolīzei izmantoja Frontier Lab (Fukushima, Japan) Micro Double-shot Pyrolyzer Py-3030D ar gāzes hromatogrāfu (GC) Shimadzu GC/MS/FID-QP ULTRA 2010 (Japāna). 2 mg homogenizēta parauga pārnesot tērauda tīģelītī, novieto to pirolīzes sistēmas automātiskajā paraugu ievadīšanas ierīcē. Pirolīzes procesu veic pie 500 ºC 15 sekundes. Pēc pirolīzes caur gāzes hromatogrāfa inžektoru gāzveida sadalīšanās produkti nonāk kapilāra tipa kolonnā (60 m*25 mm*0,25 µm ar fāzi RTX -1701. Gāzes hromatogrāfijā (Shimadzu GCMS – QP2010) gāzes – nesējs ir hēlijs, lineārs ātrums 20.0 cm s^{-1}. Hromatogrāfijas procesu veic sekojošā režīmā: termostata sasilšanas programma 1 min pie konstantas temperatūras 60 ºC, pēc tam 6 ºC min^{-1} līdz 270 ºC un beigas 10 min. pie konstantas temperatūras 270 ºC. Masas spektru uzņemšanai izmantoja elektrona trieciena jonizāciju (70 eV) un kvadrapola masas analizatoru ar skenēšanas diapazonu 15-350 m/z. Galvenās analītiskās pirolīzes priekšrocības ir minimāls analizējamā parauga daudzums un liels analīzes ātrums. Katru eksperimentu atkārto 3 – 4 reizes.

Vielu identifikācijai izmanto masspektra bibliotēku (Library MS NIST 11 un NIST 11s), pīķu relatīvie laukumi atsevišķiem savienojumiem aprēķināti, izmantojot Shimadzu programmatūru kas balstās uz GC/FID datiem.

2.2. Analīzes smiltsērkšķu biomasas kā proantocianidīnu un serotonīna ieguves avota novērtējumam

2.2.1. Smiltsērkšķu biomasas sagatavošana ekstrakcijai

Koksnes, lapu un mizas paraugi izžāvēti istabas temperatūrā, atsevišķi sasmalcināti, izmantojot naža tipa smalcināšanas iekārtu Retsch SM 100, iegūstot sasmalcinātu biomasu ar dažādu daļiņu izmēru: koksne 2–4 mm; lapas 0.1–2 mm; miza 0.5–2 mm. Sasmalcinātās koksnes, mizas un lapu mitruma saturs bija 2.0±0,1%; 7.6±0,1%; un 8.2±0,1%.

2.2.2. Smiltsērkšķu biomasas ekstrakcija

Lai novērtētu smiltsērkšķu biomasas kā bioloģiski aktīvo vielu ieguves avota potenciālu, biomasa ekstrahēta divos veidos. Pielietojot secīgu biomasas ekstrakciju, smiltsērkšķu koksne un lapas atsevišķi apstrādātas ar freonu 3-4 stundas noslēgtā sistēmā zem spiediena 4.0-4.3 Bar un temperatūrā 17–19 °C. Pēc freona frakcijas atdalīšanas sekoja 30 minūšu (10 minūtes x 3) biomasas ekstrakcija ~60 °C temperatūrā ar destilēto ūdeni un etilspirta ūdens maisījumu ar dažādu etilspirta koncentrāciju.

Pielietojot vienpakāpes biomasas ekstrakciju, biomasa ekstrahēta ar destilētu ūdeni un etilspirta ūdens maisījumiem bez biomasas priekšapstrādes ar freonu. Izmantotie šķīdinātāji

atbilst zaļās ķīmijas prasībām un ir izmantojami vērtīgu savienojumu ieguvei atbilstoši kosmētikas, farmācijas un pārtikas produktu kvalitātes, kontroles un ieguves prasībām. Lai iegūtu sausu ekstraktu, ar destilēto ūdeni iegūtie ekstrakti un ūdens suspensijas (pēc etanolu ietvaicēšanas) kolbās tika sasaldēti pie zemas temperatūras (-30 °C) un pievienoti liofilizācijas aparātam *Heto PowerDry PL3000*. Apmēram pēc 12 stundām -50 °C temperatūrā sublimācijas rezultātā iegūti sausi pulverveida ekstrakti. Ekstraktu iznākums noteikts gravimetriski, izteikts procentos no sausiem koksnes, lapu un mizas paraugiem.

2.3. Ekstraktu ķīmiskais raksturojums

2.3.1. Kvalitatīvā gāzes hromatogrāfijas analīze

Iegūtie ekstrakti ar freonu tika analizēti, izmantojot Shimadzu GC/MS/FID-QP ULTRA 2010 aparātu (Shimadzu, Kioto, Japāna) un kapilāru kolonnu RTX-1701 (garums 60 m, iekšējais diametrs 0.25 mm, slāņa biezums 0.25 μm. Sausie ekstrakti tika izšķīdināti heksānā, koncentrācijā 0.1 g $_{\text{heksāna}}^{-1}$, un nofiltrēti, izmantojot neilona filtru ar poru izmēru 0.45 μm. Savienojumu identificēšana tika veikta, pamatojoties uz GC/MS/FID datiem, izmantojot bibliotēku MS NIST 11 un NIST 11s, savukārt atsevišķu savienojumu pīķa relatīvais laukums tika aprēķināts, izmantojot Shimadzu programmatūru. Visu pīķu laukumi summēti, pieņemti kā 100%, un attiecīgi summētam laukumam aprēķināts katra identificēta individuālā savienojuma relatīvais saturs procentos. Mērījumu ticamības intervāls nepārsniedza 3%.

2.3.2. Kvantitatīvā proantocianidīnu noteikšana ekstrakta sastāvā

Kvantitatīvā proantocianidīnu analīze veikta pēc Porter metodes, kā standartu izmantojot procianidīna dimēru (*procyanidins B2*). Šīs metodes pamatā ir kolorimetriskā reakcija, kur skābes klātbūtnē notiek proantocianidīnu oksidatīvā depolimerizācija. 1 mL analizējama parauga alikvotai (0.02 mg mL^{-1}) pievieno 6 mL butanol-HCl reaģenta (konc. sālsskābi izšķīdina n-butanolā, 1:19 v/v) un 0.2 mL $FeNH_4(SO_4)_2 \cdot 12\ H_2O$ reaģenta ($FeNH_4(SO_4)_2 \cdot 12\ H_2O$ izšķīdināts 25 ml 2 M HCL šķīdumā). Sakratot mēģeni ar pagatavoto saturu, inkubēja 50 minūtes pie 60 °C temperatūras. UV absorbcija iegūtiem šķīdumiem tika mērīta pie 570 nm, rezultāts izteikts procentos uz sausnu. Katru analīzi atkārtoja 3 reizes.

2.3.3. Kvantitatīva polifenolu noteikšana ekstrakta sastāvā

Kvantitatīvā polifenolu analīze veikta pēc Folina-Čikalto metodes, kā standartu izmantojot galluskābi. 20 mg ekstrakta izšķīdina 250 mL etanolā. 120 mg galluskābes izšķīdina 1 L ūdens un no tās pagatavo standartšķīdumus 0.0075, 0.015, 0.030, 0.060, 0.090 mg mL^{-1}. 1 mL galluskābes standartšķīdumam un 1mL ekstraktam pievieno 5 mL 10% Folina-Čikolto šķīduma un 4 mL 7.5 % nātrija karbonāta šķīduma. Salīdzināšanas šķīduma pagatavošanai galluskābes vietā tika ņemts destilēts ūdeni un 50% EtOH. UV absorbciju mērīja pēc 30 min pie λ=765 nm.

2.3.4. Kvantitatīva flavonoīdu noteikšana ekstrakta sastāvā

Analizējamie paraugi 5–10 mg daudzumā izšķīdināti 25 mL 96% EtOH, 50% EtOH vai ūdenī. Iegūto šķīdumu tilpumā 0.4 mL pārnesa 10 ml mēģenē, kas saturēja 2 mL destilētā ūdens un 0.12 mL 5% nātrija nitrīta šķīduma. Labi sajaucot komponentus, tos inkubēja 5 minūtes istabas temperatūrā, pēc tam maisījumam pievienoja 0.24 mL 10% alumīnija nitrāta šķīduma, izturēja vēl 6 minūtes, un pievienoja 0.8 mL 1M nātrija hidroksīda. UV absorbcija mērīta pie 420 nm, rezultāts izteikts kvercetīna ekvivalentā mg g^{-1} sausnas. Katru analīzi atkārtoja 3 reizes.

2.3.5. UHPLC-ESI-MS/MS kvalitatīva ekstraktu analīze un kvantitatīva serotonīna noteikšana ekstrakta sastāvā

Hidrofīlie ekstrakti analizēti, izmantojot šķidruma hromatogrāfiju ar UV detektoru (Acquity UPLC) un augstas izšķirtspējas masspektometru (UPLC/SYNAPT G2Si HDMSQ-TOFMassSpectrometer (Waters Corp., ASV) tandēmā ar kvadrapola lidojuma laika masspektru (Q-TOF-MS), ar elektrosmidzināšanas jonizācijas (ESI) avotu). Paraugi tika izšķīdināti acetonitril-ūdens (v/v 50:50) šķīdumā koncentrācijā 2 mg mL^{-1}. Injekcijas tilpums bija 1.0 µL. Sadalīšanai tika izmantota Acquity UPLC BEH C18 (1.8 µm, 2.1 x 50 mm, Waters) kolonna ar plūsmas ātrumu 0.35 mL L^{-1}. Kā eluenti tika izmantoti: skudrskābe ūdenī (0.1 %, v/v) un acetonitrils.

Galvenie Q-TOF MS parametri: jonizācijas režīmi: negatīvs un pozitīvs, datu ieguves diapazons, m/z 50 to 1200; kapilāra spriegums 2.5 kV (−); konusa spriegums: 60 V; konusa gāzes plūsma: 50 L/h; kolīzijas enerģija: 6 eV; avota temperatūra: 120 °C; desolvatācijas temperatūra: 350 °C; sadursmes gāze, argons; desolvatācijas gāze: slāpeklis; plūsmas ātrums: 500 L/h. Q-TOF MS parametri serotonīna noteikšanai: jonizācijas režīms: pozitīvs, kapilārais spriegums 2.0 kV; konusa spriegums – 60 V; konusa gāzes plūsma – 100 L/h; sadursmes enerģija, 6 eV; avota temperatūra, 120 °C; desolvatācijas temperatūra, 450 °C; sadursmes gāze, argons; desolvatācijas gāze, slāpeklis; plūsmas ātrums, 750 L h^{-1}; datu ieguves diapazons, m/z 50–1200 Da. Katru analīzi atkārtoja 3 reizes.

Serotonīns identificēts un kvantificēts, izmantojot analītisko standartu (Sigma Aldrich, Mw=176.22 g moL^{-1}). Pozitīvā elektroizsmidzināšanas jonizācijas režīmā serotonīns protonēts, veidojot jonus formā $[M+H]^+$, ar m/z 177. Pamatojoties uz konstatēto serotonīna fragmentāciju, izstrādāts daudzkārtējas reakcijas monitoringa režīms konkrētajām m/z pārejām 177→160 (intensīvākais šķelšanās jons), 177→132 un 177→115. Katru analīzi atkārtoja 3 reizes.

2.4. Mērķsavienojumu izdalīšana un ķīmiskais raksturojums

2.4.1. Proantocianidīnu izdalīšana no ekstrakta

Proantocianidīnu izdalīšana no ekstrakta veikta, izmantojot sorbentu Sephadex LH-20, kā eluentu – 96% EtOH. Ekstraktu eluē caur Sephafex LH-20 kolonnu (1.5 x 8 cm) ar ātrumu 1 mL min^{-1}. Attīrīšanas laikā proantocianidīni adsorbējas uz sorbenta un kolonnā ir redzami kā brūna josla. Proantocianidīnu attīrīšanu no piemaisījumiem uzrauga ar UV spektriem, attīrot tik ilgi, kamēr absorbcija pie 280 nm vairs nemainās un ir tuvu bāzes līnijai. Kad visi piemaisījumi ir atdalīti, tad tīrus proantocianidīnus eluē ar 70% acetona šķīduma. Pēc proantocianidīnu atdalīšanas, serotonīnu saturošā frakcija tiek attīrīta pēc patentēta paņēmiena (patenta pieteikums Nr. LVP2023000055 atbilstoši likumam tiks publicēts 20.12.2024.).

2.4.2. Izdalīto proantocianidīnu LC-DAD-ESI-MS/MS analīze

Attīrīti PAC tika izšķīdināti ūdens/metanolā (v/v 20:80) ar koncentrāciju 0.1 mg mL^{-1}, nofiltrēti un izmantoti MS/MS analīzēm. Proantocianidīnu MS spektri tika reģistrēti ar Waters Acquity UPLC HClass ar PDA detektoru un Micromass QuattroMicro masas spektrometru (Waters Corp., Milford, MA, ASV), izmantojot Acquity UPLC BEH amīda kolonnu (1.7 µm, 3.0 × 100 mm). Masspektrometrs tika darbināts negatīvo jonu elektrosmidzināšanas jonizācijas režīmā ar –40 V konusa spriegumu, izmantojot tiešu infūziju. Avota un desolvatācijas temperatūra tika iestatīta attiecīgi 130 un 300 °C, konusa un slāpekļa gāze tika izmantoti ar plūsmas ātrumu 96 un 395 L h^{-1}. Kustīgā fāze sastāvēja no 0.1% skudrskābes, ūdens (A) un

acetonitrila (B), gradienta programma 85–85% (B) 0.3 min., 60–60% (B); 15–17 min., 25-75% (B), 6-7 min., 85-85% (B), 17.5-20 min., injekcijas tilpums 10.0 µL.

2.5. Antioksidatīvā aktivitāte

2.5.1. DFPH un ABTS metode

Analizējamo paraugu antioksidatīvās aktivitātes noteikšanai tika izmantotas metodes, kas balstās uz reducēšanas principu, par antioksidatīvās aktivitātes indikatoriem izmantojot stabili brīvos radikāļus: 2,2-difenil-1-pikrilhidrazilradikāli (DFPH$^{\bullet}$) un 2,2'-azino-bis(3-etilbenzotiazolin)-6-sulfonskābes ($ABTS^{+\bullet}$) katjona radikāli. Antioksidatīvā aktivitāte paraugiem noteikta, aprēķinot IK_{50} vērtību – antioksidantu masas koncentrāciju, kas ir nepieciešama, lai panāktu 50% brīvo radikāļu inhibēšanu. Jo zemāka ir IK_{50} vērtība, jo augstāka ir antioksidatīvā aktivitāte. Katru analīzi atkārtoja 3 reizes.

2.5.2. Lipīdu oksidēšanas tests

Lai novērtētu paraugu spēju aizkavēt lipīdu oksidēšanos, analīzei tika izmantotas divas krēma bāzes ar dažādu lipīdu saturu (19 un 35%), kas nesatur nekādus konservantus, stabilizatorus un antioksidantus. Lipīdu saturošo produktu oksidēšanas stabilitātes noteikšanai tika izmantota ML OXIPRES (Mikrolab Aarhus) iekārta.

Pētāmie paraugi krēma bāzes sastāvā tika ievadīti koncentrācijā no 0.05 līdz 4%, rēķinot uz lipīdu saturu substrātā, maisīti 2 minūtes, līdz izveidojās homogēns maisījums. Iegūtie homogēnie maisījumi tiek pārnesti speciālās šūnās un stingri aizskrūvēti, uzpildīti ar skābekli līdz 5 bar. Pēc skābekļa uzpildes šūnas tiek ievietotas termostatā ar uzstādīto temperatūru 120 °C. Spiediena izmaiņas atkarībā no laika tiek reģistrētas ar datorprogrammas "Paralog-Version 3.10" palīdzību. Katru analīzi atkārtoja 3 reizes. Salīdzināšanai krēma bāze bez pievienotiem analizējamiem paraugiem tika analizēta tādos pašos apstākļos. Indukcijas periods tika aprēķināts kā laiks, kurā lēni sāk krist spiediens. Kā references antioksidanti pētījumos tika izmantoti galluskābe un askorbīnskābe.

2.6. Antimikrobiālā aktivitāte

Antimikrobiālās aktivitātes *in vitro* analīzes veiktas Latvijas Universitātes Bioloģijas fakultātē, un Rīgas Stradiņa universitātē, balstoties uz Eiropas standarta protokoliem.

Paraugu antimikrobiālā aktivitāte noteikta, izmantojot diska difūzijas un agara bedrīšu metodi, kas izstrādāta 1940. gadā ikdienas antimikrobiālās jutības pārbaudei.

Antimikrobiālās aktivitātes noteikšanai izmantoti vairāki references mikrobu celmi, kas saņemti no Latvijas Universitātes Latvijas Mikrobu celmu kolekcijas (MSCL): *Pseudomonas aeruginosa* MSCL 334*, Staphylococcus aureus* MSCL 330*, Escherichia coli* MSCL 332*, Bacillus cereus* MSCL 330*, Streptococcus pyogenes* MSCL 620, *Cutibacterium acnes* MSCL 1521 un *Candida albicans* MSCL 378, un četriem klīniskiem baktēriju izolātiem: meticilīnrezistentais *Staphylococcus aureus* (MRSA), paplašināta spektra beta laktamāzes producējošā *E. coli* (ESBL) un *P. aeruginosa*. Visi klīniskie izolāti iegūti ar Rīgas Stradiņa universitātes Ētikas komitejas apstiprinājumu (25.10.2022, Nr. 4/462/2022 un Nr. 4/465/2022). Visi pacienti parakstīja informētas piekrišanas veidlapu.

Antimikrobiālā aktivitāte pētīta 96 bedrīšu platēs ar divkāršu sērijas buljona mikroatšķaidīšanas metodi, kas ļāva noteikt minimālo inhibējošo (MIC) un minimālo baktericīdo/fungicīdo koncentrāciju (MBC/MFC). Katru analīzi atkārtoja 3 reizes.

2.7. Pretiekaisuma aktivitāte

In vitro analīzes tika veiktas Latvijas Universitātes Bioloģijas fakultātē, balstoties uz Eiropas standarta protokoliem.

Asinis no veseliem donoriem ievāktas speciālos vakuteineros, kas saturēja etilēndiamīntetraetiķskābi (EDTA). Asinis ņemtas ar Latvijas Universitātes Kardioloģijas un reģeneratīvās medicīnas institūta Pētniecības ētikas komitejas saskaņojumu, atšķaidot asinis ar 0.9% nātrija hlorīda šķīdumu (1:2, v/v), kas bija papildināts ar 10 U mL^{-1} heparīna un mononukleāro šūnu frakciju. Atšķaidīti asins paraugi uzklāti uz Ficoll-Paque šķīduma (GE Healthcare, ASV) un veikta blīvuma gradienta centrifugēšana ar ātrumu 800 × g 20 minūtes istabas temperatūrā. Mononukleārās šūnas aspirētas un divas reizes mazgātas ar fosfāta-buferšķīdumu un centrifugētas 20 minūtes istabas temperatūrā. Šūnu nogulsnes suspendētas Dulbeko modificētās Īgla barotnēs (Dulbecco's modified Eagle Medium, DMEM), tos papildinot ar 1% penicilīnu (100 U mL^{-1}) – streptomicīnu (100 µg/ml) un 10% teļa serumu. Šūnas, kas iesētas 24 bedrīšu plāksnēs ar blīvumu 3 x 10^5 šūnas vienā iedobē, inkubētas 37 °C, 5% CO_2 vidē. Pēc 12 stundām šūnām pievienoti analizējamie paraugi koncentrācijās 0.5 un 0.25 mg mL^{-1}, 10 µg mL^{-1} poliinozīnskābe-policitidilskābe (Poly I:C) vai abu kombinācijā. Šūnas inkubēja 4 vai 24 stundas 37 °C temperatūrā 5% CO_2 vidē, un inkubācijas barotnes savāca un uzglabāja -80 °C temperatūrā turpmākai analīzei.

Interleikīnu IL-8 vai IL-6 koncentrācija, ko perifēro asiņu mononukleārā šūna (PBMNC) izdalīja kultivēšanas vidē, tika noteikta, izmantojot ar enzīmu saistīto imūnsorbcijas testu (ELISA). Cilvēka IL-8 DuoSet ELISA komplekti (RnD Systems, Mineapolisa, ASV) izmantoti saskaņā ar ražotāja ieteikumiem.

2.8. Iedarbība uz gremošanas fermentu aktivitāti

In vitro analīzes veiktas Rīgas Stradiņa universitātes Cilvēka fizioloģijas un bioķīmijas katedrā, balstoties uz Eiropas standarta protokoliem.

2.8.1. Analizējamo paraugu iedarbība uz amilāzes aktivitāti siekalās

Pētījumam izmantotās siekalas ziedoja studenti bez hroniskām vai akūtām saslimšanām, pēdējā ēdienreize bija 2 stundas pirms izmeklējuma. Analizējamie paraugi pārbaudīti koncentrācijā no 0.1 līdz 0.4 mg mL^{-1} siekalu. Paraugu ietekme uz siekalu amilāzi mērīta, balstoties uz polisaharīdu sadalīšanos cietē. Amilāzes aktivitāti raksturoja amilolastiskais spēks, tas ir 0.1% cietes šķīduma tilpums mililitros, kas tiek hidrolizēts ar 1 ml siekalu mēģenēs, tos inkubējot 38 °C temperatūrā 30 minūtes. Pēc tam tika pievienots 1% joda šķīdums (kā marķieris cietes klātbūtnei, mainot krāsu). Amilolastiskais spēks ir apzīmēts kā D 30/38 °C. Siekalas bez parauga klātbūtnes tika izmantotas salīdzināšanai. Katru analīzi atkārtoja 3 reizes.

2.8.2. Analizējamo paraugu iedarbība uz lipāzes aktivitāti

Lipāzes aktivitāte noteikta, izmantojot divpadsmit pirkstu zarnas gremošanas fāzes standarta modeli. Maisījumam, kas saturēja 3.8% tauku saturoša piena, aizkuņģa dziedzera šķīduma (pankreatīns no cūku aizkuņģa dziedzera), cūku žults ekstraktu (patoloģisko apstākļu veidošanai) un fenolftaleīnu, pievienoja analizējamo paraugu dažādos daudzumos. Visas mēģenes inkubētas 40 minūtes 38 °C temperatūrā, kam sekoja titrēšana ar 0.1N NaOH šķīduma, līdz krāsa mainījās uz dzeltenīgi brūnu krāsu, nosakot nepieciešamo NaOH daudzumu triglicerīdu hidrolīzei. Katru analīzi atkārtoja 3 reizes.

2.9. Hemolīzes analīze

Lai novērtētu analizējamo paraugu hemosaderību (ietekme uz asins eritrocītiem), veikts hemolīzes tests. Asinis no veseliem donoriem ievāktas Monovette stobriņos, kas saturēja etilēndiamīntetraetiķskābi (EDTA). Asinis ņemtas ar Latvijas Universitātes Kardioloģijas un reģeneratīvās medicīnas institūta Pētniecības ētikas komitejas saskaņojumu. Asinis atšķaidītas ar 0.9% nātrija hlorīda šķīdumu (4:5 v/v). Analizējamos paraugus pārvietoja 15 ml stobriņos, kas saturēja 9.8 mL fosfātu buferšķīdumu (phosphate buffered saline – PBS), inkubēja 37 °C, 5% CO_2 atmosfērā, 30 minūtes, un katram stobriņam pievienoja 0.2 mL atšķaidītu asiņu un inkubēja tajos pašos režīmos 1 stundu un 8 stundas. PBS tika izmantots kā negatīva kontrole un dejonizēts ūdens – kā pozitīva kontrole. Pēc inkubācijas veicot 5 minūšu centrifugēšanu (2000 apgriezieni minūtē), tika iegūti supernatanti, kuriem tika izmērīta absorbcija pie viļņa garuma 545 nm mikroplašu lasītājā Tecan Infinite® 200 Pro (Tecan Group Ltd., Mannedorf, Šveice). Hemolītiskā attiecība (HR) aprēķināta kā aprakstīts VI. publikācijā. Veikti 3 atkārtojumi katram paraugam.

2.10. Balb/c 3T3 (ATCC) šūnu līnijas kultivēšana

Balb/c 3T3 (American Type Culture Collection, ATCC, ASV) šūnu līnija iegūta no peļu embriju fibroblastiem. Šūnas kultivētas barotnē (DMEM, kas ir papildināta ar 1% penicilīnu (100 U/ml) – striptomicīnu (100 µg/ml) un 10% teļa serumu (Sigma, C8056, St Louis, MO, ASV). Šūnu līniju kultivēšana tika veikta inkubatorā, 5% CO_2 atmosfērā, 37 °C temperatūrā.

2.11. Citotoksicitātes noteikšana

Analizējamo paraugu toksicitāte tika testēta, izmantojot BALB/C3T3 peles fibroblastu šūnu līniju NR (neirālais sarkanais) testu. Šūnas tika kultivētas 96-lauciņu šūnu kultivēšanas platēs ar blīvumu 5 × 103 šūnas vienā lauciņā. Pēc 24 stundu inkubācijas tika pievienoti paraugi koncentrācijas diapazonā no 0.125 līdz 4 mg mL^{-1}. Atšķaidījumi tika veikti šūnu kultivētā vidē. Kultivēšana analizējamo paraugu klātbūtnē tika veikta 48 stundas. Pēc tam plātnes mazgā ar fosfātu buferšķīdumu un pievieno 25 µg mL^{-1} NR šķīduma, kas atšķaidīts 5% liellopu augļa seruma saturošā vidē. Pēc 3 stundu inkubācijas 5% CO_2 atmosfērā, 37 °C temperatūrā šūnas atmazgā ar fosfāta buferšķīdumu (PBS) un saistīto krāsvielu ekstrahē ar etiķskābes, etalona un ūdens maisījumu (1:50:49 v/v). Absorbcija pie 540 nm tika mērīta, izmantojot mikroplašu lasītāju Tecan Infinite® 200 Pro. Citotoksicitāte tika izteikta kā NR absorbciju samazinājums atkarībā no analizējamo paraugu koncentrācijas, salīdzinot ar neapstrādātām šūnām (kontroli). Rezultāti tika aprēķināti, izmantojot GraphPad 9 programmatūru. Šūnu līnija un testa metode atbilst Ekonomiskās sadarbības un attīstības organizācijas (ESAO) vadlīnijām. Tika veikti 3 atkārtojumi katram paraugam.

2.12. Biomasas atlikuma ķīmiskā raksturošana

Sekojot biorafinērijas koncepcijai, tika novērtēta koksnes atlikuma pēc ekstrakcijas un lapu izmantošanas potenciāls. Pamatojoties uz reģiona pieprasījuma dažādību un loģistikas risinājumu, tiks izstrādāta un piedāvāta smiltsērkšķu audzētājam elastīga tehnoloģiskā shēma, piedāvājot ražoto produktu klāstu.

2.13. Sasmalcinātas biomasas papildus apstrāde analīzēm

Koksnes (pirms un pēc ekstrakcijas) atlikumi un lapu paraugi bija papildus sasmalcināti piestā, izmantojot Retch SM 200 iekārtu, uzlabojot bioloģisko vielu pieejamību.

2.14. Elementanalīze

Paraugu elementanalīze, nosakot kopējo oglekļa (C), slāpekļa (N), ūdeņraža (H), skābekļa un sēra (S) saturu, veikta, izmantojot firmas ELEMENTAR iekārtu Vario MACRO. Vakuumā izžāvēts paraugs nosvērts (50 mg) un ievietots alvas folijā, kam sekoja sadegšanas katalizatora (WO_2 pulvera) pievienošana, attiecībā 1:1 w/w, iegūtais maisījums sapresēts tabletē ar sekojošo ievietošanu automātiskajā paraugu padevējā. Ierīces darbs vadīts un fiksēts datorizētā režīmā, izmantojot programmatūru VARIOEL V5.16.10. Rezultātus programma attēlo procentuālajā izteiksmē. Katram paraugam veikti 3 atkārtojumi.

Barības elementu (kalcijs, kālijs, nātrijs, fosfors) un smago metālu (kadmijs, svins, dzīvsudrabs) saturs biomasā tika noteikts saskaņā ar izstrādāto RSU procedūru, izmantojot induktīvi saistītās plazmas trīskāršā kvadrupola masspektrometra (Inductively coupled plasma triple quadrupole mass spectrometer) ICP-MS/MS, iCAP TQe iekārtu.

2.15. Vitamīnu noteikšana

Askorbīnskābes (C vitamīna) saturs biomasā noteikts, izmantojot augstas izšķirtspējas šķidruma hromatogrāfijas (HPLC) metodi, kā aprakstīts M. Ciulu rakstā (Ciulu et al., 2011), izmantojot HPLC-UV-Vis/-RI sistēmu (augstas veiktspējas šķidruma hromatogrāfs ar UV-Vis un RI detektoru) un Zorbax Eclipse XDB-C18 (Agilent, 5 µm, 150 cm x 0.46 cm i.d.) kolonnu. Kolonnas temperatūra noregulēta uz 35 °C. Kā eluenti izmantoti: A - trifluoretiķskābe (0.025 % v/v) (A) un B - acetonitrils (B). Kustīgā fāze: 100% līdz 60% A 20 minūtēs, plūsmas ātrums 1 mL min^{-1} un injekcijas tilpums 20 µL. Paraugu šķīdumi sagatavoti, izšķīdinot no biomasas iegūto ekstraktu metanolā. Rezultāti pārrēķināti uz biomasu, ņemot vērā ekstrakta iznākumu no biomasas. UV detektors iestatīts uz 254 nm.

E vitamīna kā α-tokoferola un A vitamīna kā retinola saturs biomasā noteikts, izmantojot HPLC-UV-Vis/-RI sistēmu un kolonnu Hichrom 5 C18 (i.d. 25 cm x 4.6 mm). Kā kustīgā fāze izmantots metanols ar plūsmas ātrumu 2 mL min^{-1}. Kolonnas temperatūra noregulēta uz 40 °C. Paraugu šķīdumi sagatavoti, izšķīdinot no biomasas iegūto ekstraktu metanolā, injekcijas tilpums 10 µL. UV detektors iestatīts uz 292 nm. Rezultāti pārrēķināti uz sausu biomasu, ņemot vērā ekstrakta iznākumu no biomasas.

2.16. Kvantitatīva koptauku analīze

Koptauku noteikšana paraugiem veikta, izmantojot paraugu 30 minūšu ekstrakciju (10 min x 3) ar heksānu, zem atteces. Nedaudz atdzesējot, saturu filtrē un pārnes to nosvērtajā apaļkolbā. Lai atdestilētu heksānu, izmantots rotācijas ietvaicētājs *Heidolph Instruments*, un pēc ietvaicēšanas iegūto ekstraktu žāvē žāvskapī 80 °C temperatūrā līdz konstantam svaram. Koptauku iznākums no biomasas noteikts gravimetriski.

2.17. Kvantitatīva kokšķiedru analīze

Skābi skalotās kokšķiedras (*Acid Detergent Fiber* – ADF) saturs analizējamos paraugos noteikts gravimetriski, veicot skābo hidrolīzi ar sērskābi (1.25%, w/v) ogļhidrātu atdalīšanai,

kam sekoja sarmainā hidrolīze ar NaOH (1.25%, w/v) olbaltumvielu, daļēji hemicelulozes un lignīna atdalīšanai. ADF rādītājam ir svarīga nozīme, jo, pieaugot ADF saturam barībā, samazinās barības sagremojamība.

2.18. Kvantitatīva kopproteīna analīze

Kopproteīna (KP) saturs analizējamos paraugos noteikts, izmantojot Kjeldāla metodi, un noteiktais slāpekļa (N) daudzums reizināts ar koeficientu 6.25 (N % × 6.25 = KP%/sausnu). Noteiktā kopproteīna sastāvā bija gan olbaltumvielas, gan citas slāpekli saturošas vielas.

2.19. *In vitro* gāzes emisijas analīze

In vitro gāzes emisijas noteikšanai tika izmantota ANKOM RF gāzes veidošanas sistēma (*Gas Production System*). Šī analīze balstās uz saistību starp fermentāciju spureklī un izveidotajām gāzēm. Spurekļa šķidrums ievākts no atgremotājiem kautuvē. Gāzes emisija izteikta mL g^{-1} inkubēta parauga. Gāzes emisijas uzraudzības sistēma sastāv no 50 moduļiem, kas aprīkoti ar spiediena sensoriem (spiediena diapazons: - 69 līdz 3.447 kPa; izšķirtspēja: 0.27 kPa; precizitāte: ± 0.1% no izmērītās vērtības). Nepieciešamais barības parauga daudzums bija 0.5 g katrā modulī. Katram modulim nepieciešamais spurekļa šķidrums bija 25 mL. Gāzes spiediena izmaiņas 24 un 48 stundu fermentācijas laikā uzkrājās (ΔP) un pārveidotas tilpuma vienībās, izmantojot ideālās gāzes likumu, kā aprakstīts V. publikācijā.

2.20. Sagremojamība

Sagremojamības novērtēšanai tika izmantots Ankom Daisy inkubators. Šajā testēšanā izmantotais spurekļa šķidrums ievākts, kā aprakstīts 2.19. sadaļā. Daisy inkubators ir 4 cilindru inkubators, kur 1 cilindram ir nepieciešams 1600 mL buferšķīduma un 400 ml spurekļa šķidruma kā inokulāts, kā arī filtru maisiņi (25 gab.). Filtra maisos ievietoti aptuveni 500 mg sausa parauga. Pēc tam cilindros ar šķīdumu ievietoja filtru maisiņus. Balonu 30 sekundes aerēja ar CO_2 tieši, pirms ciešās aizvēršanas un ievietoja inkubatorā uz 48 stundām. Pēc inkubācijas filtru maisiņi iztīrīti ar ūdeni un izžāvēti (105 °C, 3 stundas). *In vitro* eksperiments veikts trijos atkārtojumos, un *in vitro* sagremojamība aprēķināta kā aprakstīts V. publikācijā.

2.21. Granulēšana un granulu raksturojums

Biomasas granulēšana veikta, izmantojot laboratorijas mēroga plakanas matricas granulatoru KAHL 14-175, kas ir maza mēroga analogs rūpnieciskiem KAHL granulatoriem (Amandus Kahl GmbH & Co. KG, Reinbek, Vācija). Granulu izveidei caurules kanāla diametrs bija 6 mm, un kanāla garuma attiecība pret diametru bija 4:1. Granulēšanas sākuma temperatūra bija 50 °C. Lai novērtētu iegūto granulu kvalitāti, savstarpēji salīdzināti galvenie granulu kvalitāti raksturojošie parametri (granulu diametrs, garums, mitrums, mehāniskā izturība - nodilumizturība un tilpumblīvums) saskaņā ar Eiropas standartiem EN ISO 17831-1:2015 (“EN ISO 17831-1,” 2015) un ISO 17828 (“EN ISO 17828,” 2015). Granulu uzbriešana ūdenī noteikta vizuāli.

2.22. Koksnes atlikuma modifikācija augsnes piedevas ieguvei

Substrāta ieguvei lignīns un koksnes atlikums pēc ekstrakcijas sajauks masas attiecībā 1:1 (w/w). Tālāk substrātam pievienots Si saturošs komponents dolomīta suspensijas veidā. Si saturs substrātā sastādīja 5%/sausu masu.

2.23. Augsnes piedevas raksturojums

Augsnes piedeva ķīmiski raksturota, nosakot mitrumu ("LVS EN 13040:2008," 2008), sausnes saturu ("LVS EN 13040:2008," 2008), organisko vielu saturu ("LVS EN 13039:2012," 2012), lignīnu saturu (Klasona metode), kopējo slāpekļa (N) saturu ("LVS EN 13654-1:2003/NAC:2004.," 2003), kopējo fosforu (P_2O_5) ("LVS 398:2002," 2002), kopējo kāliju (K_2O) ("LVS ISO 110466:1995," 1995), dzīvsudraba ("LVS 346:2005," 2005), kadmija ("LVS ISO 11047:1998A," 1998), arsēna ("LVS ISO 110466:1995," 1995) saturu, augsnes pH ("LVS ISO 10390:2006," 2006).

2.24. Lauka izmēģinājumi

Lauka izmēģinājumi ierīkoti Skrīveros, Latvijas Biozinātņu un tehnoloģiju universitātes (LBTU) Zemkopības institūta bioloģiskajos laukos. Izmēģinājumā audzēta vasaras kviešu šķirne 'Vinjet' un kartupeļi 'Imanta'. Izmēģinājumu atrašanās vieta: 56º 69.4280` N un 25º 13.826` E. Lauka izmēģinājumi vasaras kviešiem bija iekārtoti četros atkārtojumos. Uzskaites lauciņa lielums bija 22.5 m^2 (2.5 m x 9 m). Izmēģinājumā trīs varianti:

1) Kontrole - bez augsnes piedevas lietošanas;
2) Augsnes piedeva 20 kg ha^{-1};
3) Augsnes piedeva 40 kg ha^{-1}.

Lai novērtētu augsnes piedevas ietekmi, izaudzētiem kviešiem noteikta ražas starpība starp izmēģinājuma variantiem un noteikti ražas kvalitātes rādītāji: kopproteīns, lipekļa un cietes saturs, Zeleny indekss, tilpummasa, izmantojot analizatoru Infratec NOVA. Ražas struktūrelementu (produktīvo stiebru skaits, vienas vārpas svars, graudu skaits vienā vārpā, 1000 graudu masa) veidošanās noteikta gravimetriski. Paraugkūļi ņemti katrā atkārtojumā ar 0.1 m^2 lielu rāmīti. Savukārt 1 000 graudu masa noteikta ar standartmetodi LVS EN ISO 520 ("LVS EN ISO 520," 2010).

Kartupeļiem lauka izmēģinājumi iekārtoti četros atkārtojumos. Uzskaites lauciņa lielums 12.6 m^2 (2.8 m x 9 m). Lauciņā četras vagas ar attālumu 70 cm, viena auga barošanās laukums 0.21 m^2. Izmēģinājumā testēti trīs varianti:

1) kontrole - bez augsnes piedevas lietošanas;
2) Augsnes piedeva 20 kg ha^{-1};
3) Augsnes piedeva 40 kg ha^{-1}.

Lauka izmēģinājumā kartupeļiem noteikta ražas starpība starp variantiem, ražas kvalitāte (cietes saturs %), preču produkcijas iznākums, bumbuļu sadalījums pa frakcijām, viena bumbuļa svars.

Izmēģinājumi ierīkoti velēnu podzolētā virspusēji glejotā mālsmilts augsnē, augsnē, kur augsnes agroķīmiskie rādītāji bija sekojoši: pH 5.6, organiskās vielas saturs 2.7 %, augiem pieejamā fosfora daudzums 105 mg kg^{-1} (zems) un kālija daudzums 201 mg kg^{-1} (augsts).

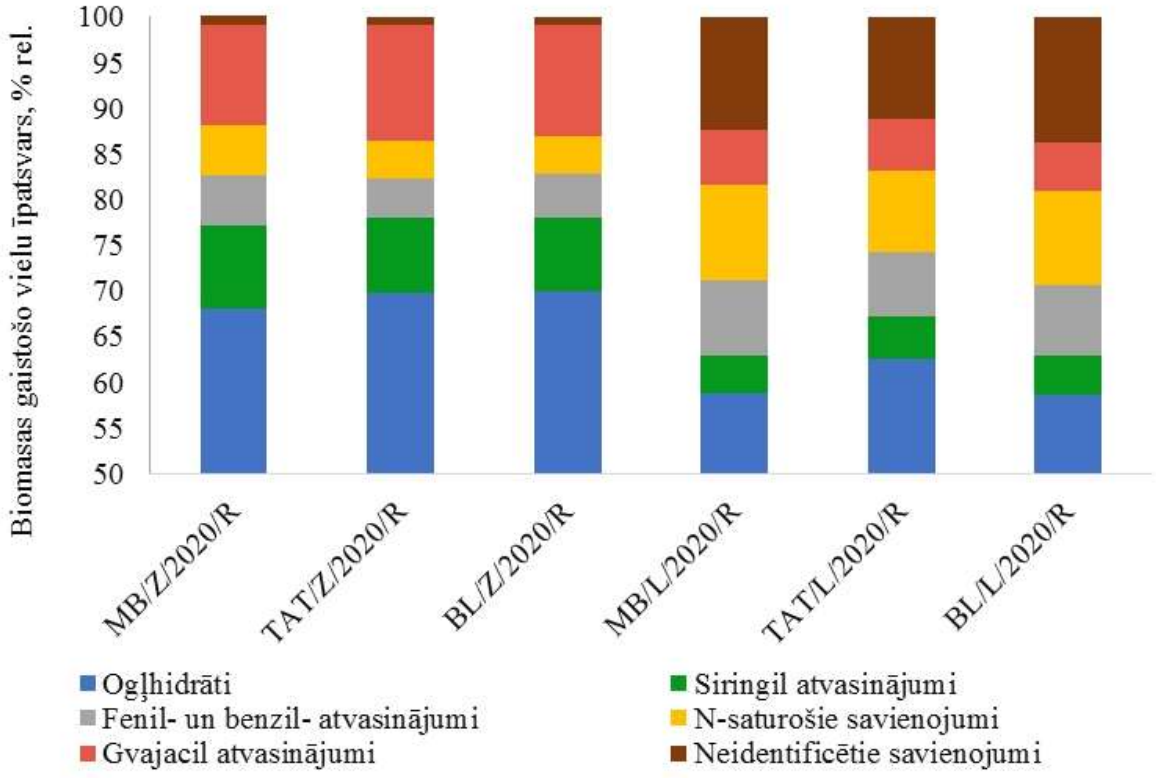
2.25. Statistikas analīze

Visi mērījumi veikti vismaz trīs eksemplāros, un rezultāti ir parādīti kā vidējā vērtība ± standarta novirze (SD). Statistiskās analīzes veiktas, izmantojot programmu Microsoft Excel 2016. Vidējie ticamības intervāli, izmantojot Stjudenta T sadalījumu, aprēķināti pie nozīmīguma līmeņa $\alpha = 0.05$. Lai kvantitatīvi noteiktu IL-8 un IL-6 izdalīšanos no cilvēka PBMNC, dati analizēti un grafiki ģenerēti, izmantojot GraphPad Prism 5.0 programmatūru (Sandjego, Kalifornija, ASV), paraugkopu salīdzināšanai izmantots ANOVA tests. Atšķirības uzskatītas par statistiski nozīmīgām, ja $p < 0.01$ un $p < 0.05$ (attiecīgais līmenis atzīmēts attēlos, kā aprakstīts VI. publikācijā).

3. REZULTĀTI UN DISKUSIJA

3.1. Smiltsērkšķu biomasas raksturojums

Smiltsērkšķu zari veido koksni ar mizas piejaukumu. Mizas saturs koksnes paraugā bija mainīgs un sastādīja ~ 8-20 % no kopējās zaru masas. Miza ir viena no izcilajām izejvielām bioloģisko aktīvo vielu ieguvei, līdz ar to zari netika mizoti un izmantoti kā vesela koksnes daļa (turpmāk tekstā saukts - koksne vai zari). Koksnes ķīmiskais sastāvs ir atkarīgs ne tikai no koka sugas, šķirnes, bet arī no koka vecuma, augšanas apstākļiem un citiem faktoriem. Smiltsērkšķu biomasas vispārīgs raksturojums veikts, izmantojot analītiskās pirolīzes metodi, kā aprakstīts 2.1. sadaļā. Koksni veidojošie galvenie komponenti ir celuloze, hemicelulozes un lignīns. Pamatojoties uz šo pamatsastāvu, to dēvē par lignocelulozes biomasu. Vadoties no analītiskās pirolīzes datiem, vislielāko daļu aizņem celuloze un hemiceluloze = 70% rel/SM, kam seko lignīns, to saturu apstiprinot ar siringila, gvajacila atvasinājumiem 20-25% rel/SM. Zaru un lapu gaistošos produktos ir arī polifenoli un N-saturošie savienojumi, kas norāda uz sekundāro metabolītu klātbūtni un koksnes augsto potenciālu izejvielas veidā bioloģiski aktīvo vielu – proantocianidīnu un serotonīnu – ieguvei. Smiltsērkšķu zaru un lapu gaistošie produkti un to relatīvais saturs ir parādīts 3.1. attēlā un I. publikācijā.



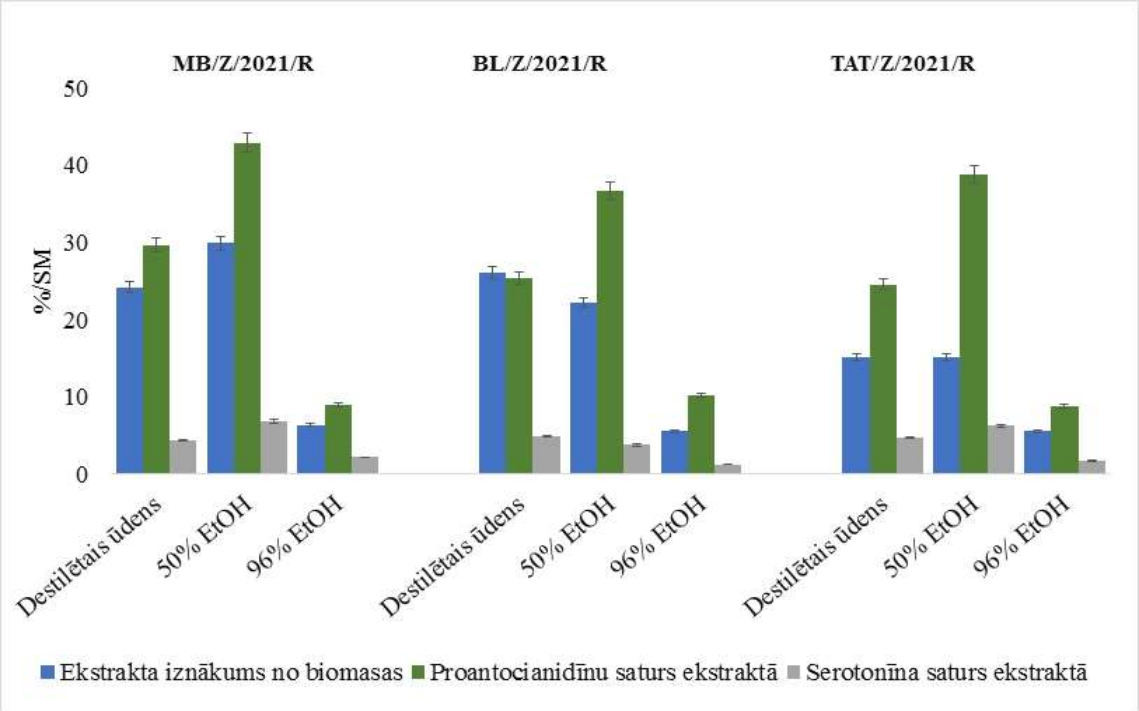
3.1.att. **Smiltsērkšķu biomasas analītiskās pirolīzes (Pa-GC-MS-FID) gaistošie produkti un to relatīvais saturs, %**

3.2. Smiltsērkšķu biomasas kā bioloģiski aktīvo vielu avota potenciāls

Lai noteiktu efektīvāko ekstrahentu mērķsavienojumu iegūšanai, laboratorijas apstākļos veikta 2.2. sadaļā aprakstītā ekstrakcija pie rekomendētajiem ekstrakcijas parametriem, izmantojot par pamatu iepriekšējos eksperimentos gūtos rezultātus, kas ietvēra biomasas un ekstrahenta masas attiecību 1:8 (w:w), ekstrakcijas temperatūru 60 °C un laiku 30 minūtes. Veicot vienpakāpes ekstrakciju zariem, izdalīto ekstraktu iznākums bija robežās no 6 līdz

30%/SM. Visaugstākais mērķsavienojumu (proantocianidīnu un serotonīnu) saturs pēc 2.3. sadaļā aprakstītas metodikas, bija smiltsērkšķu zaru ekstraktiem, kas iegūti ar 50% EtOH šķīdumu (skatīt 3.2. att.). Savstarpēji salīdzinot trīs šķirņu biomasas pēc mērķsavienojumu satura ekstraktos un biomasā, perspektīvākā šķirne bija *Maria Bruvele*.

Neskatoties uz augsto lapu ekstrakta iznākumu, proantocianidīni ekstrakta sastāvā netika konstatēti. Serotonīna saturs lapu ekstraktos bija zemāks par 0.1%/SM (II. publikācija). Tas liecina, ka efektīvākai mērķsavienojumu ieguvei iznākuma palielināšanai no zariem ir nepieciešams atdalīt lapas. Atdalītās lapas tika novērtētas konservantu un lopbarības piedevas ieguvei (skatīt 3.2. sadaļu un V. publikāciju).



3.2. att. **Trīs šķirņu smiltsērkšķu koksnes un hidrofīlo ekstraktu raksturojums**

Arvien biežāk cilvēki meklē pārtikas un kosmētikas produktus ar norādi "bez konservantiem". Bet konservantu galvenā loma ir novērst baktēriju, sēnīšu, raugu un citu patogēnu mikroorganismu vairošanos, novēršot inficēšanās varbūtību kosmētisko līdzekļu ražošanā un lietošanā. Pamatā lielākā daļa kosmētisko līdzekļu ir eļļas vai ūdens emulsija, kas ir labi apstākļi, lai vairotos patogēnie mikroorganismi. Papildus lipīdu saturošie produkti pārstrādes un uzglabāšanas laikā ir pakļauti oksidācijai, kā rezultātā uzkrājas toksiskie savienojumi, kas ne tikai pasliktina produktu īpašības, bet arī var izraisīt ilgstošus ādas bojājumus un apdraudēt cilvēka veselību. Ņemot vērā iepriekšminēto, drošai lipīdu saturošo produktu aizsardzībai būtu noderīgi 2 fāžu augu izcelsmes konservanti – lipofīlie un hidrofīlie. Lipofīlo ekstraktu ieguvei iepriekšminētajā vienpakāpes ekstrakcijas ciklā iekļauta biomasas apstrāde ar freonu R134a (IV. publikācija, patents). Šobrīd freons uzskatīts par vienu no saudzējošākiem ekstrahentiem lipofīlo vielu ieguvei no augu biomasas. Biomasas apstrāde ar freonu (skatīt 3.3. att.) veikta gan zariem, gan lapām, kā aprakstīts 2.2.2. sadaļā. Salīdzinot ar hidrofīlajiem ekstraktiem, lipofīlo ekstraktu saturs zaros bija robežās no 1.1 līdz 1.6%/SM un lapās no 2.7 līdz 3.4%/SM. Turpinot zaru atlikumu ekstrakciju ar 50% EtOH kā piemērotāko ekstrahentu hidrofīlo mērķsavienojumu ieguvei, būtiskas izmaiņas hifrofīlo ekstraktu sastāvā netika novērotas. Balstoties uz šiem novērojumiem, turpmākiem pētījumiem no iegūtiem ekstraktu paraugiem izmantoti hidrofīlie ekstrakti no zariem un lipofīlie ekstrakti no lapām.

3.3. att. **Lipofīlo ekstraktu ieguve no smiltsērkšķu lapām. Biomasas ekstrakcija ar freonu**

Pamatojoties uz GC analīzes (aprakstīta 2.3.1. sadaļā un I. publikācijā) datiem, viena no galvenajām lipofīlo ekstraktu sastāvdaļām ir taukskābes. Tieši lipīdu taukskābju sastāvs lielā mērā nosaka to uzturvērtību un bioloģisko efektivitāti. Iegūto GC datu analīze, identificējot >90% komponentu, atklāja linolēnskābes un palmitoleīnskābes pārsvaru ekstrakta taukskābju sastāvā. Saskaņā ar literatūras avotiem, šo skābju izmantošana krēmos ir svarīga sausai un sasprēgājušai ādai, kas mazina niezi, kairinājumu, palielina elastību un hidratāciju. Lipofīlo ekstraktu antimikrobiālās un antioksidatīvās īpašības lipofīliem ekstraktiem novērtētas 3.2. sadaļā.

Sekundāro metabolītu biosintēze augos ir atkarīga no ģenētiskajiem faktoriem, klimatiskajiem un ekoloģiskajiem apstākļiem. Ņemot vērā to, ka, salīdzinot ar jaunākajiem zariem, vecākie zari bija vairāk pakļauti stresam, tad pieļaujam, ka polifenolu saturs arī būs lielāks, jo tieši polifenoliem piemīt augu aizsargīpašības. Lai novērtētu zaru augšanas vecuma ietekmi uz mērķsavienojumu saturu, augļkoka kopšanas rezultātā pavasarī sagatavotas sekojošas zaru partijas – viengadīgie, divgadīgie, trīsgadīgie un četrgadīgie zari. Veicot 2.2.2. sadaļā aprakstīto vienpakāpes ekstrakciju, iegūtie ekstrakcijas dati (skatīt 3.1. tabulu) liecina, ka visaugstākais proantocianidīnu saturs bija trīsgadīgajos un četrgadīgajos zaros, savukārt serotonīna saturs – viengadīgajos un divgadīgajos zaros. Tā kā ekstraktu iznākums un mērķsavienojumu saturs ekstraktos, kas iegūti ar 96% EtOH, bija viszemākie, turpmākajos pētījumos šis ekstrahents vairs netika izmantots.

3.1. tabula.

Salīdzinošs ķīmiskais raksturojums ekstraktiem, kas iegūti no dažādiem augšanas vecuma zariem, kā ekstrahentu izmantojot destilētu ūdeni, 50% EtOH, and 96% EtOH

Maria Bruvele šķirnes paraugi	**Ekstrakta iznākums, %/SM**	**Proantocianidīnu saturs ekstraktā, %/SM**	**Serotonīna saturs ekstraktā, %/SM**
	Ekstrakcija ar destilēto ūdenī		
MB/1Z/2021/P	11.96±0.01	21.88±0.03	11.21±0.02
MB/2Z/2021/P	12.57±0.02	20.95±0.02	14.62±0.03

3.1. tabulas turpinājums

Maria Bruvele šķirnes paraugi	Ekstrakta iznākums, %/SM	Proantocianidīnu saturs ekstraktā, %/SM	Serotonīna saturs ekstraktā, %/SM
	Ekstrakcija ar destilēto ūdenī		
MB/3Z/2021/P	15.87±0.02	23.02±0.03	11.08±0.02
MB/4Z/2021/P	8.53±0.02	26.92±0.03	7.91±0.01
	Ekstrakcija ar 50% EtOH		
MB/1Z/2021/P	22.23±0.02	40.56±0.01	10.53±0.03
MB/2Z/2021/P	19.61±0.01	51.94±0.02	14.85±0.03
MB/3Z/2021/P	17.25±0.01	57.03±0.02	9.88±0.02
MB/4Z/2021/P	8.56±0.02	57.31±0.02	9.97±0.01
	Ekstrakcija ar 96%EtOH		
MB/1Z/2021/P	15.65±0.01	6.08±0.02	2.8±0.02
MB/2Z/2021/P	11.83±0.02	6.12±0.02	2.7±0.01
MB/3Z/2021/P	10.89±0.01	9.71±0.01	2.2±0.01
MB/4Z/2021/P	6.26±0.01	11.14±0.02	2.4±0.01

Lai novērtētu optimālo smiltsērkšķu kopšanas laiku un novērtētu sezonālās atšķirības biomasas sastāvā, pavasarī un rudenī no vienas un tās pašas plantācijas tika ievākti viengadīgie un divgadīgie zari. Veicot zariem vienpakāpes ekstrakciju un analizējot ekstraktu sastāvu, gūtie rezultāti liecina, ka rudenī ievāktie zari bija bagātāki gan ar serotonīna, gan ar proantocianidīnu saturu. Apkopojot iegūtos rezultātus, ir skaidrs, ka rudenī nogrieztie zari kā izejviela ir piemērotākā mērķsavienojumu ieguvei (skatīt 3.2. tabulu un II. publikāciju).

3.2. tabula.

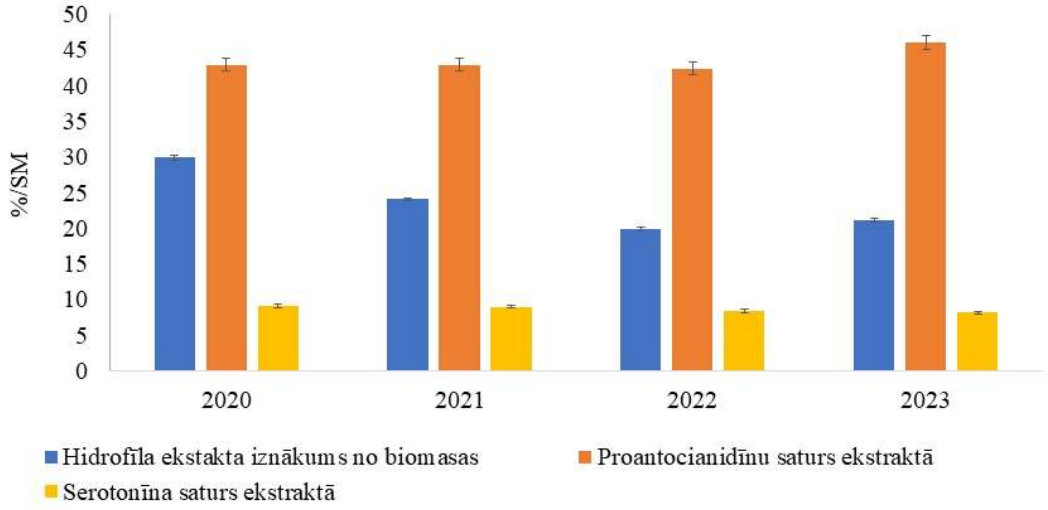
Sezonālās atšķirības ekstraktos iegūtiem no 1 un 2-gadīgo zaru maisījuma

Smiltsērkšķu šķirne	Ekstrakta iznākums, %/SM	Proantocianidīnu saturs ekstraktā, %/SM	Serotonīna saturs ekstraktā, %/SM
	Ekstrakcija ar destilēto ūdeni		
	Pavasaris / Rudens	Pavasaris / Rudens	Pavasaris / Rudens
MB/1-2Z/2022	12.1 / 15.7	21.1/ 41.3	13.0 / 13.7
BL/1-2Z/2022	10.2 / 19.4	21.5 / 41.8	11.0 / 11.6
TAT/1-2Z/2022	10.1 / 19.4	24.7 / 56.1	9.5 / 10.3
	Ekstrakciju ar 50% EtOH		
	Pavasaris / Rudens	Pavasaris / Rudens	Pavasaris / Rudens
MB/1-2Z/2022	20.2 / 26.4	50.0 / 52.9	12.8 / 14.0
BL/1-2Z/2022	18.1 / 24.0	48.2 / 64.2	11.0 / 12.6
TAT/1-2Z/2022	18.7 / 24.1	42.2 / 64.1	8.3 / 10.6

Apkopojot iegūtos rezultātus, ir skaidrs, ka rudenī ievāktā zaru biomasa ir vispiemērotākā izejviela gan serotonīna, gan proantocianidīnu iegūšanai.

Laika posmā no 2020. līdz 2023. gadam ogu novākšanas rezultātā gūtiem zaru atlikumiem šķirnei *Maria Bruvele* veikts mērķsavienojumu skrīnings, lai novērtētu šo atlikumu kā pastāvīgo tehnoloģisko izejvielu ikgadējai mērķsavienojumu ieguvei. Pēdējo trīs gadu laikā

mērķsavienojumu saturs bija līdzīgs, kas norāda uz zaru kā izejvielas augsto potenciālu (skatīt 3.4. att.).

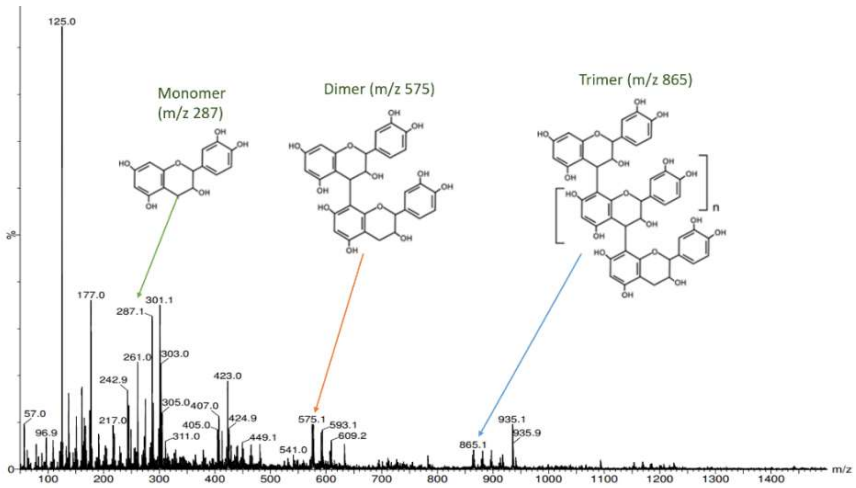


3.4. att. **Augļu novākšanas rezultātā, ievākto smiltsērkšķu zaru, kā mērķsavienojumu avota skrīnings laika periodā no 2020. līdz 2023. gadam**

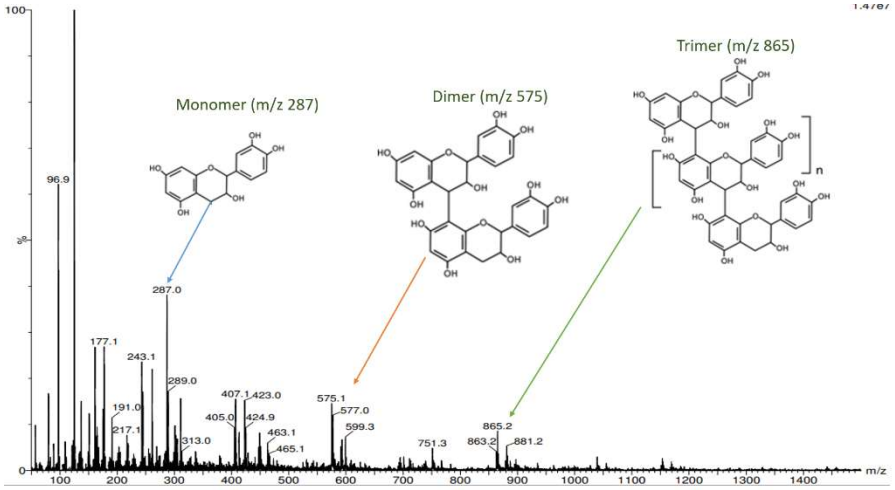
3.3. Proantocianidīnu un serotonīna izdalīšana

Serotonīna attīrīšana veikta, izmantojot preparatīvas kolonnas pildītas ar dažādu poru izmēru sorbentiem (iesniegts patentēta pieteikums) lielmolekulāro un zemmolekulāro polifenolu un to glikozīdu atdalīšanai. Visaugstākais serotonīna un proantocianidīnu saturs bija noteikts smiltsērkšķu mizas sastāvā, kas ļauj no 100 g mizas ar 50% EtOH iegūt 26 g sausa ekstrakta ar 14% serotonīna un 53 % proantocianidīnu saturu (II. publikācija), bet smiltsērkšķu zaru mizošana nav ekonomiski saprātīgs risinājums, tāpēc vispiemērotākā izejviela no analizētiem paraugiem ir nemizotie viengadīgie un divgadīgie rudens zari ar serotonīna saturu ekstraktā 13.7 %/SM. Serotonīna attīrīšana veikta secīgi, pēc 2.4. sadaļā aprakstītās metodikas, sākot ar proantocianidīnu izdalīšanu no 50% EtOH ekstrakta, izmantojot Sephadex LH-20. Proantocianidīnu izdalīšana ļāva divreiz palielināt serotonīna saturu ekstraktos. Turpinot serotonīna izdalīšanu pēc patentētā paņēmiena, daļēji atdalīti zemmolekulārie polifenoli un to glikozīdi, palielinot serotonīna saturu līdz 28.2 %/SM.

Izdalot proantocianidīnus no ekstrakta, tiem veikts ķīmiskais raksturojums, nosakot to sastāvu (MS spektroskopija) un tīrības pakāpi (Porter metode), kā aprakstīts sadaļā 2.3. Iegūto analīžu dati liecina, ka izdalīti proantocianidīni no *Maria Bruvele* šķirnes ekstraktiem ar tīrības pakāpi 91-94% PAC/SM, sastāv no A-tipa (m/z 575) un B-tipa (m/z 577) katehīna/epikatehīna dimēriem, katehīna/epikatehīna-epigalokatehīna/galokatehīna dimēriem (m/z 593), B-tipa katehīna/epikatehīna trimēriem (m/z 865), un epikatehīna-katehīna-galokatehīna trimēriem (m/z 881) (skatīt 3.5. un 3.6. att., III. publikāciju). Katehīna/epikatehīna tetramēri un pentamēri bija atrodami piemaisījuma līmenī.



3.5. att. **Izdalīto proantocianidīnu no *Maria Bruvele* 50% EtOH ekstrakta MS spektrs**

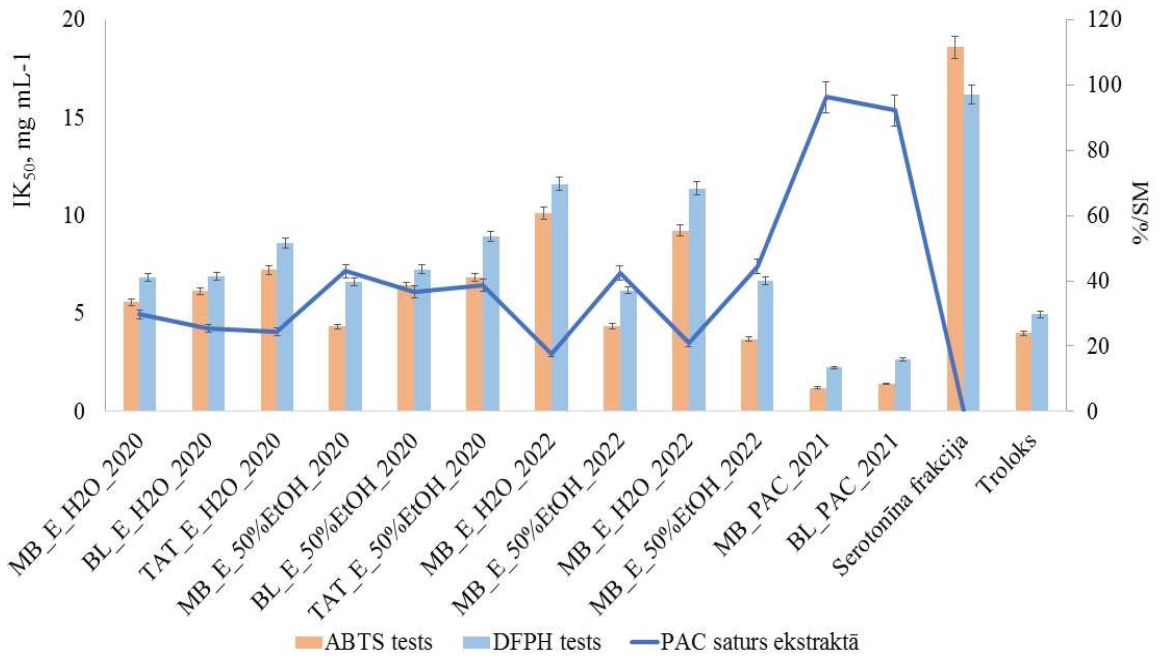


3.6. att. **Izdalīto proantocianidīnu no *Maria Bruvele* ūdens ekstrakta MS spektrs**

3.4. Ekstraktu un mērķsavienojumu raksturojums

3.4.1. Antioksidatīvā aktivitāte

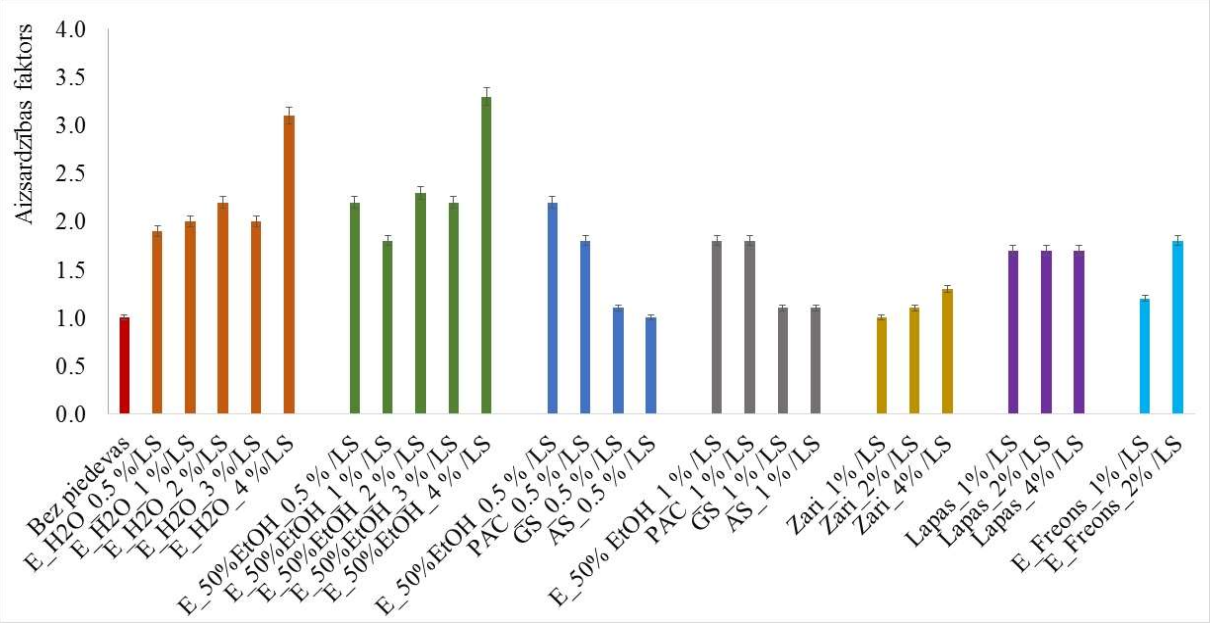
Ekstraktu un mērķsavienojumu antioksidatīva aktivitāte noteikta izmantojot DFPH$^{\bullet}$ un ABTS$^{+\bullet}$ testu (IV., VIII. publikācijas). Savstarpēji salīdzinot zaru hidrofīlo ekstraktu antioksidatīvo aktivitāti, novērota sakarība starp proantocianidīnu saturu ekstraktā un to antioksidatīvo aktivitāti – palielinoties proantocianidīnu saturam ekstraktā, palielinās radikāļu dezaktivēšanas aktivitāte ABTS$^{+\bullet}$ un DFPH$^{\bullet}$ testos (skatīt 3.7. att.). No pētītajiem ekstraktiem, 50% EtOH ekstrakti parādīja visaugstāko antioksidatīvo aktivitāti, salīdzinot ar ūdens ekstraktiem (proantocianidīnu saturs 50% EtOH ektraktā bija 42.45%/SM; IK_{50}=6.18 mg L^{-1} izmantojot DFPH$^{\bullet}$; IK_{50}=4.4 mg L^{-1}, izmantojot ABTS$^{+\bullet}$). Proantocianidīniem no MB/Z/2021/R un BL/Z/2021/R antioksidatīvā aktivitāte bija robežās no 1.2 līdz 1.4 mg L^{-1} pēc ABTS$^{+\bullet}$ testa un no 2.2 līdz 2.6 mg L^{-1} pēc DFPH$^{\bullet}$ testa, kas ir ievērojami augstāka par ekstraktiem. Troloks izmantots kā references antioksidants, kas ir ūdenī šķīstošs E vitamīna analogs. Attiecībā uz freona ekstraktu, to antioksidatīvā aktivitāte bija vāja, >30 mg L^{-1} (IV. publikācija).



3.7. att. **Analizējamo paraugu antioksidatīvā aktivitāte. Zemāka IK_{50} vērtība atbilst lielākai antioksidatīvai aktivitātei.**

Lai novērtētu analizējamo paraugu spēju aizkavēt oksidēšanos lipīdu saturošos produktos, analizējamie paraugi pievienoti kosmētisko krēmu substrātiem ar dažādu lipīdu saturu – 19% un 35% (I. publikācija). Pētāmie paraugi testēti koncentrācijā no 0.5 līdz 4% uz lipīdu saturu (LS) substrāta sastāvā. Šo analīžu veikšanai izmantota 2.5.2. sadaļā aprakstītā Oxipress metode. Analizēto paraugu aizsardzības faktors ir dots 3.8 un 3.9. attēlā. Salīdzinot analizējamos paraugus ar references materiāliem pie vienādas koncentrācijas substrātā (1%/LS), var secināt, ka 50% EtOH ekstrakts iedarbojas efektīvāk nekā references paraugi, proantocianidīni un ūdens ekstrakts. Neskatoties uz proantocianidīnu augsto antioksidatīvo aktivitāti, aizsardzības faktors substrāta oksidēšanas testā bija zemāks nekā 50 % EtOH ekstrakts. Visticamāk, tas ir saistīts ar proantocianidīnu ierobežoto šķīdību substrātā.

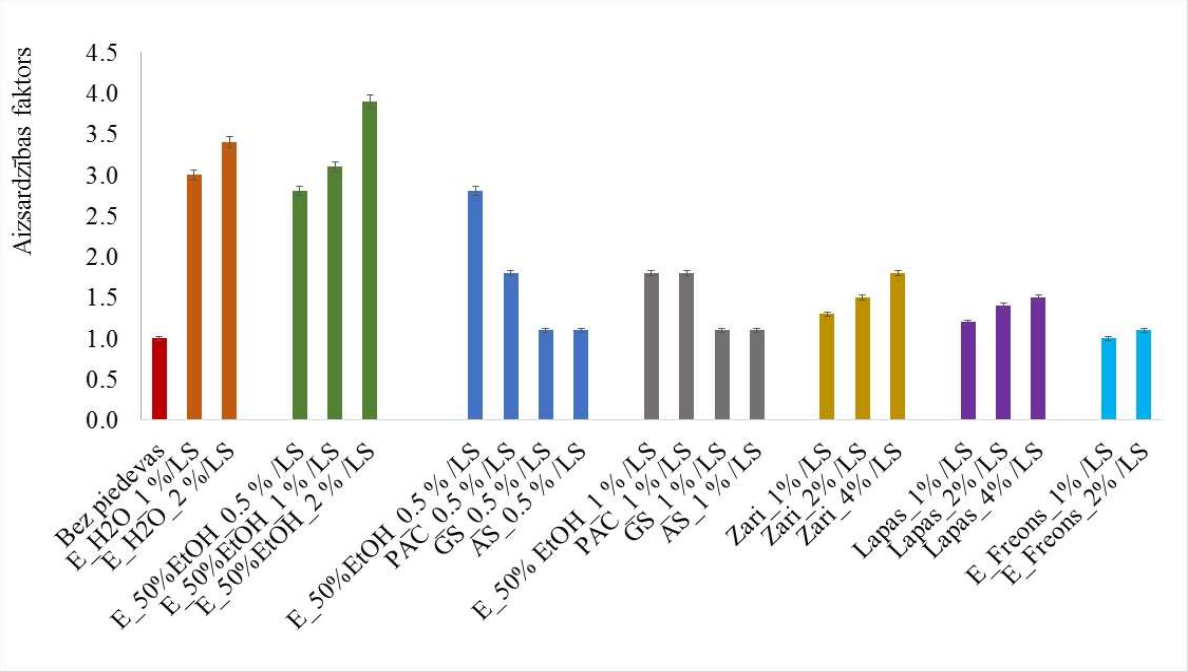
Savstarpēji salīdzinot sasmalcināto zaru un lapu ietekmi pie koncentrācijas 1%/LS, lapas ar plašu zemmolekulāro polifenolu klāstu bija līdzvērtīgas ūdens ekstrakta rezultātiem. Freona ekstrakts pie koncentrācijas 1%/LS bija līdzvērtīgs galluskābes iedarbībai, uzrādot zemu aizsardzības faktoru. Palielinot freona ekstrakta koncentrāciju līdz 2%/LS, lipīdu oksidācijas kavēšana bija līdzvērtīga lapu biomasas iedarbībai, kas norāda uz to, ka nav nepieciešama lapu biomasas ekstrakcija ar freonu, lai aizkavētu oksidēšanos lipīdu saturošos produktos.



3.8. att. **Analizējamo paraugu ietekme lipīdu oksidācijas kavēšanā, kā substrātu izmantojot krēmu ar 35 % lipīdu saturu**

E_H$_2$0 – ekstrakts, kas iegūts ar destilēto ūdeni no MB/Z/2021/R; 50%_EtOH – ekstrakts, kas iegūts ar 50% EtOH no MB/Z/2021/R; E_Freons – ekstrakts, kas izdalīts ar freonu no MB/L/2021/R; PAC - izdalītie no ekstrakta proantocianidīni; GS – galluskābe; AS – askorbīnskābe; LS – lipīdu saturs.

Ievadot analizējamos paraugus substrātā ar zemāku tauku saturu, ekstraktu darbība bija efektīvāka. Salīdzinot ūdens ekstrakta iedarbību pie koncentrācijas 1%/LS, substrātā ar 19 % lipīdu saturu, aizsardzības faktors bija 1.5 reizi augstāks, līdzīga tendence ir novērota arī 50% EtOH ekstraktam. Pārējo analizējamo paraugu efektivitāte bija zemāka.



3.9. att. **Analizējamo paraugu ietekme lipīdu oksidācijas kavēšanā, kā substrātu izmantojot krēmu ar 19 % lipīdu saturu.**

E_H$_2$0 – ekstrakts, kas iegūts ar destilēto ūdeni no MB/Z/2021/R; 50%_EtOH – ekstrakts, kas iegūts ar 50% EtOH no MB/Z/2021/R; E_Freons – ekstrakts, kas izdalīts ar freonu no MB/L/2021/R; PAC - izdalītie no ekstrakta proantocianidīni; GS – galluskābe; AS – askorbīnskābe; LS – lipīdu saturs.

3.4.2. Antimikrobiālā aktivitāte

Otra būtiska mūsdienu problēma ir baktēriju rezistence pret antibiotikām un sēņu rezistence pret fungicīdiem. Ekstraktu un mērķsavienojumu antimikrobiālā aktivitāte testēta attiecībā pret tādām baktērijām kā *Escherichia coli* (*E. coli), Pseudomonas aeruginosa (P. Aeruginosa), Staphylococcus aureus (S. Aureus), Bacillus cereus (B. Cereus)* un sēni *Candida albicans* (*C. albicans)*, nosakot minimālo inhibējošo (MIC) un minimālo baktericīdo/fungicīdu koncentrāciju (MBC/MFC) (II., VIII. publikācijas). *E. coli* baktērijas parasti dzīvo veselu cilvēku un dzīvnieku zarnās. Lielākā daļa *E. coli* veidu ir nekaitīgi, bet daži celmi, piemēram, *E. coli* O157:H7, var izraisīt smagus kuņģa krampjus un vemšanu. *P. aeruginosa* ir oportūnistisks patogēns, kas izraisa nopietnas infekcijas pacientiem ar novājinātu imunitāti, vēžu slimniekiem un pacientiem pēc smagiem apdegumiem un cistiskās fibrozes (Wu et al., 2015). *S. aureus* ir komensāls organisms, kas dzīvo ādā un gļotādā. Viegla līdz dzīvībai bīstama sepse var rasties, ja šis mikroorganisms nonāk cilvēka organismā. Tiek lēsts, ka meticilīna rezistentā *S. aureus (MRSA)* forma katru gadu izraisa aptuveni 171 200 ar veselības aprūpi saistītas infekcijas Eiropā un ir saistīta ar 5 400 papildu nāves gadījumiem. *B. cereus* ir baktēriju veids, kas rada bīstamu vielu (ko sauc par toksīnu), kas tiek pārnesta ar inficēto pārtiku. Tas galvenokārt skar pārtiku, kas pēc pagatavošanas ilgstoši uzglabāta istabas temperatūrā un tāpēc nav ātri un efektīvi atdzesēta. *C. albicans* ir oportūnistisks sēnīšu patogēns, kas ir cilvēku kuņģa-zarnu trakta floras sastāvdaļa, bet *C. albicans* spēj kolonizēt gandrīz visus cilvēka audus un orgānus, izraisot nopietnas invazīvas infekcijas (Hernday et al., 2010).

Visiem pētāmiem ekstraktiem piemīt antimikrobiālā aktivitāte. Attiecībā pret *E. coli* starp ekstraktiem visefektīvākais bija 50% EtOH ekstrakts ar kopējo polifenolu saturu 48.1 g GAE·100 g^{-1} SM. Viszemāko efektivitāti uzrādīja ūdens ekstrakts ar zemu polifenolu saturu (33.2 g GAE·100 g^{-1} SM). Līdzīga tendence ir novērota pret baktērijām *P. aeruginosa* un *S. aureus*. Ekstraktu atimikrobiālā aktivitāte pret *B. cereus* un *C. albicans* bija vājāka, novērojot tikai ekstraktu inhibējošo aktivitāti, kavējot *B. cereus* un *C. albicans* augšanu (skatīt 3.3. tabulu).

3.3. tabula.

Trīs šķirņu smiltsērkšķu ekstraktu antimikrobiālā aktivitāte

Smiltsērkšķu šķirne, 2020. gada paraugi	**Kopējais polifenolu saturs ekstraktā, g GAE·100 g^{-1} SM**	***E. coli***	***P. aeruginosa***	***S. aureus***	***B. cereus***	***C. albicans***
		MIC/MFC vai MBC, mg mL^{-1}				
Ekstrakti, kas iegūti ar destilēto ūdeni						
MB/Z/2020/R	43.62±0.03	0.39/0.39	0.39/3.13	0.39/0.78	0.78/>50	0.39/>50
BL/Z/2020/R	33.20±0.02	0.78/50	0.78/50	0.39/12.2	0.78/>50	0.39/>50
MB/Z/2020/R	35.14±0.04	0.39/0.39	0.78/1.56	0.39/0.78	0.78/50	0.39/>50
Ekstrakti, kas iegūti ar 50% EtOH						
MB/Z/2020/R	48.12±0.02	0.20/0.20	0.39/0.78	0.20/0.39	0.39/50	0.20/>50
BL/Z/2020/R	43.78±0.02	0.39/0.39	0.78/1.56	0.39/0.78	0.78/>50	0.20/>50
MB/Z/2020/R	41.36±0.03	0.39/0.39	3.13/3.13	0.20/0.78	0.78/50	0.39/>50

Līdzvērtīgi pētījumi veikti ar ekstraktiem un mērķsavienojumiem, kas iegūti no 2021. gada ievāktiem zariem. Salīdzinot ar ekstraktiem, proantocianidīni bija daudzkārt efektīvāki pret *E. coli, P. aeruginosa, S. aureus* un *Streptococcus pyogenes* (*S. pyogenes)*. No visiem paraugiem dotajās koncentrācijās neviens neuzrādīja minimālo fungicīdo koncentrāciju attiecībā pret sēni *C. albicans*.

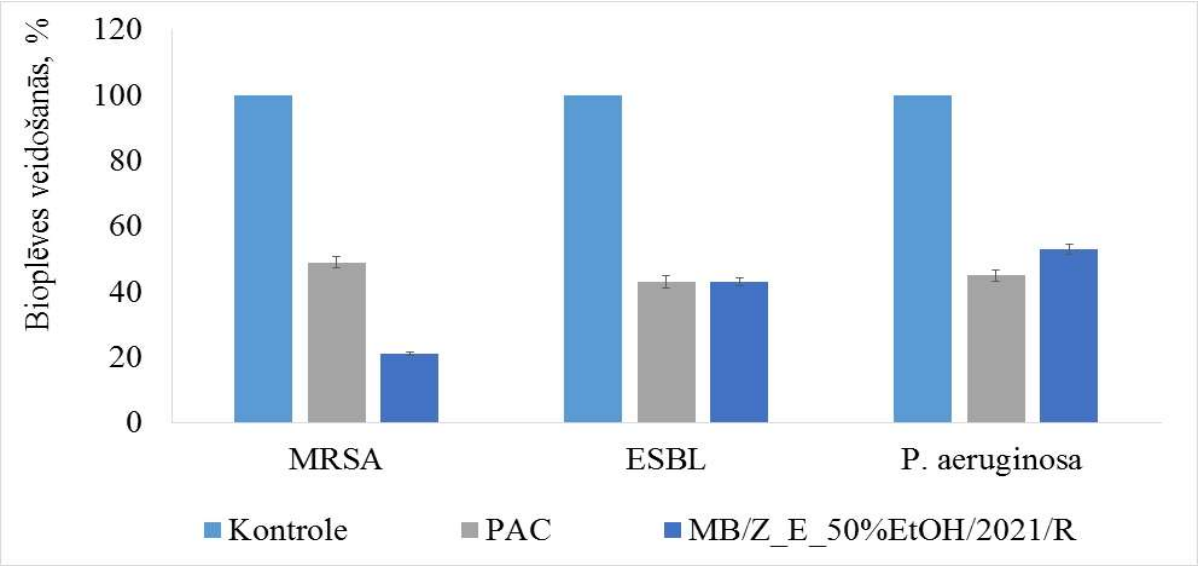
Serotonīnu saturošajai frakcijai noteikta antimikrobiālā aktivitāte attiecībā pret *S. pyogenes* un *Cutibacterium acnes* (*C. acnes)* (I. publikācija). Pamatojoties uz literatūras datiem, *S. pyogenes* izraisa dažādas akūtas infekcijas, piemēram, mīksto audu infekcijas un faringītu, kā arī smagas dzīvībai bīstamas infekcijas, piemēram, streptokoku toksiskā šoka sindromu un postošas postinfekciozas sekas, piemēram, reimatisko drudzi un glomerulonefrītu (Bryant and Stevens, 2015). Otrs patogēns, *C. acnes*, ir relatīvi lēni augoša, parasti aerotoleranta, anaeroba grampozitīva baktērija, kas saistīta ar aknes ādas stāvokli. Šis patogēns var izraisīt arī hronisku blefarītu un endoftalmītu (Corvec, 2018; Dali et al., 2001). Visi paraugi, ieskaitot serotonīnu un serotonīna standartu (references materiālu), bija efektīvi abu patogēnu nonāvēšanai. Freona ekstrakts no lapām salīdzinoši ar pārējiem ekstraktiem bija ar zemāku antimikrobiālo aktivitāti (skatīt 3.4. tabulu).

3.4. tabula.

Preparātu minimālā inhibējošā koncentrācija (MIC) un minimālā baktericīdā vai fungicīdā koncentrācija (MBC/MFC)

Paraugs; PAC saturs ekstraktā	***E. coli***	***P. aeruginosa***	***S. aureus***	***B. cereus***	***C. albicans***	***S. pyogenes***	***C. acnes***
	MIC/MFC vai MBC, mg mL^{-1}						
Ūdens ekstrakts no MB/Z/2021; 29.6%/SM	0.39/0.39	0.39/3.13	0.39/0.78	0.78/>50	0.39/ >50	0.20/0.20	0.78/0.78
50% EtOH ekstrakts no MB/Z/2021; 42.9%/SM	0.20/0.20	0.39/0.78	0.20/0.39	0.39/50	0.20/ >50	0.20/0.20	0.39/0.39
PACs no MB/Z/2021; 92.1%/SM	0.04/0.04	0.08/0.16	0.08/0.16	0.63/ 1.25	1.25/ >2.5	0.10/0.10	0.39/0.39
Serotonīnu saturošā frakcija (serotonīna saturs =28.2%/SM)	0.78/0.78	0.78/0.78	0.39/0.78	0.78/ 6.25	12.50/ >50	0.10/0.20	0.39/0.39
Freona ekstrakts no lapām	0.78/50	0.78/50	0.39/12.2	3.13/25			
Serotonīna standarts (tīrība ≥98.0%)						0.10/0.20	0.39/0.78

Ir zināms, ka bioplēves, ko veido dažādas baktēriju sugas, uzrāda paaugstinātu rezistenci pret antibiotikām un dezinfekcijas līdzekļiem, tādējādi bieži izraisa hroniskus iekaisuma procesus. Tā kā praktiski nav līdzekļu baktēriju plēvju apkarošanai, promocijas darba ietvaros novērtēta dominējošo preparātu – proantocianidīnu un 50% EtOH ekstraktu – ietekme uz bioplēvju iznīcināšanu (III. publikācija). Kā bioplēves veidotājas tika pētītas šādas baktērijas: meticilīnrezistentais *Staphylococcus aureus* (MRSA), paplašināta spektra beta laktamāzes producējošā *E. coli* (ESBL) un *P. aeruginosa*. Preparātu ietekme uz bioplēvju veidošanos parādīta 3.10. attēlā.

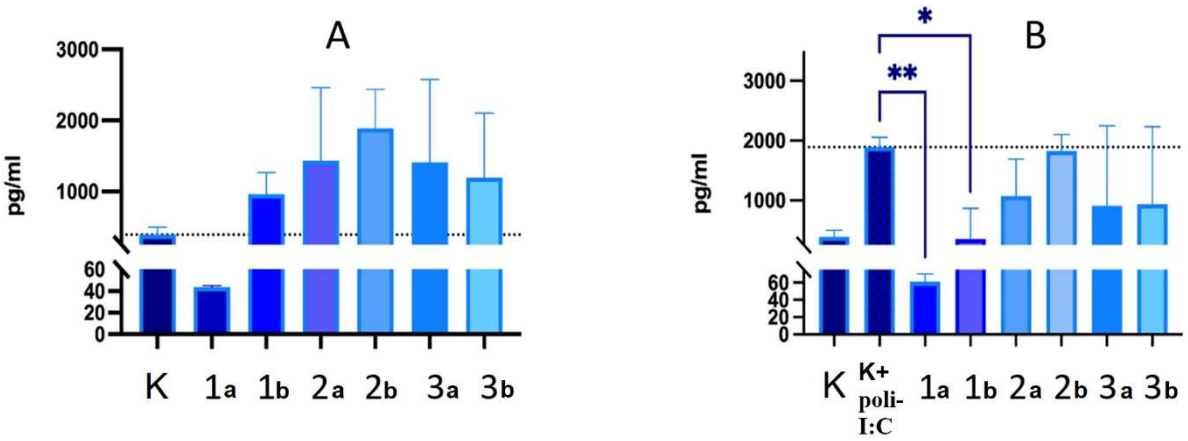


3.10. att. **Analizējamo preparātu ietekme uz bioplēves veidošanos: MRSA – meticilīnrezistentais *S. aureus*; ESBL – paplašināta spektra beta laktamāzes producējošā *E. coli*; PAC – proantocianidīni; MB/Z_E_50% EtOH/2021/R - ekstrakts, kas iegūts ar 50% EtOH no MB/Z/2021/R**

Salīdzinot ar kontroli (100% izveidota plēve), proantocianidīnu klātbūtnē plēvju veidošanās bija 2 reizes mazāka. Ekstrakta iedarbība bija līdzīga, izņemot uz MRSA bāzes veidoto bioplēvi, kuras veidošanās procentuālais lielums samazinājās 5 reizes. Pamatojoties uz gūtiem pētījuma rezultātiem, proantocianidīni un ekstrakti koncentrācijā 5 mg mL^{-1} spēj inhibēt bioplēves veidošanos un var būt izmantojami brūču dzīšanas terapijā.

3.4.3. Pretiekaisuma iedarbība

Iekaisums ir organisma atbildes reakcija uz traumām un infekcijām, un tas ir nepieciešams, lai pārvarētu to sekas. Par iekaisumu organismā liecina dažāda veida marķieru (bioķīmisku signālelementu - citokīnu) klātbūtne asinīs. Viens no galvenajiem citokīniem ir interleikīns Il-8, kas izlaists no vairākiem šūnu tipiem, reaģējot uz iekaisumu (Harada et al., 1994). Citu citokīnu IL-6 izdala baltās asins šūnas, reaģējot uz traumām vai mikrobu iedarbību, tam arī ir galvenā loma neironu reakcijā uz nervu bojājumiem (Zhang and An, 2007). Ir parādījušies pierādījumi, ka IL-6 var izmantot kā iekaisuma marķieri smagai COVID-19 infekcijai ("Raised troponin and interleukin-6 levels - COVID-19," 2020). Saskaņā ar iegūtajiem datiem, proantocianidīni koncentrācijā 0.5 mg mL^{-1} samazināja IL-8 sekrēciju no nestimulētām cilvēka perifēro asiņu mononukleārām šūnām (PBMNC). Savukārt poliinozīnskābes: policitidilskābes (poli I:C) klātbūtnē, kas atdarina ar vīrusu infekcijām saistītu iekaisumu, visi *Maria Bruvele* paraugi (proantocianidīni, 50% EtOH ekstrakts un ūdens ekstrakts) samazināja IL-8 sekrēciju no šūnām, kas norāda spēju mazināt ar vīrusu infekcijām saistītu iekaisumu (3.11. att., VI. publikācija).



3.11. att. **Izmaiņas IL-8 sekrēcijā no nestimulētām (A) un poli-I:C stimulētām (B) cilvēka perifēro asiņu mononukleārajām šūnām pēc 24 h inkubācijas ar analizējamiem paraugiem: 1 – proantocianidīni; 2 – 50% EtOH ekstrakts no MB/Z/2021/R , 3 – ūdens ekstrakts no MB/Z/2021/R. K – kontrole, K+poli-I:C –** kontrole+policitidilskābe (poli I:C); **a – koncentrācija 0.5 mg mL^{-1}, b – 0.25 mg mL^{-1}; *** $p < 0.05$, ** $p < 0.01$ vienvirziena ANOVA, n = 3.

Līdzīga situācija novērota arī pētījumos ar IL-6. Bez poli I:C stimulācijas pēc inkubācijas paraugu klātbūtnē, IL-6 sekrēcijas palielināšanās netika novērota, bet, vienlaikus pievienojot PBMNC pie parauga un poli I:C, novērots, ka proantocianidīni un 50% EtOH ekstrakts ievērojami samazina IL-6 sekrēciju. Proantocianidīnu klātbūtnē IL-6 sekrēcija samazināta līdz nestimulētam kontroles līmenim, turklāt netika novērotas atšķirības starp abām pārbaudītajām koncentrācijām. 50% EtOH ekstraktiem inhibējošā iedarbība bija atkarīga no koncentrācijas – 0.5 mg mL^{-1} samazināja IL-6 sekrēciju par 95.4%, savukārt, 0.25 mg mL^{-1} samazināja to par 63.8%. Ūdens ekstrakti nesamazināja poli-I:C izraisīto IL-6 sekrēciju (VI. publikācija). Kopumā mūsu atklājumi saskan ar citiem pētījumiem, kuros ir aprakstīta proantocianidīnu ietekme uz IL-6 un IL-8 sekrēciju iekaisuma modeļos.

3.4.4. Mērķsavienojumu iedarbība uz aizkuņģa dziedzera lipāzes aktivitāti gremošanas divpadsmitpirkstu zarnas fāzē

Normālos fizioloģiskos apstākļos (žults klātbūtnē) visi smiltsērkšķu hidrofīlie ekstrakti koncentrācijās 0.2–40 mg g^{-1} PL uzrādīja būtisku aizkuņģa dziedzera lipāzes (ADL) aktivitātes inhibēšanu. Jau pie 0.2 mg ūdens ekstrakta daudzuma, kas saturēja 43.4±0.4 g GAE·100 g^{-1} polifenolu un 17.5 ± 0.1% proantocianidīnu, lipāzes aktivitāte samazinājās par 22%. Turpmāka pakāpeniska ekstrakta daudzuma palielināšana no 0.2 līdz 40 mg g^{-1} PL nodrošināja aptuveni tādu pašu ADL inhibēšanu ticamības intervālā. Ir noteikta arī lipāzes aktivitātes inhibēšanas atkarība no proantocianidīnu satura ekstraktā: jo lielāks proantocianidīnu saturs ekstraktā, jo augstāks bija inhibēšanas procents. Ekstrakts, kas saturēja 48.6±0.2 g GAE·100 g^{-1} un 42.4±0.3% proantocianidīnu, daudzumā no 0.2 līdz 40 mg g^{-1} PL, inhibēja lipāzes aktivitāti par 33%. Proantocianidīnu galvenā loma ADL aktivitātē pierādīta, salīdzinot izdalīto proantocianidīnu no ekstrakta un eluāta (atlikušo frakciju pēc proantocianidīnu izdalīšanas), iedarbību uz ADL aktivitāti. Proantocianidīni koncentrācijas diapazonā no 1 līdz 20 mg g^{-1} PL inhibēja ADL aktivitāti par 36%. Savukārt eluāts nebija tik efektīvs un koncentrācijā 20 mg g^{-1} PL uzrādīja ADL aktivitātes palielināšanu par 6–11%. Serotonīna saturošā frakcija palielina ADL aktivitāti gremošanas divpadsmitpirkstu zarnas fāzē, un to var izmantot, lai normalizētu fizioloģisko gremošanu, īpaši triglicerīdu sadalīšanos brīvajās taukskābēs un monoglicerīdos, lai koriģētu lipīdu spektru, šūnu vielmaiņu un homeostāzi (patentēts pētījums).

Patoloģiskos apstākļos (bez žults) 50% EtOH ekstraktam un izdalītam proantocianidīnam novērota ADL inhibēšana pie 0.2 un 1 mg g^{-1} PL, taču jau pie 2 mg g^{-1} PL tika novērota

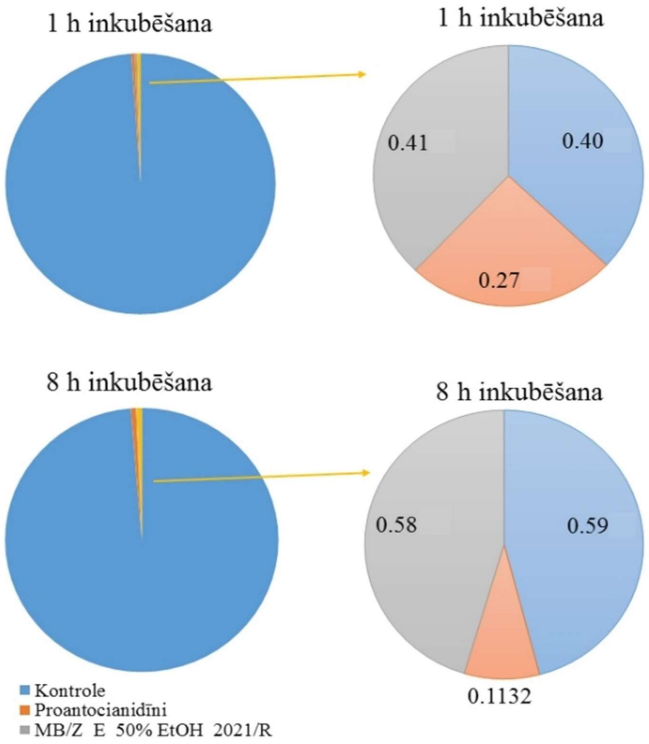
aizkuņģa dziedzera lipāzes iedarbības aktivācija. Patoloģiskos apstākļos abi mērķsavienojumi koncentrācijā no 2 līdz 400 mg g^{-1} PL katalizēja ADL iedarbību gremošanas divpadsmitpirkstu zarnas fāzē (IV. publikācija).

3.4.5. Mērķsavienojumu iedarbība uz amilāzes aktivitāti siekalās

Normālos fizioloģiskos apstākļos visi smiltsērkšķu ekstrakti un serotonīnu saturošā frakcija koncentrācijā 0.1–2 mg mL^{-1} $_{siekalu}$ uzrādīja nozīmīgu iedarbību, paātrinot cietes sadalīšanos līdz glikozei, kas var būt noderīga, lai ārstētu cilvēkus ar nepietiekamu svaru malabsorbcijas sindroma gadījumā. Izdalīti proantocianidīni tajā pašā koncentrācijā uzrāda inhibējošo aktivitāti, samazinot amilolītisko spēku no 640 uz 320 D 30/38 °C. Tas norāda, ka proantocianidīni spēj samazināt cietes ogļhidrātu sadalīšanos un uzsūkšanos organismā, kas var būt noderīga diabēta pacientiem un pacientiem ar lieko svaru (II. un VIII. publikācijas).

3.5. Analizējamo paraugu hemolīze

Visi ekstrakti pārbaudīti attiecībā uz to hemolītisko aktivitāti koncentrācijā 0.5 mg mL^{-1}. Neviens no ekstraktiem neizraisīja hemolīzi pēc 1 h vai 8 h inkubācijas, kas norāda uz ekstraktu augsto bioloģisko saderību un drošību (skatīt 3.12. att. un VI. publikāciju).

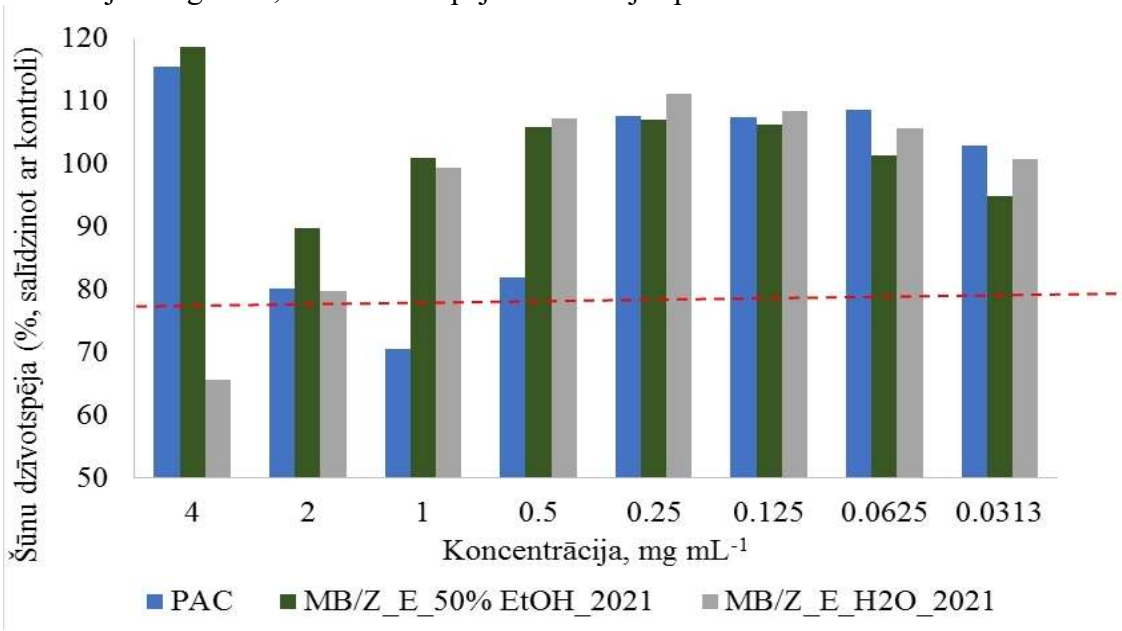


3.12. att. **Analizējamo paraugu hemolīze. PAC – proantocianidīni. MB/Z_E_50% EtOH_2021 – ekstrakts iegūts no *Marija Bruvele* zariem ievāktiem rudenī 2021. gadā ar 50% EtOH**

3.6. Citotoksicitāte

Ekstraktu un mērķsavienojumu citoksicitāte novērtēta un salīdzināta ar novēroto MIC/MBC un MIC/MFC koncentrāciju, kā arī antioksidanta IK vērtību un nepieciešamu devu amilāzes un lipāzes darbības inhibēšanai vai stimulēšanai (VI. un VIII. publikācijas).

Citotoksicitātes novērtējuma rezultāti ir atspoguļoti 3.13. attēlā. Paraugi uzskatīti par citotoksiskiem, ja šūnu dzīvotspēja bija samazināta par vairāk nekā 20%. Visi pētāmie hidrofīlie ekstrakti no zaru biomasas to darbības koncentrācijas diapazonā no 0.0313 līdz 4.0 mg mL^{-1} bija droši, neuzrādot citotoksicitāti. Proantocianidīnu droša koncentrācija bija robežās no 0.03 līdz 0.5 mg mL^{-1}, bet pie proantocianidīna koncentrācijas 1 mg mL^{-1} bija novērota neliela citotoksicitāte, samazinot šūnu dzīvotspēju par 29.56%. Ūdens ekstraktam koncentrācijā 4 mg mL^{-1}, šūnu dzīvotspēja samazinājās par 34.33%.



3.13. att. **Analizējamo paraugu citotoksicitāte**

3.7. Smiltsērkšķu biomasas novērtējums lopbarības ieguvei

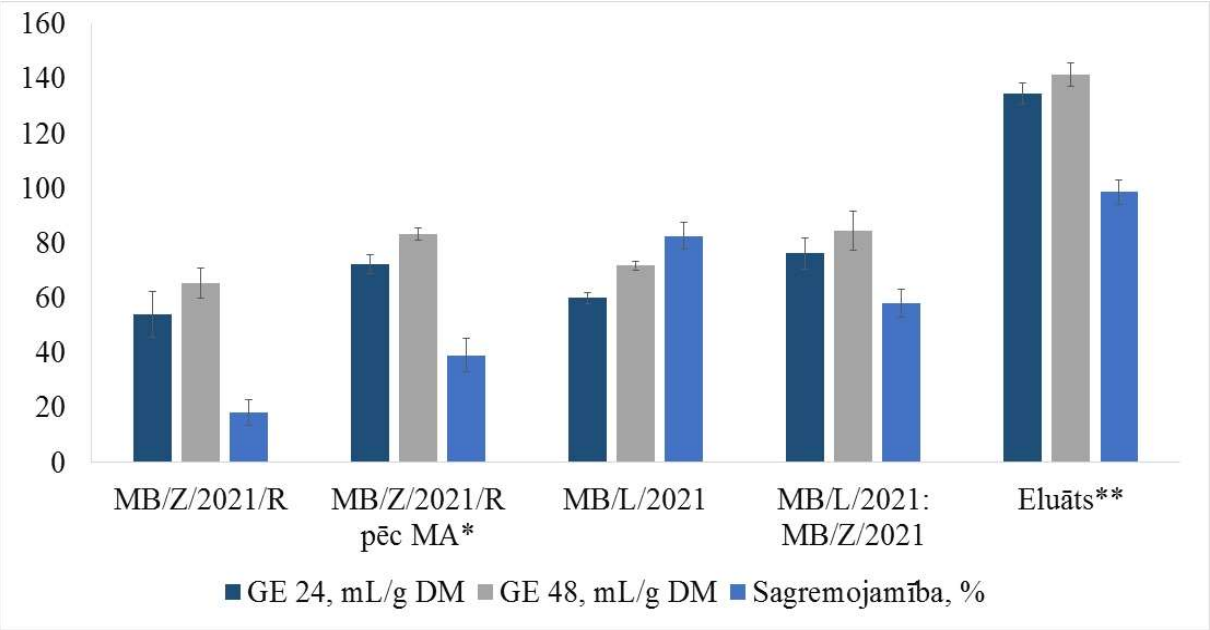
Lai novērtētu zaru atlikumu (MB/Z/2021/A, BL/Z/2021/A, TAT/Z/2021/A) un lapu (MB/L/2021, BL/L/2021, TAT/L/2021) piemērotību lopbarības ieguvei, laboratorijas apstākļos veikts paraugu ķīmiskais raksturojums, noteikts koppelnu, kokšķiedru, kopproteīna, fosfora, kālija, nātrija, kalcija un smago metālu (Pb, Cd un Hg) saturs. Lapās fosfora saturs bija robežās no 210±21 līdz 225±22 mg uz 100 g SM; kālija saturs no 1209±104 līdz 1376±113 mg uz 100 g SM; nātrija saturs no 1.72±0.40 līdz 2.25±0.52 mg uz 100 g SM; kalcija saturs no 856±205 līdz 989±237 mg uz 100 g SM. Zaros nātrija saturs bija augstāks, bet kalcija saturs zemāks: nātrija saturs no 7.83±1.80 līdz 22.5±5.2 mg uz 100 g SM; kalcija saturs no 281±67 līdz 332±80 mg uz 100 g SM. Smago metālu saturs biomasā nepārsniedz maksimāli pieļaujamo koncentrāciju un atbilst Eiropas Komisijas regulai Nr. 1275/2013. Papildus mikro- un makroelementiem smiltsērkšķu zari un lapas satur ūdenī un taukos šķīstošu vitamīnu kompleksu. C vitamīna saturs zaros bija no 8.0±3.0 līdz 178.0 ±50.0 mg uz 100g SM, lapās no 12.0±3.0 līdz15.6 mg uz 100 g SM. Salīdzinot ar zariem, visas trīs smiltsērkšķu šķirņu lapas bija bagātākas ar E vitamīnu (zaros: 14.7±2.1 mg uz 100 g SM; lapās no 30.9±4.3 līdz 42.6±2.2 mg uz 100 g SM). A vitamīns smiltsērkšķu koksnē netika konstatēts, savukārt, lapās A vitamīna saturs bija 0.86±0.07 līdz 1.29±0.02 mg uz 100 g SM (V. publikācija).

Saskaņā ar literatūras datiem, smiltsērkšķu lapas papildus satur vērtīgas 13 aminoskābes, bet koksnes daļas un miza satur 17 aminoskābes (Bekker and Glushenkova, 2001; Šnē et al., 2013).

Izejvielu kvalitāte ietekmē barības sagremojamību, kas atsaucās siltumnīcefekta gāzu veidošanās lopu zarnu traktā. Barība ar augstāku sagremojamību samazina metāna daudzuma emisiju. Ir zināms, ka liels tauku saturs biomasā (>8%/SM) var nelabvēlīgi ietekmēt spurekļa darbību, šķiedrvielu gremošanu un piena ražošanu. Tādējādi var teikt, ka tauku saturs

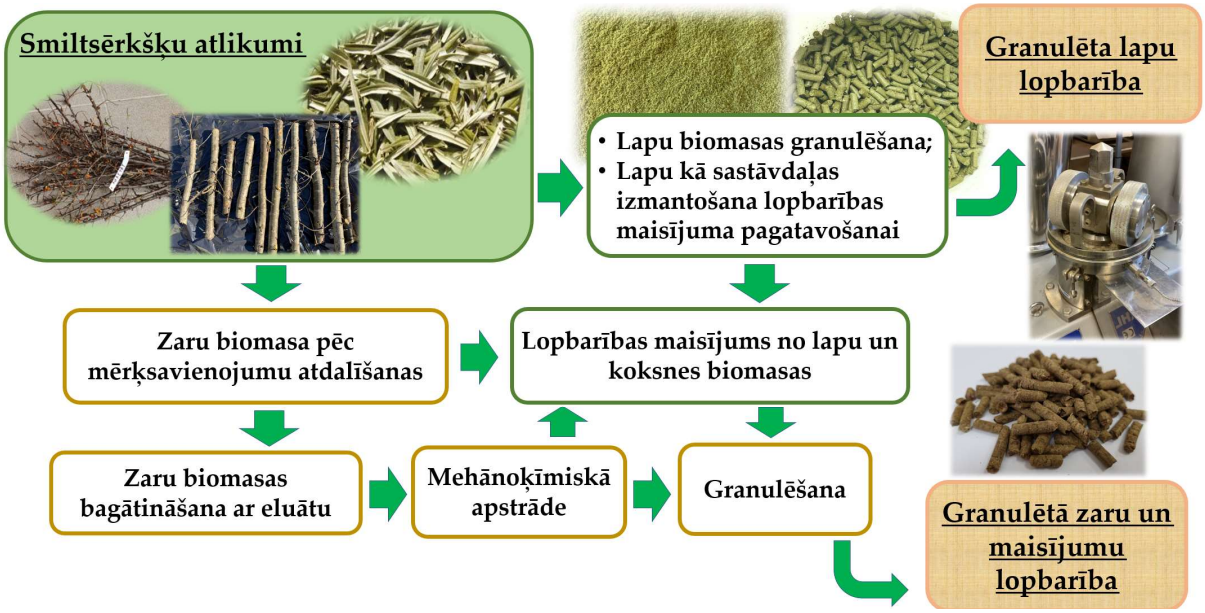
analizējamos paraugos (0.7-3.6%/SM) bija optimāls uztura barībai. ADF parasti nav sagremojama, taču tā stimulē nozīmīgu baktēriju veidošanos. Visaugstākais ADF saturs biomasā bija zariem (26.2%–27.1%/SM), lapās tās saturs bija nedaudz zemāks (18.1–19.1%/SM). Kopējais proteīna saturs zaros bija ~23%/SM un lapās robežās no 18.4% līdz 19.4%/SM.

Pētāmo paraugu sagremojamība noteikta, izmantojot *in vitro* analīzi, nosakot fermentācijas rezultātā izdalīto gāzi (V. publikācija). Jo augstāka ir biomasas sagremojamība, jo augstāka ir barības uzturvērtība un bioloģiskā vērtība. Šis tests veikts *Maria Bruveles* šķirnes biomasām MB/Z/2021/A un MB/L/2021 un atlikušai frakcijai pēc proantocianidīnu atdalīšanas (eluāts). Saskaņā ar *in vitro* testa datiem ekstrakts uzrādīja vislielāko sagremojamību pēc proantocianidīnu atdalīšanas. Savstarpēji salīdzinot biomasas, labāka sagremojamība bija lapām. Lai novērtētu mehano-ķīmiskās aktivitātes iedarbību uz gremošanas efektu, zaru atlikums papildus tiek mehano-ķīmiski apstrādāts, kā aprakstīts 2.13. sadaļā. Iegūtie rezultāti parāda to, ka mehano-ķīmiski apstrādātas biomasas sagremojamība bija 2.2 reizes labāka, salīdzinot ar paraugu bez papildus mehano-ķīmiskās apstrādes (3.14. att.).



3.14. att. ***In vitro*** **analīzes dati par biomasas sagremojamību pirms un pēc apstrādes**
* pēc MA – pēc mehano-ķīmiskās apstrādes; ** eluāts - atlikusi frakcija pēc proantocianidīnu atdalīšanas

Smiltsērkšķu biomasas pārstrāde lopbarības granulās parādīta 3.15. attēlā.



3.15. att. **Ilgtspējīgas smiltsērkšķu atlikumu pārstrādes shēma lopbarības granulu iegūšanai**

Lopbarības piedevu granulēšana ir viena no efektīvākajām metodēm produkta kvalitātes saglabāšanai gan uzglabāšanas laikā, gan to transportēšanas laikā. Sasmalcinātas lapas, salīdzinot ar koksni, ir vieglāk granulējamas, par to liecina zems enerģijas patēriņš un augsts ražīgums. Iegūto granulu nodilumizturība bija robežās no 96.9 līdz 97.7 %, mitrums 5-6%, vidējais garums 12 mm, tilpumblīvums 714-716 g cm^{-2} (3.16. att., V. publikācija).

3.16.att. **Lopbarības piedevas granulu veidā: A – lapu un zaru atlikuma maisījums (1:1 w/w); B – lapas; C – zaru atlikums**

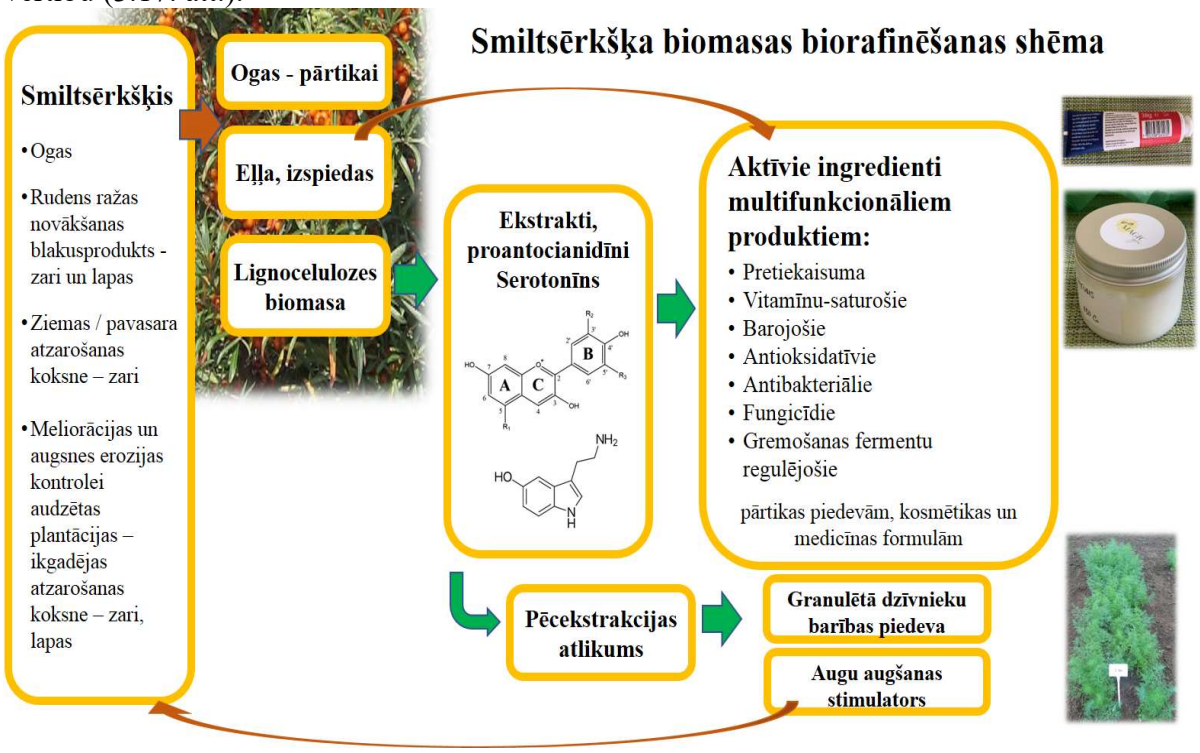
3.8. Smiltsērkšķu biomasas novērtējums augsnes piedevas ieguvei

Smiltsērkšķu zaru atlikums pēc ekstrakcijas pētīts kā lignocelulozes substrāts augsnes piedevas ieguvei, to bagātinot ar Si (VII. publikācija). Augsnes piedevas rādītāji bija sekojoši: lignīna saturs 38.5±0.5 %/SM; humīnskābes saturs – 4.3±0.1%/SM; kopējais slāpekļa saturs – 1.35±0.02 %/SM; kopējais fosfora saturs 0.06±0.01%/SM; pH – 8.7±0.1%/SM. Sadarbībā ar LBTU Zemkopības institūtu augsnes piedeva tika testēta lauka izmēģinājumos, audzējot vasaras kviešus (šķirne: 'Vinjet') un kartupeļus (šķirne: 'Imanta'). Iestrādājot augsnes piedevu augsnē, konstatēts būtisks kartupeļu ražas pieaugums. 2021. gada lauka izmēģinājumos lietojot augsnes piedevu devā 20 un 40 kg ha^{-1}, kartupeļu ražas pieaugums bija 11.2% un 13.8%, salīdzinot ar kontroles variantu. 2022. gadā karstais laiks atstāja būtisku ietekmi un kartupeļu ražas pieaugums iepriekš minētajās devās bija 8.4% un 21.4%, salīdzinot ar kontroles variantu. Audzējot vasaras kviešus, līdzvērtīgi kartupeļu izmēģinājumiem, karstais un sausais laiks

kavēja vasaras kviešu attīstību, par ko liecina zemais augu augstums (vidēji 70-73 cm), kā arī ātri nokalta lapas. Lietojot augsnes piedevu devā 20 un 40 kg ha^{-1}, 2021. gadā graudu raža pieauga par 9.5 un 11.7 %, savukārt, 2022. gadā graudu raža kopumā bija zemāka, bet salīdzinošā analīze uzrādīja būtiskāku kviešu ražas pieaugumu par 16.5 un 26.5 %, lietojot augsnes piedevu daudzumā 20 un 40 kg ha^{-1}. Sausais un karstais laiks būtiski ietekmēja arī graudu kvalitāti. Izmēģinājumā iegūtajiem graudiem ir zema tilpummasa (< 700 g L^{-1}) un 1000 g masa (27.2–28.2 g).

3.9. Smiltsērkšķu biorafinēšanas shēma

Pamatojoties uz promocijas darbā gūtiem rezultātiem tiek piedāvāta smiltsērkšķu biorafinēšanas shēma, kas ļauj racionāli izmantot visas augu daļas produktos ar pievienoto vērtību (3.17. att.).



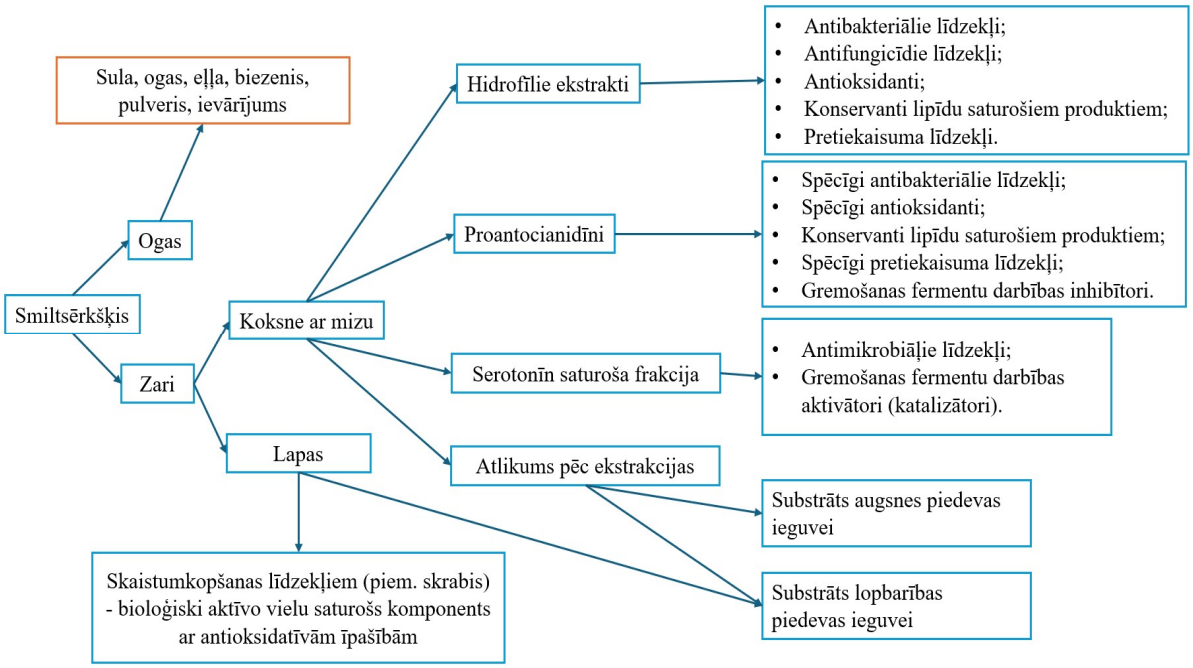
3.17. att. **Smiltsērkšķa biorafinēšanas shēma.**

SECINĀJUMI

1. Latvijā kultivēto smiltsērkšķu zari ir vērtīga izejviela proantocianidīnu un serotonīna ieguvei. Rudens ir piemērotākais sezonālais periods mērķsavienojumu ieguvei, ņemot vērā augstu mērķsavienojumu saturu zaros (proantocianidīni ~12%/SM, serotonīns ~4%/SM). Atrasts piemērotākais ekstrakcijas un attīrīšanas paņēmiens, kas nodrošina augstāko hidrofīlo ekstraktu un mērķsavienojumu iznākumu no smiltsērkšķu biomasas. Šajos apstākļos no smiltsērkšķu zariem var iegūt oligomērus proantocianidīnus (ar ~92% proantocianidīnu uz SM ekstraktu) ar polimerizācijas pakāpi 2–5 Da un serotonīna saturošo frakciju ar 28.2%/SM serotonīna.
2. Hidrofīliem ekstraktiem un izdalītiem proantocianidīniem piemīt augsta antimikrobiālā aktivitāte attiecībā pret *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus* un *Candida albicans*, radot iespēju iegūt pretmikrobu preparātus, aizvietojot vai papildinot antibiotikas cīņai pret rezistentiem mikrobiem. Serotonīns un proantocianidīni ir spēcīgi antibakteriālie līdzekļi pret ādas un mīksto audu infekciju izraisītājiem - *Cutibacterium acnes* un *Streptococcus pyogenes*, kas piesārņo ādas kopšanas produktus un attīstās sebumā. Proantocianidīni spēj aizturēt patogēno bioplēvju veidošanos par 80%, kas būtiski var samazināt hroniskus iekaisuma procesus.
3. Proantocianidīniem un to saturošiem ekstraktiem piemīt augsta pretiekaisuma aktivitāte, it īpaši samazina ar vīrusu infekcijām saistītu iekaisumu. Proantocianidīni koncentrācijā 0.5 mg mL^{-1} samazināja IL-8 sekrēciju, un koncentrācijās 0.25–0.5 mg mL^{-1} samazināja IL-6 sekrēciju. Poliinozīnskābes:policitidilskābes (poli I:C), kas atdarina vīrusa divpavedienu RNS, stimulētajās perifēro asiņu mononukleārā cilvēku šūnās gan proantocianidīni gan 50% EtOH ekstrakts būtiski samazināja IL-8 un IL-6 sekrēciju.
4. Pierādīta ekstraktu un proantocianidīnu augsta antioksidatīvā aktivitāte un to spēja aizsargāt lipīdu saturošas sistēmas no oksidācijas, kas paver iespēju tos izmantot kā dabīgus antioksidantus medicīniskajos un kosmētiskajos krēmos. Izdalītiem proantocianidīniem antioksidatīvā aktivitāte bija vidēji 3 reizēs augstākā salīdzinot ar E vitamīna analoga antioksidatīvo aktivitāti. 50% EtOH ekstrakts visefektīvāk aizkavē lipīdu saturošo produktu oksidēšanos. Substrātā ar zemāku lipīdu saturu ekstraktu darbība bija efektīvāka.
5. Hidrofīlie ekstrakti no zaru biomasas darbības koncentrācijas diapazonā no 0.03 līdz 4.0 mg mL^{-1} un mērķsavienojumi diapazonā no 0.03 līdz 0.5 mg mL^{-1} neuzrādīja citotoksicitāti un neizraisīja cilvēku asins hemolīzi, kas norāda uz to augsto bioloģisko saderību un drošību lietošanai medicīnisko un kosmētisko krēmu sastāvos, kā arī veselības aprūpē.
6. Proantocianidīni ir spēcīgi lipāzes un alfa-amilāzes darbības inhibitori, kas paver iespējas tos izmantot pretaptaukošanās terapijā, savukārt, serotonīns ir cilvēku gremošanas fermentu aktivators, kas paver iespēju to izmantot malabsorbcijas problēmu risināšanā.
7. Pēc mērķsavienojumu izdalīšanas lignocelulozes biomasas atlikumu ķīmiskais raksturojums un *in vitro* analīzes mazo atgremotāju gremošanas sistēmā parādīja iespēju tos izmantot lopbarības piedevas ieguvei. Bagātinot lignocelulozes biomasas atlikumu ar silīciju saturošo komponentu, tiek iegūta augsnes piedeva, kas veicina augu augšanu un attīstību, palielinot ražas īpatsvaru par 27%.
8. Izstrādāta bezatkritumu smiltsērkšķa biomasas biorafinēšanas shēma, kas ļaus smiltsērkšķu audzētājiem izmantot gan ogas, gan zarus - lignocelulozes biomasu, paplašinot sortimentu, izvēloties pielietošanas virzienus.

REKOMENDĀCIJAS

Izstrādāta elastīga smiltsērkšķa biomasas izmantošanas shēma ļaus smiltsērkšķu audzētājiem paplašināt sortimentu, izvēloties ražošanai sev piemērotākos produktus (3.18 att.).



3.18. att. **Izmantošanas virzienu shēma smiltsērkšķu audzētājiem**

PATEICĪBA

Izsaku visdziļāko pateicību Dr. habil. chem. Gaļinai Teliševai, lignīna laboratorijas vadītājai, par idejām, iedvesmu, zināšanām un apmācībām. Esmu pateicīga saviem zinātniskā darba vadītājiem Dr. sc. ing. Sarmītei Jancevai un Dr. sc.ing. Uldim Spulle par darba vadīšanu, neaizstājamu atbalstu promocijas darba izstrādes laikā, savlaicīgu palīdzību visos darba etapos un zinātnisko rakstu un prezentāciju sagatavošanā, vērtīgiem padomiem un efektīvu darba koordinēšanu.

Esmu pateicīga institūta un laboratorijas kolēģiem, kas mani praktiski atbalstīja promocijas darba izstrādāšanas laikā.

Esmu pateicīga Dr. biol. Vizmai Ņikolajevai, Mg. biol. Annai Ramata-Stundai, Dr. Mihailam Červenkovam (Bulgārija), Mg. biol. Mārim Seņkovam un profesorei Dr. Krasiļņikovai par atbalstu un palīdzību iegūto analizējamo paraugu raksturošanā.

Sirsnīgs paldies manai ģimenei par neizsmeļamo pacietību, saprotošo attieksmi un lielo atbalstu.

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Sea Buckthorn, Aronia, and Black Currant Pruning Biomass as a Source of Multifunctional Anti-aging Cosmetic and Pharmaceutical Creams Ingredients

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Abstract: Fruit trees' lignocellulosic biomass remaining as waste either after harvesting of the berries or after yearly pruning is an underutilized little-explored bioresource. Berries of sea buckthorn (*Hippophae rhamnoides* L.), aronia (*Aronia melanocarpa*) and blackcurrant (*Ribes nigrum*) are known for their very rich content of biologically active compounds with antioxidative and antimicrobial properties, and it was reasonable to study their woody waste biomass and it's derivatives for the same characteristics. One of the prospective applications of practical importance could be as ingredients in medicinal and anti-aging cosmetic topical formulations. Thus the study investigated influence of the sea buckthorn (SBT), aronia (AR) and blackcurrant (BC) pruning waste biomass and its derived proanthocyanidins' (PACs) on the oxidative stability of the lipid-based systems by the accelerated oxidation method at elevated pressure, as well as their antimicrobial activity against pathogenic bacteria that contaminate skincare products and develop in sebum: *Streptococcus pyogenes* MSCL 620, *Cutibacterium acnes* MSCL 1521, *Pseudomonas aeruginosa* MSCL 3314, *Staphylococcus aureus* MSCL 3340, *Escherichia coli* MSCL 332, and *Bacillus cereus* MSCL 330. The study established that the biomass, lipophilic extracts obtained using liquefied freon, and hydrophilic extracts obtained by aqueous ethanol solution (v/v 50:50) increased the oxidative stability of lipid formulations as well as had anti-oxidative properties important for healing and anti-aging effect of the creams. Hydrophilic extracts and PACs from SBT and AR had high antimicrobial activity against the above-mentioned bacteria. The study confirmed that these fruit-trees waste biomass derivatives are prospective multipurpose ingredients for application in cream formulations. The scheme of usage of the fruit trees' waste pruning and harvesting lignocellulosic biomass will help with the sustainable development of fruit production and increase the efficiency of the use of resources. At the same time, it will create additional work possibilities in non-season periods in rural areas which will optimize the use of the human capital.

Keywords: *lignocellulosic agro-waste, sea buckthorn, aronia, blackcurrant; oxidative stability; anti-aging cosmetic cream; topical medicinal formulations*

1. Introduction

Sea buckthorn (*Hippophae rhamnoides,* L., SBT) and aronia (*Aronia melanocarpa,* AR), the deciduous shrub trees, can grow in extreme climates and difficult soils, both wet and sandy, adapting to each environment. AR tolerates both wet and dry soils, survives in sandy dunes, dry rocky slopes, dry bluffs and balds. In 2020 SBT had been reported to grow in 52 countries, with a total area of 2.33 million hm^2 (Z. Wang et al., 2022). Canada, Thailand, and Poland are the top SBT berries exporters in 2022 (export values $443.57M, $378.17M and $267.77) ("Tridge," 2024). AR is originally a small shrub native to eastern North America (Kask, n.d.), it's orchards' trees grow 1.8 to 2.4 m tall, and now this tree with extremely healthy berry is gaining increased attention in Europe and USA due to extremely high content of polyphenols in its berries (Kapasakalidis et al., 2006). The industrial harvesting of SBT berries by cutting the whole branch results in a large amount of agro-waste (20% of berries mass). Both SBT and AR bush-tree demands yearly pruning for easier harvesting and better quality of the berries. Another non-studied fruit crop waste lignocellulosic biomass is blackcurrant (BC), which demands yearly pruning starting at once after planting since the biggest amount of the berries grow on young branches (UK Royal Horticultural Society, n.d.). BC woody biomass could amount 6000 kg ha^{-1} annually (De Toro, 1994).

Finding an application for this pruning biomass is a necessary condition for the creation of waste-free SBT, AR and BC technological processing. SBT is planted also for land reclamation and soil erosion control since its extensive root system is capable of fixing nitrogen and improving marginal soils (Enescu, 2014). Pruning and harvesting agro-waste of the above-mentioned fruit trees creates a stable source of raw material available for the bioeconomy.
SBT, AR and BC berries are extremely rich in bioactive compounds, nearly 200 are reported for SBT (Chen et al., 2020; K. Wang et al., 2022). The amount of anthocyanins and flavonoids in the berry of AR is five times higher than cranberry and blueberry, and also contains strong anticancer compounds (Brand, 2010). SBT oil is used in pharmacology, food, cosmetics, and even for skin protection from cosmic radiation (Jaśniewska and Diowksz, 2021; Yang and Kallio, 2002). SBT contains both lipophilic antioxidants (mainly carotenoids and tocopherols) and hydrophilic antioxidants (flavonoids, tannins, phenolic acids, and ascorbic acid) in remarkably high quantities. (Ciesarová et al., 2020) Antioxidant compounds, including catechin, quercetin, p-coumaric acid, caffeic acid, L-ascorbic acid, gallic acid etc. AR rarely seems to be affected by insects and thus could be not only a low-maintenance crop but also a source of powerful antimicrobials (Brand, 2010). BC berries have proven anti-microbial activity (Trajković et al., 2023). Antioxidant properties of these plants' berries are reported, including protection against UV-radiation, revitalization of wounds and skin burns (Gęgotek et al., 2018; Gorbatsova et al., 2007; Guo et al., 2017; Tkacz et al., 2019, (Faggiano et al., 2022).

SBT seed oil prevented a UV-induced decrease in the antioxidant capacity of skin cells (Guo et al., 2017). Pulp and oil are used in cosmetic preparations and for treating various skin disorders (Alam Zeb, 2004; Bal et al., 2011).

Recently, antioxidant activity of SBT leaf, bark, stem, root and seed and their extracts, was confirmed, with found correlation between activity and phenolic compounds (Handique, Pratap and Saikia, Mousmi, n.d.; Michel et al., 2012). In the previous studies, it was found that SBT and AR biomass also contain a wide range of bioactive compounds, including the ones with antioxidant properties (Janceva et al., 2022; Andersone et al, 2023; Upadhyay et al., 2011). It has very limited amount of research even for these fruit trees berries, and almost no studies for their lignocellulosic waste biomass. Their application in skin care formulations seems promising since skin care and medical formulations need replacement of the oil-based ingredients with reported side effects. The skin serves as the primary interface between body and environment and as a barrier against the entry of microbes (Yue et al., 2017). Global skincare products' market size was valued at USD 130.50 billion, for topical formulations – at USD 97.42 billion in 2021, with expected growth of 4.6% and 10.1% until 2030, correspondingly ("Grandviewresearch," n.d.; "Grandviewresearch," n.d.). Lipids, one of the main ingredients in creams, are susceptible to oxidation that declines efficiency and shelf life (Musakhanian et al., 2022; Wang et al., 2023). Volatile lipid oxidation products can affect product odor (Thomsen, B. R., 2018). Antioxidants in creams formulations serve two purposes: protecting the cream from oxidative damage, and protecting the skin. The most common synthetic antioxidants today are butylated hydroxyanisole and hydroxytoluene, and the "green" ones – tocopherols and ascorbic acid (Hoang et al., 2021). European Green Deal concept of avoiding loss of resources promotes finding natural functional ingredients without harm during production and in the application ("European Commission," n.d.).

Another emerging problem in the skin care and topical formulations industry is microbial contamination (Behravan et al., 2005). It can result both in breaking down active ingredients, and a serious health threat to consumers (Zeitoun et al., 2015), especially with atopic dermatitis, susceptible to recurrent microbial infections (Khanum and Thevanayagam, 2017). Studies have shown that the most frequently found microorganisms in cosmetics are *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Bacillus* species (Kim et al., 2020; Lundov et al., 2009; Neza and Centini, 2016). *Streptococcus pyogenes* (*S. pyogenes*) is one of the main bacterial causes of skin and soft tissue infections worldwide (Stevens Dennis L. and Bryant Amy E., 2016). Oxidation of sebum and *Cutibacterium acnes* (*C. acnes*) bacteria are the main causes of inflammatory acne (Hwang et al., 2022). The antibacterial activity of SBT, AR and BC agro-waste biomass derivatives against these bacteria was under study.

Microbial infections, as well as oxidative stress in most cases, is accompanied by inflammation. Interleukin-8 (IL-8) is one of the major mediators of the inflammatory response (Bishara, 2012). In the authors' previous research (Andersone et al., 2023), it was shown that SBT agro-waste-derived proanthocyanidins (PACs) reduce the IL-8 protein secretion. Accumulation of IL-8 in the skin in response to inflammatory stimuli causes damage to epidermal stem cells, decreases the expression of bleomycin hydrolase, a moisturizing factor-producing enzyme, and, thus, deteriorates skin barrier function and accelerates cell aging ("Cosmetics and toiletries," n.d.; Kemény et al., 1994), so adding PACs in cream could have anti-inflammatory and anti-aging effects. It was also found that SBT biomass contains serotonin (Janceva et al., 2022) which is reported to have vasoconstriction/vasodilator properties (Van Nueten et al., 1985; Vanhoutte, 1987) and, thus, could be beneficial for the treatment of, for example, rosacea. Similarly, AR berries polyphenols have improved arterial function in prehypertensive patients (Le Sayec et al., 2022).

Thus, the main aim of this study was to characterize and validate SBT, AR and BC agro-waste biomass and its' derived compounds for multipurpose application in cosmetic and topical formulations, allowing cascading use of SBT while obtaining various added-value products replacing various synthetic oil-derived ingredients (Figure 1).

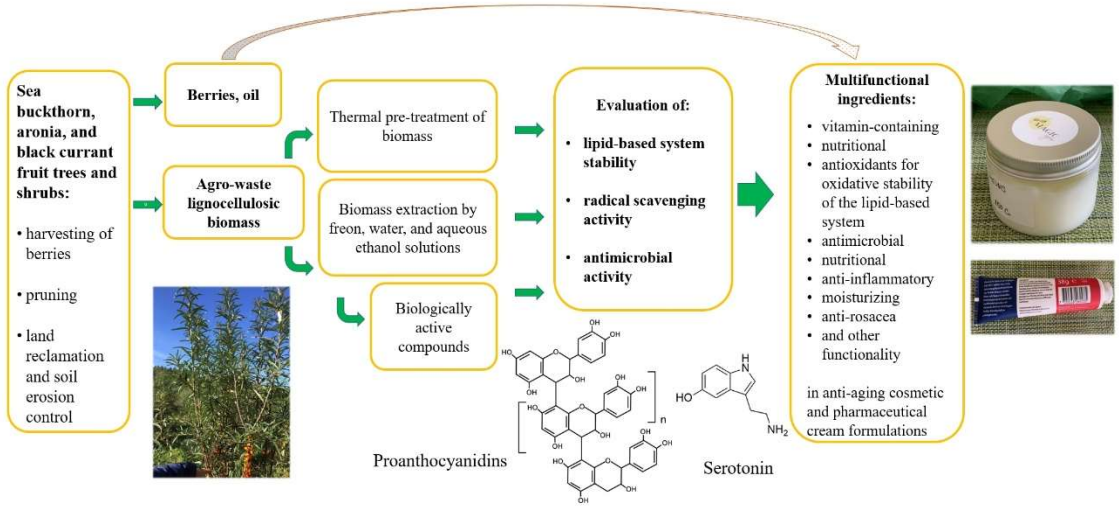

Figure 1. Validation of SBT, AR and BC biomass as a source of multifunctional ingredients in topical formulations.

For this purpose, the following research tasks were fulfilled:

- chemically characterization of the composition of SBT, AR and BC biomass, its lipophilic and hydrophilic extracts, and purified proanthocyanidins was done;
- an innovative extraction method by freon was tested and compared with extraction by hexane;

- the biomass and obtained extracts/compounds were validated for the prevention of lipid oxidation in creams with different lipid content (35% and 19%);
- above-mentioned samples were tested as antibacterial agents against the bacteria contaminating creams (*S. aureus*, *E. coli*, *P. aeruginosa*, *B. cereus*) and causing acne and infections in the skin (*S. pyogenes*, *C. acnes*).

To the best of the authors' knowledge, no research has been carried out on the SBT, AR and BC agro-waste biomass activity against lipid oxidation in creams and acne-causing bacteria.
Above mentioned complex properties of SBT, AR, and BC agro-waste and its derived components will allow replacing several cosmetic ingredients with natural ones with multi-functional properties. A cascading waste-free technological approach will provide additional income sources in rural areas, which is especially important in the agricultural sector off-season. Overall, it will make today's fruit-trees-connected business based mostly on berries more economically feasible.

2. Materials and Methods

2.1. Reagents

The solvents (high purity), $DPPH^{\cdot}$ (2,2-diphenyl-1-picrylhydrazyl), $ABTS^{+\cdot}$ (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)), the reference antioxidants Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and analytical standards gallic (purity ≥ 97.5%) and ascorbic acid (purity 99%), procyanidin B2 (purity ≥90%), and serotonin (purity ≥98%) were purchased from Sigma-Aldrich (St. Louis, USA). HPLC-grade methanol was purchased from Honeywell (CHROMASOLV, Seelze, Germany), and reagent-grade formic acid, puriss Ph.Eur≥98%, was purchased from Sigma-Aldrich (Darmstadt, Germany). Ultrapure water Type 1 was prepared using the Stackpure purification system (OmniaTap 6, Niederahr, Germany) for the mobile phase and sample preparation. All solutions were degassed by sonication for 30 minutes. Reference standards for bioactive compounds for quantitative analysis were used. (+)-Catechin analytical standard ≥ 99.0% (Supelco, Buchs,Switzerland), kaempferol ≥ 97.0 % (HPLC) (Sigma-Aldrich, Buchs, Switzerland), quercetin ≥ 95% (HPLC) (Sigma-Aldrich, St. Louis, Missouri, US), caffeic acid ≥ 98.0 % (HPLC) (Sigma-Aldrich, St. Louis, Missouri, US), (-)-epicatechin ≥ 98.0 % (HPLC) (Sigma-Aldrich, Wuxi, China CN), (-)-epigallocatechin ≥ 95 % (HPLC) (Sigma-Aldrich, St. Louis, Missouri, US), myricetin ≥ 96.0% (HPLC) (Sigma-Aldrich, Buchs, Switzerland), p-coumaric acid ≥ 98.0% (HPLC) (Sigma-Aldrich, Buchs, Switzerland) and rutin ≥ 95.0% (HPLC) (PhytoLab, Vestenbergsgreuth, Germany).

Reference microbial strains: *S. aureus* MSCL 3340*, P. aeruginosa* MSCL 3314, *E. coli* MSCL 332, *B. cereus* MSCL 330, *S. pyogenes* MSCL 620, *C. acnes* MSCL 1521 were received from

the Microbial Strain Collection of Latvia (MSCL, Riga, Latvia). Cosmetic and medicinal cream bases (with 19% and 35% lipid content, correspondingly) without added antioxidants were received from private companies producing cosmetic creams and medicinal topical formulations.

2.2. Collection of SBT, AR, BC Plant Material

The twigs (TW) and leaves (LV) plant material of SBT were collected from the SBT plantation area near Engure, in Seme parish, Tukums county of Latvia (DD: 57.1444093, 23.108156), in the summer of 2023. Twigs after separating the berries and leaves, as well as leaves, were dried at room temperature. The TW plant material of AR and BC was collected from the fruit-tree/shrub plantation area of Baldone parish, Kekava county of Latvia (DD: 56.82065, 24.27653), in early spring of 2023.

Dried twigs (were ground with a knife mill Retsch SM100 (Retsch, Haan, Germany) to the particle size of 2–4 mm, and mixed well to obtain a homogeneous biomass mixture. The leaves of SBT were ground with a knife mill Retsch SM100 (Retsch, Haan, Germany) to the biomass particle size of 1-2 mm.

2.3. Chemical Characterization of Biomass

2.3.1. Py-GC/MS/FID Analysis

Analytical pyrolysis was performed on Frontier Lab Micro Double-shot Pyrolyser Py-2020iD (pyrolysis temperature 500 °C, heating rate 600 °C s^{-1}) directly coupled with gas chromatography-mass spectrometry Shimadzu GC/MS/FID-QP ULTRA 2010 (Shimadzu, Kyoto, Japan), equipped with a capillary column RTX-1701 (Restec, Metairie, Louisiana, USA) and a 60 m × 0.25 mm × 0.25 μm film (injector temperature of 250 °C, ion source with EI of 70 eV, MS scan range m/z of 15-350, carrier gas helium at the flow rate of 1 mL min^{-1} and a split ratio of 1:30). The mass of the sample probe (residual moisture content < 1%) was 1.0–2.0 mg. The oven program: 1 min isothermal at 60 °C, followed by 6 °C min^{-1} to 270 °C, and the final hold at 270 °C for 35 min. The mass spectrometer was operated in electron impact mode using 70 eV electron energy. The identification of the individual compounds was performed based on GC/MS using Library MS NIST 11 and NIST 11s, whereas the relative area of the peak of individual compounds was calculated using Shimadzu software based on GC/FID data. The area that originated from CO_2, which overlaps the peaks from carbohydrates, lignin, and minor products, was not used in the calculations (Alves et al., 2006). The summed molar areas of the relevant peaks were normalized to 100%, and the data for four repetitive pyrolysis experiments were averaged. The variation coefficient of measurement was ≤ 5%.

2.4. Preparation of Biomass Extracts and PACs

Lipophilic extracts of TW and LV biomass were isolated in two ways: 1) at 50 °C, 30 min using hexane; 2) at 17—20 °C, using 1,1,1,2-Tetrafluoroethane (freon R134a), multiple cycles, 24 h. Hydrophilic extracts were obtained at 60 °C, 30 min using distilled water and ethanol-water solutions (v/v 50:50). Obtained extracts were dried by lyophilization using Heto Power Dry PL3000 (Thermo Fischer Scientific, Waltham, Massachusetts, USA). The yield is presented as a percentage based on DB, Confidence interval (CI): CI ≤0.7% at $\alpha = 0.05$.
The purification of PACs from non-tannin compounds and sugar was carried out using a crosslinked dextran-based solvent-resistant resin Sephadex LH-20 column with 96% EtOH and 70% (v/v) acetone as the respective purification solvents. In the purification process, low-molecular-weight phenolics were eluted with 96% EtOH, and the PACs were eluted with 70% (v/v) acetone. Purified PACs were evaporated using a rotary evaporator (Heidolph Instruments, Schwabach, Germany) before being freeze-dried using lyophilization equipment Heto Power Dry HS3000 (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -8 °C.

2.5. Chemical Characterisation of Lipophilic Extracts by GC/MS/FID Analysis

Lipophilic extracts were analyzed by GC-MS chromatography analysis using Shimadzu GC/MS/FID-QP ULTRA 2010 apparatus (Shimadzu, Kyoto, Japan), capillary column RTX-1701 (Restec, Metairie, Louisiana, USA) as described in Andersone, 2023.

2.6. Chemical Characterization of Hydrophilic Extracts

2.6.1. Determination of Total Polyphenols Content in the Extracts

The total content of polyphenols (TP) in the hydrophilic dry extracts (DE) was quantified by the Folin-Ciocalteu method using gallic acid as a reference compound. Amounts of 5 mL of 10% Folin–Ciocalteu reagent and 4 mL of 7.5% sodium carbonate solution were added to 1 mL of the extract. After 30 min, the absorbance of the mixture was measured against a blank water solution at 765 nm using a UV/VIS spectrometer Lambda 650 (Perkin Elmer, Shelton, CT, USA). Gallic acid was used to calibrate the standard curve. Each extract was analyzed in triplicate, and the results were expressed in g of gallic acid per 100 g of DE (g GAE·100 g^{-1} DE). Confidence interval (CI) was ≤ 0.4 g GAE·100 g^{-1} DE, at α=0.05.

2.6.2. Determination of PACs Content in the Extracts and Purified PACs Samples

The total content of PACs in the hydrophilic extracts and in purified PACs samples was measured by the butanol-HCl assay using procyanidin dimer B2 as a reference compound (Janceva et al., 2022). Amounts of 6 mL of acid butanol (5% (v/v) concentrated HCl in n-butanol) and 0.2 mL of iron reagent (w/v) ($FeNH_4(SO_4)_2 \cdot 12\ H_2O$ in 2 M HCl) were added to 1 mL of the extract aliquots while stirring the tube and heated in a water bath at 80 °C for 50 min.

After 50 min, the absorbance of the mixture was measured against a blank solution at 550 nm using UV/VIS spectrometer Lambda 650 (Perkin Elmer, Shelton, CT, USA). Each extract was analyzed in triplicate, and assay results were expressed as a percentage per DE. CI ≤ 0.4% at α=0.05.

2.6.3. UHPLC-UV-TOF/MS Analysis for Serotonin Determination in Extracts

Identification of the compounds in the hydrophilic extract was performed by the following procedure. Dry extracts were dissolved in aqueous acetonitrile (v/v 50:50) with an approximate concentration of 2 $mg \cdot mL^{-1}$ and filtered (Nylon filter, 0.45 μm pore size), after which they were used for UHPLC-UV-TOF/MS experiments. LC analyses of the samples were performed on the Acquity UHPLC system (Waters Corp., Milford, MA, USA) coupled with a quadrupole-time of flight (Q-TOF) MS instrument (UHPLC/Synapt Q-TOF MS, Waters, Milford, MA, USA) equipped by an electrospray ionization (ESI) source as described in Janceva, 2024.

2.6.4. Qualitative analysis of extracts by liquid chromatography-mass spectrometry (LC-MS)

The UHPLC-MS/MS analyses were conducted using a Vanquish Flex UHPLC system (Thermo Fisher Scientific, Germering, Germany) equipped with a Vanquish Binary Pump F and a Vanquish Split Sampler FT. Chromatographic separation was achieved on a Zorbax Eclipse Plus C18 column (2.1 × 150 mm, 5 μm; Agilent, US). Mobile phase A consisted of 0.1% formic acid in ultrapure water, and mobile phase B contained 0.1% formic acid in methanol. The flow rate of the mobile phase was set at 0.4 mL min^{-1}, and the column temperature was maintained at 40 °C. The gradient program was set as follows: 0 min, 5% B; 1.0 min, 5% B; 15.0 min, 30% B; 20.0 min, 50% B; 25.0 min, 70% B; 26.0 min, 95% B; 28.0 min, 95% B; 29.0 min, 5% B; and 30.0 min, 5% B. Equilibration time was 3 minutes. The injection volume was 1 μL. Mass spectrometric analysis was performed using an Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionisation (HESI-II) probe (Thermo Fisher Scientific). The instrument operated in both negative and positive ion modes within the m/z range of 100 to 1500. For the mass spectrometer, the spray voltage was 2.5 kV (−) and 3.5 kV (+); the sheath gas flow rate was 50; the auxiliary gas flow rate was 10; the ion transfer tube temperature was 325 °C; the vaporizer temperature was 350 °C; the S-lens RF level was 70%; the scan mode was full MS (resolution 30,000) and ddMS2 (15,000), with absolute collision energy and HCD collision energy set to 15, 40, and 70. Data were processed using Xcalibur 4.6 (Thermo Fisher Scientific, Waltham, MA, USA) instrument control and data handling software. Compound profiling was applied to the UHPLC-HRMS raw files of the studied extracts using TraceFinder 5.1 software (Thermo Fisher Scientific, Waltham, MA, USA). A database of 110 compounds for the identification of

individual components using LC-MS was created by using various literature sources, including mzCloud, PubChem, FoodDB, and KNApSAcK.

2.7. Determination of the Radical Scavenging Activity

2.7.1. DPPH· (2,2-diphenyl-1-picrylhydrazyl radical) assay

Hydrophilic extracts and purified PACs were tested for their radical scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) using UV/VIS spectrometer Lambda 650 (Perkin Elmer, Shelton, CT, USA). The $DPPH^{\cdot}$ assay was measured according to the procedures described by Dizhbite et al. (Dizhbite, 2004). A range of different concentrations of the obtained dried hydrophilic extracts (section 2.4) in DMSO was prepared. The absorbance at 515 nm was measured 15 min after the mixing of 30 µL of extract (or antioxidant standard) with 3.0 mL DPPH· ($1 \cdot 10^{-4}$ $mol \cdot L^{-1}$) solution. DMSO was used as a control and Trolox as a reference antioxidant standard.

The measurements were done in triplicate. The free radical scavenging activity is expressed as the concentration of antioxidant, $mg \cdot L^{-1}$, required for a 50% inhibition of the free radicals (IC_{50}). $DPPH^{\cdot}$ inhibition (decrease in absorbance at 515 nm) was calculated according to equation (1):

$$I=(Aa-Ab)/Ab \cdot 100, \quad (1)$$

where: I–$DPPH^{\cdot}$ inhibition, %; Ab–absorbance of DPPH· solution without sample or a reference antioxidant standard after 15 min; Aa–absorbance of $DPPH^{\cdot}$ solution with sample or a reference antioxidant standard after 15 min. $CI \leq 0.3$ $mg \cdot L^{-1}$.

2.7.2. $ABTS^{+\cdot}$ (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assay

$ABTS^{+\cdot}$ was produced by the reaction of 2 $mmol \cdot L^{-1}$ ABTS stock solution with 70 $mmol \cdot L^{-1}$ potassium persulfate ($K_2S_2O_8$) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{+}$ solution (stable for 2 days) was diluted with phosphate-buffered saline (pH 7.4) to an absorbance of 0.80 ±0.02 at 734 nm. The absorbance at 734 nm was investigated 10 min after the mixing of 30 µL of extract (or purified PACs and antioxidant standard - Trolox) was diluted in DMSO of five different concentrations with 3.0 mL $ABTS^{+\cdot}$ solution, using a UV/VIS spectrometer Lambda 650 (Perkin Elmer, Shelton, CT, USA). DMSO was used as a control and Trolox as the antioxidant standard. $ABTS^{+\cdot}$ inhibition (decrease in absorbance at 745 nm) was calculated according to equation (2):

$$I=(Aa-Ab)/Ab \cdot 100, \quad (2)$$

where: I– $ABTS^{+\cdot}$ inhibition, %; Ab–absorbance of $ABTS^{+\cdot}$ solution without sample or a reference antioxidant standard after 10 min; Aa–absorbance of $ABTS^{+\cdot}$ solution with sample or a reference antioxidant standard after 10 min. CI ≤ 0.3 $mg \cdot L^{-1}$.

2.8. Determination of the Oxidative Stability of the Lipid-Based System (LBS)

The effect of samples (biomass after thermal pre-treatment, biomass extracts, and PACs) on the oxidative stability of the LBS was performed with cosmetic cream and topical drug bases (basic composition without antioxidant additives), using Oxipress apparatus (Mikrolab Aarhus, Højbjerg, Denmark). The lipid content in cosmetic and medicinal creams was 19% and 35%, respectively. Creams were kindly provided by "Madara Cosmetics" and "Magic You" (Latvia). The oxidative stability was determined under the optimum conditions described by Trojakova et al. (Trojakova, L et al., 2001), with slight modifications. The cream base was placed into reaction vessels in the amount corresponding to 5 g of the lipid phase (26.3 g and 14.3 g for 19% and 35% creams, correspondingly). The sample was mixed in the reaction vessel with the lipid-based substrate, and then thoroughly mixed for 10 min. The reaction vessel was connected to oxygen, the air was expelled and then filled with oxygen to the defined pressure. The oxygen pressure was set as 0.5 MPa, and the temperature was set as 120 °C. The LBS without antioxidants was used as a control. The changes of O_2 pressure depending on time were recorded. The protection factor (PF) of the samples was calculated according to equation (3):

$$PF=IPx/IPc, \quad (3)$$

where IPx and IPc are the induction period (time, h) of substrate oxidation in the presence of the sample and one of the blank, respectively (Figure 2). CI is shown in the results section under the Tables.

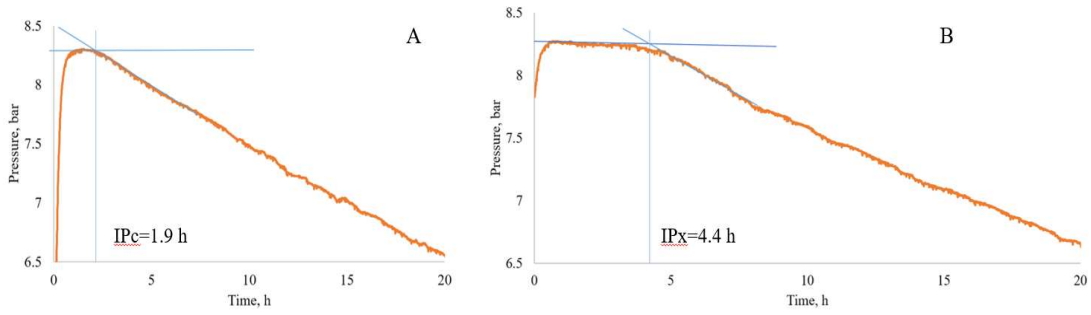

Figure 2. Influence of the sample on the oxidation time of cream (A – cosmetic cream base without antioxidant; B – with the addition of the antioxidant sample into cosmetic cream.

2.9. Determination of the Antimicrobial Activity

Several reference microbial strains, received from the Microbial Strain Collection of Latvia (MSCL), University of Latvia, were used: *P. aeruginosa* MSCL 3314, *S. aureus* MSCL 3340, *E. coli* MSCL 332, *B. cereus* MSCL 330, *S. pyogenes* MSCL 620, *C. acnes* MSCL 1521. Evaluation of the antimicrobial activity of the extracts, purified PACs, and serotonin of the SBT, carried out according to the method for determining the sensitivity of microorganisms to antimicrobial drugs. Antimicrobial activity was studied in 96-well plates by the twofold serial broth microdilution method, which provides for the determination of the minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC). Stock suspensions of extracts and PACs were prepared in dimethyl sulfoxide at a concentration of 10 $mg \cdot mL^{-1}$. The inoculum of bacteria was prepared in sterile water with a density of 0.08–0.10 at A625 and diluted 100-fold in appropriate broth. Then, 96-well plates were incubated at 37 °C for 24 h for *P. aeruginosa, S. aureus*, *E. coli,* and *B. cereus* or 48 h for *S. pyogenes* and *C. acnes*. Mueller-Hinton broth and agar were used for aerobic cultivation of *P. aeruginosa*, *S. aureus*, *E. coli*, *B. cereus,* and *S. pyogenes*, and Wilkins-Chalgren Anaerobe broth and agar was used for anaerobic cultivation (BD GasPak EZ) of *C. acnes*. The MIC was determined as the lowest concentration of studied material which showed no visible growth. From wells, where growth was not detected 4 µL of media was seeded on appropriate solidified media for MBC determination.

2.10. Statistical Analysis

All experiments were conducted in triplicate, except Py-GC/MS/FID and GC/MS/FID analyses where four repetitive experiments were done. The results were expressed as means. Statistical analyses were performed using Microsoft Excel 2016. Confidence intervals (CI) for a mean using Student's T distribution were calculated at a significance level of 5% ($\alpha = 0.05$). Pearson's correlation coefficient was evaluated for the relationship between LBS oxidation stability and PACs content in the cream. A significance level of $p < 0.05$ was used.

3. Results and Discussion

3.1. Chemical Composition of Biomass

3.1.1. Analytical Pyrolysis Data

The Py-GC/MS/FID data represent volatiles formed from cellulose, hemicellulose, lignin, proteins, and extractives. Aliphatic acids and esters, aliphatic alcohols, aliphatic aldehydes, ketones, furan and pyran derivatives, cyclopentane derivatives, and sugars attributed to carbohydrates-derived volatiles represented 73–78% of SBT, AR, and BC total twigs biomass and 59% of SBT/LV biomass volatile products (TVP) (Figure 3).

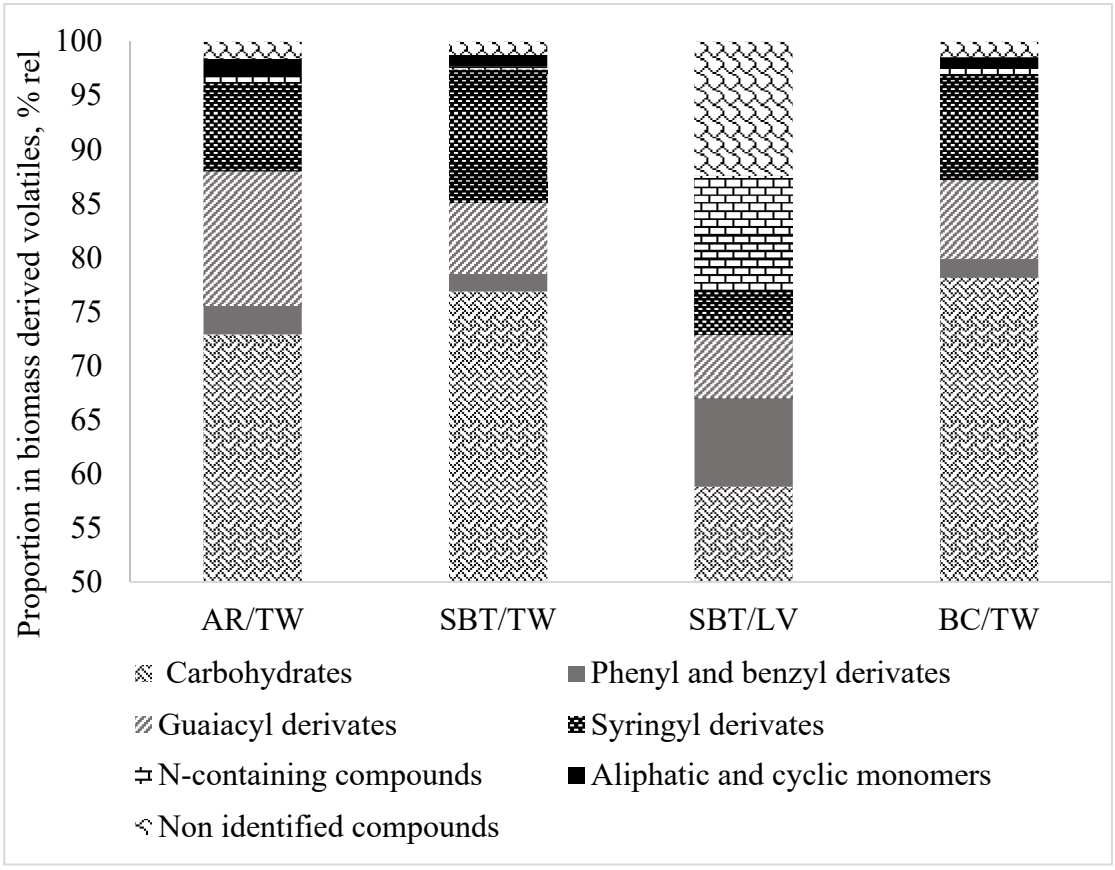

Figure 3. Py-GC/MS/FID data of SBT, AR, and BC twigs and SBT leaves biomass-derived volatiles. The variation coefficient of measurement was ≤5%.

Besides the carbohydrates derived, twigs and leaves also contained N-containing volatiles, showing the presence of proteins and other nitrogen-containing compounds. According to Py-GC/MS/FID, nitrogen-containing compounds in SBT/LV were 10 times more than in the twigs. The serotonin presence in SBT/TW and SBT/LV plant material compositions was confirmed by UHPLC analysis in p. 3.3.3.

Using Py-GC-MS-FID analysis data, structural features of lignins of the composition of twigs and leaves biomass associated with antioxidant and antimicrobial activity were characterized. Literature data on the content of lignin in SBT, AR and BC twigs biomass, and the relative proportions of syringyl (S) and guaiacyl (G) derivatives in it determined by analytical pyrolysis were not found, so to the best of the authors' knowledge, this is the first analysis of SBT, AR and BC twigs biomass lignins. The thermal decomposition of the lignin starts with the cleavage of the weak bonds (α-ether and β-ether bonds), releasing a mixture of methoxylated phenol, G, and S-type derivatives. The total lignin-derived volatiles content in SBT/LV and all twigs composition ranged from 10% to 21%/TVP that is within the usual range for deciduous trees. In comparison to leaves, the SBT/TW twigs had 1.9 times higher content

of lignin-related G and S-type phenols. The content of lignin-related G and S-type phenols in all twigs samples was 6.6 – 12.5 % TVP and 8.3 – 12.3% TVP, respectively.

It is known that the presence of ortho-methoxyl groups (G and S) has a positive effect associated with antioxidant activity (Aadil et al., 2014; Anouar et al., 2013; Dizhbite, 2004; Pan et al., 2006; Zhao et al., 2018). The amount of G+S products describes the amount of phenylpropane units (FPV) containing $–O-CH_3$ groups. The difference between S/G proportion in leaves and twigs is statistically insignificant. FPV in SBT leaves' TVP is 10%, which is almost half as much as in twigs (17–21%). This indicates a higher antioxidant potential of the twig's plant materials. However, the readily available phenols (represented by phenyl and benzyl derivatives detected by analytical pyrolysis) content in leaves (8%TVP) is higher than in twigs (2% TVP for SBT/TW), which corresponds to the literature data (Brinkmann et al., 2002) indicating the additional antioxidant effect of this biomass. It could be said that biomass types are potent antioxidants. Since aging and inflammatory processes are commonly related to oxidative stress, it could be proposed that fruit trees lignocellulosic biomass have the potential as multifunctional anti-aging, anti-inflammatory as well as LBS system oxidation preventing component for creams, but the activity will depend also on the solubility in LBS (Barsberg et al., 2014). According to the analytical pyrolysis data, the relative proportion of the twigs biomass-derived volatiles had an insignificant difference in the relative proportion of chemical constituents considering CI (analytical pyrolysis data, section 3.1.1.). SBT/LV differed more in the relative proportion of chemical components from twigs, so leaves of SBT were further investigated as antioxidants of lipid systems (section 3.2.) as well.

3.2. Biomass Influence on the Oxidative Stability of the Lipid-Based System (LBS)

It can be seen that samples based on leaves work better for preventing lipid oxidation in the LBS with higher content of lipids, but samples based on twigs – in the LBS system with lower lipids content (Table 1). It could be connected with a bigger content of lipophilic compounds in the leaves that have a better affinity to the system with higher lipids content. Hydrophilic compounds of the twigs have better solubility in the system with lower lipids content.

Table 1. Influence of leaves and twigs biomass on the oxidative stability of creams.

Sample	**IP, h**	**PF**	**IP, h**	**PF**
	Lipid content in cream–19%		**Lipid content in cream–35%**	
Cream base without antioxidants	11.0	1.0	1.9	1.0
Cream base + 1% BC/TW on LBS	13.2	1.2	1.9	1.0
Cream base + 2% BC/TW on LBS	14.6	1.3	2.2	1.2
Cream base + 4% BC/TW on LBS	14.6	1.3	2.4	1.3
Cream base + 1% AR/TW on LBS	14.1	1.3	2.1	1.1
Cream base + 2% AR/TW on LBS	18.2	1.7	2.6	1.2

Cream base + 4% AR/TW on LBS	20.1	1.8	2.8	1.5
Cream base + 2% SBT/TW on LBS	13.7	1.3	1.9	1.0
Cream base + 2% SBT/TW on LBS	16.8	1.5	2.1	1.1
Cream base + 4% SBT/TW on LBS	19.8	1.8	2.4	1.3
Cream base + 1% SBT/LV on LBS	13.2	1.2	3.2	1.7
Cream base + 2% SBT/LV on LBS	15.1	1.4	3.2	1.7
Cream base + 4% SBT/LV on LBS	16.8	1.5	3.2	1.7

The CI for all the results did not exceed 0.2 at $\alpha = 0.05$.

With the addition of 4% antioxidant (SBT/TW, SBT/LV, BC/TW or AR/TW), the protection factor of the cream further increased only 1.1-1.2 times in comparison with 2% of antioxidant on LBS, which indicates a supersaturation of the lipophilic and hydrophilic solution in the cream composition with the active components from biomass. It makes it impossible to fully assess the influence of lipid and hydrophilic components in the composition of biomass on the protection of the cream bases from oxidation. However, according to Oxypress data, the SBT and AR twigs work well as antioxidants, and in the amount of 2%/LBS increased the protection of the cream base with 19% lipid content from oxidation by 1.5-1.7 times compared to control. SBT/L in the amount of 2%/LBS increased the protection factor of the cream base by 1.4 (for the cream base with 19% lipid content) and 1.7 (for the cream base with 35% lipid content), respectively.

To study possibilities to increase antioxidant activity, lipophilic and hydrophilic extracts of biomass were further evaluated.

3.3. Lipophilic and Hydrophilic Extracts Influence on the Oxidative Stability of the LBS

3.3.1. Yield and Chemical Composition of the Lipophilic Extracts

The yields of lipophilic extracts obtained with hexane from the entire studied twigs biomass were quite close and varied from 1.1 to 1.4% per DB. The yield of lipophilic extract from twigs is very low and it's clear that it's economically unreasonable to offer it as an antioxidant in cream formulations. However, lipophilic compounds as active nutrients could be further evaluated for improving overall skin health since they contain valuable active compounds (e.g., linolenic acid participates in the formation of vitamin F providing structure and flexibility to the outer layer of the cells, helps with reducing of the negative effect of UV radiation, important for ageing skin (Medic, 2023; Michalak et al., 2021). The yield of lipophilic extract from SBT/LV was 3.4%/DB.

Ozone-friendly 1,1,1,2-Tetrafluoroethane (freon R134a) and hexane were tested for comparison of the process effectivity, on the example of SBT/LV. The yield of lipophilic extracts from SBT/LV was 2.7% and 3.4%, correspondingly (CI $\leq$0.7% and 0.4% at $\alpha = 0.05$ for extraction by freon and hexane, correspondingly). The difference between the freon and hexane yield of lipophilic compounds is statistically significant. Therefore, testing of SBT

lipophilic extracts from leaves on lipid oxidation was done assuming the multi-functionality of these ingredients.

The composition of lipophilic extracts of SBT/LV obtained by freon and hexane was evaluated by GC/MS/FID (Table 2). The composition of SBT/LV lipophilic extracts obtained by freon and hexane was evaluated by GC/MS/FID (Table 2).

Table 2. GC/MS/FID data of identified compounds in lipophilic extract of SBT/LV composition isolated by freon and hexane.

Identified compounds	**Freon extract, % rel**	**Hexane extract, % rel**
2-Propenoic acid, 2-methyl-, 1,4-butanediyl ester	4.5	-
Linolenic acid	2.1	-
Stearic Acid	2.4	1.9
Phthalic acid, diisobutyl ester	0.6	1.0
Palmitic acid		5.1
Phthalic acid, diisooctyl ester	11.5	2.0
9,12-Octadecadienoic acid, methyl ester	1.5	1.5
Palmitoleic acid		1.4
Phthalic acid, dibutyl ester	1.1	8.9
9-Octadecenoic acid, ethyl ester	4.4	8.4
9,12-Octadecadienoic acid, methyl ester	-	3.5
Stearolic acid	-	1.7
Behenic acid	-	1.5
(E)-13-Docosenoic acid	-	7.8
Tridecanedioic acid, diethyl ester	-	5.2
Docosanoic acid, ethyl ester	-	3.9
Erucic acid (13-Docosenoic acid, (Z)	2.2	1.9
Total acid / ester relative content in ODE	**30.3**	**55.7**
Cyclobutane, 1-butyl-2-ethyl-	0.5	-
Undecanal	0.8	-
1-Dodecene	2.4	-
1-Pentadecene	3.8	-
Cyclopropane, 1-methyl-2-octyl-	0.7	-
1-Hexadecene	3.5	-
2-Tridecenal, (E)-	2.3	-
(2E,6E)-3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol	-	23.7
Pentadecanal-	1.5	0.9
Cyclopentadecanol	5.8	3.0
2-Pentadecanone, 6,10,14-trimethyl-	3.0	1.1
1-Octadecene	1.6	-
Phytol	6.5	-
5-Pentadecen-7-yne, (Z)-	3.4	-
4,8,12,16-Tetramethylheptadecan-4-olide	7.3	1.2
Total aliphatic and cyclic monomers in ODE	**43.1**	**29.9**

It can be seen that the relative content of easily oxidized components in the hexane extract of leaves was greater (55.7% rel /ODE) than in the freon extract (30.3% rel/DE), but the freon extract contained a greater amount of aliphatic and cyclic monomers (43.1% rel/DE).

These differences in extracts composition could influence lipophilic extracts activity in the LBS (section 3.3.2).

3.3.2. Influence of Lipophilic Extracts on the Oxidative Stability of the LBS

Tests with the lipophilic extracts of the SBT/LV showed that only the ones obtained by freon improved the oxidative stability of cream with lipids content of 35%, in the concentration of 2% on LBS (Table 3).

Table 3. SBT/LV lipophilic extracts influence on the oxidative stability of creams.

Sample	**IP, h**	**PF**	**IP, h**	**PF**
	Lipid content in cream–19%		**Lipid content in cream–35%**	
Cream base without antioxidants	11.0	1.0	1.9	1.0
Cream + 1% SBT/LV/FR* on LBS	11.1	1.0	2.2	1.2
Cream + 2% SBT/LV/FR* on LBS	11.6	1.1	3.5	1.8
Cream + 2% SBT/LV/HX** on LBS	11.4	1.0	2.2	1.2

*SBT/LV/FR lipophilic extract obtained by freon from SBT/LV
**SBT/LV/HX lipophilic extract obtained by hexane from SBT/LV
The confidence interval for all the results did not exceed 0.2 at $\alpha = 0.05$.

This could be explained by the relative content of easily oxidized components in the hexane extract which were greater (55.7% rel /ODE) than in the freon extract (30.3% rel/DE); the freon extract also contained a greater amount of aliphatic and cyclic monomers (43.1% rel/DE) (Table 2). However, several following factors have to be considered. 1,1,1,2-Tetrafluoroethane (freon R134a) has insignificant ozone depletion potential and negligible acidification (acid rain) potential. At the same time, its global warming potential exceeds the regulations established in 2021, and after January 2030 it has to be replaced by or mixed with the newer freon R1234yf, which is much more expensive. But hexane is a toxic solvent that, after a while, can be banned for application in EU. Therefore, since the effect of the lipophilic extract on the oxidative stability of the LBS is comparatively low, it's preferable to use biomass without extraction, or its hydrophilic extracts. The lipophilic extract can be validated for other potential benefits in cream composition.

3.3.3. Yield and Chemical Composition of the Hydrophilic Extracts

The yield of hydrophilic extracts from the twigs' biomass, obtained using distilled water as the most environmentally friendly solvent varied from 14.0 to 15.0% per DB, correspondingly (CI $\leq$0.3%/DB). The yield of hydrophilic extract from SBT/LV was

19.2%/DB. With 50% EtOH, the yield of extracts increased significantly, suggesting that the extractives are more soluble in the ethanol-water solution: AR/TW 17.8% > SBT/TW 15.5 > BC/TW 15.5%/DB. The SBT/TW and AR/TW differ among the studied tree species not only by the highest total yield of hydrophilic extracts but also by the high content of polyphenols in them (46.2-48.6 g GAE·100 g^{-1} DE). The content of polyphenols in the SBT/LV was 2 times lower (22.6 g GAE·100 g^{-1} DE). According to Porter's analysis data, oligomeric polyphenols (proanthocyanidins, PACs) are dominant polyphenolic compounds in the composition of hydrophilic extracts. It is known that PACs are active metabolites of cellular metabolism and play an important role in various physiological processes of plant life. The function of PACs includes protection against microbial attack, pathogens, mechanical damage, and infectious diseases, increasing the resistance of trees to decay. The PACs content of hydrophilic extracts obtained with 50% EtOH from the entire biomass under study were: AR/TW 74.0% > SBT/TW 36.2% > BC/TW 33.9% (Table 4).

Table 4. Chemical characterization of the hydrophilic extracts from twigs and leaves biomass

Sample	Yield of extract from biomass, %/DW	TP content in extract, g GAE·100 g^{-1} DE	PACs content in extract, %/DE	Serotonin content in extract, %/DE
Water extracts				
SBT/TW	14.42±0.02	26.2 ±0.2	16.4±0.1	7.5±0.1
SBT/LV	19.18±0.03	24.2±0.1	0	n.d.
BC/TW	15.03±0.04	16.4±0.1	12.9±0.2	n.d
AR/TW	13.99±0.04	38.6±0.2	23.6±0.1	n.d
50% ETOH extracts				
SBT/TW	15.54±0.05	46.2±0.1	36.2±0.2	8.2±0.1
SBT/LV	22.41±0.02	22.6±0.1	0	n.d.
BC/TW	15.08±0.02	36.1±0.1	33.9±0.2	n.d
AR/TW	17.76±0.02	48.6±0.1	74.0±0.3	n.d

According to UHPLC-MS/MS data, oligomeric PACs of hydrophilic extracts was mainly composed of procyanidin B-type dimer, trimer, tetramer and procyanidin glycoside.

Oligomeric PACs have better light and heat resistance, as well as higher stability (pH 2.0–6.0) than polymeric PACs (Qi et al., 2022). The presence of low molecular weight polyphenolic compounds such as catechin/epicatechin, quinic acid, gallic acid, salicylic acid, rutin, myricetin, quercetin, kaempferol was found in all extracts (Table 5). The serotonin content in 50% EtOH extract was close to the water extract within the CI. Some research showed that serotonin could promote the biological activity of other compounds when applied together (Erland et al., 2019).

Table 5. Tentative identification of the chemical constituents of SBT/TW, AR/TW and CB/TW extracts by UHPLC-MS/MS under negative ionization

No.	tR (min)	Tentative compound	m/z	Error (ppm)	MS/MS
1	0.96	Gluconic acid	195.0509	-0.81	177,04; 129,02
2	1.02	Disaccharide	341.1086	-0.93	202,07; 179,05; 89,02; 59,01
3	1.02	Quinic acid	191.0561	-0.10	111,01;87,01; 85,03
4	1.11	Malic acid	133.0142	-0.45	115,00; 71,01
5	1.53	Citric acid	191.0195	-1.36	111.01
6	2.61	Gallic acid	169.0142	-0.53	125.02
7	4.87	Protocatechuic acid 3-glucoside	315.072	-0.44	631,15; 315,07; 153,02; 152,01; 109,03, 108,02
8	4.99	Protocatechuic acid	153.0193	0.12	153,02; 135,01; 109,03
9	5.02	L-DOPA 3'-glucoside	358.1141	-0.77	312,11; 177,05; 161,05; 150,06; 119,04; 113,03; 101,02; 89,02
10	5.26	Galloyl glucose	331.0671	-0.44	313,06; 169,01 168,01; 125,02
11	6.05	Vanillic acid glucoside	329.0878	0.06	659,18; 167,03
12	6.63	Chlorogenic acid	353.0876	-0.64	191,05; 179,03
13	6.86	Salicylic acid	137.0244	0.18	93.03
14	7.83	3-Methoxy-4-hydroxyphenylglycol glucuronide	359.0981	-0.63	197,05; 182,02; 153,06; 138,03
15	8.53	Epigallocatechin	305.0666	-0.30	261,08; 219,07; 219,07; 179,03; 167,04; 137,02; 125,02
16	8.63	Catechin	289.0718	0.06	245,08; 205,05; 201,07; 179,04; 125,02; 123,05; 109,03
17	10.02	Neochlorogenic acid	353.0876	-0.64	191.05
18	10.24	Procyanidin B-type dimer	577.1348	-0.56	407,07; 289,07; 245,08; 202,08; 161,02; 125,02
19	10.31	Caffeic acid	179.0349	-0.38	201,07; 135,05
20	10.82	Caffeoylquinic acid	353.0876	-0.64	191.05
21	12.15	Procyanidin B-type trimer	865.1978	-0.84	695,15; 577,14; 451,11; 407,08; 289,07
22	12.23	Epicatechin	289.0716	-0.57	245,08; 205,05; 201,07; 179,04; 125,02; 123,05; 109,03
23	12.41	Procyanidin B-type tetramer	1153.2623	0.35	577,13; 525,08; 449,09; 407,08; 287,06; 243,03; 161,02
24	13.70	p-coumaric acid	163.0401	0.09	119.05
25	18.95	Rutin	609.1457	-0.69	163,00; 151,00; 148,02; 135,00
26	19.87	Myricetin	317.03	-0.73	151,00; 137,02; 109,03
27	21.80	Quercetin	301.0351	-1.05	273,04; 201,07; 151,00; 121,03
28	23.28	Kaempferol	285.04	-0.19	159,05; 151,00; 143,05; 117,03

Based on the results of chemical characterization (Figure 3 and Tables 4-5), and the influence of the biomass on the oxidative stability of the LBS, twigs and leaves rich in polyphenols can be considered as a potential ingredient to protect the LBS from oxidation.

3.4. Evaluation of Extracts and Target Compounds on LBS Stability and Their Antimicrobial Activity

3.4.1. PACs Influence on the Oxidative Stability of the LBS

The results of DPPH and $ABTS^+$ radical scavenging activity showed that PACs isolated from the SBT/TW 50% EtOH extract (92.1 g·100 g^{-1} DE by Butanol-HCl assay method) showed higher antioxidant activity (IC_{50} = 2.4 mg·L^{-1} by $DPPH^{\cdot}$ test and IC_{50} = 1.1 mg·L^{-1} by $ABTS^{+\cdot}$ test) than that of Trolox (IC_{50}=4.6 mg·L^{-1} by $DPPH^{\cdot}$ test and IC_{50} = 4.0 mg·L^{-1} by $ABTS^{+\cdot}$ test) and hydrophilic extracts (IC_{50}=3.8–10.2 mg·L^{-1} by $DPPH^{\cdot}$ test and IC_{50} = 2.6–5.6 mg·L^{-1} by $ABTS^{+\cdot}$ test), with CI ≤0.3 mg·L^{-1} at α = 0.05. The hydrophilic extracts from AR/TW had the highest antioxidative activity among all extracts (IC_{50}=3.8 mg·L^{-1} by $DPPH^{\cdot}$ test and IC_{50} = 2.6 mg·L^{-1} by $ABTS^{+\cdot}$ test).

Comparing the values of the PF in LBS for extracts at the same concentration (2%/LCS), the efficiency of 50% ethanol-water extract from SBT/TW was significantly higher (PF=3.9 for a cream with lipid content of 19%) than for SBT/TW biomass (PF=1.5, Table 1).

Although the antioxidant activity of purified PACs against DPPH and ABTS+ radicals is much better than for the extracts, it could be seen that influence of PACs on the oxidative stability of the creams is similar (within the CI). As it was mentioned the effect of extracts could be associated not only with the presence of polyphenols, which are considered to be the most active compounds responsible for antioxidant properties but also with their solubility in the tested system. The combined action of low and high molecular polyphenols gave a better effect in protecting the cosmetic cream's lipids from oxidation than purified PACs which dissolve in the lipid system only partially. Nevertheless, the Pearson's correlation coefficient between LBS oxidation stability and PACs amount in the cream composition (introduced as a PACs-containing extract) was quite high (r = 0.85).

Among hydrophilic extracts, AR had the highest effect on LBS stability. Compared to ascorbic acid, gallic acid, and tert-butylhydroquinone – TBHQ, the water extract, 50% EtOH extract, and PACs more effectively protected the creams from oxidation (Table 6).

Table 6. Hydrophilic extracts and individual compounds influence the oxidative stability of creams.

Sample	**IP, h**	**PF**	**IP, h**	**PF**
	Lipid content in LBS–19%		**Lipid content in LBS–35%**	
Cream base without antioxidants	11.0	1.0	1.9	1.0

SBT/AR/BC twigs water extracts				
Cream + 0.5% extract on LBS	28.2/ 31.6/26.4	2.6/2.9/2.4	3.6 /4.3/3.2	1.9 /2.3/1.7
Cream + 1% extract on LBS	32.5/34.2/28.2	3.0/3.1/2.6	3.8 /4.8/3.6	2.0 /2.5/2.1
Cream + 2% extract on LBS	37.3/39.6/36.7	3.4 /3.6/3.3	4.2 /4.6/3.8	2.2 /2.4/2.0
SBT/AR/ BC twigs 50% EtOH extract				
Cream + 0.5% extract on LBS	29.5/31.6/27.4	2.8/3.0/2.6	4.1/4.4/3.8	2.2/2.3/2.0
Cream + 1% extract on LBS	34.1/36.6/32.2	3.1/3.3/2.9	3.4/3.6/3.2	1.8/1.9/1.7
Cream + 2% extract on LBS	36.8/43.2 /32.4	3.4/3.9/3.0	4.4/4.7/4.0	2.3/2.5/1.6
SBT/AR purified PACs				
Cream + 0.5% PACs on LBS	n. a	n. a	3.5 /3.6	1.8 /1.9
Gallic acid (GA)				
Cream + 0.5% GA on LBS	n. a	n. a	2.0	1.1
Cream + 1% GA on LBS	n. a	n. a	3.9	2.1
Ascorbic acid (AA)				
Cream + 0.5% AA on LBS	n. a	n. a	1.9	1.0
Cream + 1% AA on LBS	n. a	n. a	2.1	1.1
TBHQ				
Cream + 1% TBHQ on LBS	-	-	-	1.3 (Janceva et al., 2017)
Cream + 2% TBHQ on LBS	-	-	-	1.8 (Janceva et al., 2017)
Cream + 3% TBHQ on LBS	-	-	-	2.4 (Janceva et al., 2017)

The confidence interval for all the results did not exceed 0.2 at $\alpha = 0.05$.
Comparing the data on antioxidant activity in cream with 35% and 19% lipid content, the extracts work better in a system with a lower lipid concentration.

3.4.2. The Impact of PACs and Serotonin-Rich Extracts on Antimicrobial Activity

PACs and the extracts that contain them showed promising results for LBS stabilization and therefore can be used for preventing lipid oxidation in creams. Serotonin is a valuable compound whose content in SBT biomass is quite high (could reach 8-10% depending on the season), and, therefore, its isolation for specific medicinal purposes could be done. But considering its quite complicated isolation, its application for preventing lipids oxidation in creams will not be tested, since it is of little practical value. The isolation of serotonin is the following step after the extraction of PACs, so in the biomass cascading application scheme, PACs could be used for LBS stabilization, and serotonin – for other purposes. In this article, it will be tested for anti-bacterial activity. To prove multi-functionality, serotonin, and PACs were both evaluated as agents against bacteria that most often appear in creams and topical formulations, as well as bacteria causing a range of skin problems and acne. Serotonin was concentrated to 26%/DE according to the know-how process elaborated in the laboratory (patent application pending). All extracts inhibited gram-positive and gram-negative pathogenic bacteria: *P. aeruginosa*, *S. aureus*, *E. coli*, *B. cereus*, *S. pyogenes*, and *C. acnes* (Table 7).

Table 7. Antimicrobial activity of PACs, hydrophilic extracts and serotonin in comparison with synthetic antibiotics

Samples		*E. coli*	*P. aeruginosa*	*S. aureus*	*B. cereus*	*S. pyogenes*	*C. acnes*
		MIC/MBC, mg·mL^{-1}					
SBT/TW water extract		0.39/0.39	0.39/3.13	0.39/0.78	0.78/>50	0.20/0.20	0.78/0.78
AR/TW water extract		5.0/5.0	2.5/>5.0	0.039/0.31	0.16/0.16	n.d.	n.d.
BC/TW water extract		5.0/5.0	1.25/1.25	0.63/0.63	1.25/>5.0	n.d.	n.d.
SBT/TW 50% EtOH extract		0.20/0.20	0.39/0.78	0.20/0.39	0.39/50	0.20/0.20	0.39/0.39
AR/TW 50% EtOH extract		2.5/2.5	0.63/2.5	0.31/0.63	0.16/>5.0	n.d.	n.d.
BC 50% EtOH extract		2.5/2.5	0.63/0.63	2.5/2.5	1.25/>5	n.d.	n.d.
SBT/TW purified PACs		0.04/0.04	0.08/0.16	0.08/0.16	0.63/1.25	0.10/0.10	0.39/0.39
AR/TW purified PACs		0.63/1.25	0.16/0.63	0.08/0.08	0.04/0.08	n.d.	n.d.
SBT/TW serotonin-rich extract (26.6%/ODE)	-	0.78/0.78 (Janceva et al., 2023)	0.78/0.78 (Janceva et al., 2023)	0.39/0.78 (Janceva et al., 2023)	0.78/6.25 (Janceva et al., 2023)	0.10/0.20	0.39/0.39
Serotonin standard (purity≥98.0%)	-	-	-	-	-	0.10/0.20	0.39/0.78
Amoxicillin	-	-	-	0.13/0.13	0.008/0.016*	-	0.0001***
Bactroban	-	-	0.16/0.18**	0.20/0.20**	-	0.20/0.40**	-
Gentamicin	-	0.001/0.004	0.0003/0.004	0.0003/0.004	-	0.40/0.40**	0.004***
Chlor-amphenicol	-	-	0.40/0.40**	0.40/0.40**	-	0.40/0.40**	-

The confidence interval for all the results did not exceed 0.05 mg·mL-1at α = 0.05.
* (Olajuyigbe, 2012); ** (Okunye et al., 2020); *** (Rollason et al., 2013).

Considering the complexity of serotonin purification, the serotonin-rich extract, alone or together with purified PACs or 50% EtOH extract, can be used against *S. pyogenes* and *C. acnes* without further SBT-derived serotonin purification. Along with the confirmed *in vitro* and mentioned earlier anti-inflammation properties of biomass-derived PACs, and vasoconstriction/vasodilator properties of serotonin, clinical trials described in literature confirmed the ability of PACs extract obtained from SBT berries to support stem cell types involved in regenerative and reparative functions (Drapeau et al., 2019), as well as AR berries anti-inflammatory, hypotensive, antiviral, anticancer properties (Jurendić and Ščetar, 2021),

and ability of BC berries anthocyanins to increase the level of collagen (Nanashima et al., 2018). These findings supports the concept of the application of multi-purpose ingredients based on SBT/TW, AR/TW and BC/TW extracts and biomass-derived PACs and SBT-derived serotonin in topical formulations after clinical testing for biomass-derived compounds. One or the other ingredient and biomass source can be chosen depending on the target properties of the cream formulation.

4. Conclusions

The study confirmed that SBT, AR and to lesser extern BC lignocellulosic biomass (twigs and leaves remaining as agro-waste after harvesting and pruning) as well as its derivatives can be effectively used as antioxidants in lipid-based systems, decreasing the oxidation rate from 1.3 to 4 times, as well as antimicrobial agents against pathogenic bacteria that contaminate skincare products and develops in sebum (*P. aeruginosa, S. aureus, E. coli, B. cereus, S. pyogenes, and C. acnes*). SBT, AR and BC lignocellulosic biomass was valorized as the active ingredient in its different treatment stages, till purified compounds, for application in anti-aging cosmetic and pharmaceutical creams. Along with the anti-inflammatory properties studied by authors earlier for SBT, and confirmed presence of biologically active compounds described in literature as ant-aging and anti-inflammatory agents, SBT, AR and BC biomass, its extracts, PACs and serotonin are prospective for multipurpose application in topical formulations. Wasteless fruit trees processing scheme offered in this study on the example of SBT, AR and BC will help with the development of the sustainable economy based on the available natural resources in rural areas.

CRediT authorship contribution statement

Anna Andersone: Writing- Original draft preparation, Conceptualization, Methodology, Investigation, Validation, Writing- Reviewing and Editing. **Sarmite Janceva**: Writing- Original draft preparation, Conceptualization, Methodology, Investigation, Data curation, Writing- Reviewing and Editing, Supervision, Formal Analysis. **Liga Petersone**: Methodology, Investigation, Writing- Reviewing and Editing. **Vizma Nikolajeva**: Methodology, Investigation, Writing- Reviewing and Editing: **Natalija Zaharova**: Investigation, Writing- Reviewing and Editing. **Gints Rieksts:** Investigation, Resources. **Galina Telysheva***:* Conceptualization, Methodology, Resources, Writing- Original draft preparation, Supervision. **Uldis Spulle**: Investigation.

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Declaration of Competing Interest

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

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Data Availability

Research data is available upon request.

Abbreviations:

Abbreviation	Meaning
SBT	sea buckthorn
AR	aronia
BC	blackcurrant
TW	twigs
LV	leaves
LBS	lipid-bases system
PACs	proanthocyanidins
GA	gallic acid
AA	ascorbic acid

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A Comparative Assessment of Sea Buckthorn (*Hippophae rhamnoides* L.) Pruning Waste as a Potential Source of Serotonin

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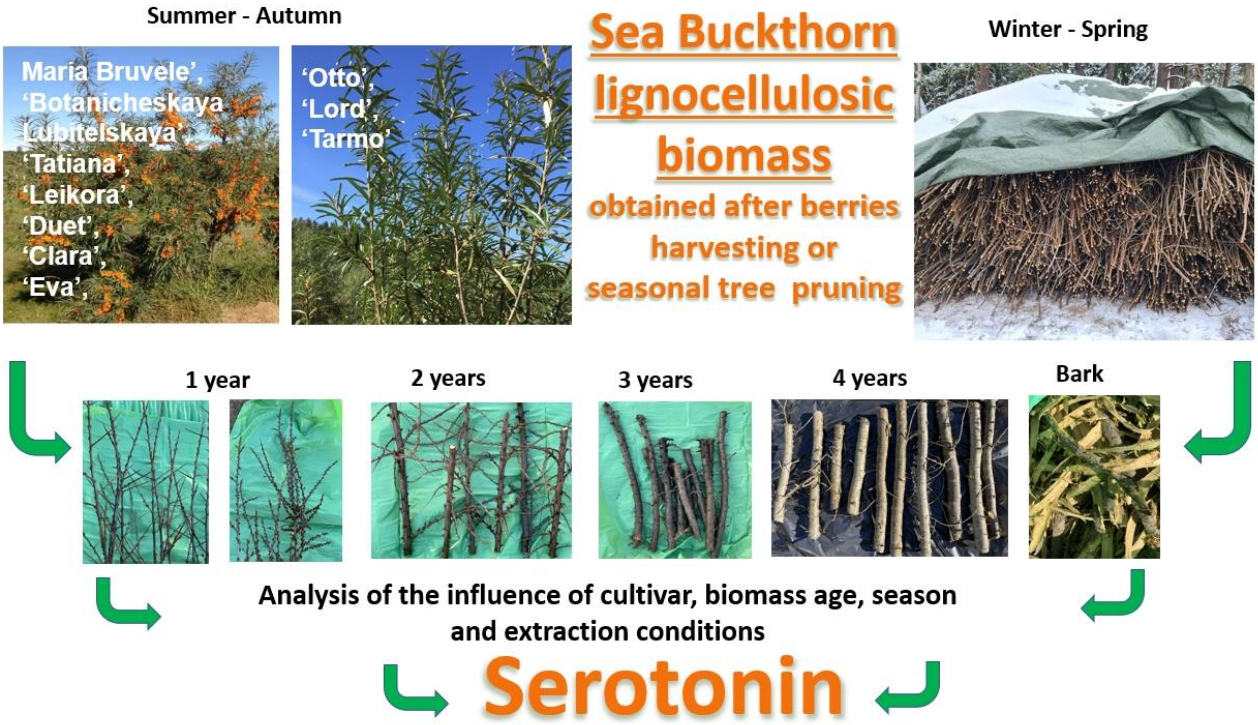
GRAPHICAL ABSTRACT



A Comparative Assessment of Sea Buckthorn (*Hippophae rhamnoides* L.) Pruning Waste as a Potential Source of Serotonin

Sarmite Janceva,[a] Anna Andersone,[a,b,*] Liga Lauberte,[c] Natalija Zaharova,[a,b] Galina Telysheva,[a] Jelena Krasilnikova,[d] and Gints Rieksts [a]

Sea buckthorn (*Hippophae rhamnoides* L.) twigs, remaining after harvesting and pruning, are an underutilized and little-explored biomass resource. This study investigated the content of serotonin in 10 sea buckthorn cultivars ('Maria Bruvele', 'Botanicheskaya Lubitelskaya', 'Tatiana', 'Otto', 'Leikora', 'Duet', 'Clara', 'Lord', 'Eva', 'Tarmo') for the first time, and for further adjustment of the extraction conditions, cultivar 'Maria Bruvele' was extracted by water and water/ethanol solution with 20-25, 50, 70, and 96% ethanol at different temperatures. The results showed that 50% water/ethanol solutions are the most suitable for extraction, which makes it possible to increase the yield of serotonin. The 2-year-old twigs and bark from 'Maria Bruvele' collected in autumn contained higher serotonin content compared to spring-collected biomass. Serotonin sequential purification allowed the serotonin content in the fraction to increase to 26%/DM. The serotonin-rich fraction showed antimicrobial activity against gram-positive and gram-negative bacteria. In tests with salivary amylase, a serotonin-rich fraction at the amount of 0.1-0.4 mg/mL of saliva, under normal physiological conditions, tended to increase amylase activity, resulting in acceleration of starch degradation to glucose. Thus, the results support further study of the serotonin fraction for the treatment of people having underweight, malnutrition, and malabsorption conditions.

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Keywords: Sea buckthorn; Serotonin; Twigs; Bark; Freon; Antimicrobial activity; Amylase activity

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INTRODUCTION

Natural products, in the form of pure compounds or in the form of complex plant extracts, open unlimited possibilities for the discovery of new nutraceuticals due to the unsurpassed chemical diversity. According to the World Health Organization (WHO), 60% of the world's population relies on herbal medicine, and about 80% of the population in developing countries depends almost totally on it for their primary health care (Ahmad Khan and Khan 2019). In the USA, approximately 49% of the population has tried natural medicines for the prevention and treatment of diseases. Plants used in traditional medicine contain a wide range of biologically active substances that can be used to treat various chronic and infectious diseases. Natural compounds can be obtained from any part of the

plant, such as bark, leaves, twigs, berries, flowers, roots, fruits, and seeds (Gradt *et al.* 2017). The synergy of various secondary metabolites activity in plant extracts has been described elsewhere (Janceva *et al.* 2017; Abegaz and Kinfe 2019).

Sea buckthorn (*Hippophae rhamnoides* L.) (SBT) is a unique and valuable plant due to its medicinal and nutritional potential. Approximately 40 countries cultivate SBT, with a total area of cultivation worldwide of about 3 million hectares. China, Northern Europe, Canada, Romania, Russia, and Mongolia cover almost 90% of the world's SBT stocks. China is the world's leading producer. The total annual SBT harvest is 8.5 million tons (Nawaz *et al.* 2019). In Latvia, SBT is mainly grown on private plantations, on area of about 400 hectares, with a production of 12 to 50 kg of fruits per plant (Klovane 2014).

Due to its valuable composition and high biological activity, the most known product of SBT processing is oil. Interest in it has been increasing in the pharmacology, food, and cosmetology industries (Ivanova *et al.* 2019). The therapeutic effect of SBT oil is explained by the presence of vitamins, carotenoids, tocopherols, and a number of other biologically active substances (Kallio *et al.* 2002; Andersson *et al.* 2009). Juice, syrup, and tincture of fresh fruits are also recommended as a multivitamin additive for the prevention of beriberi, and other vitamin-deficiency diseases.

Currently, special attention is paid to the complex and waste-free processing of SBT with the maximum extraction of biologically active substances and the expansion of the range of preparations from the SBT (Janceva *et al.* 2022). In folk medicine, a decoction of leaves is used to treat gastrointestinal diseases, ulcers, and microbial infections (Suryakumar and Gupta 2011; Yue *et al.* 2017; Letchamo *et al.* 2018). There are reports that the extract of bark and shoots has antitumor activity (Christaki 2012; Olas *et al.* 2018). The authors' previous studies revealed anti-inflammatory activity of SBT twigs extracts and the possibility of the extracts to influence pancreatic lipase and salivary amylase activities (Janceva *et al.* 2021; Andersone *et al.* 2023a,b).

The chemical characterization of the extracts of vegetative parts of the SBT is relevant and will help to create a waste-free processing scheme for SBT cultivation. Preliminary studies by the authors, as well as literature reported that SBT twigs contain a complex of nitrogenous compounds, including serotonin in the cortex (Gradt *et al*. 2017) in greater amounts than in the biomass of other plants. Research on serotonin in SBT has just started, and the data are very limited. In plants, serotonin is synthesized differentially whereby tryptophan is first catalyzed into tryptamine by tryptophan decarboxylase, followed by the catalysis of tryptamine by tryptamine 5-hydroxylase to form serotonin (Ramakrishna *et al.* 2011). However, another pathway of transformation of tryptophan to serotonin in plants cannot be ruled out. The function of serotonin is not yet clear as well. The biggest concentration of serotonin in plants so far has been found in walnuts and hickory; it was reported to be implicated in the responses to biotic and abiotic stress, as an antioxidant and growth regulator (Erland *et al.* 2019; Mandal 2023). Serotonin as a neurotransmitter is involved in the regulation of a number of important functions in humans, including sleeping, hunger, thirst, and mood (Ramakrishna *et al.* 2011).

The purpose of this work was to evaluate the twigs of ten SBT cultivars, as well as leaves, bark, and twigs of different ages (1 to 4 years) of the 'Maria Bruvele' cultivar, collected in spring and autumn seasons, as a raw material for obtaining serotonin. A further goal was to find the optimal conditions for the extraction of plant material and fractionation of biomass, in terms of obtaining serotonin. Additionally, the study was aimed at testing the serotonin-rich fraction's antimicrobial activity and effect on the activity of the amylolytic enzyme alpha-amylase in saliva, for assessment of the practical application possibilities.

EXPERIMENTAL

Materials

The different age twigs after harvesting of ten SBT cultivars - 'Maria Bruvele', 'Botanicheskaya Lubitelskaya' ('Bot. Lub.'), 'Tatiana', 'Otto', 'Leikora', 'Duet', 'Rumania', 'Lord', 'Eva', and 'Tarmo' were collected from the SBT plantation area in Latvia, Tukums, with the same growing conditions, in August of 2020. The twigs were dried at 22 to 26 °C temperature, and ground with a knife mill Retsch SM100 (Retsch, Haan, Germany) and sieved to select the particles between 2 and 4 mm. These fractions were stored at −8 °C. Additionally, the one, two, three, and four-year-old twigs, leaves, and bark samples of the SBT cultivar - 'Maria Bruvele' were collected from the same plantation area, in March and September of 2021 and 2022.

Isolation of the Serotonin-Rich Extracts from Ten SBT Biomass

For the first comparative evaluation of the serotonin content in ten SBT cultivars, and as a way to select the target cultivar with the biggest serotonin content, serotonin-containing extracts were isolated from twigs according to the scheme shown in Fig. 1.

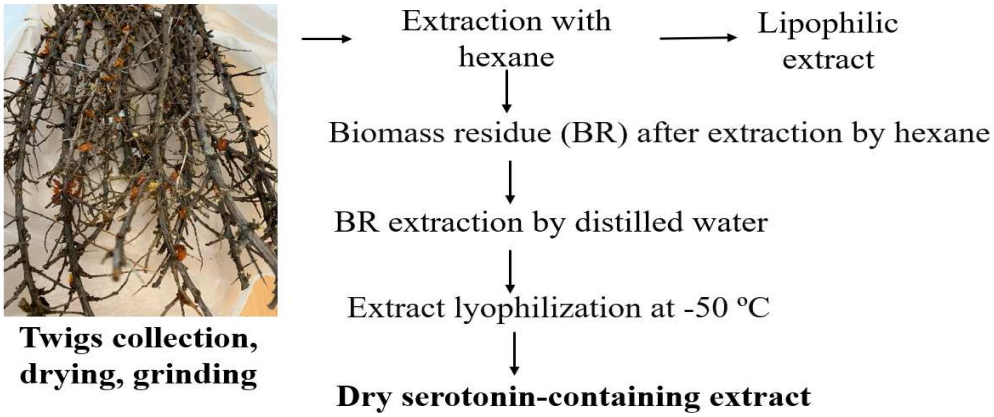

Fig. 1. Scheme of serotonin isolation from SBT twigs for initial comparative assessment of its content in ten SBT cultivars

Lipophilic compounds were separated by biomass extraction at 50 to 60 °C for 30 min by *n*-hexane. The extracts after hexane evaporation were dried at 40 °C to yield a dry extract. The yield of the lipophilic extracts was presented as % of the DM of biomass.

Hydrophilic extracts from residues after lipophilic compounds separation were isolated by extraction at 60 °C for 30 min (3 x 10 min) using distilled water. For study of the influence of extraction conditions on the serotonin yield and its content in the extract, extraction of SBT 'Maria Bruvele' biomass was carried out using aqueous solutions with different percentages of ethanol (20, 50, 70, 96%) and at different temperatures (22-25, 50, and 70 °C). The extracts after ethanol evaporation were freeze-dried at -50 °C for 12 h to obtain a dry powder. The yield of the extracts is presented as % of the DM of biomass. The CI for the results did not exceed 3% at $\alpha = 0.05$.

Maria Bruvele Biomass Extraction by 1,1,1,2-Tetrafluoroethane

For assessment of the influence of solvent at the first stage of serotonin-rich biomass extraction, 1,1,1,2-tetrafluoroethane (freon R134a) was used as an alternative solvent, for the isolation of non-polar and semi-polar compounds from 'Maria Bruvele' samples (leaves, twigs, and bark). Extraction was performed in a Nectacel 1L pilot extractor (Celsius, France), within a closed system, under pressure of 4.0 to 4.3 bar and temperature of 17 to 19 °C. The yield of the extracts isolated by freon R134a was presented as a percentage based on the DM of biomass. The CI for the results did not exceed 3% at $\alpha = 0.05$.

Extraction of Hydrophilic Compounds from Biomass Residue After Extraction by 1,1,1,2-Tetrafluoroethane

After 'Maria Bruvele' biomass (twigs, bark, leaves) extraction by freon R134a, biomass residue was extracted by distilled water and ethanol-water solution (1:1, v/v) at 60 °C for 30 min.

Identification and Quantification of Serotonin Content in Extract

Dry crude extracts were dissolved in aqueous acetonitrile (v/v 50:50) with an approximate concentration of 2 mg/mL and filtered (Nylon filter, 0.45 µm pore size), and used for UHPLC-UV-TOF/MS experiments. LC analysis of the samples were performed on the Acquity UPLC (Waters Corp., Milford, MA, USA) coupled with a quadrupole-time of flight (Q-TOF) MS instrument (UPLC/Synapt Q-TOF MS, Waters, Milford, MA, USA) equipped with an electrospray ionisation (ESI) source. The separation was carried out on a U-HPLC column (2.1 mm x 50 mm i.d., 1.7 µm, BEHC18) (Waters Acquity) at a flow rate 0.35 mL/min. The eluent was 0.1% formic acid, water (A), and acetonitrile (B). A gradient solvent system was used: 0 to 1 min, 5% to 20% (B); 1 to 5 min, 20% to 25% (B); 5 to 6 min, 25% to 75% (B), 6 to 7 min, 75% to 80 % (B), 7 to 8.5 min, 80% to 7% (B), 8.5 to 10 min, 5% to 5% (B), and the injection volume was 1.0 µL. The major operating parameters for the Q-TOF MS were set as follows: capillary voltage, 2.5 kV (-) and 2.0 kV (+); cone voltage, 60 V; cone gas flow, 100 L/h; collision energy, 6 eV; source temperature, 120 °C; desolvation temperature, 450 °C; collision gas, argon; desolvation gas, nitrogen; flow rate, 750 L/h; data acquisition range, *m*/*z* 50 to 1200 Da; ionization mode – positive. Serotonin was identified with its analytical standard (Sigma Aldrich, M_w=176.22 g/moL) (Fig. 2).

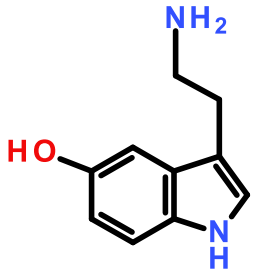

Fig. 2. Chemical structure of serotonin (Mw=176.22 g/moL)

In positive electrospray ionization mode, serotonin was protonated to produce ions in the form $[M+H]^+$, with *m*/*z* 177. On the basis of detected serotonin fragmentation, a multiple reaction monitoring mode (MRM) was developed for the specific *m*/*z* transitions 177→160 (the most intensive cleavage ion), 177→132, and 177→115 (Fig. 3).

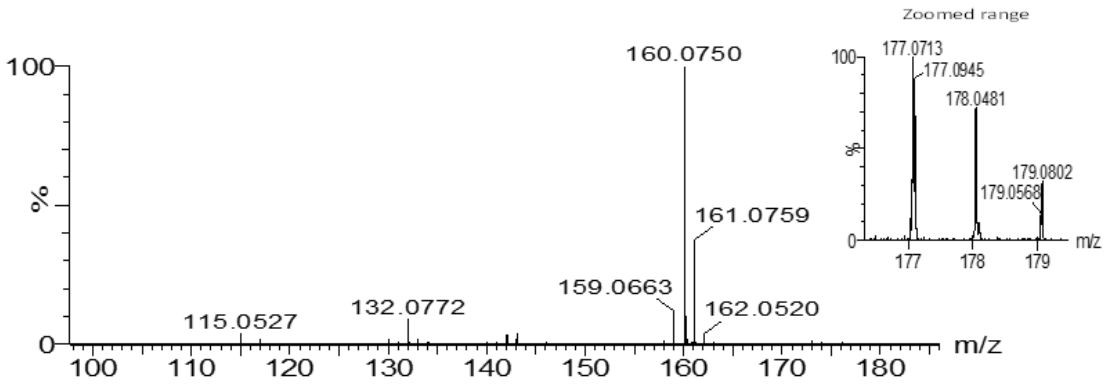

Fig. 3. Serotonin identification by UHPLC-UV-TOF/MS

Serotonin Purification

The serotonin purification was carried out using size exclusion and ion exchange resins (patent pending) for high- and low-molecular-weight polyphenol separation from extracts.

Antimicrobial Analyses

Antimicrobial activity was studied in 96-well plates by the two-fold serial broth microdilution method, which allowed the determination of the minimum inhibitory (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC), as described by Andersone *et al.* (2023b).

In-vitro Analysis of Alpha-Amylase Activity

In-vitro analyses were performed at the Department of Human Physiology and Biochemistry of Riga Stradins University based on European standard protocols as described by Krasilnikova *et al.* (2013). The saliva used for research was donated by students with no record of chronic or acute illness, the last meal was 2 hours before the examination to get clean results. The extracts were tested in amounts from 0.1 to 0.4 mg/mL of saliva. The influence of the extracts on salivary amylase was measured by the breakdown of polysaccharides containing linear α-1,4 glucose bonds in starch. The amylase activity was characterized by the amyloclastic force (AF); that is, the volume of the 0.1% starch solution in milliliters that is hydrolyzed by 1 mL of saliva in the test tubes at 38 °C for 30 min. Then, 1% iodine solution was added (as a marker for the presence of starch by color changes). The amyloclastic force is denoted as $D_{30/38^{\circ}C}$. Saliva without extract was used as a reference. The amyloclastic force of the reference sample was $D_{30/38^{\circ}C}$ 640.

Statistical Analysis

All measurements were conducted in triplicate (n=3). The results are presented as the mean value ± confidence interval (CI). Statistical analyses were performed using Microsoft Excel 2016. CIs were calculated for a mean using a Student's T distribution at a significance level $\alpha = 0.05$.

RESULTS AND DISCUSSION

Chemical Characterization of Twigs from Ten SBT Cultivars

For the first evaluation of the yield of extracts and content of serotonin in SBT twigs (further in the text – biomass) of ten different cultivars ('Maria Bruvele', 'Bot. Lub', 'Tatiana', 'Otto', 'Leikora', 'Duet', 'Clara', 'Lord', 'Eva', and 'Tarmo') collected in August of 2020 were studied. The yield of lipophilic extracts obtained with hexane from the entire studied biomass was quite close and varied from 1.2 to 1.9% per DM. Further on, distilled water was used as an extractant to test its suitability on serotonin and as the most environmentally friendly, low-cost simple solvent. The yield of hydrophilic extracts from biomass, obtained using distilled water differed statistically significantly and ranged from 19 to 29% per DM. The content of serotonin in hydrophilic extracts ranged from 1.5 to 7.9%/DM (Fig. 4).

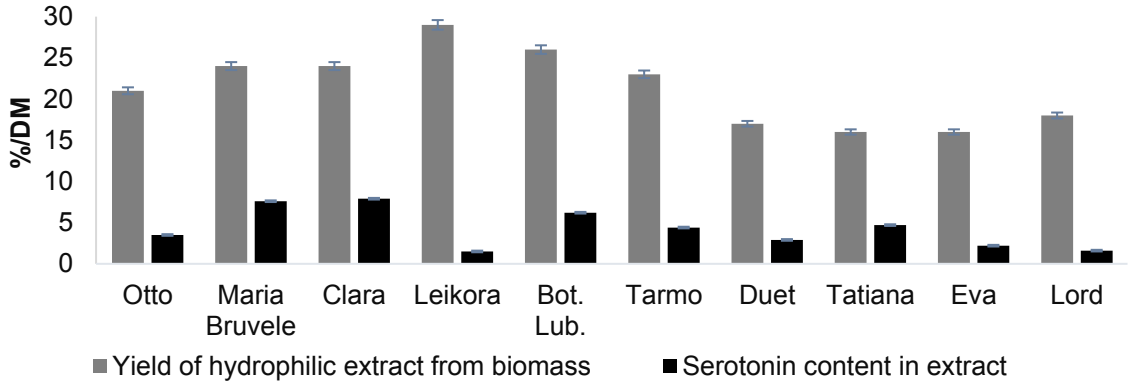

Fig. 4. Comparison of yield of water extracts and serotonin content in the extract for 10 SBT cultivars

The highest content of serotonin in the water extract was in 'Maria Bruvele' (7.6%/DM) and 'Clara' (7.9%/DM). Based on the increase in the growing of 'Maria Bruvele' in the Baltic region and on the results of the chemical characterisation above, 'Maria Bruvele' biomass collected in 2020 was chosen for further extraction optimization experiments.

Extraction Conditions for Serotonin Isolation from 'Maria Bruvele' Biomass

To determine the suitability of ethanol-water solutions for the serotonin extraction, 'Maria Bruvele' biomass with a particle size of 2 to 4 mm was extracted at 60 ºC with a duration time of 30 min, as solvents using ethanol-water solutions (20, 50, 80, and 96% of ethanol, further in the text: 20% EtOH, 50% EtOH, 80% EtOH and 96% EtOH). The content of serotonin in all 'Maria Bruvele' hydrophilic extracts varied from 7.5 to 10.4%/DM. The 50% EtOH ethanol-water solution provided the highest yield of serotonin from biomass (2.2%/DM) with 8.2%/DM of the serotonin in the extract. The serotonin yield from 20 and 80% ethanol-water solutions was similar within the CI. Despite the high content of serotonin in the extract (10.4%/DM) isolated with 96% EtOH, the serotonin yield from the biomass was only 1.1%/DM (Fig. 5).

Generally, high extraction temperature increases the efficiency of extraction. Serotonin isolation from twigs by 50% EtOH at 70 to 80 °C showed a significant serotonin content decrease (2.6 times) in extract composition, which indicated that serotonin is a

thermolabile compound. Therefore, to continue the studies, biomass extraction was carried out at 60 °C.

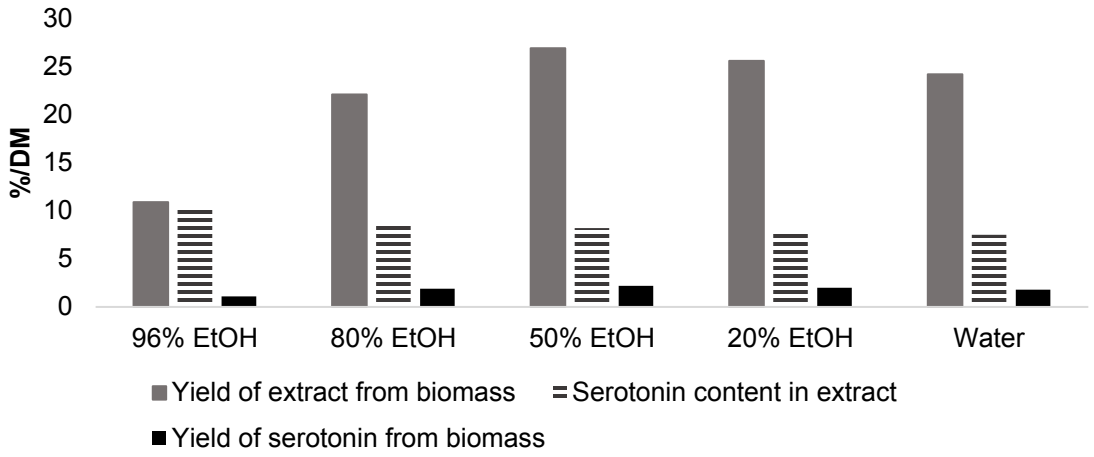

Fig. 5. Effect of the ethanol concentration in extractant on the efficiency of serotonin isolation from SBT 'Maria Bruvele' twigs (extraction time 30 min., temperature 60 °C).

Comparison of Extracts Yield and Serotonin Content in 'Maria Bruvele' 1–4-Year-Old Twigs

The yield of freon extracts from one, two, three, and four-year-old twigs collected in March 2021 ranged between 0.6 and 1.2%/DM. The yield of an extract isolated by freon from 'Maria Bruvele' twigs collected in 2021 was 1.8 times lower than by hexane. This could be due to the lower extraction temperature (up to 20 °C) allowed in the freon extraction equipment for the freon used (R134a).

The yield of hydrophilic extracts from twigs collected in March 2021 ranged between 6 and 22% /DM (Table 1).

Table 1. Serotonin Content in SBT Twigs Depending on Age (Collected in March 2021) and solvent. Extraction Condition: Mass Ratio of Biomass and Solvent (1:8, w/w), Extraction Temperature of 60 °C, Time of 30 min

Samples	Yield of Extract from Biomass (%/DM)	Serotonin Content in Extract (%/DM)	Yield of Serotonin from Biomass (%/DM)
	Extraction with Distilled Water		
1-Year-Old Twigs	11.96±0.05	11.21±0.02	1.34±0.01
2-Year-Old Twigs	12.57±0.06	14.62±0.03	1.84±0.01
3-Year-Old Twigs	15.87±0.05	11.08±0.03	1.76±0.01
4-Year-Old Twigs	8.53±0.04	7.91±0.02	0.67±0.01
	Extraction with 50% EtOH		
1-Year-Old Twigs	22.23±0.04	10.53±0.03	2.34±0.01
2-Year-Old Twigs	19.61±0.03	14.85±0.02	2.91±0.01
3-Year-Old Twigs	17.25±0.02	9.88±0.02	1.70±0.01
4-Year-Old Twigs	8.56±0.04	9.97±0.03	0.85±0.01

The highest yield of hydrophilic extracts was obtained by ethanol-water solution (1:1; v/v or 50% EtOH at 60 °C, 30 min). Based on the Table 1 data, 1- and 2-year-old twigs extracts had the highest content of serotonin (11-15%/DM of extract, 2.3 and 2.9%/DM of biomass). These results were close to the data of other authors for different cultivars, which indicated that the content of serotonin in SBT twigs was 2.0 to 3.16%/DM (Galitsyn *et al.* 2014).

Leaves, 2-year-old twigs, and bark from 2-year-old twigs, collected in September after picking berries, were also tested as the raw materials for serotonin. Compared to the twigs collected in March, the yield of extract and content of serotonin in 1- and 2-year-old twigs collected in autumn was significantly higher. The yield of hydrophilic extract was ~1.3 times higher. The serotonin content in the extract isolated by 50% EtOH increased to 14.02%/DM. The yield of serotonin from twigs extracted by 50% EtOH was 3.7%/DM. This indicated that the twigs pruned in autumn have more potential as a raw material for the isolation of serotonin.

Table 2. Serotonin Content in Biomass Collected in September 2021

Samples	Yield of Hydrophilic Extract from Biomass (%/DM)	Serotonin Content in Extract (%/DM)	Yield of Serotonin from Biomass (%/DM)
	Extraction with Distilled Water		
1- and 2-Year-Old Twigs (Mix)	15.73±0.03	13.67±0.02	2.15±0.01
Bark from 2-Year-Old Twigs	18.02 ±0.04	13.84 ±0.01	2.49±0.01
	Extraction with 50% EtOH		
1- and 2-Year-Old Twigs (Mix)	26.42±0.03	14.02±0.02	3.70±0.01
Bark from 2-Year-Old Twigs	26.07±0.03	14.16±0.03	3.69±0.01

The leaves contained an insignificant amount of serotonin – 0.04%/DM or less. These results were consistent with the literature data, which showed that the content of serotonin in leaves ranged from 0.03 to 0.36%/DM (Galitsyn *et al.* 2014). The authors' previous studies showed that trees debarking biomass had a high amount of biologically active compounds. This was also confirmed by the serotonin data in this study. The high amount of hydrophilic extract from bark made it possible to obtain 26 g of extract/100 g DM of bark with a content of serotonin of 14%/DM (in September 2021). This high amount was also confirmed for bark collected in 2022 from 2-year-old twigs (13.4%/DM of serotonin content in hydrophilic extract).

However, debarking of SBT twigs (with the highest serotonin content) is not economically reasonable at an industrial scale since this process requires much labor and time. Thus, the most suitable raw material for serotonin obtaining could be 1-and 2-year twigs which have bigger content of bark than 4-year-old twigs.

The hydrophilic extracts of 1-and 2-year-old twigs collected in September 2021 contained a high amount of polyphenols, mainly proanthocyanidins (52.98%/DM). Based on the results of our previous studies, where proanthocyanidins are strong inhibitors of amylase activity, their separation from serotonin was essential for this study. Their isolation from extract, as one of the main stages of serotonin purification allowed for twice the increase of serotonin content in the remaining extract.

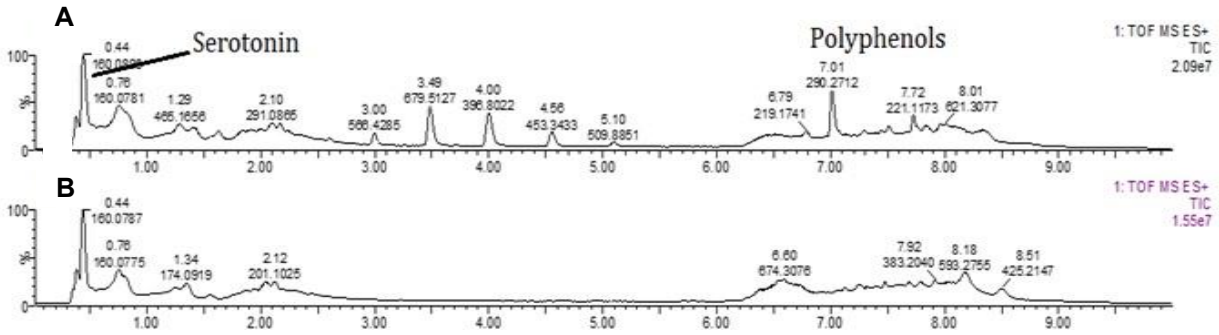

Fig. 6. UHPLC-TOF/MS chromatograms of the 50% EtOH extracts (A: 1- and 2-year-old twigs (mix); B – bark from twigs)

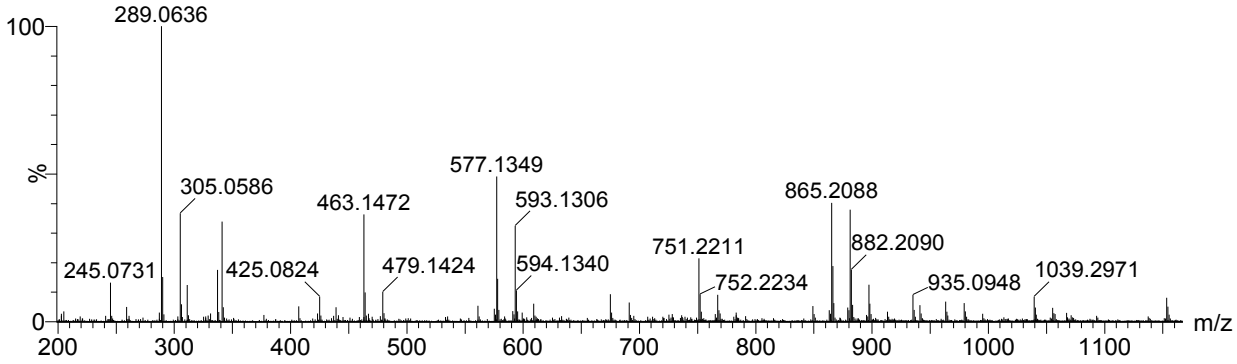

Fig. 7. Mass spectrum of proanthocyanidins from 50% EtOH extract isolated from 1- and 2-year-old twigs of 'Maria Bruvele'

After sequential serotonin purification, serotonin content in fraction increased to 26.1%.

Antimicrobial Activity of Serotonin-rich Fraction

In the case of physiological disorders, pathogenic bacteria can linger, multiply, and cause pathological processes in human body. In purulent inflammatory processes, representatives of the genus *Pseudomonas* are often found.

Table 3. Antimicrobial Activity of Serotonin-purified Fraction

Samples	*E. coli*	*P. aeruginosa*	*S. aureus*	*B. cereus*	*C. albicans*
Serotonin-rich Fraction (Serotonin Content in Fraction: 26.6%/DM)	MIC/MBC or MIC/MFC (mg/mL)				
	0.78/0.78	0.78/0.78	0.39/0.78	0.78/6.25	0.20/0.20

A certain role is assigned to yeast-like fungi of the genus *Candida*, which in normal flora of healthy people are either absent or found in very small quantities. Serotonin-purified fraction showed significant antimicrobial activity against gram-positive and gram-negative pathogenic bacteria, such as *P. aeruginosa*, *S. aureus*, *E. coli*, *B. cereus,* and fungus *C. albicans*.

Serotonin-rich Fraction Influence on Alpha-Amylase Activity

Under normal physiological conditions, a serotonin-rich fraction at amounts of 0.1 to 0.4 mg showed a significant activation (two times) of amyloclastic force (Table 4).

Table 4. Influence of Serotonin-Rich Fraction (SRF) on Amylase Activity in Normal Physiological Conditions

Sample	SRF Amount In Saliva (mg/mL)	Amyloclastic Force (Saliva pH 7)
Human Saliva Without Extract (Control)	-	640
Serotonin-rich Fraction (Serotonin Content in Fraction: 26.6%/DM)	0.1	1280
	0.2	1280
	0.4	1280

Increased α-amylase activity accelerates the degradation of starch to glucose, which may be useful in the treatment of people having conditions of underweight, malnutrition, and malabsorption.

CONCLUSIONS

1. Sea buckthorn twigs and bark of investigated cultivars are the valuable source of serotonin with an average yield of 2-6% /DM.
2. Biomass extraction with 50% EtOH at 60 °C and the following sequential purification allowed to obtain extract with a serotonin content of 26% /DM.
3. Serotonin-rich fraction had antimicrobial activity showing its perspective as an antimicrobial agent.
4. Serotonin-rich fraction had the ability to activate the amylase activity in normal physiological conditions that could be useful for the treatment of persons with underweight, malnutrition, and malabsorption. Further research is needed.

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A comparative analysis of the proanthocyanidins from fruit and non-fruit trees and shrubs of Northern Europe: Chemical characteristics and biological activity

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ABSTRACT

The comparative analysis of the chemical composition and biological activity of extracts and proanthocyanidins (PACs) isolated from harvesting and pruning agro-waste of fruit shrub-trees (*Hippophae rhamnoides* L. and *Aronia melanocarpa*) and forest cleaning waste of non-fruit trees and shrubs (*Alnus incana* L., *Alnus glutinosa* and *Salix caprea*) of Northern Europe was carried out. *Aronia melanocarpa* and *Hippophae rhamnoides* L. biomass had the highest proanthocyanidins content (up to 12%) on dry biomass. Fruit trees-derived purified PACs which contained structures with higher polymerization degree had slightly higher antioxidant activity. Both fruit and non-fruit trees PACs can be successfully used in antimicrobial preparations and as antioxidants, and could be interchangeable in the compositions of the antimicrobial preparations, especially against *E. coli*, *P. aeruginosa* and *S. aureus*. *Salix caprea,* which contained wider range of gallocatechin subunits, and *Alnus* spp., which contained diarylheptanoids, had shown better antimicrobial activity against *B. cereus.* Non-fruit trees PACs are desirable in the preparations against *C. albicans.* PACs from *Hippophae rhamnoides* L. and *Aronia melanocarpa* inhibited biofilm formation to 50%, but the extract from *Hippophae rhamnoides* L. showed the most significant MRSA biofilm growth inhibition (onto 80%). Preparations on the basis of waste biomass-derived compounds will contribute to sustainable pharmacy since their application will allow to diminish the use of antibiotics and to have less toxic side effects both for humans and the environment.

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[1] Sadly, Professor Dr. habil. chem. Galina Telysheva passed away in November 2021. She was the head of the project until November 2021, and took part in the article conceptualization, writing, choice of methodology, and investigation. Anna Andersone, Sarmite Janceva, Liga Lauberte, Natalija Zaharova and Gints Rieksts express their gratitude to Professor, Dr. habil. chem. Galina Telysheva, an appreciated research leader, for her ideas and passion in the work with lignocellulosic biomass that opened for us the amazing world of science.

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Abbreviation

SBT	sea buckthorn
PACs	proanthocyanidins
EtOH	ethanol
IC_{50}	the concentration of 50% inhibition of free radicals
CI	confidence interval
DP	degree of polymerization
MIC	minimum inhibitory concentration
MBC	minimum bactericidial concentration
MFC	minimum fungicidal concentration
MSCL,	Microbial Strain Collection of Latvia
CFU	colony-forming units
MRSA	Methicillin-resistant *Staphylococcus aureus*
ESBL	extended-spectrum beta-lactamases producing *Escherichia coli*

1. Introduction

After analyzing both: the latest scientific studies and indigenous people's experience, it can be said that each plant could be useful for human society in one or another way, and, moreover, be a source of beneficial molecules produced in a sustainable way. The latest literature and our previous research data showed that plant-derived polyphenolic compounds and, particularly, proanthocyanidins (PACs) are prospective secondary metabolites with high biological activity. PACs are chemically heterogeneous oligomers of polyhydroxy-flavan-3-ol monomer units linked mainly by C4–C6 or C4–C8 bonds (B-type PACs). Less widespread are the A-type PACs, characterized by the presence of flavanol units doubly linked by C4–C8 and C2–O7 or C4–C6 and C2–O7 bonds (Panzella and Napolitano, 2022) (Fig. 1).

The most studied PACs for pharmaceutical applications are the ones derived from grape seeds (genus *Vitis*), maritime pine bark (*Pinus pinaster*), lingonberry (*Vaccinium vitis-idaea*), cranberries (*Vaccinium macrocarpon*), and Quebracho tree (*Schinopsis balansae*) (Morazzoni et al., 2021; Tian et al., 2018; Unusan, 2020). Commercial medicinal preparations are available on their basis ("Atrantil Capsules," n.d.; "Pine Bark Extract: Uses, Benefits, and Side Effects," n.d.; Lee, 2013). However, PACs obtained from the vegetative part of the trees which appear as forest cleaning residues and as agro-waste are much less studied. It was later shown that they can be applied as antioxidants, antimicrobial, and anti-inflammatory agents (Andersone et al., 2023; Coşarcă et al., 2019; Janceva et al., 2022a; Panzella and Napolitano, 2022; Rauf et al., 2019). A study of their possible interchangeability, where a comparison of their chemical characteristics and biological activity for producing pharmaceutical preparation was carried out, will create a basis for a sustainable application of different biomass waste available for producers at the moment. Since it was shown that PACs obtained from sea buckthorn (SBT) lignocellulosic biomass have antimicrobial as well as adhesive (Janceva et al., 2022b) properties, one of the possible directions could be the application of PACs as anti-microbial preparations, including for the prevention of biofilms formation which is rapidly becoming a primary objective of wound care (Attinger and Wolcott, 2012). Antibacterial activity against different strains could be regulated by PACs composition coming from different biomass sources.

According to Global Antimicrobial Resistance (AMR) and Use Surveillance System Report of 2021 ("World Health Organization and European Centre for Disease Prevention and Control report: antimicrobial resistance remains a health threat in Europe," n.d.), although there are a lot of synthetic antimicrobial preparations, many high-priority pathogens gradually mutate due to antibiotics overuse and misuse and become resistant to them. New resistance mechanisms spread internationally, creating a growing global

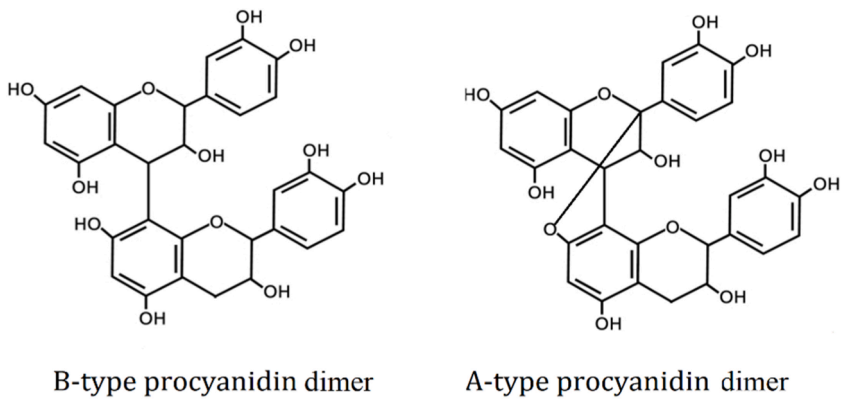

Fig. 1. Structures of the A- and B-type of PACs.

healthcare crisis. As a consequence, it is often necessary to increase the dose or combine different antibiotics, which adversely affects the human body and leads to higher medical expenditure, lengthier hospitalization, and increased death ("Antibiotic resistance," n.d.).

In addition, the manufacturing of synthetic antibiotics has an unacceptable risk of environmental contamination (Nijsingh et al., 2019). The same goes for their residues after consumption (antibiotics in active form through urine and excreta contaminate soils, waters, plants, etc., favoring resistant strains), and disposal, which demands strict control and resources (Bengtsson-Palme et al., 2018; Polianciuc et al., 2020).

The situation in animal husbandry is even worse, it was estimated that the total antibiotic consumption in husbandry (228 countries) in the 2010–2030 will rise by 67% (from 63,151 tons in 2010 to 105,596 tons in 2030), or even by 99% for middle-income countries (Bengtsson-Palme et al., 2018; Nijsingh et al., 2019; Polianciuc et al., 2020; "The Center for Disease Dynamics Economics & Policy. Animal use and resistance," n.d.; Van Boeckel et al., 2015).

On the other side, to address sustainability concerns, in the activity connected with forest and agricultural resources, it is important to apply the principle of the cascading use of biomass and find the most valuable application of each part of the plant before it will be used for energy production (Didier Bourguignon, 2015; European Commission. Directorate General for Internal Market, Industry, Entrepreneurship and SMEs, 2018). It was shown that preliminary extraction of the biologically active compounds will not influence or even have a positive effect on the calorific value of the remaining biomass after extraction (Stolarski et al., 2022). Therefore, plant secondary metabolites extraction could be considered a valuable step for the bioeconomy subsistence and plant biomass application efficiency. Extraction and purification of the target valuable chemical compounds are important for the side-stream valorization of forestry and agricultural waste and retaining land ecosystem stability in the long term (Müller and Laibach, n.d.; Coşarcă et al., 2019; Fierascu et al., 2019; McElroy et al., 2018; Zhang et al., 2018).

Apart from above mentioned widely known sources of PACs, other biomass types can be considered as raw materials for obtaining of PACs. Grey alder (*Alnus incana*), black alder (*Alnus glutinosa*), and goat willow (*Salix caprea*) are fast-growing tree species of Northern Europe that overgrow on agricultural fields and melioration ditches and form a big mass of wood-waste used today mainly for burning. At the same time, in horticulture industry, agrotechnical measures (harvesting and pruning) result in a large stock of lignocellulosic biomass of fruit trees – sea buckthorn (*Hippopae rhamnoides* L.), and aronia (*Aronia melanocarpa)* (Janceva et al., 2022a). Both of these biomass types – of non-fruit and fruit trees origin – at the moment are considered as lignocellulosic waste but could become an interchangeable raw material for PACs production. There are many studies on the fruit-trees reproductive part PACs and pomaces, showing a major contribution of PACs and flavan-3-ols to the antioxidative activities of the berries, but the research of the vegetative part of fruit shrubs is very limited, although some studies confirm that PACs extracted from twigs have a similar effect (Denev et al., 2019; Tian et al., 2018). Our previous research showed that PACs from lignocellulosic biomass of sea buckthorn have no cytotoxic effect within the frames of their antimicrobial and anti-inflammatory effect concentrations (Andersone et al., 2023; Janceva et al., 2022a). A range of clinical trials is available for PACs activity ("Puredia CyanthOx™30 Clinical Trial," n.d.; Sengupta et al., 2011), although their effect has to be further investigated on humans.

The biomass source, its treatment, and the degree of purification influence biological activity (Coşarcă et al., 2019; Fierascu et al., 2019; McElroy et al., 2018). For the extraction, ethanol/water solution is preferable as GRAS (Generally Recognized As Safe) solvent. For comparison, all biomasses have to be extracted at the same conditions.

The purpose of this study was a comparative analysis of the chemical composition and biological activity of extracts and PACs isolated from lignocellulosic biomass obtained as a harvesting and pruning waste of fruit trees and forest cleaning non-fruit trees/shrubs of Northern Europe: sea buckthorn - *Hippophae rhamnoides* L., black chokeberry - *Aronia melanocarpa*, as well as grey alder - *Alnus incana*, black alder - *Alnus glutinosa*, and willow - *Salix caprea*. Two groups of microbials were used for this research – from Microbial Strain Collection of Latvia (MSCL) University of Latvia and from clinical/human origin isolates. Such innovative study will not only allow to choose the most prospective types of PACs or their mixes for antibacterial and antioxidant treatment, but also will create a basis for the choice of the raw material for PACs production to be more flexible, depending on the availability of the different waste biomass or its mixtures from farmers and foresters. Lignocellulosic biomass waste doesn't compete for land with food PACs sources, and all mentioned trees are all native for Europe. The results of this study will allow farmers to increase the sustainability of fruit production, will contribute to waste-free agriculture and forestry in Europe and other countries, and create possibility for making value-added products on the basis of available biomass.

2. Materials and methods

2.1. Materials

2.1.1. Forest and ditch cleaning waste

Undergrowth (2–7 years old trees) obtained as a result of forest cleaning measures from the grey alder (*Alnus incana* (L.) Moench, further in the text – *Alnus incana*) and black alder (*Alnus glutinosa* (L.) Gaertn, further in the text – *Alnus glutinosa*) were collected in a forest of Baldone parish, Kekava county of Latvia (Decimal degrees (DD): 56.82065, 24.27653). Trees were cut from an area of about 5 ha, in September 2021.

Willow (*Salix caprea*) young trees were obtained from ditch cleaning of agricultural fields of Baldone parish, Kekava county of Latvia (DD: 56.77306/24.30162), in September 2021.

The biomass was dried at room temperature, leaves were removed, twigs and stem biomass was ground with a mill (Retsch SM100, RETSCH, Haan, Germany), where size reduction takes place by cutting and shearing forces, and sieved to select the particles between 2 and 4 mm. The biomass samples were stored at −8 °C.

2.1.2. Sea buckthorn plant material

Lignocellulosic residues after harvesting berries and pruning plant material of the four years old sea buckthorn fruit tree-shrubs (family Elaeagnaceae, *Hippopae rhamnoides* L. cultivar 'Maria Bruvele', further in the text – *Hippopae rhamnoides* L.) were collected from sea buckthorn plantation area in Seme parish, Tukums county of Latvia (DD: 57.1444093, 23.108156), in autumn 2021. The plant material was dried at room temperature and ground with a mill (Cutting Mill SM100, Retsch, Haan, Germany). The particle size of the ground *Hippopae rhamnoides* L. biomass was between 1 and 4 mm. The biomass samples were stored at −8 °C.

2.1.3. Aronia twigs

The stems with twigs of 7 years old *Aronia melanocarpa* 'Mulatka' shrubs (further in the text – *Aronia melanocarpa)*) were collected in the early beginning of autumn 2021, from Baldone parish, Kekava county of Latvia (DD: 56.77306/24.30162), after berries were harvested. The twigs were dried at room temperature and ground with a mill (Cutting Mill SM100, Retsch, Haan, Germany). The particle size of the ground twigs was between 1 and 4 mm. The twigs biomass samples were stored at −8 °C. Forest and ditch cleaning waste, as well as aronia and sea buckthorn twigs/stems, are referred to as "biomass" further in the text.

2.2. Chemicals

The solvents (high purity), DPPH· (2,2-diphenyl-1-picrylhydrazyl), ABTS+· (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), reference antioxidants Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), analytical standards procyanidin B2, $FeNH_4(SO_4)_2$•$12H_2O$, n-butanol (purity ≥99.4%), and crosslinked dextran-based resin Sephadex LH-20 were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All chemicals were analytical-reagent grade.

2.3. Microbial strains

The clinical isolates of bacteria and fungus were isolated from pus, sputum, and urine samples, and identified with the VITEK2 system (bioMérieux, France). To confirm bacterial resistance disc diffusion method according to the European Committee on antimicrobial susceptibility testing (EUCAST) was used. For all the experiments four clinical isolates - Methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamases producing *Escherichia coli* (ESBL), *Pseudomonas aeruginosa* and *Candida albicans* were used. All clinical isolates were obtained with approval from the Ethics Committee of Riga Stradiņš University (25.10.2022) (No. 4/462/2022 and No. 4/465/2022). All patients signed an informed consent form.

The several reference microbial strains, received from the Microbial Strain Collection of Latvia (MSCL), University of Latvia, were used: *Pseudomonas aeruginosa* MSCL 331, *Staphylococcus aureus* MSCL 334, *Escherichia coli* MSCL 332, *Bacillus cereus* MSCL 330, and *Candida albicans* MSCL 378.

2.4. Methods

2.4.1. Proanthocyanidins-rich extract isolation from biomass

The PACs-rich extracts were obtained from biomass by reflux extraction with ethanol (EtOH)-distilled water solution at 60 °C for 60 min. The extracts were freeze-dried using lyophilization equipment Heto Power Dry HS3000 (Thermo Fisher Scientific, Waltham, MA, USA) to yield a dry weight (DW) extract. The yield of the extracts is presented as a percentage based on DW. The PACs-rich extract was stored at −8 °C.

2.4.2. Purification of proanthocyanidins

The purification of PACs from non-PACs compounds was carried out using a crosslinked dextran-based solvent-resistant resin Sephadex LH-20 column with 96% EtOH and 70% (v/v) acetone as the purification solvents. In the purification process, low-molecular-weight phenolics were eluted with 96% EtOH, and the PACs were eluted with 70% (v/v) acetone. Purified PACs were evaporated using a rotary evaporator (Heidolph Instruments, Schwabach, Germany) prior to being freeze-dried using lyophilization equipment Heto Power Dry HS3000 (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −8 °C.

2.4.3. UHPLC-ESI-MS/MS qualitative analysis

The identification of compounds was performed by an Acquity UPLC system (Waters Corp., Milford, MA, USA) coupled with a quadrupole-time of flight (Q-TOF) MS instrument (UPLC/Synapt Q-TOF MS, Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) source. The UHPLC separation was carried out using a Waters Acquity BEHC18 column (2.1 mm × 50 mm i.d., 1.7 μm). The analytical standards of Procyanidin B2 (90%, HPLC grade) and catechin (98%, HPLC grade) were used for confirmation of the suitability of the UHPLC-ESI-MS/MS method for identification of proanthocyanidins.

The mobile phase consisted of 0.1% formic acid, water (A), and acetonitrile (B), with a flow rate of 0.35 mL/min under the gradient program of 5%–20% (B) for an initial 1 min, 20% - 25% (B); 5–6 min, 25% - 75% (B), 6–7 min, 75% - 80% (B), 7–8 min, 80% - 5% (B), 8–10 min, 5% (B), the injection volume was 2.0 μL.

Mass spectrometric analysis was operated in negative and positive ion mode and the full scan mass spectral data were collected over a range from *m/z* 50 to 1200. The optimum source parameters were as follows: capillary voltage, 2.5 kV (−); cone voltage, 60 V; cone gas flow, 50 L/h; collision energy, 6 eV; source temperature, 120 °C; desolvation temperature, 350 °C; collision gas, argon; desolvation gas, nitrogen; flow rate, 500 L/h.

2.4.4. Determination of proanthocyanidins content in the sample

PACs content in the extracts and purified PACs samples was measured by oxidative depolymerization to anthocyanidins in acid butanol (butanol–HCl method) (Andersone et al., 2023) using procyanidin dimer B2 as a reference compound. Amounts of 6 mL of

acid butanol (5% (v/v) concentrated HCl in *n*-butanol) and 0.2 mL of iron reagent (w/v) ($FeNH_4(SO_4)_2$•$12H_2O$ in 2 M HCl) were added to 1 mL of the extract aliquots whilst stirring the tube without heating and allowing it to be heated in a water bath at 80 °C for 50 min. After 50 min, the absorbance of the mixture was measured against a blank solution at 550 nm using a UV/VIS spectrometer Lambda 650 (PerkinElmer, Inc., Waltham, MA, USA).

Each extract was analyzed in triplicate, and assay results were expressed as a percentage per dry extract (DM). The results' confidence interval (CI) did not exceed 3% at $\alpha = 0.05$.

2.4.5. FTIR analysis

FTIR spectra were recorded in KBr pellets (1.6 mg of the sample in 200 mg of KBr (IR grade, Sigma Aldrich), in the range of 440 0-400 cm^{-1} (resolution of 4 cm^{-1}, 32 scans), using a Nicolet iS50 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA. Spectrum v5.0.1 program was used for processing the spectrum.

2.4.6. LC-DAD-ESI-MS/MS analysis of purified proanthocyanidins samples

Purified PACs were dissolved in aqueous methanol (v/v 20:80) with an approximate concentration of 0.1 mg/mL and filtered and then used for MS/MS experiments. The MS spectra of PACs were recorded with a Waters Acquity UPLC HClass with PDA detector and Micromass QuattroMicro Mass spectrometer (Waters Corp., Milford, MA, USA) using Acquity UPLC BEH Amide column (1.7 μm, 3.0 × 100 mm). The mass spectrometer was operated in negative ion electrospray ionization mode with −40 V cone voltage using direct infusion. The source and desolvation temperatures were set at 130 and 300 °C, respectively, and the cone and nitrogen gas were employed at flow rates of 96 and 395 L/h. The mobile phase consisted of 0.1% formic acid, water (A), and acetonitrile (B), the gradient program of 85–85% (B) for an 0.3 min, 60–60% (B); 15–17 min, 25–75% (B), 6–7 min, 85–85% (B), 17.5–20 min, the injection volume was 10.0 μL.

2.4.7. Determination of antioxidant activity: DPPH· (2,2-diphenyl-1-picrylhydrazyl radical) assay

Extracts and purified PACs were tested for their radical scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl (DPPH·) using UV/VIS spectrometer Lambda 650 (PerkinElmer, Shelton, CT, USA). The DPPH· assay was measured according to the procedures described by Dizhbite (2004). A range of different concentrations of the obtained dried hydrophilic extracts (Section 2.4) in DMSO was prepared. The absorbance at 515 nm was measured 15 min after the mixing of 30 μL of extract (or antioxidant standard) with 3.0 mL DPPH· ($1{\cdot}10^{-4}$ mol L^{-1}) solution. DMSO was used as a control and Trolox as a reference antioxidant standard.

The measurements were done in triplicate. The free radical scavenging activity is expressed as the concentration of antioxidant, mg•L^{-1}, required for a 50% inhibition of the free radicals (IC_{50}). DPPH· inhibition (decrease in absorbance at 515 nm) was calculated according to Eq. (1):

$$I = \frac{(Aa - Ab)}{Ab} \bullet 100, \quad (1)$$

where: I–DPPH· inhibition, %; A_b–absorbance of DPPH· solution without sample or a reference antioxidant standard after 15 min; A_a–absorbance of DPPH· solution with sample or a reference antioxidant standard after 15 min. CI ≤ 0.3 mg L^{-1}.

2.4.8. Determination of antioxidant activity: $ABTS^{+\cdot}$ (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assay

$ABTS^{+\cdot}$ was produced by reaction of 2 mmol L^{-1} ABTS stock solution with 70 mmol L^{-1} potassium persulfate ($K_2S_2O_8$) allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{+}$ solution (stable for 2 days) was diluted with phosphate-buffered saline (pH 7.4) to an absorbance of 0.80 ± 0.02 at 734 nm. The absorbance at 734 nm was investigated 10 min after the mixing of 30 μL of extract (or purified PACs and antioxidant standard – Trolox) was diluted in DMSO of five different concentrations with 3.0 mL $ABTS^{+\cdot}$ solution, using a UV/VIS spectrometer Lambda 650 (PerkinElmer, Shelton, CT, USA). DMSO was used as a control and Trolox as the antioxidant standard. $ABTS^{+\cdot}$ inhibition (decrease in absorbance at 745 nm) was calculated according to Eq. (2):

$$I = \frac{(Aa - Ab)}{Ab} \bullet 100, \quad (2)$$

where: I–$ABTS^{+\cdot}$ inhibition, %; A_b–absorbance of $ABTS^{+\cdot}$ solution without sample or a reference antioxidant standard after 10 min; A_a–absorbance of $ABTS^{+\cdot}$ solution with sample or a reference antioxidant standard after 10 min. CI ≤ 0.3 mg L^{-1}.

2.4.9. Determination of the antimicrobial activity

Minimum inhibitory concentration (MIC) determination by microtitre broth dilution method was used. Stock solutions of the respective plant extracts were prepared in 10 mL microcentrifuge tubes by dissolving dry plant extract in dimethylsulphoxide (DMSO) to a final concentration of 100 mg/mL. The serial dilutions from the stock solution were made ranging from 50 mg/mL to 0.01 mg/mL using Mueller–Hinton broth (Becton Dickinson, Sparks, MD, USA) for bacteria and Malt extract broth for *C. albicans* in 96-well microplates. The microbial suspension containing approximately 5×10^5 colony-forming units (CFU)/mL was prepared from a 24 h culture plate. From this suspension, 100 μL was inoculated into each well. The microtiter plates were incubated at 37 °C, 24 h. After incubation, 40 μL of a 0.4 mg/mL solution of INT was added to each well as an indicator of microbial growth. The plates were incubated for 30 min and the MIC values were determinated.

Minimum bactericidal and fungicidal concentration (MBC/MFC) was recorded as the lowest extract concentration killing 99.9% of the bacterial/fungus inocula after 24 h incubation at 37 °C. MBC/MFC was performed on all extracts and purified PACs samples.

10 μL were taken from the well obtained from the MIC experiment (MIC value) and two wells above the MIC value well and spread on Mueller–Hinton agar plates for bacteria and Malt extract agar plates for *C. albicans*. The number of colonies was counted after 18–24 h of incubation at 37 °C. The concentration of sample that produces <10 colonies was considered as MBC/MFC value.

2.4.10. Anti-biofilm assay

The effect of extracts and purified PACs on the biofilm formation of three new clinical bacterial isolates, was evaluated by using the microdilution method. For culturing the biofilm of each strain of bacteria, the freshly obtained culture was inoculated in ratio of 1:100 in 96 well microtiter plate of polypropylene material. The plate was covered and incubated at 37 °C for 24 h to culture the biofilms. All experiments were conducted in replicates of four. Crystal violet (CV) assay was performed to assess the biofilm-inhibiting activity of plant extracts. For biofilm inhibition assay the medium and planktonic cells were discarded after 24 h of static growth at 37 °C. Each well was rinsed thrice until the complete removal of all media and planktonic cells. Adhered cells were stained with 125 μL of CV (0.1%) for 30 min at room temperature. Then the CV was washed out while using deionized H_2O. The CV bound to biofilm was re-solubilized in 30% acetic acid and its absorbance was measured at 550 nm.

2.5. Statistical analysis

All measurements were conducted in triplicate, and the results are presented as the mean value. Statistical analyses were performed using Microsoft Excel 2016. Confidence intervals (CI) for a mean using Student's T distribution were calculated at a significance level of 5% ($\alpha = 0.05$).

3. Results and discussion

3.1. Proanthocyanidins content in fruit and non-fruit trees biomass

It was found that the yield of extracts from all biomass types varied from 12.1% to 19.9% on the biomass's dry weight (DW). The highest yield of extract was 19.9% on DW for *Hippophae rhamnoides* L. and 17.2% on DW for *Aronia melanocarpa* twigs. The content of PACs was from 28.2% to 71.0% on extract DW. The extract isolated from *Aronia melanocarpa* biomass has the highest content of PACs (71.0% on extract DW; 12.2% on biomass DW), followed by *Hippophae rhamnoides* L. (42.4% on extract DW; 8.4% on biomass DW) (Fig. 2).

Our earlier studies showed that PACs content in the extracts of the European deciduous trees wood and bark, which usually has the highest content of PACs in the whole tree, was not exceeding 43% (Janceva et al., 2015). Therefore, it can be seen that the fruit trees biomass is very prospective for PACs isolation due to the high content of PACs.

Since the bioactivity of PACs is generally recognized to be dependent on their structure, including the degree of polymerization (DP), the linking type of flavan-3-ol units, and the hydroxylation of constitutive units (Neilson et al., 2016; Sójka et al., 2013), the UHPLC-ESI-MS/MS analysis was done.

3.2. UHPLC-ESI-MS/MS analysis of proanthocyanidins-rich extracts

The compounds in proanthocyanidins-rich extracts were tentatively identified according to their retention times and mass behavior with those of authentic standards or literature data. The alder bark proanthocyanidins were identified according to Symma and Hensel (2022) The inter flavonoid bonds are not stable (Xu et al., 2015), and proanthocyanidins characteristically form clusters with themselves (Symma and Hensel, 2022). The cluster ions $[2M–H]^-$ (*m/z* 683, 675, 1015, 959, 955, 923, 987, 927), and $[3M–H]^-$ (*m/z* 1025) were identified in *Alnus glutinosa* and *Alnus incana* extracts.

The results showed that the PACs in non-fruit biomass extracts composition were identified as a complex of B-type of procyanidin dimers, trimers, and tetramers. A-type PACs in extracts composition were not detected. Among the identified compounds in the hydrophilic extracts (Table 1), coumaryl quinic acid, procyanidins monomers (catechin, epicatechin), hirsutanolol, oregonin, hydroxyoregonin, and other compounds were also found, in addition to PACs. The presence of oregonin and hydroxyoregonin was not detected in *Salix caprea*, they were presented only in *Alnus* species. The composition of the *Salix caprea* extract differed from other extracts by the presence of salicylic acid derivatives, procyanidin trimer digallate, epigallocatechin units complex epigallocatechin-epigallocatechin-epicatechin and epigallocatechin-epigallocatechin-epigallocatechin-epicatechin, indicating to the presence of prodelphinidin in the PACs composition, catechin-pentoside, isolariciresinol-pentoside, etc.

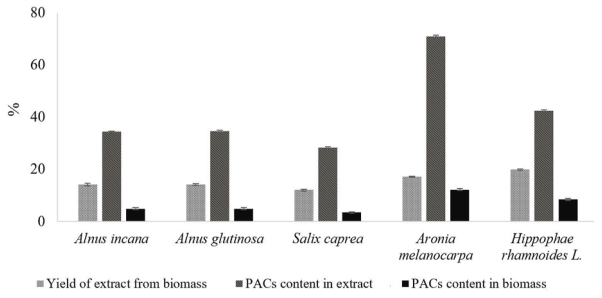

Fig. 2. Yield of the extracts and PACs from non-fruit and fruit trees biomass. The data is presented with CI for a mean at $\alpha = 0.05$.

Table 1
The chemical compounds in the extracts from non-fruit trees biomass identified by UHPLC-ESI-MS/MS.

Extract' raw material	t^R (min)	$[M-H]^-$ (*m/z*)	MS/MS ions (*m/z*)	Identified compounds
Alnus glutinosa	2.41	337.0961	163.0; 191.1; 675.2	Coumaryl quinic acid
	2.45	865.1918	577.2; 451.9; 305,1	Epicatechin-epicatechin-epicatechin
	2.64	865.1929	577.1; 289.1	Epicatechin-epicatechin-epicatechin
	2.83	431.1924	289.1; 245.1	Catechin derivative
	2.96	865.1943	509.2; 403.2; 289.1	Epicatechin-epicatechin-epicatechin
	4.07	507.1870	327.1; 1015.2	Hirsutenone hexoside
	4.31	479.1908	959.0	Hydroxyoregonin
	4.61	477.1773	327.1; 955.4	Oregonin
	5.18	345.1362	311.1; 179.1	Hirsutanolol
	5.83	461.1783	311.1; 923.4	Aceroside VII or 1-(4-Hydroxyphenyl)-7-(3.4-dihydroxyphenyl)-heptan-3-one-5-O-
	5.97	461.1777		pentoside
	6.34	625.2516	493.2; 477.2; 463.1; 327.1	1.7-Bis-(3.4-dihydroxyphenyl)-3-heptanyl 3-O-β-D-glucopyranosyl-β-D-xylopyranoside or rubranoside C
	6.45	493.2060	295.1; 987.3	Rubranoside A
	6.54	463.1997	331.2; 927.3	Rubranol xyloside
	6.73	327, 1165	245.9	Hirsutenon
Alnus incana	0.42	341.1123	683.2; 1025.2	3-glucopyranosyloxy-1-(4-hydroxyphenyl)- butanone
	2.33	1153.2507	865.2; 577.2; 289.1	Epicatechin-epicatechin-epicatechin-epicatechin
	2.44	337.0965	163.1; 191.0; 675.2	Coumaryl quinic acid
	2.51	865.2003	577.2; 451.9; 305.1	Epicatechin-epicatechin-epicatechin
	2.68	577.1353	451.8; 405.8; 289.1	Epicatechin-epicatechin
	2.90	289.0749	245.1	Catechin/Epicatechin
	3.04	1153.2540	865.2; 289.1	Epicatechin-epicatechin-epicatechin-epicatechin
	4.29	507.1867	327.1; 1015.4	Hirsutenone hexoside
	4.57	479.1921	959.2	Hydroxyoregonin
	4.88	477.1768	327.1; 955.3	Oregonin
	5.42	345.1360	311.1; 179.2	Hirsutanolol
	6.15	461.1776	311.1; 923.2	Aceroside VII or 1-(4-Hydroxyphenyl)-7-(3.4-dihydroxyphenyl)-heptan-3-one-5-O-
	6.26	461.1783		pentoside
	6.42	625.2496	493.2; 477.1; 463.1; 327.0	1.7-Bis-(3.4-dihydroxyphenyl)-3-heptanyl 3-O-β-D-glucopyranosyl-β-D-xylopyranoside or rubranoside C
	6.50	493.2060	295.2; 987.2	Rubranoside A
	6.58	463.1977	331.2; 927.3	Rubranol xyloside
	6.66	593.2615	477.2; 1187.3	(3R)-1.7-Bis-(4- dihydroxyphenyl)- 3-heptanol 3-O-β-D-glucopyranosyl-(1 → 3)-β -D-xylopyranoside
	6.75	327.1169	245.2	Hirsutenon
Salix caprea	0.56	191.0614	111.1	Quinic acid
	1.59	331.0681	315.1; 169.0; 153.0	Gallic acid glucoside
	1.59	331.0681	315.1; 169.0; 153.0	Dihydroxybenzoic acid glucoside
	2.64	1201.0157	897.1; 593.0; 305.1	Epigallocatechin-epigallocatechin-epigallocatechin-epicatechin
	2.70	421.1021	289.1; 245.1; 203.3; 843.5	Catechin pentoside
	2.71	897.1869	305.1; 593.1; 1185.4	Epigallocatechin-epigallocatechin-epicatechin
	2.80	299.1209	137.1; 119.1; 113.3; 93.0	Salicylic acid sugar derivative
	2.81	337.0957	191.0; 163.1; 119.1; 93.0	Coumaroyl-quinic acid
	2.88	289.1209	245.1; 205.3	Catechin
	2.92	431.0986	137.0; 119.1; 93.0	Salicylic acid derivative
	2.93	881.4042	135.1; 343.0; 395.1; 441.2; 577.1	Epigallocatechin-epicatechin-epicatechin
	3.00	1169.1404	303.2; 577.1; 897.7	Procyanidin trimer digallate
	3.13	675.4169	525.2; 431.2; 289.1; 245.1	Catechin derivative
	3.18	491.1926	293.1	Isolariciresinol-pentoside isomer

For fruit trees, the UHPLC-ESI-MS/MS analysis results showed that the predominant compounds in the biomass extracts from *Hippophae rhamnoides* L. and *Aronia melanocarpa* were B-type procyanidin dimers, trimers, and tetramers. Among the identified compounds in the extracts, quinic acid, serotonin (only in *Hippophae rhamnoides* L. extract), procyanidins monomers (catechin, epicatechin), quercetin, gallocatechin or its isomer epigallocatechin, and other compounds were also found, in addition to PACs (Table 2).

The qualitative UHPLC-ESI-MS/MS analysis showed that non-fruit trees biomass extracts contained a wider range of the extractive compounds than the one of the fruit-trees. Fruit-trees and *Alnus incana* biomass extracts contained B-type procyanidin tetramers in their composition, for *Alnus glutinosa* and *Salix* the maximum degree of polymerization (DP) corresponded to trimers. Therefore, PACs, in the extracts of *Alnus glutinosa* and *Salix,* could possibly be less active than the other species under study.

For further research, PACs-rich extracts were purified from non-PACs compounds using Sephadex LH-20 as described in 2.4.2. Section. After PACs purification, the PACs content in the samples increased by 1.3–3.3 times and was 0.92–0.96 g/g DW according to the Butanol-HCl assay results.

Table 2
The chemical compounds in the extracts from fruit trees biomass identified by UHPLC-ESI-MS/MS.

Extract' raw material	t^R (min)	$[M-H]^-$ (*m/z*)	MS/MS ions (*m/z*)	Identified compounds
Aronia melanocarpa	1.43	289.0732	245.1; 205.1	Catechin derivative
	1.51	1153.2642	865.2; 577.2; 289.1	Epicatechin-epicatechin-epicatechin-epicatechin
	1.81	593.2190	425.1; 407.2; 305.3; 289.1	Gallocatechin-(4α-8)-catechin
	1.97	865.2002	577.2; 289.1	Epicatechin-epicatechin-epicatechin
	2.09	305.2608	303.1; 179.1; 125.2	Epigallocatechin
	2.12	577.1353	289.1; 245.1	Epicatechin-epicatechin
	2.19	1153.2526	865.1; 577.2; 289.1	Epicatechin-epicatechin-epicatechin-epicatechin
	2.32	1153.2608	865.2; 577.1; 289.1	Epicatechin-epicatechin-epicatechin-epicatechin
	2.45	865.2036	577.2; 289.1	Epicatechin-epicatechin-epicatechin
Hippophae rhamnoides L.	0.41	341.1094	179.1; 161.0; 143.0; 119.0; 113.0; 101.0	Sucrose
	0.41	341.1094	179.1; 161.0; 143.0; 119.0; 113.0; 101.0	Fructose
	0.41	341.1094	179.1; 161.0; 143.0; 119.0; 113.0; 101.0	Glucose
	0.47	191.0560	111.0; 173.1; 127.0; 85.0	Quinic acid
	0.98	175.0869	159.1; 147.0	Serotonin
	1.84	305.0668	179.0; 125.1	Gallocatechin or its isomer epigallocatechin
	1.89	593.1306	407.1; 425.1; 305.1; 467.2; 289.1	Epicatechin-epigallocatechin
	1.97	1185.2532	881.2; 593.1; 305.1; 289.1; 245.0	Epicatechin-epicatechin-epigallocatechin-epigallocatechin
	2.06	1055.2736	881.1; 593.0;305.1; 289.1	Galloylated procyanidin tetramer
	2.30	865.1998	577.2; 289.1; 245.1	Epicatechin-epicatechin-epicatechin
	2.38	289.0721	245.1; 125.0	Catechin/Epicatechin
	2.50	1153.2622	865.2; 577.2; 289.1; 245.0	Epicatechin-epicatechin-epicatechin-epicatechin
	3.28	609.4180	301.1; 271.0	Quercetin-3-O-rutinoside
	3.33	301.0921	286.1; 109.0	Quercetin
	7.14	487.3412	293.1; 117.0	Triterpenoid
	7.79	471.3461	452.2; 265.0; 117.1	Triterpenoid
	7.86	471.3415	265.1; 117.1	Triterpenoid
	8.07	455.3518	277.2; 117.1	Triterpenoid
	8.01	617.3837	255.2; 117.0	Acylated triterpenoid

3.3. Purified PACs characterization

3.3.1. FTIR analysis

A comparative analysis of FTIR spectra of the extracts and PACs was carried out. The extended band in the range of 3400–3200 cm^{-1} indicates the presence of –OH groups in the samples. The low-intensity peaks at 2950 and 2880 cm^{-1} indicate –C-H group stretching vibrations. Aromatic structures show absorptions in the regions 1600–1585 cm^{-1} and 1500–1400 cm^{-1} due to C–C stretching vibrations in the aromatic ring. Around 1200 cm^{-1}, another middle-intensity peak appears, which is a strong clue of polyphenol C–O group in PACs structure. It was shown that purification of PACs from non-PACs compounds significantly reduced the number of carbohydrates and low molecular weight acylated polyphenols (3400–3200 cm^{-1}; 2950 cm^{-1}; 1100–950 cm^{-1}). A weak peak at 1700 cm^{-1}, on the spectra of extracts and purified PACs, is characteristic for the carbonyl group, which may be due to the presence of a gallic acid residue in the extract and PACs (Fig. 3).

The FTIR spectra of purified PACs samples showed similarity with the reference compound B2 procyanidin spectrum (Fig. 4).

The FTIR spectra of PACs samples contain all the absorption bands characteristic for B2 procyanidin standard.

Generally, procyanidins-type oligomers show a single peak at 1540–1520 cm^{-1} in the FTIR spectra, whereas that of the prodelphinidins type shows a double peak (Sun, 2019). Based on the single peak at 1521 cm^{-1} it can be assumed that PACs samples are composed of procyanidin units or are the dominant components in their composition. The bands at 804, 798, and 795 cm^{-1} and at 1049, 1047, and 1045 cm^{-1} were assigned to the C–H and C–C stretching vibrations respectively. The bands at 822 cm^{-1} were due to the R_1

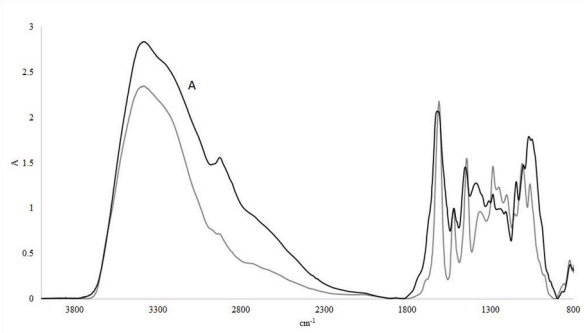

Fig. 3. FTIR spectra of *Aronia melanocarpa* samples: black line (A) – extract, grey line – purified PACs.

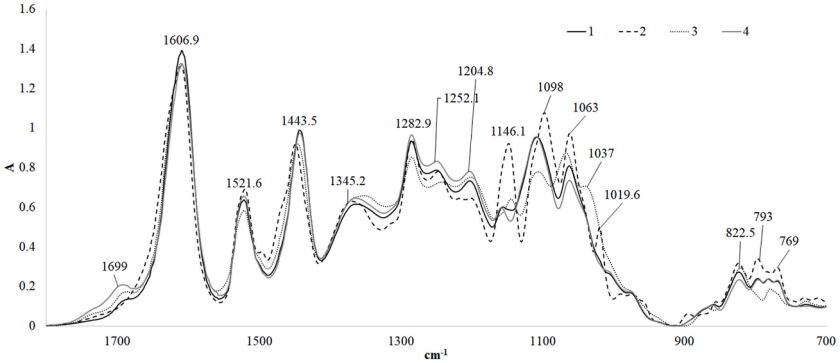

Fig. 4. FTIR spectra of purified PACs from fruit and non-fruit trees biomass: 1–*Hippophae rhamnoides* L.; 2–B2 procyanidin standard; 3–*Alnus glutinosa*; 4–*Salix caprea.*

and R_2 substituted aromatic structure (Fig. 5). The absorbance band at 1699 cm^{-1} indicates that a carbonyl group (C = O) is present in PACs samples, indicating to an impurity in the quinoid form.

The peak in the region of 1493 cm^{-1} is more pronounced for B2 procyanidin standard, but deconvolution of the spectra of PACs confirmed its presence in the analyzed samples.

3.3.2. LC-DAD-ESI-MS/MS analysis

In recent years, LC-DAD-ESI-MS/MS has been increasingly used to determine the composition of PACs. The advantage of LC-DAD-ESI-MS/MS is quick and accurate measure of the molecular weight, producing fewer fragment ions for polymer analysis without the necessity for reference standards. Nevertheless, it is still a problem that ESI–MS signal intensity and detectability of PACs are decreasing with increasing DP: a phenomenon that especially limits identification of PACs which are only present at lower concentrations in a mixture (Symma and Hensel, 2022). Therefore, in this study, the oligomeric PACs up to DP5 can be detected and analyzed. The LC-DAD-ESI-MS/MS experiments in the negative ion mode showed the presence of intense $[M–H]^-$ ion peaks corresponding to B-type procyanidins dimer (DP2, *m/z* 577), trimer (DP3, *m/z* 865), tetramer (DP4, *m/z* 1153), and pentamer (DP5 *m/z* 1441). In literature data it was noted that two flavan-3-ol monomers - catechin (C) and epicatechin (EC) showed fragmentation ions *m/z* 245, *m/z* 205, *m/z* 179, and *m/z* 125, respectively (Lin et al., 2014). The B-type procyanidin trimer of *m/z* 577 produce ions at *m/z* 451, 425, 407 and 289 (Rockenbach et al., 2012). For PACs with DP ≥ 3, further fragmentation can occur from repeated quinone methide (QM) breaks of interflavan bonds connecting the flavan-3-ols of the extension units (Fig. 6).

Tetramers and pentamers with doubly charged ions were detected, whose fragmentation gave rise to ions at *m/z* 1027, 865, 863, 739, 451, 407, 289, and 287 (Saéz and Baer, 2019).

A structurally significant fragmentation pathway for deprotonated procyanidins is heterocyclic ring fission which results in the elimination of 1,3,5-trihydroxybenzene, $[M–H–126]^-$ (Rush et al., 2018). The typical PACs fingerprints were obtained by selected monitoring with the following transitions: PC extension unit (*m/z* 287 → 125), procyanidin terminal units (*m/z* 289 → 245), prodelphinidin extension units (*m/z* 303 → 125), and prodelphinidin terminal units (*m/z* 305 → 125), galloylated procyanidin (729 → 289) (Engström et al., 2014).

Identification of the composition of PACs isolated from *Aronia melanocarpa* biomass was studied for the first time. The isolated and purified PACs from *Aronia melanocarpa* extract were a complex of procyanidin oligomers consisting of A and B-type of catechin dimers (Fig. 1), trimers, B-type of catechin tetramers, and pentamers (Fig. 7, Table 3). Thus, it can be seen that A-type procyanidin dimer and trimer and B-type pentamers which were not detected in the extract by UHPLC-ESI-MS/MS (Table 2), present in the PACs composi-

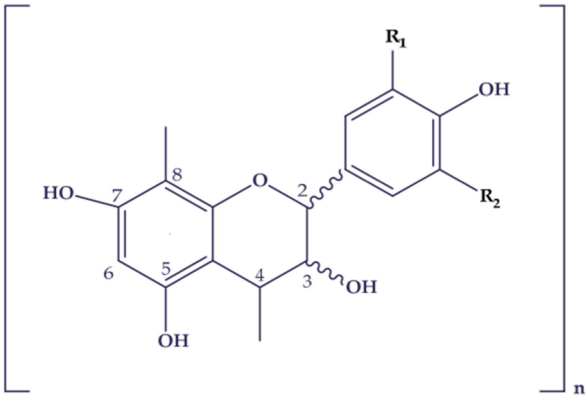

Fig. 5. Structures of the flavan-3-ol units in PACs: Procyanidins R_1 = H, R_2 = OH; Prodelphinidins R_1 = OH, R_2 = OH.

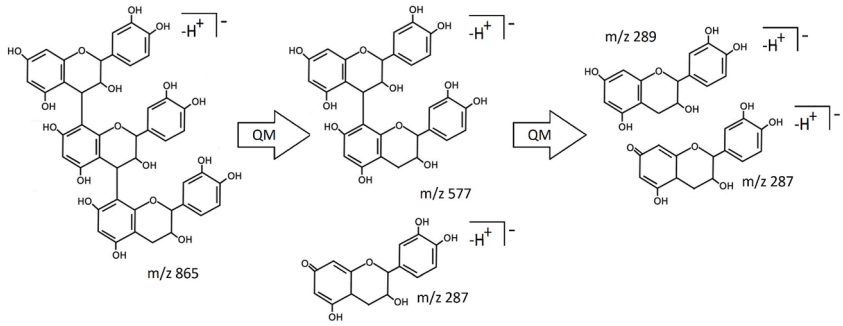

Fig. 6. PACs trimer fragmentation by quinone methide (QM) breaks of interflavan bonds.

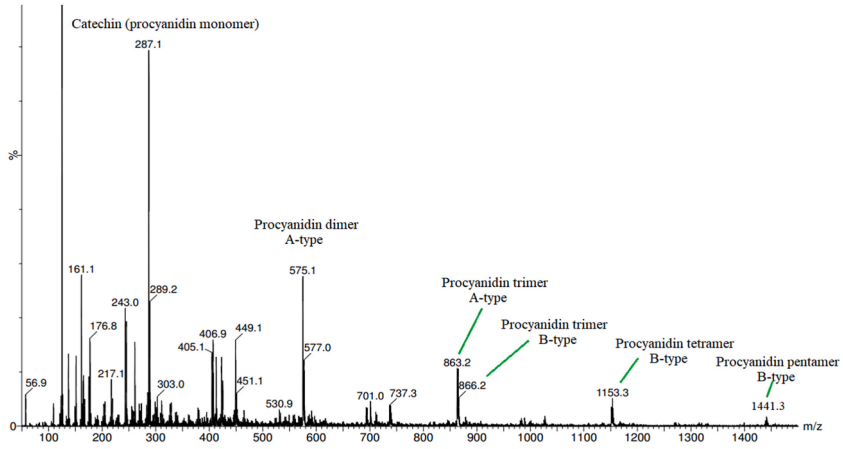

Fig. 7. Average MS spectrum of PACs from *Aronia melanocarpa*.

Table 3
Profile of PACs from fruit trees biomass identified by LC-DAD-ESI-MS/MS analysis.

PACs	$[M-H]^-$ (*m/z*)	Fragments (*m/z*)	Identified compounds
Aronia melanocarpa	289.2/287.1	245; 205; 179; 125	Catechin/epicatechin (procyanidin monomer)
	575.1	449; 423; 289	Procyanidin A-type dimer (catechin-catechin)
	577.0	559; 451; 425; 289	Procyanidin B-type dimer (catechin-catechin)
	863.2	737; 711; 591; 575	Procyanidin A-type trimer (catechin-catechin-catechin)
	865.2	695; 577; 425; 407	Procyanidin B-type trimer (catechin-catechin-catechin)
	1153.3	983; 865; 577	Procyanidin B-type tetramer (catechin-catechin-catechin-catechin)
	1441.3	1153; 865; 575	Procyanidin B-type pentamer (catechin-catechin-catechin-catechin-catechin)
Hippophae rhamnoides L.	289.2/287.1	245; 205; 179; 125	Catechin/epicatechin (procyanidin monomer)
	305.1	221, 219, 179	Gallocatechin
	575.1	449; 423; 289	Procyanidin A-type dimer (catechin-catechin)
	577.0	559; 451; 425; 289	Procyanidin B-type dimer (catechin-catechin)
	593.1	441; 467; 305	Catechin-gallocatechin
	863.2	737; 711; 591; 575	Procyanidin A-type trimer (catechin-catechin-catechin)
	865.2	695; 577; 425; 407	Procyanidin B-type trimer (catechin-catechin-catechin)
	881.1	729; 407; 711	Epicatechin-catechin-gallocatechin

tion. Probably it could be explained by bigger relative content of them in the purified PACs that allowed them to be detected. The LC-DAD-ESI-MS/MS spectra of purified PACs samples from *Hippophae rhamnoides* L., *Aronia melanocarpa*, *Alnus glutinosa, Salix caprea,* and *Alnus incana* are shown in Figs. 7–11.

The signal of tetramer on the LC-DAD-ESI-MS/MS spectra in the composition of *Hippophae rhamnoides* L. was very weak (1153.30 *m/z*), but it was detected by UHPLC-ESI-MS/MS, therefore, most probably it is present in the composition.

The PACs composition of *Hippophae rhamnoides* L. differed from *Aronia melanocarpa* by the presence of additional polyphenols units such as gallocatechin, catechin-epigallocatechin/gallocatechin and catechin-catechin-gallocatechin.

It can be also seen from LC-DAD-ESI-MS/MS spectra that *Hippophae Rhamnoides* L. PACs, separated in the same conditions that *Aronia melanocarpa,* have more admixtures.

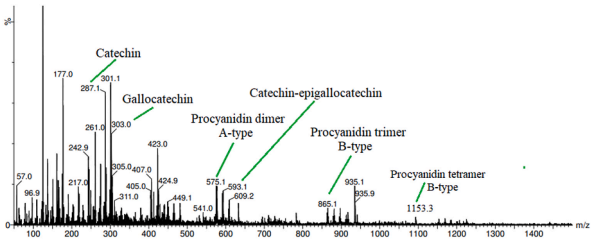

Fig. 8. Average MS spectrum of PACs from *Hippophae rhamnoides* L.

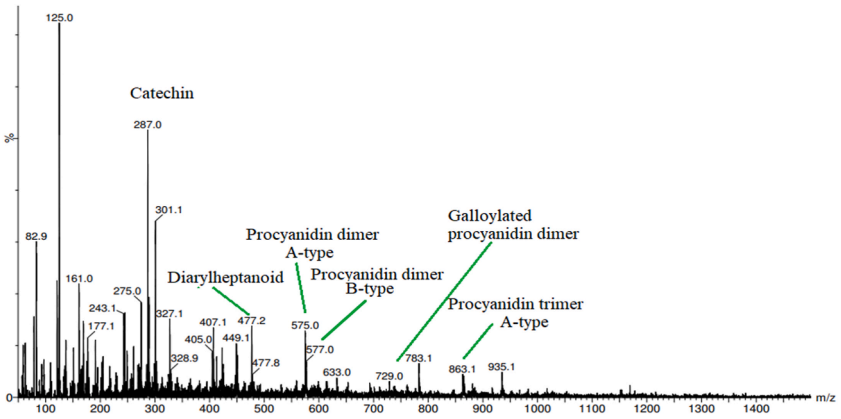

Fig. 9. Average MS spectrum of PACs from *Alnus glutinosa.*

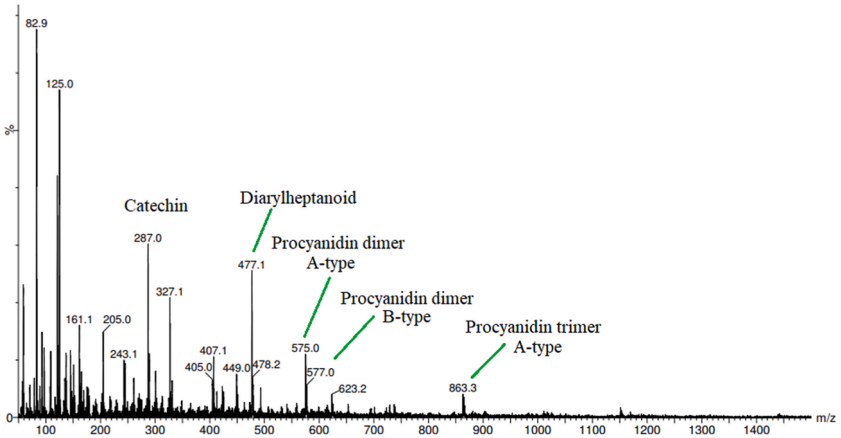

Fig. 10. Average MS spectrum of PACs from *Alnus incana.*

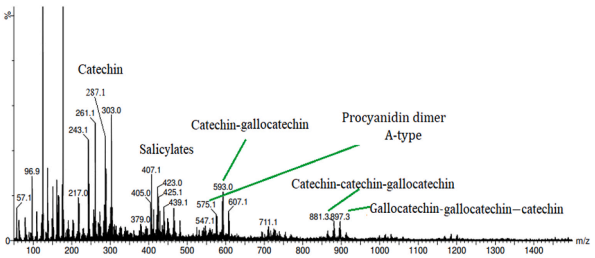

Fig. 11. Average MS spectrum of PACs from *Salix Caprea.*

The *Alnus* species PACs (Figs. 9 and 10) consisted of A and B-type procyanidins dimers, galloylated procyanidin dimer, and A-type procyanidin trimer. Along with procyanidins oligomers, diarylheptanoids with the identified molecular ion $[M-H]^-$ 477.1 and 477.2 were detected.

Compared to *Alnus species* PACs, the PACs sample from *Salix Caprea* was composed of oligomers not exceeding *m/z* 897 Da, including A-type procyanidin dimer, as well as dimer and trimers, consisted of gallocatechin subunits (see Table 4).

3.4. Influence of PACs content in extract on biological activity

3.4.1. Antioxidant activity

The evaluation of the antioxidant activity of PACs-rich extract and purified PACs was carried out using two recognized DPPH$^\bullet$ and ABTS$^{\bullet+}$ assays. The results of the radical scavenging activity analyses are presented in Fig. 12. Among all the extracts, *Aronia melanocarpa* and *Hippopae rhamnoides* L. extracts had the highest antioxidant activity (IC_{50} = 2.4 mg L^{-1} in ABTS$^{\bullet+}$ test and IC_{50} = 3.6 mg L^{-1} in DPPH$^\bullet$ test for *Aronia melanocarpa* and IC_{50} = 3.2 mg L^{-1} in ABTS$^{\bullet+}$ test for *Hippopae rhamnoides* L.), that is higher than the one of the synthetic antioxidant Trolox taken as the reference (IC_{50} = 4.0 mg L^{-1} in the test with ABTS$^{\bullet+}$ and IC_{50} = 4.7 mg L^{-1} in the test with DPPH$^\bullet$). It can be associated with a high content of PACs (71.0 and 42.4% on DW of extract) in the *Aronia melanocarpa* and *Hippopae rhamnoides* L. biomass. All purified PACs had higher antioxidant activity in ABTS$^{\bullet+}$ test, varied from IC_{50} = 1.2–1.4 mg L^{-1}, and in DPPH$^\bullet$ test, varied from IC_{50} = 2.1–2.6 mg L^{-1}, than Trolox reference.

Among all purified PACs, the ones from *Aronia melanocarpa* and *Hippopae rhamnoides* L. (0.98 g g^{-1} and 0.93 g g^{-1} PACs compounds content by butanol-HCl test, respectively) showed the highest antioxidant activity (IC_{50} = 1.1 mg L^{-1} and IC_{50} = 1.2 mg L^{-1} in ABTS$^{\bullet+}$ test and IC_{50} = 2.1 mg L^{-1} and IC_{50} = 2.2 mg L^{-1} in DPPH$^\bullet$ test, respectively (CI ≤ 0.1 mg L^{-1} at α = 0.05). All purified PACs had higher antioxidant activity than Trolox (Fig. 12).

These PACs' properties can be valuable not only in the creation of pharmaceuticals but also in food and cosmetics industries to slow down the oxidative processes occurring in raw materials and finished products at different stages of the technological processes and during storage.

3.4.2. Antimicrobial activity

Antimicrobial activity of non-fruit trees extracts and purified PACs is shown in Table 5.

Table 4
Profile of PACs from non-fruit trees biomass identified by LC-DAD-ESI-MS/MS analysis.

PACs	$[M-H]^-$ (*m/z*)	Fragments (*m/z*)	Identified compounds
Alnus incana, Alnus glutinosa	289.2/287.1	245; 205; 179; 125	Catechin (procyanidin monomer)
	577.0	559; 451; 425; 289	Procyanidin B-type dimer (catechin-catechin)
	575.1	449; 423; 289	Procyanidin A-type dimer (catechin-catechin)
	729.2	577; 407; 559	Galloylated procyanidin dimer
	863.2	737; 711; 591; 575	Procyanidin A-type trimer (catechin-catechin-catechin)
Salix Caprea	289.2/287.1	245; 205; 179; 125	Catechin
	305.1	221; 219; 179	Gallocatechin
	575.1	449; 423; 289	Procyanidin A-type dimer (catechin-catechin)
	593.1	441; 467; 305	Catechin-gallocatechin
	881.1	729; 407; 711	Catechin-catechin-gallocatechin
	897.3	593; 305	Gallocatechin-gallocatechin-catechin

LC-DAD-ESI-MS/MS analysis showed that trimers and dimers were presented in the composition of all PACs. However, since *Aronia melanocarpa* has pentamers detected, and *Hippophae rhamnoides* L. has tetramers confirmed by LC-DAD-ESI-MS/MS, it could be proposed that they will have the highest biological activity.

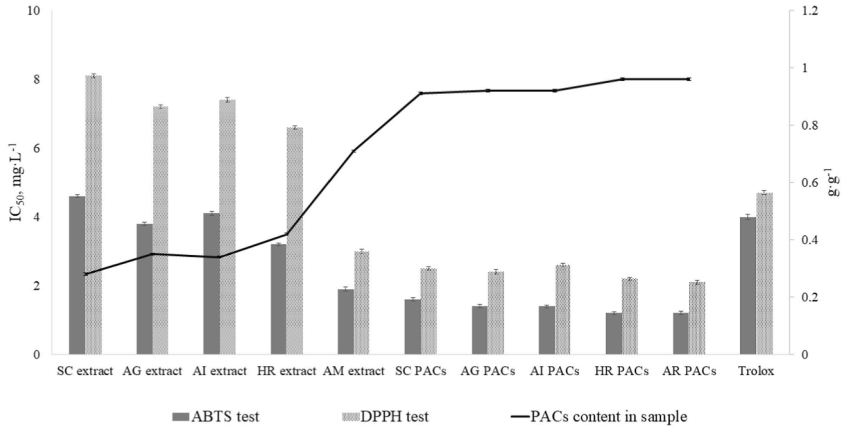

Fig. 12. Antioxidant activity of the biomass extracts and purified PACs: SC–*Salix caprea*, AG–*Alnus glutinosa*, AI–*Alnus incana*, HR–*Hippophae rhamnoides* L., AM–*Aronia melanocarpa*. The data is presented with CI for a mean at α = 0.05.

Table 5
Antimicrobial activity of PACs-rich samples from non-fruit trees.

Microorganisms	PACs from *Alnus incana*	Extract from *Alnus incana*	PACs from *Alnus glutinosa*	Extract from *Alnus glutinosa*	PACs from Salix caprea	Extract from Salix caprea
	MIC/MBC or MFC, mg·mL^{-1}					
E. coli	0.08/0.31	0.39/0.78	0.08/0.31	0.39/0.39	0.04/0.16	0.10/0.20
P. aeruginosa	0.16/1.25	0.78/>50	0.16/1.25	3.13/3.13	0.08/0.31	0.39/0.39
S. aureus	0.04/0.16	0.20/0.20	0.04/0.16	0.20/0.78	0.04/0.16	0.20/0.20
B. cereus	0.04/0.16	0.78/>50	0.04/0.16	0.78/50	0.08/0.08	0.78/>50
C. albicans	0.63/1.25	6.25/>50	0.31/1.25	0.39/>50	0.31/1.25	3.13/3.13

The confidence interval for all the results did not exceed 0.05 mg·mL^{-1}at $\alpha = 0.05$.

It can be seen that the purified PACs worked better than extracts for all microorganisms under study. Extract of *Salix caprea* had the highest or similar MIC activity for all bacteria, but as to for fungus *C. albicans, Salix caprea* started to inhibit its growth at a higher concentration than *Alnus glutinosa*. But MBC/MFC activity of *Salix caprea* was the highest for almost all microorganisms, except for *B. cereus*, where *Alnus glutinosa* was more active.

Since the content of PACs was the highest in fruit-trees, the evaluation of the antimicrobial activity of fruit-trees extracts and PACs was performed against two groups of microbial strains: reference microbial strains from the Microbial Strain Collection of Latvia (MSCL), University of Latvia (*Pseudomonas aeruginosa* MSCL 331, *Staphylococcus aureus* MSCL 334, *Escherichia coli* MSCL 332, *Bacillus cereus* MSCL 330, and *Candida albicans* MSCL 378), and four clinical/human origin isolates (*Staphylococcus aureus* MRSA, *Escherichia coli* ESBL, *Pseudomonas aeruginosa* and *Candida albicans*). Purified PACs of fruit trees biomass had the statistically the highest antimicrobial activity against clinical/human isolates and reference microbial strains. It was found that PACs were more effective against reference microbial strains derived from the MSCL. The purified PACs inhibited MSCL bacteria growth at concentration of 0.04–0.78 mg mL^{-1}, while clinical isolates' bacteria were inhibited by PACs at a higher concentration – 0.67–5.99 mg mL^{-1} (Table 6).

It was shown that, same as for non-fruit trees, the extracts of fruit trees *Hippophae rhamnoides* L. and *Aronia melanocarpa*, also had less antibacterial activity compared to purified PACs samples. It corresponds to the literature data where PACs of *Hippophae rhamnoides* L. with B-type linkage have antimicrobial activity, although obtained by the other extraction procedure (Różalska et al., 2018). However, *Aronia melanocarpa* and *Hippophae rhamnoides* L. extracts tended to have higher fungicidal activity (MFC) for the clinical isolates. This could be explained by presence of low-molecular polyphenolics compounds, such as quinic acid, catechin/epicatechin, gallocatechin or its isomer epigallocatechin, and quercetin, in the extracts, which could work better in some conditions against fungi than PACs. Further research is necessary. The data for the MICs of *Hippophae rhamnoides* L. extracts in relation to MSCL *Candida albicans* were close to the literature data for *Hippophae rhamnoides* L. twigs extracts activity, with less activity against clinical isolates, although the difference was no so significant. However, the extracts were obtained by a different procedure and the conditions for the determination of MIC differed from the present article, so the results can't be compared directly (Różalska et al., 2018).

The analysis with purified PACs showed that all purified PACs had high antimicrobial activity (Table 7).

3.4.3. Biofilm formation

The prevention and management of biofilm formation in chronic wounds is rapidly becoming a primary objective of wound care. Microorganisms in biofilms are known to exhibit increased resistance to antibiotics and disinfectants, thereby often causing chronic inflammatory processes. Over 90% of chronic wounds have a biofilm-containing bacteria and/or fungi living within biofilm construct, and this becomes a major problem in wound healing (Attinger and Wolcott, 2012; Wolcott et al., 2010).

Table 6
Antimicrobial activity of PACs-rich samples from fruit trees.

Microorganisms	PACs from *Aronia melanocarpa*	Extract from *Aronia melanocarpa*	PACs from *Hippophae rhamnoides* L.	Extract from *Hippophae rhamnoides* L.
	MIC/MBC or MFC, mg·mL^{-1}			
	Clinical isolates			
E. coli	1.50/2.99	4.26/4.26	2.99/5.99	11.16/22.32
P. aeruginosa	2.38/4.75	1.99/3.98	5.99/5.99	5.58/11.76
S. aureus	0.67/0.67	1.64/3.28	0.74/0.74	2.79/2.79
C. albicans	12.75/>50	10.56/21.12	11.98/>50	5.58/44.65
	Microbial strains from MSCL			
E. coli	0.04/0.04	0.78/0.78	0.04/0.04	0.20/0.20
P. aeruginosa	0.08/0.16	0.78/0.78	0.08/0.16	0.39/0.78
S. aureus	0.08/0.16	0.39/0.39	0.08/0.16	0.20/0.39
B. cereus	0.63/1.25	0.78/0.78	0.63/1.25	0.39/50
C. albicans	0.63/1.25	1.56/3.13	1.25/2.50	0.20/>50

The confidence interval for all the results did not exceed 0.05 mg·mL^{-1}at $\alpha = 0.05$.

Table 7
Antimicrobial activity of purified PACs samples.

Purified proanthocyanidins					
Microorganisms	Hippophae *rhamnoides* L.	*Aronia Melanocarpa*	*Alnus glutinosa*	*Alnus incana*	*Salix caprea*
	MIC/MBC or MFC, mg·mL^{-1}				
E. coli	0.04/0.04	0.04/0.04	0.04/0.16	0.08/0.31	0.04/0.16
P. aeruginosa	0.08/0.16	0.08/0.16	0.08/0.31	0.16/1.25	0.08/0.31
S. aureus	0.08/0.16	0.08/0.16	0.04/0.16	0.04/0.16	0.04/0.16
B. cereus	0.63/1.25	0.63/1.25	0.08/0.31	0.04/0.16	0.08/0.08
C. albicans	1.25/2.50	0.63/1.25	0.31/1.25	0.31/1.25	0.31/1.25

The confidence interval for all the results did not exceed 0.05 mg·mL^{-1} at $\alpha = 0.05$.

Since there are practically no means of combating bacterial films, the study of the effect of preparations on the destruction of these biofilms is one of the most relevant areas of research in pharmacology. Extracts with the highest PAC content and PACs from *Aronia melanocarpa* and *Hippophae rhamnoides* L. were chosen as inhibitors of biofilm formation. The following bacteria were studied as biofilm formers: MRSA, ESBL, and *P. aeruginosa.* Comparing with the control (100% formed film based on bacteria), the percentage formation of films of MRSA, ESBL and *P. aeruginosa* was 2 times lower in the presence of PACs. The effect of the *Hippophae rhamnoides* L. extract was similar within the CI, with the exception of the biofilm based on MRSA, the percentage formation of which decreased by 5 times (Fig. 13).

The study showed that PACs also have the potential to inhibit bacterial growth in the biofilms.

These results can be used for the creation of supportive therapy in wound healing, where PACs that have no toxicity and side effects in the range of anti-microbial effect (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) et al., 2022), could complement antibiotics in the fight against biofilms, especially in between the healing windows, and for keeping the surrounding tissue healthy. It was reported that *Hippophae rhamnoides* L. extracts obtained by the other extraction procedure had a significant inhibiting effect on biofilm formation even at slightly lower concentration (Różalska et al., 2018). Cathechins, catechin gallates and epigallocatechin gallate which are monomers of PACs were reported to be able even to reverse several antibiotics resistance (Slobodníková et al., 2016). Extracts containing polyphenols were reported to increase the efficacy of the antibiotics that led to lowering of antibiotics dose (Daglia, 2012). Since it was proven that PACs also high anti-inflammatory properties (Andersone et al., 2023; Zhang et al., 2020), PACs have the potential to become a solution in wound progress to the proliferative and remodelling phase of healing. Thanks to their adhesive properties, it is possible for PACs to interfere and compete with biofilms bacteria fimbriae adhesins, as well as to produce antiadhesive actions against bacteria in urinary tract and dental infections (De La Iglesia et al., 2010). This statement needs further experimental research. In the literature, as one of the additional or alternative ways of fighting the pathogens, is disarming of the bacteria, targeting their virulence instead of killing them completely, in order to prevent antibiotic resistance (Heras et al., 2015). Adhesion factor of PACs could be used in these anti-virulence therapies.

It could be also said that although the bactericidal concentration of the extracts and PACs is bigger than for chemically synthesized antibiotics (usually the antibiotics are necessary in μg·mL^{-1} (Upadhyay et al., 2010; Żuchowski, 2023), the natural extracts studied are able to reduce the viability of the initial microorganism inoculation by ≥99.9%. Considering that the most often they do not have toxicity in their activity diapason (Sadowska et al., 2017; Janceva et al., 2022a,b), they could be used without side effects characteris-

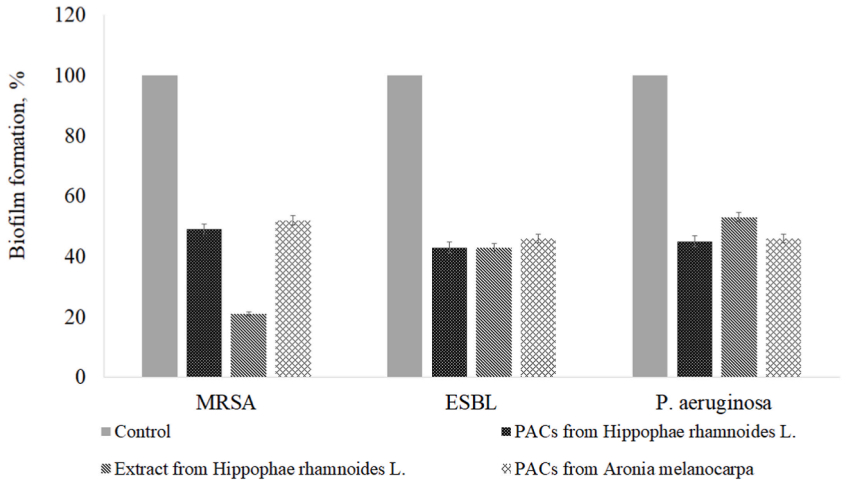

Fig. 13. Effect of extract and purified PACs from *Hippophae rhaimnoides* and *Aronia melanocarpa* on biofilm formation of MRSA, *P. aeruginosa* and ESBL at concentrations of 5 mg/mL. The error bars represent the CI at $\alpha < 0.05$. Data are presented as the percentage of biofilm formation compared to the control containing none of the extracts (100% biofilm).

tic for the allopathic medicine, or in the combined therapy for multidrug-resistant pathogens (Sadowska et al., 2017). Moreover, natural extracts could be taken for a long time, e.g., 3–6 months, whereas synthetic antibiotics at such prolonged treatment interfere with the physiological processes of human or animal organisms, and their cumulative negative effect increases with the time they are taken (Kiss et al., 2022). Proven excellent antioxidant properties of the *Aronia melanocarpa* and *Hippophae rhamnoides* L. extracts and PACs, derived from all the plants under study, will allow to make preparations with both antioxidant and anti-microbial properties.

Wastewater from antibiotic production, municipalities and hospitals are the biggest source of antibiotic resistance genes and environmental pollution (Pereira et al., 2015; "The costs and risks of AMR water pollution," n.d.). Therefore, partial replacement of antibiotics by plant-derived antimicrobials would contribute to greener and more sustainable approach.

4. Conclusions

The first comparative analysis of fruit and non-fruit trees lignocellulosic biomass-derived PACs was done and showed that the fruit trees *Aronia melanocarpa* and *Hippophae rhamnoides* L. had higher content of PACs on the extract (71.0% and 42.4%, respectively) and yield from the biomass (12.2% and 8.4%, respectively). Fruit trees-derived purified PACs, which contained structures with higher polymerization degree, had slightly higher antioxidant activity. However, all PACs and *Aronia melanocarpa* extract were more active than Trolox.

All the PACs had high activity against microbials. One can say that both: fruit and non-fruit trees PACs have the potential to be successfully used in antimicrobial preparations and as antioxidants, and could be interchangeable in the compositions of the pharmaceutical preparations, especially against *E. coli*, *P. aeruginosa* and *S. aureus*. Depending on the availability of biomass at the moment, PACs of one or another tree could be chosen in a mixture of working PACs. PACs of *Salix caprea,* which contained wider range of gallocatechin subunits, and *Alnus* spp.*,* which contained diarylheptanoids, had shown better anti-microbial activity against *B. cereus*; thus, they are preferable in anti-microbial composition against this bacterium. Non-fruit trees PACs are desirable in the preparations against *C. albicans.* PACs from *Hippophae rhamnoides* L. and *Aronia melanocarpa* inhibited biofilm formation.

High content of PACs in *Aronia melanocarpa* and *Hippophae rhamnoides* L. tree biomass extracts widens possibilities for *Aronia melanocarpa* and *Hippophae rhamnoides* L. plantations cultivation, which will complement berries production with another type of value-added product; thereby, contributing to the development of a sustainable horticulture.

Extraction of PACs from non-fruit forest and agricultural field cleaning biomass will allow its cascading use with obtainment of value-added products before biomass final application as a fuel.

Medicinal preparations on the basis of natural compounds will contribute to sustainable pharmacy since their application will allow to diminish the use of antibiotics and contribute to less toxic side effects both: for humans and the environment.

CRediT authorship contribution statement

Anna Andersone: Writing- Original draft preparation, Conceptualization, Methodology, Investigation, Validation, Writing- Reviewing and Editing. Sarmite Janceva: Writing- Original draft preparation, Conceptualization, Methodology, Investigation, Data curation, Writing- Reviewing and Editing, Supervision, Formal Analysis. Liga Lauberte: Methodology, Investigation, Writing- Reviewing and Editing. Ingus Skadins: Methodology, Investigation. Vizma Nikolajeva: Methodology, Investigation, Writing- Reviewing and Editing: Konstantins Logviss: Methodology, Investigation. Natalija Zaharova: Investigation, Writing- Reviewing and Editing. Gints Rieksts: Investigation, Resources. Galina Telysheva: Conceptualization, Methodology, Resources, Writing- Original draft preparation, Supervision.

Author statement

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Declaration of competing interest

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

Data availability

Data will be made available on request.

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Lignocellulosic Waste Compounds for Pancreatic Lipase Inhibition: Preliminary Extraction by Freon, Obtaining of Proanthocyanidins and Testing on Lipase Activity

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† Sadly, Galina Telysheva passed away in November 2021. As she was the head of the project, and took part in the conceptualization, writing, choice of methodology, and investigation, the rest of the authors decided to submit the paper with her name as co-author. This is our tribute to our dear master. Anna Andersone (2nd author) as a daughter of Galina Telysheva confirms that Galina Telysheva approved the publication, and there is no conflict of interest.

Abstract: The twigs of sea buckthorn, blackcurrant, gooseberries, quince, and grapes were evaluated as a promising source of biologically active compounds—proanthocyanidins (PACs). Sea buckthorn twigs had the highest content of PACs (9.2% on dry biomass). Preliminary pretreatment of biomass with freon R134a did not allow an increase in PACs content in the composition of hydrophilic extract but confirmed the value of freon extract as an antibacterial agent against *P. aeruginosa* and *B. cereus*. The content of PACs was used as an indicator for assessment of the influence of hydrophilic extracts on pancreatic lipase activity. Under normal physiological conditions, in the presence of bile, the extract, which contained 42.4% of PACs was more effective compared to the extract which contained 17.5% of PACs. At all concentrations (0.2–40 mg of sample/g of pancreatic lipase), it inhibited lipase activity by 33%. Purified PACs were the most effective in inhibiting lipase activity (by 36%). However, in pathological physiological conditions (without bile), the opposite effect on lipase activity was observed. Thus, PACs and extracts can be used as inhibitors of pancreatic lipase only under normal physiological conditions.

Keywords: obesity; sea buckthorn; freon R134a; blackcurrant; gooseberries; quince; grapes; polyphenols; proanthocyanidins; lipase inhibitors

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1. Introduction

It was recently proven, based on the example of sea buckthorn, that lignocellulosic biomass of fruit trees/shrubs can be as valuable as fruits in terms of bioactive compounds. Moreover, the content of some of the secondary metabolites is even bigger in their lignocellulosic biomass than in fruits [1]. As all fruit shrubs undergo pruning to a bigger or lesser extent, the volume of waste biomass increases along with increase in berry industrial production volume. Today, this biomass is mostly burned or incorporated into soil directly or through composting. Such a method of biomass application has a small added-value and, additionally, causes greenhouse gas (GHG) emission, since soil microflora induced by such biomass stimulates the production and emission of NO and N_2O, which have significantly bigger GWP than CO_2 [2]. Following the authors' research on sea buckthorn waste biomass, innovative research and comparison of other fruit shrubs' biomasses is

necessary as this opens possibilities for their future applications as a source for separate compounds or extracts complex. Regarding the underutilized lignocellulosic biomass of the range of fruit shrubs cultivated in Europe, berries, which are highly nutritional and reported to have anti-diabetic and weight-reduction properties, were chosen for this study: blackcurrant [3,4], cultivated on a large scale, especially in Poland [5]; gooseberry [6,7], grape [8], and quince [9]. The fruits, pomace, and leaves of the above-mentioned fruit shrubs are used in folk medicine, but only a few publications are available on the phenolic compounds and lipophilic extracts of these types of biomass. Preliminary studies have shown that proanthocyanidins (PACs) are the dominant polyphenols in lignocellulosic biomass [10,11], which are reported to have anti-diabetic, anti-inflammatory, anticancer, antioxidant, cardio-protective, antimicrobial and other health-related properties [11,12]. Previously published data on PACs of the above-mentioned fruit shrub lignocellulosic waste are scarce, so the comparative research of this lignocellulosic biomass was a novelty of this work.

The target PAC properties studied in this research were connected with the anti-diabetic and anti-obesity properties of PACs, since obesity is one of the most influential diseases today. This is mainly caused by excessive consumption of sugar and high fat foods when all consumed energy is not used through physical activity and is stored by the body as fat. According to the World Health Organisation, around 2 billion adults currently are overweight, of whom 650 million are affected by obesity (BMI $\geq$ 30 kg/m^2) [13,14] with side-effects of coronary heart disease, type 2 diabetes, cancers, atherosclerosis, hypertension, hyperlipidemia, dyslipidemia, stroke, and other health problems [15–21].

A symptomatic solution to prevent obesity could be the action of digestive enzymes [22,23]. Pancreatic lipase is a key enzyme for triglyceride absorption in the small intestine [24]. This enzyme is secreted from the pancreas and hydrolyses triglyceride into glycerol and fatty acids. The suppression of triglyceride absorption by lipase inhibition is one of the additional approaches for preventing obesity [25]. Many people choose medications for effective withdrawal of symptoms and rapid results [26,27]. Currently, in clinical practice, many synthetic drugs are used, characterized by various combinations of components and enzymatic activity. However, the risk of side effects and the cost of these synthetic drugs is high compared to herbal examples [27,28]. Among the commercially available drugs that inhibit the absorption of intestinal fat due to the inhibitory activity of lipase are orlistat (Xenical®), lorcaserin (Belviq®), phentermine/topiramate (Qsymia®), naltrexone/bupropion (Contrave®) and liraglutide (Saxenda and Victoza®). All these drugs are not safe, as they have such side effects as oily discharge from the rectum, gas, imperative urge to defecate, steatorrhea, increased defecation, and fecal incontinence. In addition, increased blood pressure, high pulse rate, palpitation, dry mouth, headache, insomnia, memory impairment, and paraesthesia have been reported [29].

It was proven that PACs can inhibit digestive enzymes (pancreatic lipase, α-amylase, and trypsin), with different inhibitory potency [27,30]. Since obesity is often accompanied by prolonged infectious deceases and inflammation [31], our hypothesis is that the complex properties of waste-biomass-derived extracts and PACs could be a solution to the complex problems of obesity; therefore, the anti-oxidant and anti-microbial properties of the lipophilic and hydrophilic extracts were also studied. It was shown in our previous research that agro-waste from fruit trees could serve as a raw material for a range of biologically active molecules [12,32].

Sequential extraction allows the obtaining of both non-polar and polar compounds. Lately, extraction by liquified gases, such as supercritical CO_2, butane, and freons, has started to be developed extensively, mostly for plant material with a high content of lipophilic compounds (orange peels, carrots, sunflower seeds, lavender flowers, etc.) [33,34]. Gas extraction with liquefied hydrocarbons (butane and propane) also reported less decomposition or modification of the compounds [35]. The liquified gases CO_2 and freon R134a were reported to be efficient for the extraction of cannabis, as the safest and highest-capacity processing methods [36]. It was shown that using a mixture of CO_2 and R134a

solvents can reduce the amount of supercritical fluid necessary [37]. Extraction with R134a does not demand such a high pressure as with supercritical CO_2 and is generally much cheaper. As an advantage of extraction by fluoro-hydrocarbons, the possibility of changing the yield of extractable compounds by the addition of a co-solvent is also mentioned [38]. Both CO_2 and freon are approved by the FDA as safe solvents and can replace hexane solvent, which is highly toxic and is banned for use in Europe. Using a closed loop in a freon extractor prevents the R134a global warming effect. The disadvantage of R134a freon extraction is the production stage of the freon from trichloroethylene. Therefore, supercritical CO_2 extraction seems to be much "greener". However, CO_2 extraction demands much more electricity for heating, pressure, cooling, and intensive ventilation to prevent suffocation [39]. CO_2 capturing increases the fuel needs of an electricity plant by 25–40% [40], and demands amine absorbents [41] which in vivo may form nitrosamines and nitramines, affecting health and the environment; it was found that several of the amines can be highly carcinogenic [41]. Thus, further study would be necessary for the comparison of both extraction methods, which is planned in the future. The innovative freon extraction technology has still undergone very limited research with regard to lignocellulosic biomass, and in this study this was evaluated. The influence of freon extraction conditions on further extraction of hydrophilic compounds by water and ethanol/water solutions was studied.

Based on the analysis performed, the present study aimed to obtain the lipophilic and hydrophilic extracts and PACs from the twigs of five types of fruit trees/shrubs (sea buckthorn, blackcurrant, gooseberries, quince, and grapes), widespread in the Baltic States and Europe, the chemical characterization of the obtained extracts, and the study of the effect of extracts and isolated PACs on the activity of the pancreatic lipase in normal and pathological patient conditions. The anti-microbial properties of lipophilic compounds obtained by hexane and freon were tested for preliminary validation of the concept of natural materials-based complex solutions for obesity patients. To the best of the authors' knowledge, this is the first comparative study of the above-mentioned lignocellulosic biomass. Thus, the novelties of the present study are the usage of the above-mentioned lignocellulosic agro-waste as a source of extracts and PACs; evaluation of freon extraction as a method to obtain polar and semi-polar compounds from lignocellulosic biomass; study of the effect of co-solvent; anti-microbial properties of lipophilic extracts; and analysis of the influence of hydrophilic extracts and PACs on lipase activity.

2. Materials and Methods

2.1. Collection of Plant Material

The twigs of sea buckthorn (*Hippopae rhamnoides* L.), cultivar 'Maria Bruvele', Institute of Wood Chemistry (IWC) sample storage No. 91/03-20/146-22, were collected from the sea buckthorn plantation area in Seme parish, Tukums county of Latvia (DD: 57.1444093, 23.108156); blackcurrant (*Ribes nigrum* L.), cultivar 'Selechenskaja', IWC No. 97/03-20/146-22; gooseberries (*Grossulariaceae* family), cultivar 'Kuršu Dzintars', IWC No. 98/03-20/146-22; quince (*Chaenomeles japonica*), cultivar 'Rasa', IWC No. 99/03-20/146-22; and grapes (*Vitis vinifera*), cultivar 'Zilga', IWC No. 100/03-20/146-22, were collected from the fruit-tree/shrub plantation area of Baldone parish, Kekava county of Latvia (Decimal degrees (DD): 56.82065, 24.27653). Twigs were cut in September 2022. All biomass samples are deposited in the Institute of Wood Chemistry (IWC), room 239, at the freezer, and available for analyses. The twigs were dried at 20–25 °C temperature, leaves were removed, and the twigs were grounded with a mill (Retsch SM100, RETSCH, Haan, Germany). The grounded twigs biomass (further in the text—biomass) was stored at −8 °C.

2.2. Reagents

The solvents (high purity), $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl), $ABTS^{+\bullet}$ (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), the reference antioxidants Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), as well as analytical standards gallic acid (purity $\geq$ 97.5%) and procyanidin B2 (purity $\geq$ 90%) were purchased from Sigma-Aldrich

(St. Louis, MO, USA). Reference microbial strains: *S. aureus* MSCL 3340, *P. aeruginosa* MSCL 3314, *E. coli* MSCL 332, *B. cereus* MSCL 330 were received from the Microbial Strain Collection of Latvia (MSCL, Riga, Latvia).

2.3. Isolation of the Extracts from Biomass

The scheme of extracts' isolation from dry biomass (DB) is shown in Figure 1.

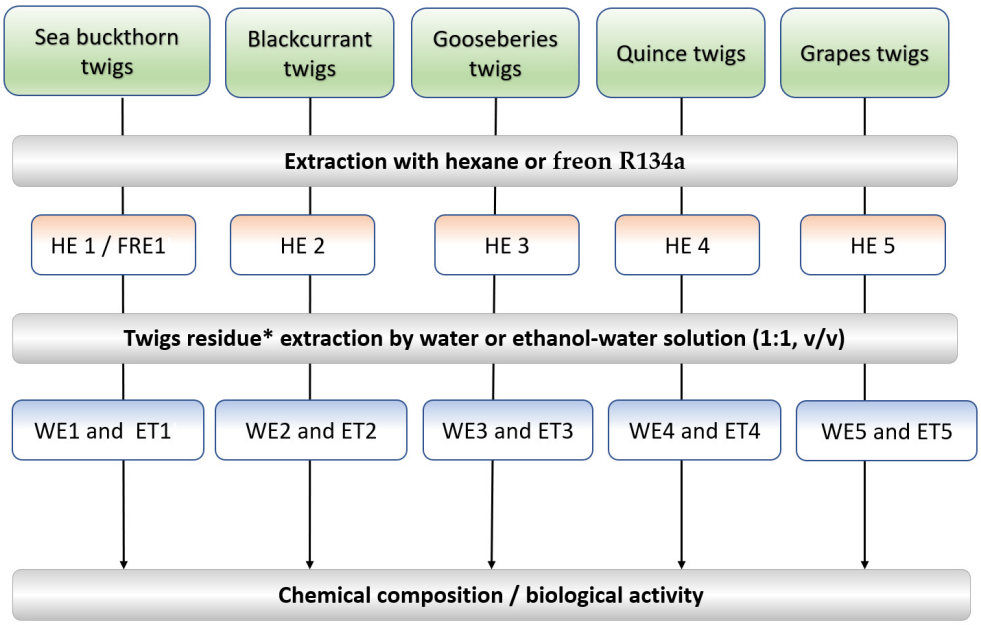

Figure 1. Principal schema of extracts isolation from biomass: * biomass residue after extraction with hexane; H-hexane extract; WE-water extract; ET-50% EtOH extract.

2.3.1. Lipophilic Extract

Lipophilic extracts (HE 1-HE 5, Figure 1) from biomass were isolated by extraction at 50–60 °C for 40 min using n-hexane as a solvent. The extracts after hexane evaporation were dried at 40 °C to yield a dry extract (DE). The yield of the extracts is presented as a percentage based on DB. The confidence interval (CI) for the results did not exceed 3% at $\alpha = 0.05$).

2.3.2. Semipolar Extracts

Semipolar extract (FRE 1, Figure 1) from DB of sea buckthorn (with/without co-solvent (ethanol) was isolated by ozone-friendly 1,1,1,2-tetrafluoroethane (freon R134a) in a closed system under a pressure of 4.0–4.3 Bar and a temperature of 17–19 °C. The yield of the extracts is presented as a percentage based on DB. The CI for the results did not exceed 3% at $\alpha = 0.05$).

2.3.3. Hydrophilic Extracts

Hydrophilic extracts (WE 1-WE 5 and ET 1-ET 5, Figure 1) from the DB were isolated by extraction at 60 °C for 40 min (20 min × 2 times) using distilled water and 50% EtOH solution (ethanol: distilled water, 1:1, *v*/*v*). The extracts, after ethanol evaporation, were freeze-dried to yield a DE. The yield of the DE is presented as a percentage based on ODB. The CI for the results did not exceed 3% at $\alpha = 0.05$.

2.4. Determination of Total Phenol Content in the Extracts

The total polyphenol (TP) content in the extracts (WE 1-WE 5 and ET 1-ET 5, Figure 1) was quantified by the Folin–Ciocalteu method using gallic acid (GA) as a reference compound. An aliquot (1 mL) of the extract was transferred into the test tube, 5 mL

of Folin–Ciocalteu reagent and 4 mL 7% aqueous sodium carbonate solution were added; the tube was vortexed and placed into a water bath at 30 °C for 1 h. The absorbance of the mixture was then recorded at 760 nm with a UV/VIS spectrometer Lambda 650 (Perkin Elmer, Inc., Waltham, MA, USA) against a blank containing 1 mL of extraction solvent. The content of TP was calculated as a GA equivalent (GAE) from the standard curve and expressed as g GAE·100 g^{-1} DE. All measurements were carried out in triplicate. The CI for the results did not exceed 3% at $\alpha = 0.05$.

2.5. Determination of PACs Content in the Extracts

The content of PACs in extracts (WE 1-WE 5 and ET 1-ET 5) and purified PACs fraction were measured by the butanol-HCl method using procyanidin dimer B2 as a reference compound. An aliquot (1 mL) of the extract was transferred into the test tube, 6 mL of acid butanol (5% (*v*/*v*) concentrated HCl in n-butanol) and 0.2 mL of iron reagent (*w*/*v*) ($FeNH_4(SO_4)_2 \cdot 12\ H_2O$ in 2 N HCl) were added; the tube was vortexed and placed into water bath at 80 °C for 50 min. The absorbance of the mixture was then recorded at 550 nm with a UV/VIS spectrometer Lambda 650 (Perkin Elmer, Inc., Waltham, MA, USA) against a blank containing 1 mL of extraction solvent. Each sample was analyzed in triplicate, and assay results were expressed as a percentage per DE. The CI for the results did not exceed 3% at $\alpha = 0.05$).

2.6. Determination of Total Flavonoid Content in Extracts

Next, 5–10 mg of the DE was dissolved in 25 mL of 96% ethanol. Then 0.4 mL of the extract solutions were added to a 10 mL test tube containing water (2 mL). After that, 5% sodium nitrite ($NaNO_2$) solution (0.12 mL) was added and incubated for 5 min at room temperature, after which 10% aluminium nitrate solution (0.24 mL) was added to the mixture. After 6 min, 1 mol·L^{-1} sodium hydroxide (0.8 mL) was added. The absorbance was measured at 420 nm, and the result was expressed as mg of quercetin equivalents per g of dry weight (DW).

2.7. Determination of Total Tannin Content in Extract

All extract aliquots were divided into two parts. To one part, phosphomolybdotungstic reagent was added. After pH adjustment with sodium carbonate solution and 30 min reaction time, absorbance was measured at 760 nm using a UV/VIS spectrometer Lambda 650 (Perkin Elmer, Inc., Waltham, MA, USA). The second part of the extract was shaken with hide powder CRS and filtered. The phosphomolybdotungstic reagent was added to the filtrate, and after pH adjustment and 30 min of reaction time, absorbance was measured at 760 nm. As a standard substance, pyrogallol was used after a reaction with a phosphomolybdotungstic reagent. Polyphenols adsorbed by hide powder (=tannin content) were calculated by subtracting the unabsorbed polyphenols from the total polyphenols, each referring to the absorption of the standard substance. The tannin content was expressed as a percentage per DE. The CI for the results did not exceed 3% at $\alpha = 0.05$).

2.8. Determination of PAC Composition by LC-UV-ESI-QTOF MS Analysis

Dry purified PAC fraction was dissolved in aqueous methanol (*v*/*v*; 2:8) with an approximate concentration of 0.1 mg·mL^{-1} after which it was filtered and then used for TOF/MS experiments. The TOF-MS spectra of PACs were recorded with Waters Acquity UPLC HClass with a PDA detector and Micromass QuattroMicro Mass spectrometer (Waters Corp., Milford, MA, USA) using the Acquity UPLC BEH Amide column (1.7 µm, 3.0 × 100 mm).

2.9. Determination of Lipophilic Extract Composition by GC-MS Analysis

Lipophilic extracts (HE 1 and FRE 1) were analyzed by GC-MS chromatography using Shimadzu GC/MS/FID-QP ULTRA 2010 apparatus (Shimadzu, Kyoto, Japan), a capillary column RTX-1701 (Restec, Metairie, LA, USA) and a 60 m × 0.25 mm × 0.25 mm film

(an injector temperature of 250 °C, an ion source with EI of 70 eV, carrier gas helium at the flow rate of 1 mL min^{-1} and a split ratio of 1:30). Dry extracts (residual moisture content <1%) were dissolved in hexane (*w*/*w* 1:10) with an approximate concentration of 0.1 g/g_{hexane} and filtered (Nylon filter, 0.45 µm pore size), after which they were used for GC-MS experiments. The oven program: 1 min isothermal at 60 °C, followed by 6 °C min^{-1} to 270 °C, and the final hold at 270 °C for 10 min. The mass spectrometer was operated in electron impact mode using 70 eV electron energy. The identification of the individual compounds was performed based on GC/MS using Library MS NIST 11 and NIST 11s, whereas the relative area of the peak of individual compounds was calculated using Shimadzu software based on GC/FID data. The summed molar areas of the relevant peaks were normalized to 100%, and the data for four repetitive pyrolysis experiments was averaged. The variation coefficient of measurement was ≤5%.

2.10. Determination of Radical Scavenging Activity

2.10.1. DPPH• (2,2-Diphenyl-1-picrylhydrazyl Radical) Assay

The dry extracts (HE 1L, FRE1, WE 1-WE 5 and ET 1-ET 5) and purified PAC fraction were tested for their radical scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) using a UV/VIS spectrometer Lambda 650 (Perkin Elmer, Shelton, CT, USA). The DPPH• assay was measured according to the procedures described by Dizhbite et al. [42]. A range of different concentrations of the obtained DE in DMSO was prepared. The absorbance at 515 nm was measured 15 min after the mixing of 30 µL of extract (or antioxidant standard) with 3.0 mL DPPH• (1·10–4 mol·L^{-1}) solution. DMSO was used as a control and Trolox as a reference antioxidant standard. CI ≤ 0.3 mg·L^{-1}.

2.10.2. $ABTS^{+\bullet}$ (2,2′-Azinobis (3-Ethylbenzothiazoline-6-sulfonic Acid) Assay

The dry extracts (WE 1-WE 5 and ET 1-ET 5) and purified PAC fraction were tested for their radical scavenging activity against the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{+\bullet}$) using UV/VIS spectrometer Lambda 650 (Perkin Elmer, Shelton, CT, USA). $ABTS^{+\bullet}$ was produced by the reaction of 2 mmol·L^{-1} ABTS stock solution with 70 mmol·L^{-1} potassium persulfate ($K_2S_2O_8$), allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{+}$ solution (stable for 2 days) was diluted with phosphate-buffered saline (pH 7.4) to an absorbance of 0.80 ± 0.02 at 734 nm. The absorbance at 734 nm was investigated 10 min after the mixing of 30 µL of extract (or purified PACs fraction and antioxidant standard—Trolox), diluted in DMSO of five different concentrations with 3.0 mL $ABTS^{+\bullet}$ solution. DMSO was used as a control and Trolox as the antioxidant standard. CI ≤ 0.3 mg·L^{-1}.

2.11. Antimicrobial Activity of Lipophilic Extracts

Minimum inhibitory concentration (MIC) determination by microtiter broth dilution method was used. Stock solutions of the respective plant extracts were prepared in 10 mL microcentrifuge tubes by dissolving dry plant extract in dimethyl-sulfoxide (DMSO) to a final concentration of 100 mg·mL^{-1}. The serial dilutions from the stock solution were made, ranging from 50 mg·mL^{-1} to 0.01 mg·mL^{-1}, using Mueller–Hinton broth (Becton Dickinson, Sparks, MD, USA) in 96-well microplates. The microbial suspension containing approximately 5×10^5 colony-forming units (CFU)/mL was prepared from a 24 h culture plate. From this suspension, 100 µL was inoculated into each well. The microtiter plates were incubated at 37 °C, for 24 h. After incubation, 40 µL of a 0.4 mg·mL^{-1} solution was added to each well as an indicator of microbial growth. The plates were incubated for 30 min and the MIC values were determined.

Minimum bactericidal concentration (MBC) was recorded as the lowest concentration of an antimicrobial substance that reduces the viability of the initial microorganism inoculation by ≥99.9% after 24 h incubation at 37 °C. MBC was evaluated for extracts and purified PACs fraction. 10 µL were taken from the well obtained from the MIC experiment (MIC value), as well as two wells above the MIC value well, and spread on Mueller–Hinton

agar plates. The number of colonies was counted after 18–24 h of incubation at 37 °C. The concentration of sample that produces <10 colonies was considered as MBC value. CI $\leq$ 0.3 mg·L^{-1}.

2.12. Determination of the Influence of Extracts and Purified PACs on Lipase Activity

The test tubes were prepared composed of 4.0 mL milk (3.8% fat), 1 mL pancreatic solution (pancreatin from porcine pancreas, Sigma-Aldrich), 1 mL bile (bile extract porcine, Sigma-Aldrich), and 1 mL phenolphthalein. Orlistat (purity ≥98%, Sigma-Aldrich), a known inhibitor of pancreatic lipase, was used as a reference compound. The first test tube was used as a control tube without the investigated components (PACs, extracts, and reference). To the following tubes, 100–2000 µL of the analyzed sample (PACs, extract or Orlistat) at the concentrations of 2, 20, and 200 mg·L^{-1} (0.2–400 mg of sample/g of pancreatic lipase—further in the text—mg·g^{-1} PL) were added. All tubes were put in the incubator for 40 min at 38 °C. Afterwards, each solution from the tubes was titrated with 0.1 N NaOH solution until the colour changed into yellowish-brown, and the needed amount of NaOH was determined. CI $\leq$ 0.3 mg· g^{-1} PL.

2.13. Statistical Analysis

All measurements were conducted in triplicate and the results were presented as the mean value. Statistical analyses were performed using Microsoft Excel 2016, version No. 16. CI for a mean using Student's *T* distribution was calculated at a significance level of 5% (α = 0.05).

3. Results and Discussion

3.1. Lipophilic and Hydrophilic Extracts from Fruit-Tree Biomass

The yields of lipophilic extracts obtained with hexane from the entire studied biomass were quite close, but statistically different, and varied from 0.92 to 1.44% per DB. The yields of hydrophilic extracts from the same biomass, obtained using distilled water as the most environmentally friendly solvent, differed statistically significantly and varied from 7.1 to 15.7% per DB. With 50% EtOH, the extracts yield increased significantly, suggesting that the extractives are more soluble in the ethanol–water suspension. The yields of hydrophilic extracts obtained with 50% EtOH from the entire biomass under study were: sea buckthorn 21.7% > quince 14.3% > grapes 13.9% > black currant 13.5% > gooseberry 7.5% (Table 1). The biomass of sea buckthorn differs from the other studied tree species not only by the highest total yield of hydrophilic extracts (21.7% per DB), but also by the high content of polyphenols (48.6 g GAE·100 g^{-1} DE).

Table 1. The yield of lipophilic and hydrophilic extracts from fruit trees biomass, %/DB.

Solvents	Sea Buckthorn	Blackcurrant	Gooseberries	Quince	Grapes
Hexane	1.44 ± 0.03	0.92 ± 0.01	1.18 ± 0.01	1.02 ± 0.03	1.16 ± 0.02
50% EtOH (*v*/*v*)	21.71 ± 0.03	13.52 ± 0.03	7.50 ± 0.03	14.29 ± 0.03	13.88 ± 0.02
Water	15.73 ± 0.05	10.77 ± 0.04	7.08 ± 0.05	13.79 ± 0.03	11.71 ± 0.03

Gooseberry biomass had the lowest yield of hydrophilic extracts (7.1% per DB by distilled water and 7.5% per DB by 50% EtOH). Polyphenols played the main role in the biological activity of all hydrophilic extracts. The amount of them in the 50% EtOH hydrophilic extracts was higher and varied from 30.3 to 48.6 g GAE·100 g^{-1} DE. A comparative analysis of the solvent dependence on the yield of polyphenols and between biomasses is shown in Table 2.

The content of TP of sea buckthorn was very similar in years 2020 and 2022, considering the CI (e.g., for 50% EtOH extract: 48.1 ± 0.2 g GAE·100 g^{-1} extract in 2020 [12] against 48.6 ± 0.2 g GAE·100 g^{-1} DE in 2022). The literature data on TP of twigs of the other biomasses under study is very limited. Gooseberry berries were reported to contain TP in

the amount of around 3 g GAE·100 g^{-1} biomass [43], which correlates well with our data calculated on biomass (around 2.9 g GAE·100 g^{-1} biomass for 50% EtOH extract). Quince berries were reported to be rich in PACs [44]. But information on lignocellulosic biomass is absent.

Table 2. The composition of hydrophilic extracts obtained from fruit tree biomass.

Biomass	TT Content in Extract, % per DE	TP Content in Extract, g GAE·100 g^{-1} DE	PACs Content in Extract, % per DE	TF Content in Extract, g RU·100 g^{-1} DE
		Water extracts		
Sea buckthorn	32.2 ± 0.3	38.4 ± 0.4	17.5 ± 0.1	2.7 ± 0.1
Blackcurrant	14.8 ± 0.2	32.1 ± 0.3	10.6 ± 0.3	4.6 ± 0.2
Gooseberries	10.7 ± 0.5	22.7 ± 0.3	9.0 ± 0.2	2.2 ± 0.3
Quince	18.3 ± 0.3	31.2 ± 0.1	14.0 ± 0.4	9.9 ± 0.1
Grapes	19.2 ± 0.2	18.6 ± 0.5	10.6 ± 0.5	2.5 ± 0.3
		50% EtOH extracts		
Sea buckthorn	44.1 ± 0.5	48.6 ± 0.2	42.4 ± 0.3	4.9 ± 0.1
Blackcurrant	38.2 ± 0.2	33.6 ± 0.1	14.2 ± 0.2	5.1 ± 0.1
Gooseberries	12.6 ± 0.4	38.9 ± 0.2	10.2 ± 0.3	4.2 ± 0.1
Quince	22.5 ± 0.3	42.2 ± 0.2	18.7 ± 0.2	12.6 ± 0.2
Grapes	16.9 ± 0.4	30.3 ± 0.1	11.4 ± 0.2	3.1 ± 0.1

Tannins are the dominant polyphenols in all hydrophilic extract compositions. The number of PACs, also known as condensed tannins, from the total amount of tannins ranged from 54 to 83% per DE. The highest content of PACs was observed for sea buckthorn 50% EtOH extracts (42.4% per DE). However, 50% EtOH extract from quince biomass was richer than sea buckthorn (4.9 g RU·100 g^{-1} per DE) in total flavonoid content (12.6 g RU·100 g^{-1} DW). All these polyphenols are biologically active natural antioxidants and antimicrobial agents, which can be used as ingredients in the formulation of different medications. The synergistic effect of the polyphenolic mixtures additionally leads to the simultaneous action on various disease pathways, which consequently contributes to a faster and more effective treatment outcome.

It is known that polyphenols are predominantly present in glycosylated forms. This is the main reason for their low absorption in the stomach, since only aglycones and some glucosides can be absorbed in the small intestine, and the rest are absorbed in the large intestine. The effectiveness of polyphenols absorbed in the colon reaches only 15–20% of the total content of polyphenols absorbed in the intestine. Thus, glucosides in dietary sources of polyphenols provide faster and more efficient absorption of polyphenols [45].

In order to reduce the number of polyphenolic glycosides and free sugars, fractionation of the extract was carried out using Sephadex®LH-20 (Cytiva, Uppsala, Sweden). The results were obtained in two fractions: a fraction consisting of low molecular polyphenols and their glycosides and free sugar, and a fraction consisting of PACs. The composition of PACs (Figure 2) was determined by LC-UV-ESI-QTOF MS analysis.

3.2. Sea Buckthorn Biomass Extraction by Freon R134a

Evaluation of pre-purification of biomass from impurities was carried out using 1,1,1,2-tetrafluoroethane (Freon R134a). Based on the highest content of PACs in the extract (Table 2), the sea buckthorn was used for analysis. To compare with the extraction by hexane, the extraction with freon was carried out in a closed system at 17–19 °C from 8 to 24 h. The yield of freon extract (1.6% per DB) was insignificantly higher, but the composition of extracts obtained differed. When adding a co-solvent (ethanol) from 5 to 10% on dry biomass and carrying out the extraction without freon circulation, but by maceration for 36 h at 17–19 °C, two extracts were separately obtained with a yield of 1.8 and 2.4% per DB. Based on the GC analysis data, the freon extract was richer in total aliphatic and cyclic monomers. The hexane extract was richer in total acid/ester content. The changes in

chemical composition between hexane extract and freon extract of sea buckthorn is shown in Table 3.

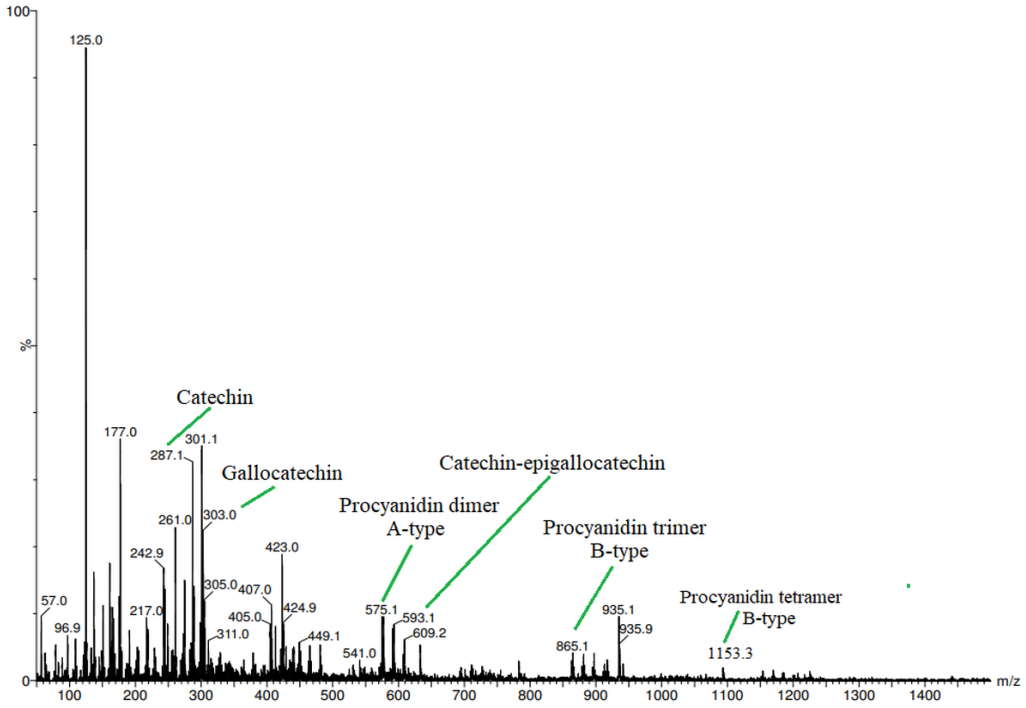

Figure 2. LC-UV-ESI-QTOF-MS analysis of PACs from sea buckthorn biomass.

Table 3. Comparison between extracts obtained by hexane and freon.

Identified Compounds Group	Freon Extract, % rel	Hexane Extract, % rel
Total acid/ester	30.3	55.7
Total aliphatic and cyclic monomers	43.1	29.9

Since this is the first research on freon extraction from twigs, there were study limitations consisting of seasonal and geographical changes in lipophilic compound content, which have to be further studied. The other types of freon (e.g., R1234yf or R1234ze) also have to be evaluated for the extraction. Although freon R134a is used in a close loop and has 0 ozone depletion, it has a high GHP (1300 vs. 7 vs. of the R1234ze) and its production and application could be banned after a while.

Sea buckthorn biomass residue, after freon extraction with/without co-solvent, was extracted by 50% EtOH. The yield of hydrophilic extracts from all biomass residues slightly decreased. Small changes were seen only in the reduced content of polyphenols in 50% EtOH extract (Table 4).

Table 4. Influence of freon on hydrophilic extracts yield and composition.

Sea Buckthorn Biomass	Yield of 50% EtOH Extract, % per DB	TP Content in Extract, g GAE·100 g^{-1} DE	PACs Content in Extract, % per DE
Residue after hexane extraction	21.71 ± 0.03	48.6 ± 0.2	42.4 ± 0.3
Residue 1 (without co-solvent)	20.36 ± 0.02	47.2 ± 0.3	42.2 ± 0.3
Residue 2 (5% of co-solvent)	20.44 ± 0.02	46.8 ± 0.2	41.9 ± 0.3
Residue 3 (10% of co-solvent)	19.98 ± 0.03	46.2 ±0.3	42.5 ± 0.3

3.3. LC-UV-ESI-QTOF-MS Analysis of Proanthocyanidins Fraction from Sea Buckthorn Hydrophilic Extract

According to *LC-UV-ESI-QTOF-MS* analysis (Figure 2), the purified PAC fraction consists of A-type of catechin/epicatechin dimer (m/z 575), B-type of catechin/epicatechin trimer (m/z 865), gallo-catechin units (m/z 303), and catechin-epigallocatechin dimer (m/z 593) (Figure 3).

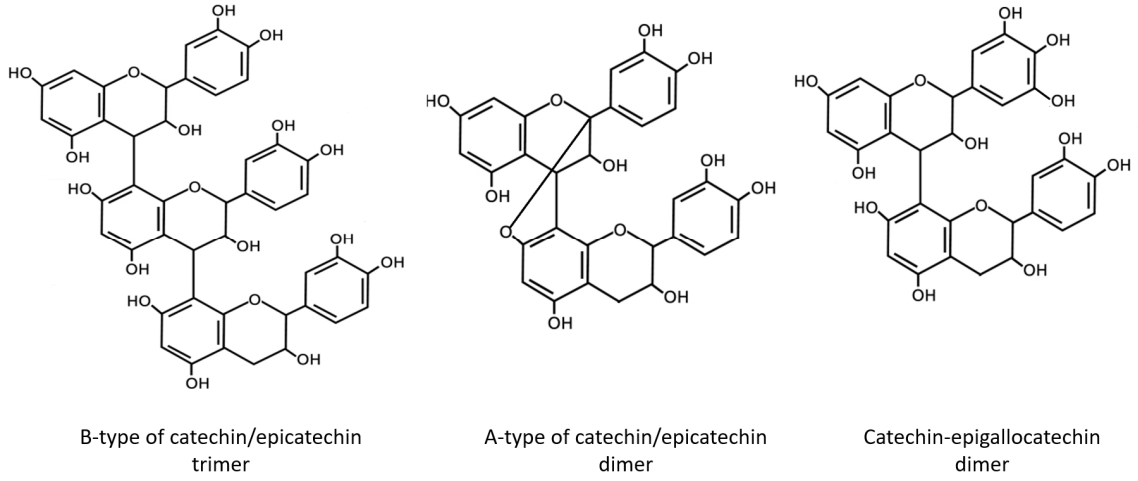

Figure 3. Chemical structures of identified compounds of PACs composition.

3.4. Radical Scavenging Activity of Extracts and Purified PACs

The radical scavenging activity of all biomass extracts was evaluated by $ABTS^+$ and $DPPH^\bullet$ assays. In comparison to Trolox as a reference, which is a water-soluble derivative of vitamin E (IC_{50} = 4.0 mg L^{-1} in $ABTS^{+\bullet}$ test and IC_{50} = 4.7 mg L^{-1} in $DPPH^\bullet$ test), the 50% EtOH extracts from sea buckthorn and quince biomass showed the highest radical scavenging activity (IC_{50} = 3.2 and 3.5 mg L^{-1} in $ABTS^{+\bullet}$ test and IC_{50} = 6.1 and 6.6 mg L^{-1} in $DPPH^\bullet$ test, respectively) (Table 5).

Table 5. Radical scavenging activity of lipophilic and hydrophilic extracts.

Solvent	Biomass	TP Content in Extract, g GAE·100 g^{-1} DE	IC_{50}, mg·L^{-1} by DPPH Test, CI $\leq$ 0.3 mg·L^{-1}	IC_{50}, mg·L^{-1} by ABTS Test, CI $\leq$ 0.3 mg·L^{-1}
Hexane	Sea buckthorn	n.d.	>30	-
Freon	Sea buckthorn	n.d.	>30	-
Water	Sea buckthorn	38.4 ± 0.4	9.8	5.6
	Blackcurrant	32.1 ± 0.3	14.1	7.5
	Gooseberries	22.7 ± 0.3	16.6	8.2
	Quince	31.2 ± 0.1	14.4	7.9
	Grapes	18.6 ± 0.5	22.1	10.6
50% EtOH	Sea buckthorn	48.6 ± 0.2	6.1	3.2
	Blackcurrant	33.6 ± 0.1	12.6	6.9
	Gooseberries	38.9 ± 0.2	9.4	5.2
	Quince	42.2 ± 0.2	6.6	3.5
	Grapes	30.3 ± 0.1	15.2	8.6
PACs fraction from sea buckthorn extract			2.3	1.4
Trolox			4.7	4.0

Due to the low content of polyphenols in blackcurrant, gooseberries, and grape extracts, the radical-scavenging activity was also low. Previous research has shown that purified PACs are powerful antioxidants. The radical scavenging activity of PAC fraction

with PACs content 92.1% on dry weight (by Buthanol-HCl assay), was 2.3–2.7 times higher (IC_{50} = 1.4 mg·L^{-1} in $ABTS^{+\bullet}$ test and IC_{50} = 2.3 mg·L^{-1} in $DPPH^{\bullet}$ test) than 50% EtOH extract from sea buckthorn (IC_{50} = 3.2 mg·L^{-1} in $ABTS^{+\bullet}$ test and IC_{50} = 6.1 mg·L^{-1} in $DPPH^{\bullet}$ test). The results for sea buckthorn twigs-derived PACs correlated well with the antioxidant activity of sea buckthorn twigs-derived PACs in year 2020: IC_{50} = 2.6 mg·L^{-1} in $DPPH^{\bullet}$ test, with CI = 0.1 at α = 0.05 [23]. Biomass of the other plant origin has not been previously studied. Freon and hexane extract at a concentration of 30 mg·L^{-1} did not show the ability to inhibit DPPH and ABTS radicals.

3.5. Antimicrobial Activity of Lipophilic Extracts from Sea Buckthorn Biomass

Since obesity has a risk of recurrent soft-tissue infections or prolonged hospitalization [46], the antimicrobial activity of lipophilic extracts was tested with Gram-negative and Gram-positive bacteria (Table 6). The results showed that the antimicrobial activity of both extracts was different; for example, according to the MBC value, which showed the complete inhibition of bacteria, the freon extract is more effective against *P. aeruginosa* and *B. cereus.* The hexane extract was more effective against *E. coli* and *S. aureus* bacteria.

Table 6. Antimicrobial activity of lipophilic extracts.

Sample	*E. coli*		*P. aeruginosa*		*S. aureus*		*B. cereus*	
	mg·mL^{-1}; CI $\leq$ 0.3 mg·mL^{-1}							
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Hexane extract	6.25	6.25	>50	>50	1.56	3.13	6.25	>50
Freon extract	0.78	50	0.78	50	0.39	12.2	3.13	25

3.6. Inhibitory Effects of PACs and Other Polyphenol-Containing Samples on Pancreatic Lipase Activity In Vitro

Our previous studies have shown that the bioactivity of PACs was strongly influenced by the degree of purity of the isolated PACs. It was also pointed out that the biological activity of PACs is higher than that of monomeric flavan-3-ols, because as the number of monomeric units bound in oligomeric or polymeric PACs increases, the number of hydroxyl groups also increases, which can donate electrons to neutralize free radicals [47]. The degree of polymerization (DP) appears to affect the ability of PACs to inhibit enzymes in the same way. The relationship between PACs' structure and function still remains a controversial issue; although there is evidence to support that DP must be high to increase their biological activity, other authors suggest that PACs must have a low DP range in which their biological activity is highest. For example, oligomeric PACs from apples with DP = 5 were good inhibitors of pancreatic lipase activity but their inhibitory activity decreased as their DP increased to decamers [48]. Oligomeric PACs with DP = 2–10 isolated from cocoa beans (Theobroma cacao) also had the highest inhibitory activity (compared to polymeric fractions) over pancreatic amylase, lipase, and phospholipase A2 [49]. Similar effects have been found for PACs from rowan (Sorbus aucuparia): it was found that PAC fraction (containing oligomeric and polymeric PACs) was a better inhibitor of pancreatic α-amylase than polymeric PAC-enriched fractions, although both were effective as enzyme inhibitors [47]. This confimed our previous investigation of PACs from deciduous trees' biomass as inhibitors of α-amylase [10].

Under normal physiological conditions (in the presence of bile), all hydrophilic extracts of sea buckthorn at all concentrations (0.2–40 mg·g^{-1} PL) showed significant inhibition of pancreatic lipase activity. Already at the amount of 0.2 mg of water extract containing 43.4 $\pm$ 0.4 g GAE·100 g^{-1} of polyphenols and 17.5 $\pm$ 0.1% of PACs, the lipase activity decreased by 22%. Further gradual increase of the amount of the water extract from 0.2 mg·g^{-1} PL to 40 mg·g^{-1} PL gave approximately the same lipase inhibition, within the confidence interval. The dependence of lipase activity inhibition on the content of PACs in the extract has also been established: the higher the content of PACs in the extract,

the higher the percentage of inhibition. An extract containing 48.6 ± 0.2 g GAE·100 g^{-1} and 42.4 ± 0.3% PACs at the amount of 0.2 mg·g^{-1} PL to 40 mg·g^{-1} PL inhibited lipase activity by 33%. A PACs fraction (PACs content in extract 92.1% on dry weight) was the best pancreatic lipase inhibitor; at a concentration 1 mg·g^{-1} PL, pancreatic lipase activity decreased by 36%. At the same concentration, Orlistat was 1.4 times more effective (inhibited lipase activity by 52%). However, it has to be considered that Orlistat has side effects, described above in the Introduction. At the concentrations of PACs until 20 mg·g^{-1} PL, lipase inhibition was similar in inhibited value taking into account confidence interval. Therefore, in practical applications, the concentration 0.2–1 mg·g^{-1} PL is sufficient for lipase inhibition and there is no need to increase the concentration. When comparing all tested samples, the fraction of the extract after PAC separation (admixture) was not effective as a pancreatic lipase inhibitor, and at a concentration of 20 mg·g^{-1} PL contributed to the increase of lipase activity (by 6–11%) (Figure 4). This confirms that PACs are the main active compounds inhibiting lipase activity in studied concentrations.

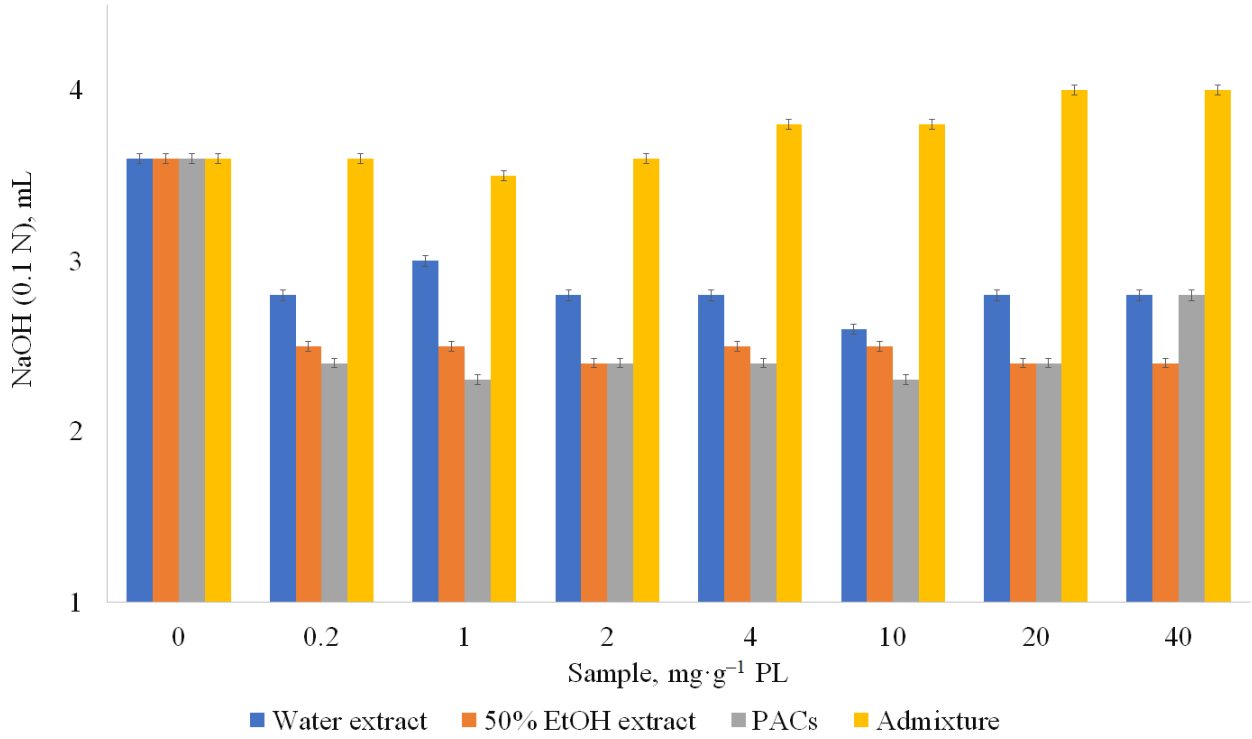

Figure 4. Influence of samples on pancreatic lipase (PL) activity in the presence of bile (normal physiological conditions). CI ≤ 0.3 mg·g^{-1} PL.

Under pathological conditions (without a bile), a similar tendency was observed for lipase inhibition in the 50% EtOH extracts and purified PACs fraction at 0.2 and 1 mg·g^{-1} PL; however, already at 2 mg·g^{-1} PL, pancreatic lipase activity activation was observed (Figure 5).

In the PAC-free extract (admixture), the lipase activity under pathological conditions was higher (10–15%) compared to the results under normal conditions (6–11%). At the amount of samples' of 0.2 and 1 mg·g^{-1} PL, PACs and extracts showed slight lipase inhibition activity. However, at 2 mg·g^{-1} PL almost all samples showed the neutral or lipase activation effects.

The purified PACs were further tested at the amount of 0.2–400 mg·g^{-1} PL. The purified PAC sample at the amount of 2–400 mg·g^{-1} PL had the activation tendency of lipase activity in pathological conditions (Figure 6).

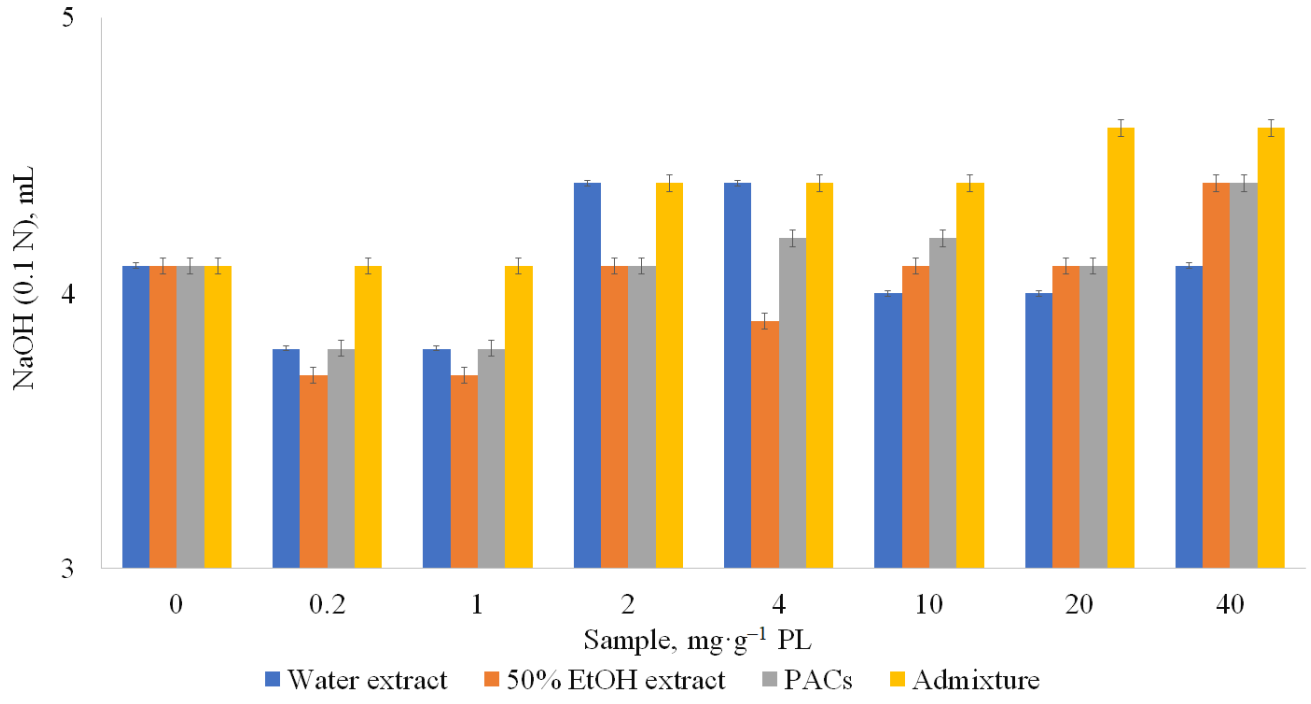

Figure 5. Influence of samples on pancreatic lipase (PL) activity without bile (pathological conditions). CI ≤ 0.3 mg·g^{-1} PL.

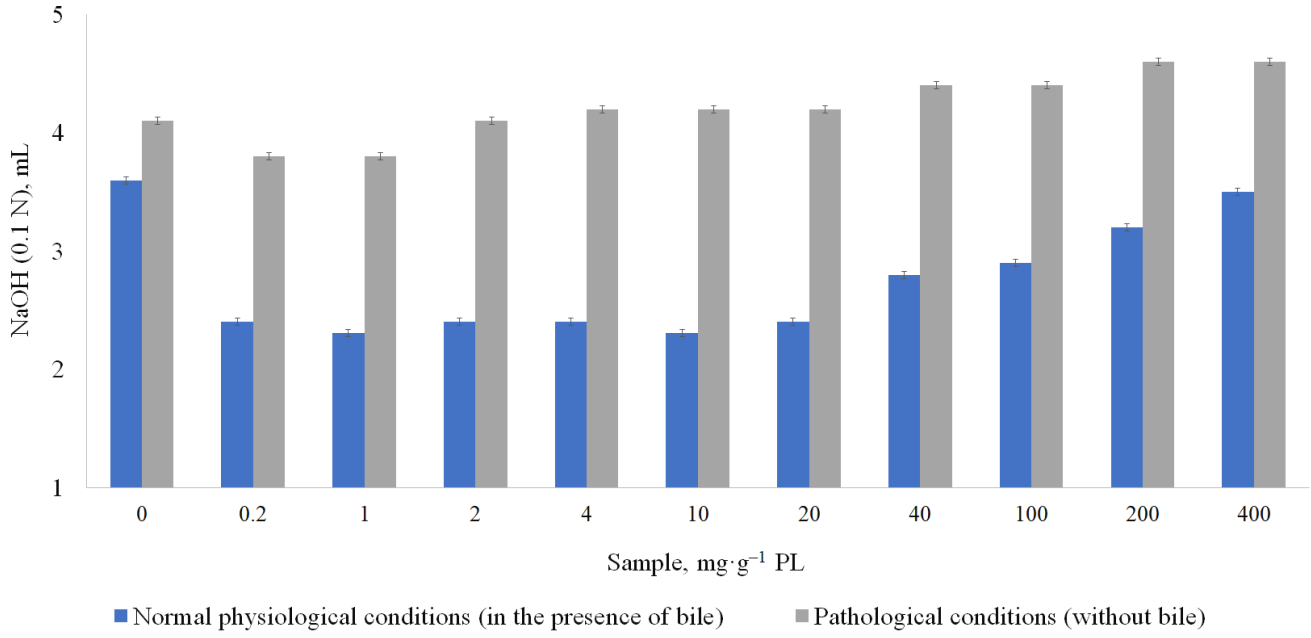

Figure 6. Influence of purified PACs on pancreatic lipase activity. CI ≤ 0.3 mg·g^{-1} PL.

Authors' earlier studies showed that PACs are the main component in inhibiting the activity of digestive enzymes in certain concentrations. Under normal conditions, PACs were the most effective at the amount 0.2–20 mg·g^{-1} PL, inhibiting lipase activity by 33–36%. Under pathological conditions, PACs in all concentrations from 2 to 400 mg·g^{-1} PL were lipase action activators.

Future perspectives are the evaluation of the influence of the PACs' composition on the lipase activity (structure-activity relationship) and clinical studies, which are missing for almost all discovered lipase inhibitors.

4. Conclusions

Several fruit shrubs pruning plant material was evaluated and compared for the first time. It was shown that the lignocellulosic biomass originating from agro-waste of fruit shrubs is a source of valuable metabolites. Innovative freon extraction of non-polar and semi-polar compounds, as well as the influence of EtOH as co-solvent on further extraction of hydrophilic compounds, were tested. Lipophilic extracts obtained by freon had a different composition than those obtained by hexane and had a low antioxidant activity (IC_{50} > 30 mg·L^{-1}), but showed anti-microbial activity. The freon extract was more effective against *P. aeruginosa* and *B. cereus*, but the hexane extract was more effective against *E. coli* and *S. aureus* bacteria.

The 50% EtOH extracts from sea buckthorn and quince biomass showed the highest radical scavenging capability among all the extracts, but it was still lower than for purified PACs. The sea buckthorn PACs and 50% EtOH extracts had the strongest inhibiting effect on pancreatic lipase (33–36%) in normal physiological conditions. In pathological conditions, both extracts, as well as PACs, tended to slightly increase lipase activity.

Lignocellulosic biomass-derived PACs' influence on pancreatic lipase proved in the study, along with PACs' and the lipophilic extracts' anti-microbial activity, can be used in complex treatments for obesity and its accompanying microbial infections. It has to be pointed out that due to inhibition of lipase, absorption of fat-soluble vitamins and nutrients is inhibited as well. Thus, lipase inhibition could be a temporary solution in fighting obesity, when patient's pathological molecular mechanisms already developed and it's not easy to change them just by transition to healthier lifestyle.

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Granulated Animal Feed and Fuel Based on Sea Buckthorn Agro-Waste Biomass for Sustainable Berry Production

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Abstract: The industrial harvesting of sea buckthorn (SBT) berries with twigs and subsequent pruning creates a large volume of lignocellulosic agro-waste. This study aimed to valorize this agro-waste as a raw material for animal feed and fuel granules, for developing a sustainable cascading SBT production scheme. Five SBT cultivars' biomasses were characterized by analytical pyrolysis, mass spectrometry, and GC analysis. Condensed tannins, which are undesirable components for animal feed, were separated by extraction. The residue was analyzed for total protein, vitamins (A, C, and E), ash, crude fat, wood fiber, and macroelements (P, K, Ca, and Na), and showed great potential. The heavy metal (Cd, Hg, and Pb) content did not exceed the permitted EU maximum. Granulation regimes were elaborated using a flat-die pelletizer, KAHL 14-175. The digestibility and the amount of produced gas emissions were determined using in vitro systems that recreate the digestion of small ruminants. The investigation proved that SBT leaves and stems are a unique underutilized source of animal feed, used alone or in combination with others. Twigs, due to their thorns, were granulated and valorized according to standards for application as fuel. The scheme offered in this study enables SBT agro-waste utilization and sustainable SBT berry production.

Keywords: sea buckthorn; agro-waste; lignocellulosic biomass; condensed tannins; animal feed; digestability; greenhouse gas emissions; small ruminants; granulated feed; granulated fuel

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1. Introduction

The world population is rapidly increasing: it is expected that there will be nearly 10 billion people on Earth by 2050 [1]. Agricultural production grows accordingly [2], and sustainability is the only way for mankind to survive, minimize negative effects on the environment, and keep the planet's population healthy [3].

Animal farming demands 70% of agricultural land and 30% of the earth's land surface [4–6]. An increase of 50% in animal feed is necessary by the year 2050; thus, to prevent the expansion of pasture areas with more than 500 mln hectares (hm^2) [1] and save the forests, it is extremely important to study the huge amount of agricultural waste and use it in the best way possible. Agro-waste, as a source of animal feed and feed

additives, could be one of the possible ways to sustainable agriculture. Feed accounts for 60–70% of total expenses for livestock and poultry [7]. Insufficient amounts of raw materials and growing costs have led to an imbalance in the animals' diets and a decrease in zootechnical indicators.

People's growing interest in a vegetarian diet nevertheless supports the demand for milk products. If everyone were vegan, the land use for agriculture would decrease by 75% [8]. However, it is highly unlikely to happen as vegans today comprise a mere 1–2% of the world population [9,10]. The problem of waste-free production is real one in any case.

Hippophae rhamnoides L. (sea buckthorn, SBT) of the family *Elaeagnaceae* is a unique fruiting shrub tree that can survive in extreme temperatures (from –43 °C to 55 °C) and grows well under drought conditions. According to legend, people in ancient Greece discovered the plant and its benefits in feeding racehorses [11]. Today, the industrial economically viable harvesting of SBT berries is possible only by cutting the whole berried branch. An SBT plantation can yield 25 tons of berries from 1 hm^2 bi-yearly, which is 12.5 tons per year and hm^2 [12]. Waste lignocellulosic biomass amounts to around 20–30% of the berries' mass. Foliage yield from the whole SBT tree could reach 16 tons/ha which is more than from any shrubs and grasses [13]. Moreover, berry-producing trees must be cut every four years, otherwise, berries will be difficult to harvest. SBT trees that are used for land reclamation and the improvement of soil quality, thanks to their roots' nitrogen-fixing ability (an SBT plantation with 8–10-year-old trees is able to fix nitrogen in the amount of 180 kg/hm^2 in a year [14]), also have to be pruned. As a result, lignocellulosic biomass, containing twigs, stems, leaves and even roots, is appearing as agro-waste in large amounts. SBT grows in 52 countries, on a total area of 3 mln hm^2, according to a 2023 report [15]. The SBT berry has a high nutritional and pharmaceutical value [11,15–22]; however, its economic potential is still underdeveloped due to the high expenses of SBT berry harvesting [23]. The human labor expenses for SBT harvesting were determined to be 58% of the total production costs [24,25]. Therefore, the application of side streams is necessary both for sustainability and economic feasibility. "Fodder trees" can be considered as new multipurpose solutions since the side-product—agro-waste—grows in the same cultivation area and does not demand new agricultural lands for producing feed [26,27].

Currently, SBT wood residues (twigs and stems) are mainly used as a renewable energy source. A 6-year-old SBT orchard can provide tons of fuel wood, whereas, one ton of SBT wood is equal to 0.68 tons of conventional coal [28]. Out of the total SBT biomass, leaves are valorized for other applications in some countries. In China, Mongolia, Scandinavian countries, Germany, the Czech Republic, Latvia, Russia, and Greece, SBT leaves are used for the preparation of tea with antioxidant properties [11,29–31]. In India, leaves are used as a feed additive for chicken and cattle [32], and in Mongolia—for the treatment of colitis and enterocolitis in humans and animals [33]. It was found that SBT leaves are the richest source of protein compared to other tree leaves [34], and SBT berries have a stimulating influence on the growth, immunity, and production performance of poultry and livestock without toxicity effects [17,35]. The body weight and egg production of chicken increased greatly after being supplemented with SBT leaves, seeds, and fruit residues [13]; however, further research is necessary in this area [36,37]. SBT pomace can be added successfully to the diet of ram lambs as well [38]. No toxic or carcinogenic side effects of berry-based products were reported [18]. There are some commercially available animal digestive supplements based on berries [39,40]. However, the research on the SBT agro-waste biomass profile of bioactive components and research on its influence on ruminants is still very limited [19,36]. There are no data available on small ruminant feed based on SBT lignocellulosic biomass, and there is no commercial animal feed production based on SBT agro-waste biomass.

Condensed tannins (CTs), except when in small concentrations, could have a negative effect on animals' digestibility [41]. Moreover, animals may not like their bitter taste. Therefore, they could be isolated and, as shown in previous studies, could find applications in health care, cosmetics, and food industries due to their anti-inflammatory and anti-bacterial properties [42].

Measurements of gas production could help to find correct feed compositions to minimize the negative greenhouse gas (GHG) impact on the environment. Digestive health remains one of the key factors in the high productivity of farm animals. The leaves and stems of SBT can be compared with grass in terms of a wide range of biologically active compounds and nutrients; thus, they can be used as a valuable food source for animals during winter. Twigs that have sharp thorns which are dangerous for animals even after grinding could be investigated for granulated fuel production. The densification of SBT biomass residues by granulation after CT extraction enables better transportation, dosing, and storage properties.

The objective of this study was the evaluation of SBT agro-waste as a raw material to obtain granulated animal feed and fuel granules for the development of a sustainable cascading production scheme in SBT cultivation (Figure 1).

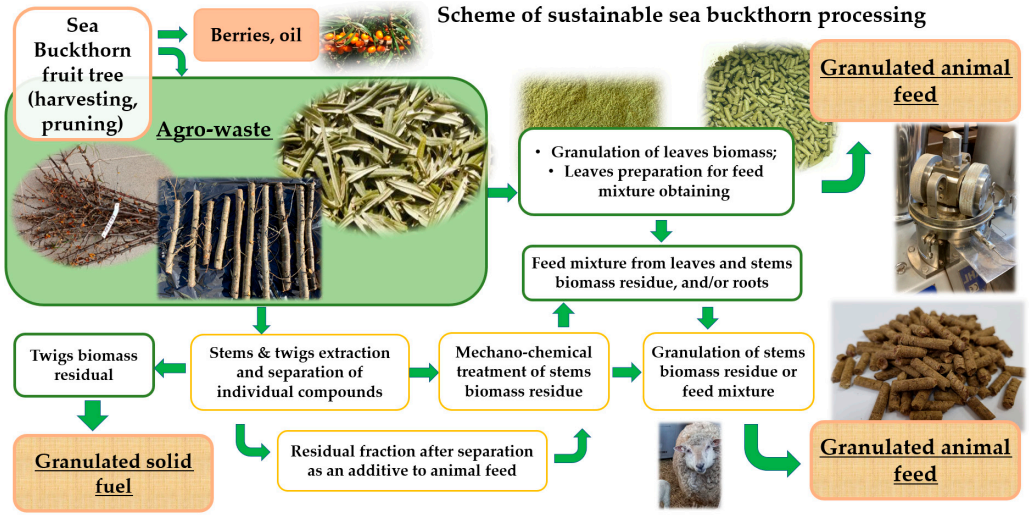

Figure 1. Scheme of sustainable sea buckthorn processing with the production of animal feed.

Such a scheme for cascading sustainable SBT processing, as well as experimental work on nutritional values, digestibility, and feed/fuel granule compositions, are novelties of the present research: all vegetative parts of the SBT tree (stem, leaves, twigs, and roots) were evaluated for the production of granulated feed for small ruminants; twig extracts were studied for the production of feed functional ingredients with antimicrobial properties; and extraction residues were evaluated as granulated fuel.

The development of alternative feed and feed additives on the market is also necessary to optimize livestock feeding costs and to provide a backup supply chain in times of economic instability and fluctuating energy prices. Building berry-producing plantations requires considerable investment [12]—and, thus, innovative agro-waste utilization turning it into valuable products is necessary.

2. Materials and Methods

2.1. Collection of Agro-Waste Biomass

The stems (ST), twigs (TW), leaves (LV), and roots (R) comprising the agro-waste biomass (further in the text—biomass) of SBT cultivars 'Maria Bruvele' (MB), 'Botanicheskaya Lubitelskaya' (BL), 'Tatiana' (TAT), 'Tarmo' (TM), and 'Otto' (OT) were collected from the four-year-old trees of an SBT plantation area in Latvia (80 trees of each cultivar) during the summer of 2021. The trees grew on the same land, and they were treated the same. The biomass was dried at room temperature. A knife mill, Retsch SM100 (Retsch, Haan, Germany), was used for grinding, and the LV particle size after grinding was 1–2 mm, and those of ST, TW, and R—2–4 mm.

2.2. Isolation of the Condensed Tannins from the ST and TW Biomass

An ethanol–water solution (80:20 vol.% ethanol:water solution, further in the text—80% EtOH) was used for the biomass extraction followed by CT isolation from the extract; the temperature of the extraction solution was 60–70 °C, and the mass ratio of biomass sample to 80% EtOH = 1:8, *w*/*w*. CT separation from the extract was carried out using a Sephadex LH-20 as described in Janceva et al. [42]. Confidence interval: CI $\leq$ 0.5% at α = 0.05.

2.3. Mechanochemical Treatment of SBT Biomass

The mechanochemical treatment of SBT initial ST biomass and ST biomass samples after CT extraction was carried out for evaluation of its effect on digestibility. Mechanochemical treatment was carried out separately for each sample in an original trituration-type mill (original construction, Riga, Latvia). The trituration of ST was carried out for 20 min, at 100 rpm.

2.4. Initial and Treated Biomass Characterization

All determinations are expressed on a dry matter (DM) basis (moisture content of initial and treated biomass less than 1%).

2.4.1. Crude Fiber Content Determination

The content of crude fiber in SBT biomass samples was determined gravimetrically by acid hydrolysis with H_2SO_4 (1.25%, *w*/*v*), used for the extraction of sugars and starch, followed by alkaline hydrolysis with NaOH (1.25%, *w*/*v*) which removes proteins, some hemicellulose, and lignin, as described by Joslyn et al. [43]. The weight of the biomass sample for one analysis was 20 g of DM. Each experiment was performed in triplicate. CI $\leq$ 0.3% at α = 0.05.

2.4.2. Total Protein Content Determination

The Kjeldahl method was applied for the determination of the total protein content in the SBT biomass [44]; a sample of 2 g was taken for analysis, and an appropriate nitrogen factor (NF—6.25) was used for the estimation of the total protein content. Each experiment was performed in triplicate. CI $\leq$ 0.3% at α = 0.05.

2.4.3. Determination of Crude Fat, Crude Ash, Macro-Elements, and Heavy Metal Content

To ascertain the content of crude fat, the extraction of fat from the SBT biomass samples by hexane was used. The weight of the obtained fat was measured, and the content of fat was expressed in % of the weight of the biomass sample. A sample of 10 g was taken for each analysis. The crude ash content (sample of 5 g for each experiment) was determined after biomass sample ignition at 550 °C in a Carbolite ELF 11/6B furnace while measuring the weight of the residue. The content of ash was expressed as % of the weight of the biomass sample. Organic matter content was calculated as the difference between the dry biomass content (taken as 100%) and the content of ash in %.

The contents of the macroelements and heavy metals were determined by ICP-MS analysis using a Thermo Fisher Scientific iCAP TQe (Bremen, Germany) fitted with a nebulizer, a quartz spray chamber, with a sampling cone made of nickel, and a skimmer cone with platinum tip, as described in Naccarato et al. [45]. A peristaltic pump and an autosampler ASX-560 (both from Thermo Fisher Scientific, GmbH, Bremen, Germany) were used to pump the solutions from the tubes. Following a 20 to 30 min period of ICP-MS stabilization, the working capacity was adjusted before the analyses to maximize the signal and minimize interference effects by applying a tuning solution based on the torch's horizontal and vertical location, the extraction lens, and the CCT (collision cell technology) focus lens. The highest purity argon and helium gas (99.99%) was employed as the carrier gas at 0.8 mL/min in auxiliary flow, at 1.0 mL/min, and 5.3 mL/min in nebulizer flow. Nitric acid (65%), Suprapur® for the trace analysis (Supelco), and hydrogen peroxide (30%) were all used in the sample digestion process. Calibration curves for quantitative analysis

were elaborated with the diluting of multielement solutions (10 mg/L); Cd, Ca, Pb, K, and Na (10 mg/L, Merck, Germany); and Hg element solution (1000 mg/L, Merck, Germany). The calibration standards, the procedure blanks, and the samples made up each batch of analysis. The weight of each sample was 100 mg. The majority of the elements under investigation were examined in kinetic energy discrimination mode (KED-mode) at the operational helium gas collision cell. Each experiment was performed in triplicate.

CI for crude fat: CI $\leq$ 0.6% at α = 0.05; for ash and organic matter: CI $\leq$ 0.9% at α = 0.05.

CI for heavy metal content is given in the Results Section 3.5, under corresponsing Table.

2.4.4. Determination of The Total Amount of Carbohydrates

Gas chromatography (GC) analysis before and after hydrolysis, reduction, and acetylation was used to determine the total amount of carbohydrates in the extracts, as well as their composition. For each experiment, a 10 mg sample was used. The analysis was performed using a GC System of the Agilent 6850 Series (Agilent Technologies, Inc., Santa Clara, CA, USA) as described in Blakeney et al. [46]. A DB-1701 column was used (the length of the column: 30 m; internal diameter: 0.25 mm; layer thickness: 0.25 µm). The analysis was repeated 3 times for each sample. CI $\leq$ 0.8% at α = 0.05.

2.4.5. Determination of Vitamin Content

The content of vitamin C (ascorbic acid) in the SBT biomass was determined by high-performance liquid chromatography (HPLC) as described in Ciulu et al. [47]. An HPLC-UV-Vis/-RI system (high-performance liquid chromatograph with UV–vis and RI detector) was used (Vanquish CORE, Dionex Softron GmbH, Part of Thermo Fisher Scientific, Germering, Germany). The extracts were rapidly dissolved in a purified water mixture of 2 M NaOH and 1 M phosphate buffer. Separation was performed on an Eclipse XDB-C18 Zorbax column (5 µm, 150 cm $\times$ 0.46 cm i.d., Agilent); the column was heated to 35 °C, and as a mobile phase, trifluoroacetic acid aqueous solution (0.025%, *v*/*v*) (A) and acetonitrile (B) were used. The gradient elution was applied (100% to 60% A in 20 min), at a flow rate of 1 mL/min. The injection volume was 20 µL. The UV detector settings: 254 nm.

The content of vitamin E as α-tocopherols and vitamin A as retinol in the biomass were determined by HPLC analysis, as described by Sibel Konyalıoğlu et al. [48] using an HPLC-UV-Vis/-RI system (Dionex Softron GmbH, Part of Thermo Fisher Scientific, Germering, Germany). A Hichrom 5 C18 column (25 cm $\times$ 4.6 mm i.d.) was used; methanol was used at the mobile phase, and the flow rate was 2 mL/min. The column was heated to 40 °C. The dry extracts were dissolved in methanol. Ten microliters of each aliquot were injected into the HPLC column. Detection was at 292 nm. Each experiment was performed in triplicate. The CI is given in the Results Section 3.5, under corresponsing Table.

2.4.6. Analytical Pyrolysis

The analytical pyrolysis (Py-GC/MC/FID) method was applied for the chemical characterization of SBT biomass. The temperature of pyrolysis was 500 °C and the heating rate was 600 °C/s. A Frontier Lab Micro Double-shot Pyrolyzer Py-3030D directly coupled with a Shimadzu gas chromatograph GC/MS/FID-QP ULTRA 2010 (Fukushima, Japan) was used. The capillary column was RTX-1701 (Restec, Metairie, Louisiana, USA), 60 m $\times$ 0.25 mm $\times$ 0.25 mm film. The injector temperature was 250 °C; ion source with EI of 70 eV. The MS scan range was 15–350 m/z. Helium was used as a carrier gas, the flow rate was 1 mL/min, and the split ratio was 1:30. A sample of 1.20 mg was taken for each analysis. The individual compounds were identified by GC/MS with the help of library MS NIST 11 and NIST 11s. On the basis of GC/FID data, the relative peak areas for the individual compounds were calculated using Shimadzu software. The relevant peaks' summed molar areas were normalized to 100%. The pyrolysis analysis was repeated four times and the data were averaged. The variation coefficient of measurement was $\leq$5%.

2.4.7. Elemental Analysis

The elemental composition (C, H, and N) of the SBT biomass samples was determined using a Vario MACRO CHNS elemental analyzer with a heat conduction detector (Elementar Analysensysteme GmbH, Langenselbold, Germany). The dry sample was weighed in a foil (weight of sample: 50 mg DM). The WO_2 powder was used as a combustion catalyst, in a ratio of 1:1 (w/w). The obtained sample/catalyst mixture was pressed into a tablet and placed in the automatic sample feeder (carousel). The equipment was controlled in a computerized mode and VARIOEL V5.16.10 software was used for data processing. The results were expressed as percentages of DM. Three repetitive analyses were performed for each sample. CI $\leq$ 0.2% at α = 0.05.

2.5. Preparation of Animal Feed Compositions

Stem (ST) biomass in compositions of animal feed was used as the residual fraction after extraction and CT separation and after mechanochemical pre-treatment (MT; mechanochemically treated stems further in the text—ST/MT). Leaves (LV), roots (RT), ST/MT, and mixes of LV with ST/MT were investigated as feed additives. The ratios of ST, LV, and RT used for analysis were as follows: LV 100%, ST 100%, ST/MT 100%, LV:ST/MT (1:1; w/w), and LV:ST (1:1; w/w). In addition, for the granulation experiment, the mix with roots was used, LV: ST/MT (1:1, w/w) + 5% of roots.

2.6. Determination of Released Gas Emissions, In Vitro Analysis

The amount of the in vitro gas production (GP) was determined using an ANKOM RF Gas Production System (AGPS; ANKOM Technology, Macedon, NY, USA), which is designed for analysis of different feed sources and feed additives. The in vitro gas production method is based on the relationship between the fermentation in the rumen and the gases formed and can also be used to measure and quantify nutrient utilization. Rumen fluid was collected from slaughterhouse animals (rams) following the protocol of Fortina et al. (2022) [49], with small modifications. It was decided to use rumen fluid collected from slaughterhouse animals, first, because a significant difference in in vitro digestibility has not been found when the fluid was obtained from slaughtered or fistulated ruminants [50], and, second, because this was a more ethically acceptable approach [51]. It was reported that it is possible to store the rumen fluid without significant quality changes by putting it in thermic bottles wrapped in a thermic bag, for a period of up to 300 min after collection [49]. The methodology and test conditions were in accordance with the prescriptions provided by the manufacturer and following the protocol of Videv [52]. In short, a feed sample in the amount of 0.500 $\pm$ 0.001 g, 25 mL of rumen fluid, and 50 mL of incubation medium, made as described by Theodorou et al. [53], were placed in each of the modules of the system. Each of the 50 modules of the ANKOM system has pressure measuring sensors installed (the range is: $-$69 to 3.447 kPa; resolution: 0.27 kPa; accuracy: $\pm$ 0.1% of the measured value). Specialized software received the data from every module through a wireless connection and recorded it every 30 s. GP was expressed as mL/g incubated DM. The changes in gas pressure accumulated during 24 and 48 h of fermentation (ΔP) were converted into volume units by applying the ideal gas law:

$$\text{GP (mL/g DM)} = (\Delta P/Po) \times Vo, \text{(mL/g incubated DM)}, \quad (1)$$

where ΔP is the change in the accumulated pressure (expressed in kPa) at the top of the module, Vo is the volume of the bottle at the top (235 mL), and Po is the atmospheric pressure which was recorded by the apparatus before the beginning of the experiment.

For taking into account the final volume of released gas from the rumen fluid itself, a blank module without a sample of the feed was used. The zero module, placed above the incubator, took into account the atmospheric pressure in the room and corrected the data according to atmospheric pressure. The samples were analyzed in triplicate.

2.7. Determination of Digestibility of the SBT Biomass Samples

For the evaluation of digestibility, an Ankom Daisy incubator was used (Ankom Technology, Macedon, NY, USA). The rumen fluid, used in this testing, was collected as described in Section 2.6. The methodology and test conditions were in accordance with the manufacturer's prescriptions and followed the protocol of Kiliç et al. [54]. Four rotating digestion jars (or cylinders) were placed in the specially designed and controlled Daisy incubator where a constant, uniform heat (≈39 °C) was maintained and agitation was provided. A buffer solution (1600 mL) and rumen fluid (400 mL) were used as inoculums for each cylinder. The dry samples in the amount of approximately 500 mg were placed in filter bags (25 pcs). Then, the filter bags were placed into the cylinders with the inoculum. Aeration of the cylinder was performed for 30 s using CO_2, and then the cylinders were tightly closed (immediately after aeration) and placed in the incubator for 48 h. After 48 h of incubation, the filter bags were taken out from the incubator. Water was used for cleaning the bags, and then the bags were dried at 105 °C, for 3 h. Analysis for neutral detergent fiber (NDF) digestibility of the contents of the bags was performed with a fiber analyzer. In vitro true digestibility was calculated according to the following equation:

$$\text{IVTD, \%} = 100 - ((\text{W3} - (\text{W1xC1})) \times 100)/\text{W2} \quad (2)$$

where IVTD is the in vitro true digestibility of feed, W1 is the weight of the filter bag, W2 is the weight of the sample put in the bag, W3 is the weight of the bag with the sample after NDF analysis, and C1 is the correction coefficient for the weight of the bag without a sample.

Three repetitions of the in vitro experiment were performed. The confidence intervals are given in the Results Section 3.7.

2.8. Antimicrobial Activity of the SBT Fraction

Analysis of the antimicrobial activity of the residual fraction after CT separation from the extracts of both stems and twigs (MB/ST+TW) was performed at the University of Latvia, Faculty of Biology. Several reference microbial strains were used, which were obtained from the Latvian Microbial Strain Collection (MSCL), University of Latvia: *Pseudomonas aeruginosa* MSCL 3314, *Staphylococcus aureus* MSCL 3340, *Escherichia coli* MSCL 332, *Bacillus cereus* MSCL 330, and *Candida albicans* MSCL 378. Antimicrobial activity was analyzed in 96-well plates by the two-fold serial broth microdilution method [55]. As a result, the values of the minimum inhibitory (MIC) and minimum bactericidal/fungicidal concentrations were ascertained (MBC/MFC). CI $\leq$ 0.01 at α = 0.05.

2.9. SBT Biomass Granulation and Characterization of the Pellets

2.9.1. Biomass Granulation

For the simulation of the pelletizing in real production conditions, a laboratory pelletizer KAHL 14-175 (Amandus Kahl GmbH & Co. KG, Reinbek, Germany) equipped with a flat die, analogous to the factory-scale KAHL granulators, was used for granulation. The channel diameter was 6 mm, and the channel length-to-diameter ratio was 4:1. Each experiment was performed in triplicate. Preliminary granulation of sawdust until the equilibrium temperature in the pelletizer reached 50 °C was performed. The weight of the studied biomass used for one granulation experiment was 2 kg.

2.9.2. Characterization of the Pellets

The measurement of the ash content was performed, expressed as a % of the weight of the residue after the ignition of solid biomass samples at 550 °C in a muffle furnace to the initial weight of the solid biomass sample, according to the EN ISO 18122:2023 standard [56]. Higher heating value (HHV) was determined experimentally by burning granules in a calorific bomb of original construction (Li-104, Latvia), according to the standard ISO 18125:2017 (solid biofuels) [57] and calculated on a DM basis. A sieve with a 3.15 mm cell

size was used for the separation of fines (ISO 3310) [58]. After the fines separation, the determination of the durability (DU) and bulk density (BD) was performed based on the European standards EN ISO 17831-1:2016 [59] and ISO 17828 [60], correspondingly. The CI for the HHV and LHV of the pellets: ±0.6 MJ/kg; the CI for durability: ±0.7%; the CI for bulk density: ±11 kg/m^3; the CI for ash content: ±0.6%; the CI for the average length of pellets: ±10 mm.

2.10. Statistical Analysis

All experiments were conducted in triplicate, except for analytical pyrolysis (Py-GC/MS/FID) and gas chromatography (GC/MS/FID) analysis where four repetitive experiments were performed. The results are expressed as means. Microsoft Excel 2016 was used for statistical analyses. Confidence intervals (CI) were calculated for a mean using Student's *t*-distribution, and a significance level of 5% was applied ($\alpha = 0.05$). For the evaluation of the strength of the linear relationship between two different variables, Pearson's correlation coefficient was calculated. A significance level of $p < 0.05$ was applied.

3. Results and Discussion

3.1. Chemical Composition of SBT Biomass

3.1.1. Organic Matter in the Biomass

It was found that the biomass samples had a high content of organic matter (95.5–98.1%/DM), which included such components as proteins, fats, fiber, and non-structural carbohydrates (sugar and starch). The ash content of the SBT biomass samples ranged from 1.9 to 5.2%/DM. The highest ash content was in leaves (4.8–5.2%/DM) and roots (4.0–4.5%/DM).

3.1.2. Relative Composition of SBT Biomass by Py-GC/MS/FID

According to the results of analytical pyrolysis analysis, the main components of organic volatile products of SBT biomass DM are carbohydrates, including low-molecular-weight sugars, starch, and various non-starch polysaccharides, which are the most important sources of energy for non-ruminants and ruminants. The total carbohydrates-derived volatile contents in the SBT stems, leaves, and roots were 66.7–68.4% rel, 56.7–60.6% rel, and 72.8–75.9% rel/TVP, respectively (Figure 2).

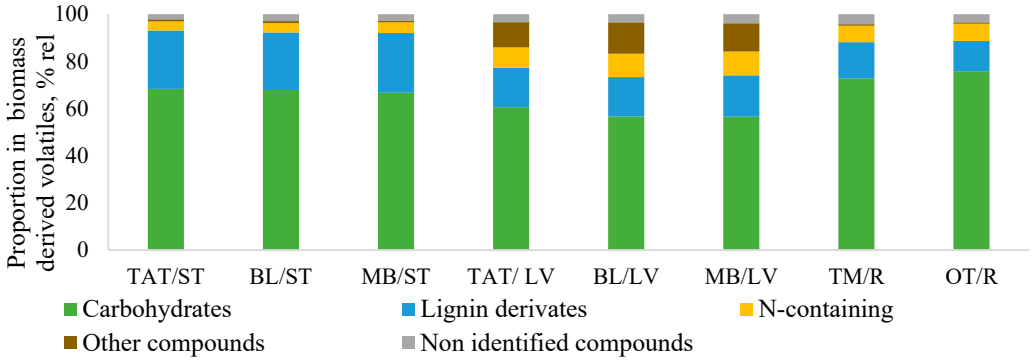

Figure 2. Py-GC/MS/FID data of organic volatile products of SBT biomass: TAT—Tatiana; BL—Botanicheskaya Lubitelskaya; MB—Maria Bruvele; OT—Otto; TM—Tarmo; ST—stem; LV—leaves; R—roots.

The carbohydrate concentration of the SBT roots was 1.1 and 1.3 times higher in comparison to the stems and leaves biomasses, respectively.

3.1.3. Carbohydrate Composition by GC-MS

Based on the results of gas chromatography analysis, the main sugar monomer units of roots' carbohydrate composition were glucose (73.6–77.1%/total carbohydrate content of root DM) and mannose (10.1–12.9%/total carbohydrate content of root DM). The total

contents of galactose, xylose, and arabinose were 2.1–3.1%, 3.2–4.6%, and 6.5–6.8%/total carbohydrate content of root DM, respectively (Figure 3).

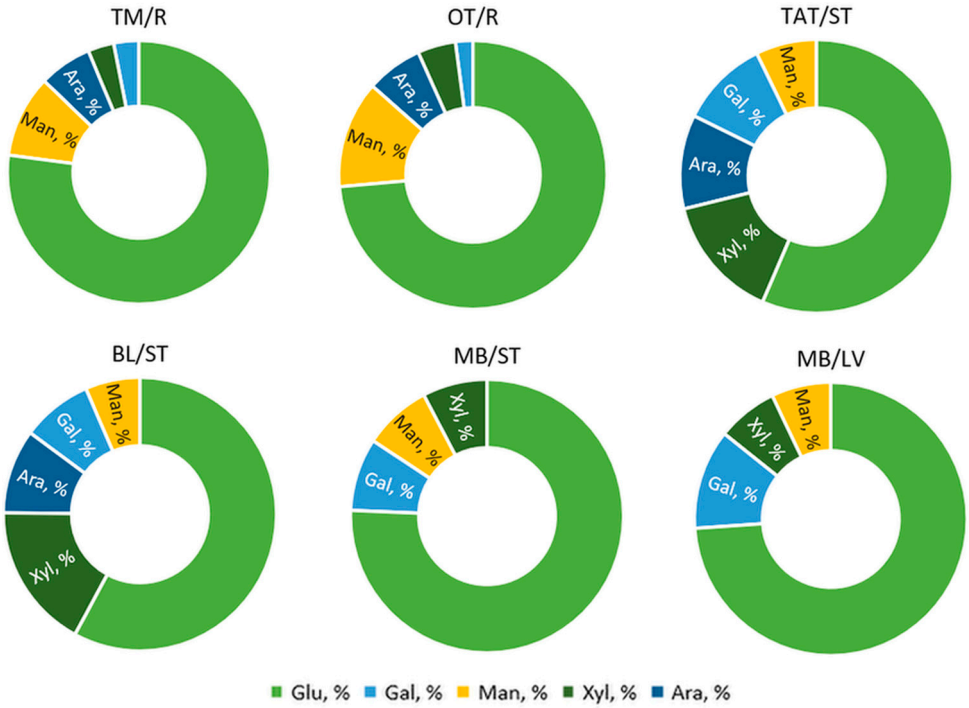

Figure 3. The sugar composition in total carbohydrates of SBT biomass (GC data): TAT—Tatiana; BL—Botanicheskaya Lubitelskaya; MB—Maria Bruvele; OT—Otto; TM—Tarmo; ST—stem; LV—leaves; R—roots.

The amount of glucose in the composition of total identified sugars of the SBT stems, roots, and leaves was close for each vegetative part between the five cultivars. The amount of xylose was the highest for the stems of BL and TAT. Xylose is not a desirable component in feed: it was proven that in high amounts it could reduce ruminal digestibility of various animal feeds [61,62]. Therefore, for the subsequent first experiments, stem samples of MB with comparatively smaller content of xylose were chosen. The structural (free) carbohydrate (monosaccharide) content in SBT biomass did not exceed 1%/DM. To make carbohydrates more available as an energy source, pretreatment was considered. It was reported that it is possible to increase the surface area of cellulose up to 10^6 times by decreasing its particle size [63], and thus improve the nonmotile cellulolytic microbe penetration into the cell lumen [64].

3.1.4. Relative Composition of SBT Biomass Phenol/Lignin Part by Py-GC/MS/FID

The total phenol/lignin-derived volatile (Ph/L-DV) contents in the SBT stems, leaves, and roots were 24.4–25.4% rel, 16.7–17.6% rel, and 12.8–15.3% rel/TVP, respectively. The phenol/lignin-derived pyrolysis products can be divided into phenyl (Ph) and benzyl (B), guaiacyl (G), and syringyl (S) derivatives in SBT biomass. The Ph/L-DV of SBT stems have the highest content of G derivative units (43.8–50.3% rel/Ph/L-DV), and fewer S (32.0–35.0%/ rel Ph/L-DV) and Ph and B units (17.4–21.2% rel/Ph/L-DV). The Ph/L-DV of leaves and roots presented more Ph and B derivative units, 40.8–44.9% and 69.7–71.9%, respectively (Figure 4).

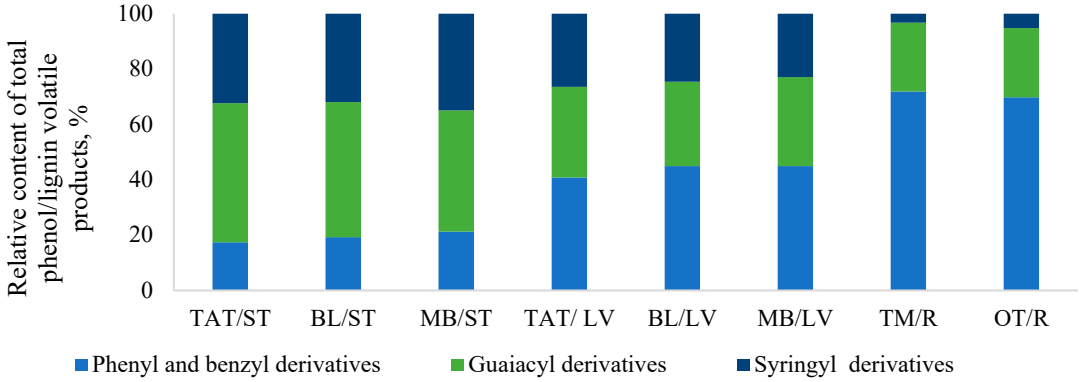

Figure 4. Relative contents (%) of phenyl and benzyl, guaiacyl, and syringyl derivatives in the Ph/L-DV released after Py-GC/MS of SBT biomass: TAT—Tatiana; BL—Botanicheskaya Lubitelskaya; MB—Maria Bruvele; OT—Otto; TM—Tarmo; ST—stem; LV—leaves; R—roots.

The phenyl and benzyl derivatives came from polyphenolic compounds that have antioxidant activity [65] and could serve for the oxidative stability of animal feed. Since phenolic compounds will remain in the residual biomass fraction after the separation of CTs, their antibacterial activity will be tested (Section 3.1.2). Lignin is a hardly digestible source and its strong bonds in lignin–carbohydrate complexes are the main obstacle to wood-containing part application in animal feed [66]. Therefore, mechanochemical pre-treatment was further investigated in this study (Section 3.3) for the possibility of degrading the cell wall.

3.2. CT Separation

According to the literature data, CTs, which are found among polyphenolic compounds in SBT biomass, are also anti-nutritional since they bind proteins. However, CTs are strong antioxidant and antimicrobial agents and can be used in cosmetics, the production of adhesives, and other related industries [19,67]. Among the studied biomass samples (stems, twigs, roots, and leaves) only SBT stems and twigs contained CTs in an amount of 6 to 11%/DM. Only the stems could be used for animal feed production since the twigs have sharp thorns, and therefore twigs will be tested for granulated fuel production. However, CT, as a valuable compound, was preliminarily isolated from both stems and twigs. The correctly chosen extractant made it possible to completely remove CTs from the stem and twig biomasses.

3.3. Mechanochemical Pre-Treatment for the Improvement of Digestibility

In animal nutrition, lignin cannot be readily fermented by rumen microbes. The solution to this was the use of mechanochemical processing. The mechanochemical treatment disrupts the cell wall of the plant, thereby facilitating the digestibility of valuable components. The digestibility results of SBT stems after CT separation before and after mechanochemical processing are shown in 3.7.

3.4. The Anti-Microbial Properties of the Residual Fraction after CT Separation

The residual fraction after CT separation contained serotonin, low-molecular-weight polyphenolic compounds (quinic acid, catechin, etc.), and their glycosides [19]. In this study, this residual fraction's antimicrobial activity was evaluated against Gram-positive and Gram-negative bacteria as well as pathogenic fungi. The lowest MIC/MBC values for the fraction were the following: 0.78/0.78 mg/mL against *E. coli*, 1.56/3.13 mg/mL against *P. aeruginosa*, 1.56/>50 mg/mL against *B. cereus*, and 0.78/1.56 mg/mL against *S. aureus*. The lowest MIC/MFC against *C. albicans* was 12.50/>50 mg/mL. This showed that enriching the biomass with the above-mentioned low-molecular-weight components and

returning them to the biomass is a way to create feed additives with special antimicrobial target properties (Figure 5).

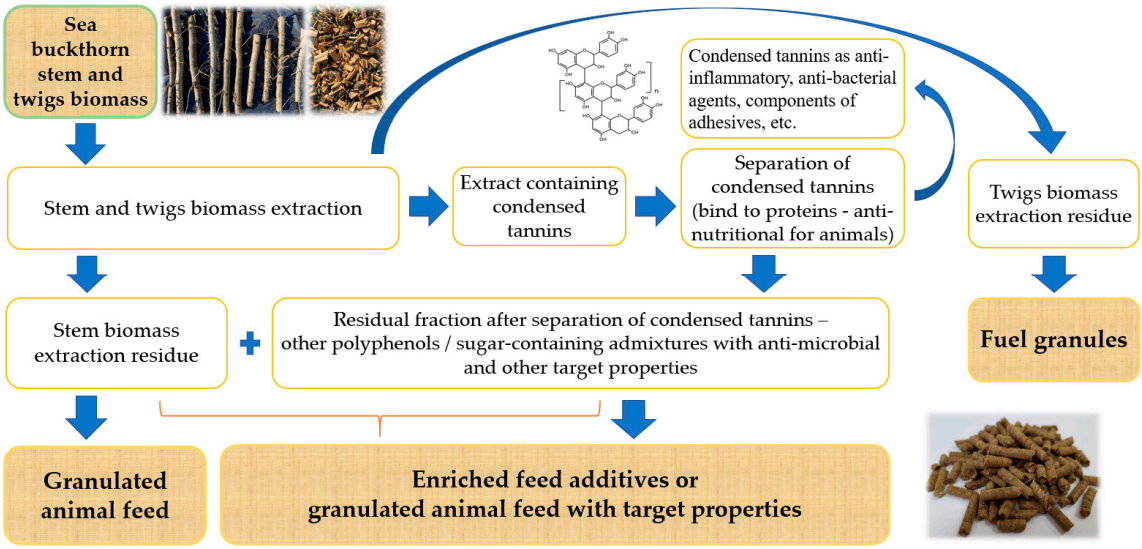

Figure 5. SBT stems and twigs application scheme.

3.5. Macro-Nutrients and Vitamins in SBT Biomass

The physiological and functional processes of an animal are influenced not only by organic matter but also by the inorganic components in the feed additive. The results of the analyses are shown below in Table 1.

Table 1. Contents of macronutrients and heavy metals in SBT biomass.

Macronutrients and Heavy Metals *	MB/LV	MB/ST	BL/LV	BL/ST	TAT/LV	TAT/ST
P, mg/100 g DM	225 ± 22	220 ± 22	210 ± 21	199 ± 20	212 ± 18	217 ± 26
K, mg/100 g DM	1376 ± 113	1109 ± 107	1209 ± 104	1037 ± 56	1216 ± 114	1119 ± 108
Na, mg/100 g DM	1.72 ± 0.40	22.5 ± 5.2	2.25 ± 0.52	7.83 ± 1.80	1.88 ± 0.36	11.4 ± 3.5
Ca, mg/100 g DM	989 ± 237	281 ± 67	856 ± 205	332 ± 80	917 ± 162	306 ± 46
Cd **, mg/kg DM	0.011 ± 0.003	0.027 ± 0.006	0.011 ± 0.003	0.011 ± 0.002	0.014 ± 0.002	0.018 ± 0.004
Hg **, mg/kg DM	0.0069 ± 0.0012	0.0031 ± 0.0006	0.0067 ± 0.0012	0.0022 ± 0.0004	0.0058 ± 0.0004	0.0028 ± 0.0005
Pb **, mg/kg DM	0.086 ± 0.0022	0.086 ± 0.0022	0.10 ± 0.030	0.037 ± 0.010	0.0042 ± 0.0021	0.073 ± 0.0016

** Does not exceed the permitted maximum: Cd—1 mg/kg DM; Hg—2 mg/kg DM; Pb—10 mg/kg DM (the values in Regulation No. 1275/2013). * The results are shown as mean ± CI at $\alpha = 0.05$.

Determination of the content of heavy metals in the feed is necessary since heavy metals have toxic effects on animal health. The analysis showed that heavy metal content did not exceed the permissible norms mentioned in the Commission Regulation (EU) No. 1275/2013 [68] (Table 1).

It was shown that SBT biomass contains fat-soluble and water-soluble vitamins. Vitamin C content was much higher in the stems than it was in the roots and leaves. The leaves of all three SBT cultivars are richer in vitamins E and C. The A vitamin was not found in the SBT stems and roots (n.f.) (Table 2).

Table 2. Contents of vitamins in SBT biomass.

Samples *	Vitamin C, mg/100 g DM‘	Vitamin E (α-tocopherol), mg/100 g DM	Vitamin A (Retinol), mg/100 g DM
MB/LV	15.6 ± 4.4	30.9 ± 4.3	1.29 ± 0.02
MB/ST	178.0 ± 50.0	17.3 ± 2.4	n.f.
BL/LV	12.0 ± 3.0	44.3 ± 6.2	1.14 ± 0.03
BL/ST	9.2 ± 3.6	14.7 ± 2.1	n.f
TAT/LV	13.3 ± 3.6	42.6 ± 2.2	0.86 ± 0.07
TAT/ST	8.0 ± 3.0	16.2 ± 2.6	n.f.
TM/R	n.d.	n.d.	n.f.
OT/R	n.d.	n.d.	n.f.

TAT—Tatiana; BL—Botanicheskaya Lubitelskaya; MB—Maria Bruvele; OT—Otto; TM—Tarmo; ST—stem; LV—leaves; R—roots. * The results are shown as mean ± CI at $\alpha = 0.05$.

Moreover, it has been reported that SBT leaves contain thirteen different amino acids, and wood and bark contain seventeen amino acids [69].

3.6. Main Compounds in SBT Biomass and Their Role in Rumen Digestion

In a complete diet, the amount of crude fiber, protein, and crude fat is of great importance. The total protein content in the dry SBT biomass samples ranged from 18% to 24%/DM (Figure 6).

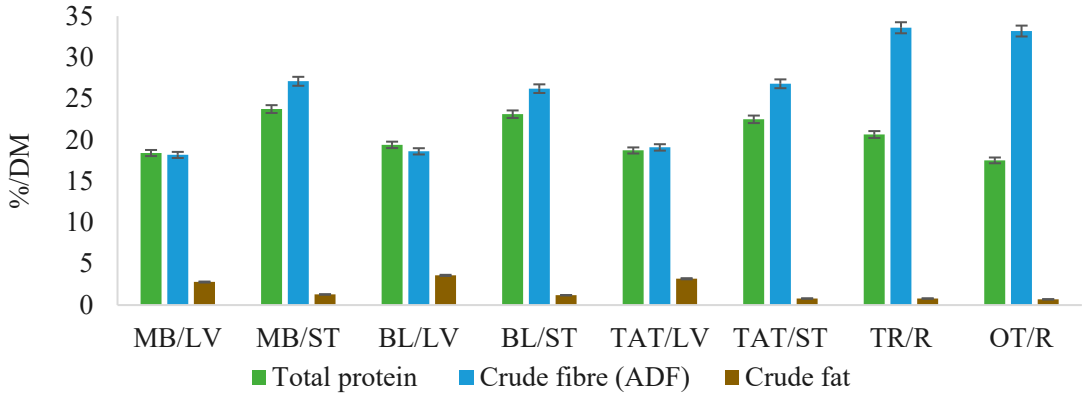

Figure 6. Percentage of total protein, crude fiber, and crude fat in dry SBT biomass (TAT—Tatiana; BL—Botanicheskaya Lubitelskaya; MB—Maria Bruvele; OT—Otto; TM—Tarmo; ST—stem; LV—leaves; R—roots).

The highest content of total protein was in SBT stems ~23%/DM, followed by leaves (18.4–19.4%/DM) and roots (17.5–20.7%/DM).

The content of total fat in SBT biomass was as follows: stems (0.7–1.2%/DM), leaves (2.8–3.6%/DM), and roots (0.7–0.8%/DM). Dairy cows and sheep usually have a pasture-based diet with a low fat content of 2–6% on a DM basis. However, the energy content in fat is more than twice that of carbohydrates, calculated based on weight. Dietary fat contents over 8% can negatively impact rumen function, fiber digestion, and milk production [70]. Thus, it can be said that the fat content in SBT stems and leaves is optimal for nutritional feed.

The crude fiber content in the biomass was 18–27%/DM. Crude fiber is usually indigestible or barely digestible, but it stimulates the production of important gut bacteria. With a deficiency of crude fiber in the diets of cows, an upset of pre-gastric digestion occurs, and the productivity of milk production deteriorates [71]. The highest content of crude fiber in biomass was in SBT roots (33.2–33.6%/DM), followed by stems (26.2–27.1%/DM) and leaves

(18.1–19.1%/DM). Since the crude fiber concentration of SBT roots was ~2 times higher than in leaves, roots could be used for the production of fiber-containing feed additives.

3.7. Determination of the in vitro Gas Production and Digestibility of SBT Biomass

For the evaluation of the feed, methods of measuring digestion by in vitro techniques are ethically preferable, less expensive, and faster than in vivo methods [72]. In the in vitro released gas measurements, the amount of gas that arises from the fermentation process is measured. The high potential of a feed's nutritional and biological value is realized through proper digestion.

The testing was performed on samples from the MB cultivar because it shows better overall composition of the main nutritive compounds. According to in vitro test data, the extract showed the greatest digestibility after the separation of CT. The leaves have a much higher digestibility than the stems. The stems after mechanochemical treatment had a 2.2 times greater digestibility in comparison to the stems before treatment (Table 3).

Table 3. In vitro true digestibility (IVTD) of SBT biomass samples.

Sample *	GP24, mL/g DM	GP48, mL/g DM	IVTD, %/DM
MB/LV	59.97 ± 1.94	71.76 ± 1.61	82.60 ± 4.80
MB/ST/MT	72.38 ± 3.46	83.18 ± 2.21	39.12 ± 6.06
MB/ST	53.99 ± 8.19	65.33 ± 5.56	18.11 ± 4.61
MB/LV: MB/ST/MT (*w*/*w*; 1:1)	76.29 ± 5.73	84.65 ± 7.18	58.11 ± 5.05
MB/LV: MB/ST (*w*/*w*; 1:1)	76.73 ± 5.51	81.93 ± 8.97	49.83 ± 5.16
MB residual fraction after CT separation	134.58 ± 3.94	141.51 ± 4.10	98.69 ± 4.44

ST—stems after CT separation; LV—leaves; R—roots; MB/ST/MT—mechanochemically treated Maria Bruvele stems after CT separation; GP24—gas pressure of 24 h incubated DM; GP48—gas pressure of 24 h incubated DM; IVTD—in vitro true digestibility of feed. * The results are shown as mean ±CI at $\alpha = 0.05$.

It can be seen that the digestibility of the leaves is a bit lower than that of the MB residual fraction after CT separation, but at the same time, the gas emissions are much lower for the leaves. Therefore, for a reduction in GHG emissions, the SBT leaves should always be in the composition of the SBT-based animal feed. The leaves can be combined with other types of biomasses.

The samples from the MB/LV (IVTD = 82.60%) and MB residual fraction after CT separation (98.69%) showed higher digestibility than canola (64.15%), mustard (73.54%), and turnip hays (61.2%), obtained under similar conditions [54], as well as *Quercus robur* L. oak tree leaves (56.22%), alfalfa hay (71.60), giant fennel hey (70.47%) [73], corn silage (61.95%), perennial ryegrass (71.67%), and common vetch/oat hay (66.04%) [74].

Under similar conditions to those in our experiments, the GP24 and GP48 in traditionally used cereal grain forages were as follows: barley—289.5 mL/g DM and 405.8 mL/g DM; wheat—339 mL/g DM and 448.1 mL/g DM; and maize—421.3 mL/g DM and 491.5 mL/g DM, for 24 h and 48 h, respectively. Meanwhile, in the SBT-based samples, the GP for both 24 and 48 h of incubation was several times lower, with only the MB residual fraction after CT separation showing a slightly higher production of gasses (see Table 3); however, even in that case, the gas production was at least two times lower than that of the cereal grain forages [52].

The in vitro testing showed promising results with regard to the future use of SBT biomass as animal feed since all samples had low GHG production accompanied by high digestibility in the leaves and biomass residual fraction after CT separation. Future in vivo experiments will be needed to prove the possibility of the sustainable use of these biomass products as a substitute for some of the traditionally used plant feeds. Moreover, the use of plant biomass with a lower GP will reduce the negative CO_2 imprint from livestock breeding and, thus, will have a positive ecological effect.

3.8. Caloric Value of SBT Biomass

The contents of carbohydrates, lipids, and proteins are stoichiometrically connected with the contents of carbon, hydrogen, and nitrogen. Therefore, based on the results of CHN elemental analyses of biomass, it is possible to calculate a caloric value [75]. The carbon content in the SBT biomass samples varied from 40.5% to 50.9% (Table 4).

Table 4. Elemental analysis and caloric value data of SBT biomass.

SBT Biomass	C	H	N	Organic Matter	Calorific Value, MJ/kg DM		Caloric Value, kcal/g DM	
					HCV	LCV	HCV	LCV
	%/DM; CI ≤ 0.2% at α = 0.05				CI ≤ 0.03% at α = 0.05			
MB/ST	50.6	5.6	3.8	97.2	20.47	19.34	4.89	4.62
BL/ST	49.9	5.6	3.7	98.1	20.21	19.07	4.83	4.55
TAT/ST	50.9	5.5	3.6	97.6	20.52	19.41	4.90	4.64
MB/LV	49.1	5.9	2.7	94.8	19.79	18.67	4.73	4.46
BL/LV	49.3	5.8	3.1	96.2	19.90	18.80	4.75	4.49
TAT/LV	49.5	5.9	3.0	94.8	20.02	18.89	4.78	4.51
TM/R	46.5	4.8	3.3	95.5	18.63	17.51	4.45	4.18
OT/R	40.5	5.0	2.8	96.0	16.60	15.48	3.96	3.70

TAT—Tatiana; BL—Botanicheskaya Lubitelskaya; MB—Maria Bruvele; OT—Otto; TM—Tarmo; ST—stem; LV—leaves; R—roots.

The calorific values of the plant samples were all in the range of 16.6 MJ/kg to 20.52 MJ/kg. The data confirmed that the higher the carbon content, the higher the caloric value, for all the SBT samples. Considering that 1 MJ is 238.85 kcal, the caloric value of the studied biomass was in the range of 4–5 kcal/g DM. The relationship between the carbon content and caloric value with a correlation coefficient of 0.99 is shown in Figure 7.

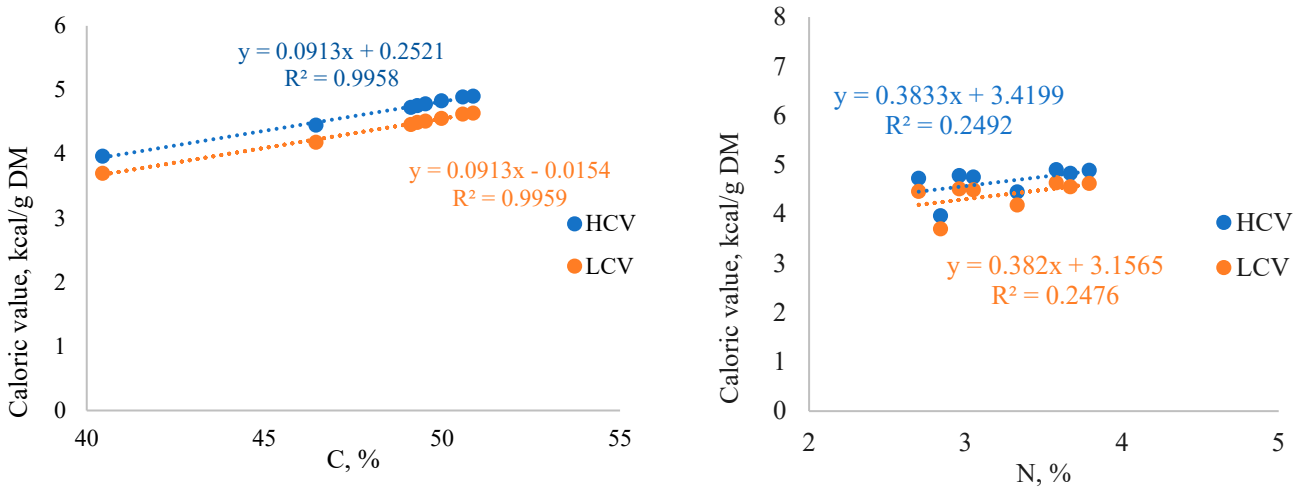

Figure 7. Correlations between C (on the (**left**)) and N (on the (**right**)) element content and caloric value (HCV and LCV).

It can be seen that the N content in the biomass samples does not correlate well with the caloric values of the feed.

3.9. Granulation of SBT Biomass Samples

The granulation of biomass is an effective method for preserving stable quality indicators during the storage of feed and for the improvement of technological characteristics.

MB stems after MT and the separation of CTs and leaves were granulated as described in Section 2.9. Granulation with the roots added (5% of total biomass, correspondingly) was tested to improve the quality of the granules (Figure 8).

Figure 8. Feed pellets: (**A**)—MB/LV: MB/ST/MT (1:1, *w*/*w*) + 5% TM/R; (**B**)—MB/LV; (**C**)—MB/ST/MT.

The characteristics of the obtained pellets are shown in Table 5. In the presence of roots, the biomass became stickier, the durability of the pellets showed a tendency to improve (although insignificantly), and the amount of fines in the pellets was diminished. The roots were also provided to intercalate a sweet taste to the feed. The disintegration of the granules in water was evaluated visually. The time of swelling for the granules made of 100% stems or leaves was 30–60 min.

Table 5. The characteristics of feed granules obtained on the basis of SBT biomass.

Samples	Pellets Durability, %	Pellets Moisture, %	Bulk Density, kg/m^3	Average Length, mm
MB/ST/MT	96.9	5.8	714.8	12
MB/LV	97.7	5.4	715.7	12
MB/LV: MB/ST/MT (1:1, *w*/*w*)	97.2	5.5	714.2	12
MB/LV: MB/ST/MT (1:1, *w*/*w*) + 5% TM/R	98.1	5.6	714.8	8

MB—Maria Bruvele; TM—Tarmo; ST—stem; LV—leaves; R—roots.

The twigs of BL, MB, and TAT, after CT separation, were granulated to obtain fuel pellets. The HHVs of the pellets were 19.8–20.5 MJ/kg (LHV: 18.5–19.6 MJ/kg); durability: 96.8–97.2%; bulk density: 713–715 kg/m^3; ash content: 3.5–3.9%; and the average length of pellets: 12 mm. According to the specifications of the EN ISO 17225 standard [76], the twigs after the separation of CTs can be used for the production of granulated fuel for district heating and power stations. Adding some amount of sawdust would help to diminish the ash content to the level of less than 2%, as required for pellets for non-industrial applications (ISO 17225-2 standard, class B). This study confirmed that the residue of twigs after the isolation of CTs can be used as a granular fuel.

4. Conclusions

This study confirmed that SBT agro-waste biomass, after the separation of condensed tannins, could be a unique and valuable raw material for ruminant feed and feed additive production. Agro-waste biomass, as a side-product of the SBT berry industry, demands no additional agricultural land for the production of the animal feed that supports sustainable agriculture. The high amount of protein, wood fiber, macronutrients, and vitamins in SBT plant material can provide livestock with alternative feed options when other sources of feed are limited. Feed on an SBT basis can also be valuable for animals during winter, and dry seasons, or serve as a supplement to low-protein forage. The anti-microbial properties of the residual fraction after CT separation are useful for particular animal health conditions and for

safe feed storage. The twig fraction, after the separation of CT, is suitable for the production of pellets for district heating and power stations. SBT biomass utilization for animal feed additives and solid fuel allows the creation of a scheme for sustainable SBT berry production, where each target product residual fraction has an added-value application.

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Institutional Review Board Statement: Ethical review and approval were waived for this study since the rumen fluid was obtained from slaughterhouse rams to avoid interfering with animal welfare.

Informed Consent Statement: Not applicable.

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Anti-Inflammatory, Anti-Bacterial, and Anti-Fungal Activity of Oligomeric Proanthocyanidins and Extracts Obtained from Lignocellulosic Agricultural Waste

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Abstract: It has now been proven that many pathogens that cause infections and inflammation gradually mutate and become resistant to antibiotics. Chemically synthesized drugs treating inflammation most often only affect symptoms, but side effects could lead to the failure of human organs' functionality. On the other hand, plant-derived natural compounds have a long-term healing effect. It was shown that sea buckthorn (SBT) twigs are a rich source of biologically active compounds, including oligomeric proanthocyanidins (PACs). This study aimed to assess the anti-pathogenic and anti-inflammatory activity of water/ethanol extracts and PACs obtained from the lignocellulosic biomass of eight SBT cultivars. The anti-pathogenic activity of extracts and PACs was studied against pathogenic bacteria *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Bacillus cereus* and fungus *Candida albicans* in 96-well plates by the two-fold serial broth microdilution method. The anti-bacterial activity of purified PACs was 4 and 10 times higher than for water and water/ethanol extracts, respectively, but the extracts had higher anti-fungal activity. Purified PACs showed the ability to reduce IL-8 and IL-6 secretion from poly-I:C-stimulated peripheral blood mononuclear cells. For the extracts and PACs of SBT cultivar 'Maria Bruvele' in the concentration range 0.0313–4.0 mg/mL, no toxic effect was observed.

Keywords: anti-inflammatory; anti-microbial activity; anti-pathogenic activity; anti-fungal; proanthocyanidins; extracts; lignocellulosic biomass; sea buckthorn

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1. Introduction

Following the global response to SARS-CoV-2, emerging antibiotic resistances were evaluated as a "silent pandemic", putting the ability to effectively combat prevalent infectious diseases at risk [1]. Estimates from the European Union/European Economic Area (EU/EEA) alone show that each year, more than 670,000 infections occur due to bacteria being resistant to antibiotics, and approximately 33,000 people die as a direct consequence [2]. Infections caused by bacteria forming biofilms are much less susceptible to antibiotics [3,4]. Bacterial infections are a common complication after primary infection with respiratory viruses such as influenza viruses, rhinoviruses, and coronaviruses and are often characterized by severe disease and high mortality [5]. New powerful anti-microbial agents are necessary, but stronger antibiotics of synthetic origin cause more severe side effects and "sweep away" both pathogenic and beneficial microorganisms.

Moreover, one of the side effects of antibiotics overuse is the development of resistant fungal infections. The polymorphic fungus *Candida albicans* is a member of the normal

human microbiome, but under certain circumstances, especially among immunocompromised populations, it causes infections that range from superficial infections of the skin to life-threatening systemic infections in the bloodstream or internal organs [6]. *Candida* is the fourth most common cause of hospital-acquired systemic infections in the United States, with crude mortality rates of up to 50% [7]. Treatment for these fungal infections has adverse effects and is slowly becoming obsolete due to varying mutation rates and rising resistance in multiple species. For example, about 7% of all *Candida* blood samples tested at the CDC are resistant to the most common anti-fungal drug fluconazole [8].

Finding natural compounds that would effectively act on bacterial and fungal infections, independently or synergistically with anti-bacterial or anti-fungal treatments, and increasing the body's own ability to overcome the infection without exerting toxicity on internal organs are challenging emerging tasks today.

Infections can become more dangerous if there is an excessive inflammatory response. Many kinds of cells and active ingredients (cytokines, chemokines, and bioactive amines) participate in inflammation [9]. The accompanying SARS-CoV-2 cytokine storm—a group of related medical conditions in which the immune system produces too many inflammatory signals—can lead to organ failure and death. Inflammation has a protective function, but excess inflammation can induce host tissue damage, chronic diseases, and even cancer [10,11]. Currently, anti-inflammatory drugs are mainly steroidal and non-steroidal drugs, but they have frequent clinical side effects. The development of safer alternatives has attracted widespread attention.

One of the possible solutions is the use of natural molecules with anti-bacterial, anti-fungal, and anti-inflammatory activity. Plant extracts are complex mixtures containing a wide variety of primary and secondary metabolites, and their action may be the result of the synergy of different chemical components. Plant preparations have a number of advantages in particular due to the absence of side effects and a decrease in toxic effects for the body, while they cause complex pharmacological effects.

Plant-derived compounds, including alkaloids, phenolic acids, flavonoids, carotenoids, coumarins, terpenes, proanthocyanidins (PACs), and some primary metabolites (amino acids, peptides, organic acids) exhibit anti-microbial and anti-inflammatory properties [4,5,12–14]. Dietary polyphenols such as flavonoids, phenolic acids, and PACs in large quantities in foods of plant origin exhibit many beneficial effects and play an important role in the prevention of chronic and degenerative diseases. These dietary polyphenols are also found in lignocellulosic biomass (twigs, bark) in both deciduous and fruit trees/bush species.

PACs in plants represent the first biochemical defense against external injuries and infections [15,16]. Chemically, PACs are oligomers (degree of polymerization (DP) = 2–5) and polymers (DP > 5) or polymers of monomeric flavan-3-ols produced as an end product of the flavonoid biosynthetic pathway. Studies have demonstrated the biological activities of PACs [17–21].

The most widely studied PACs of grape seeds have been reported to exhibit anti-inflammatory activity by reducing the accumulation of pro-inflammatory cytokines [22], reducing aerobic and anaerobic microorganisms' colonies in plaque [23] and preventing gastrointestinal bacterial infections [24]. The latest studies showed that cranberry PACs prevent the evolution of resistance to tetracycline in *Escherichia coli* and *Pseudomonas aeruginosa*, rescue antibiotic efficacy against antibiotic-exposed cells, and inhibit biofilm formation [25]. PAC oligomers isolated from peanut skin (*Arachis hypogaea* L., Fabaceae) were reported to have the potential to reduce inflammation and melanogenesis [26]. PACs' properties are related to their chemical structure, as they have phenolic rings that can bind to a wide range of molecules and act as electron scavengers by capturing ions and radicals [24].

Our preliminary studies of the chemical composition of extracts isolated from the waste biomass of sea buckthorn (SBT) and other wood species (grey alder, black alder, willow, pine) by water and aqueous ethanol solutions showed that the PACs are the dominant polyphenolic compounds in the extracts [27]. In previous research, it was shown that SBT twigs are a valuable and cheap source of PACs, and they have higher anti-microbial

activity than extracts by themselves [28]. In a range of European countries such as Latvia, Estonia, Romania, and Germany, as well as in Canada and China where SBT is cultivated on plantations, a large volume of underutilized lignocellulosic biomass waste forms as a result of agrotechnical measures carried out for SBT twice per year, at yearly industrial harvesting of the berries, which includes cutting the whole branch (20% of the berries' mass) and pruning (90% of SBT plantation biomass every fourth year, almost a full cut of the whole shrub tree) [28].

It is known that PACs can prevent bacteria from attaching to cell or biomaterial surfaces [3,4,29,30] by impairing bacterial motility [5,9–11,13,14]. It is also suggested that PACs may inhibit biofilm formation and enhance the effect of gentamicin against *P. aeruginosa*. There is evidence that in the presence of PACs, the acquisition of resistance in *E. coli* and *P. aeruginosa* after treatment with tetracycline is completely stopped. Thus, PACs have the ability to interfere with the mechanisms of intrinsic resistance, thus suppressing the typically inevitable long-term evolution of acquired antibiotic resistance [15]. Due to their antioxidant and anti-inflammatory properties, PACs reduce inflammation in an animal model of gastric and colonic inflammation [31].

Herbal products were discarded from conventional medical use in the mid-20th century, not necessarily because they were ineffective but because they were not as economically profitable as the newer synthetic drugs. In spite of this, the global herbal medicine market was valued at USD 151.91 billion in 2021 and projected to grow from USD 165.66 billion in 2022 to USD 347.50 billion by 2029 [32].

The aim of this study was the assessment the role of PACs in the anti-inflammatory and anti-microbial activity of the ethanol/water extracts obtained from lignocellulosic agricultural waste of eight SBT cultivars: 'Maria Bruvele', 'Tarmo', 'Tatiana', 'Duet', 'Leikora', 'Clara', 'Otto', and 'Botanicheskaya Lubitelskaya'. The anti-inflammatory effect was evaluated by the reduction in IL-8 and IL-6 secretion, one of the major mediators of the inflammatory response, an early-phase biomarker, in the presence of extracts and PACs.

The anti-bacterial activity was tested toward Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus cereus*, as well as Gram-negative bacteria, *Pseudomonas aeruginosa* and *Escherichia coli*, and fungus *Candida albicans*. *S. aureus* is a leading pathogen associated with a number of diseases, including osteomyelitis, pneumonia, endocarditis, and septicemia. *B. cereus* produces toxins, causing two types of gastrointestinal illness: emetic (vomiting) syndrome and diarrheal syndrome. Multidrug-resistant *P. aeruginosa* is the one that most often causes infections in humans and causes infections and deaths among hospitalized patients [33]. Several strains of *E. coli* are enteric pathogens associated with hemorrhagic colitis and the development of the life-threatening condition hemolytic uremic syndrome (HUS) [34].

2. Results and Discussion

2.1. Chemical Composition

The results of extraction by 50% ethanol (50% EtOH) and water show that the yields of hydrophilic extract substances of all SBT twigs' biomass under study varied from 19% to 29%. UHPLC-ESI-MS/MS profile of twigs' extracts from all SBT cultivars contained the complex phenolic fingerprint with different phenolic compounds identified, comprising oligomeric and monomeric flavonoids. Oligomeric flavonoids—PACs (mainly B-type PACs)—were the dominant polyphenols in the extract's composition. The PAC content in 50% EtOH extracts was higher than that found in water extracts and ranged from 34.8 to 42.9% in 50% EtOH extracts and from 23.8 to 29.6% in water extracts of the cultivars tested (Figure 1).

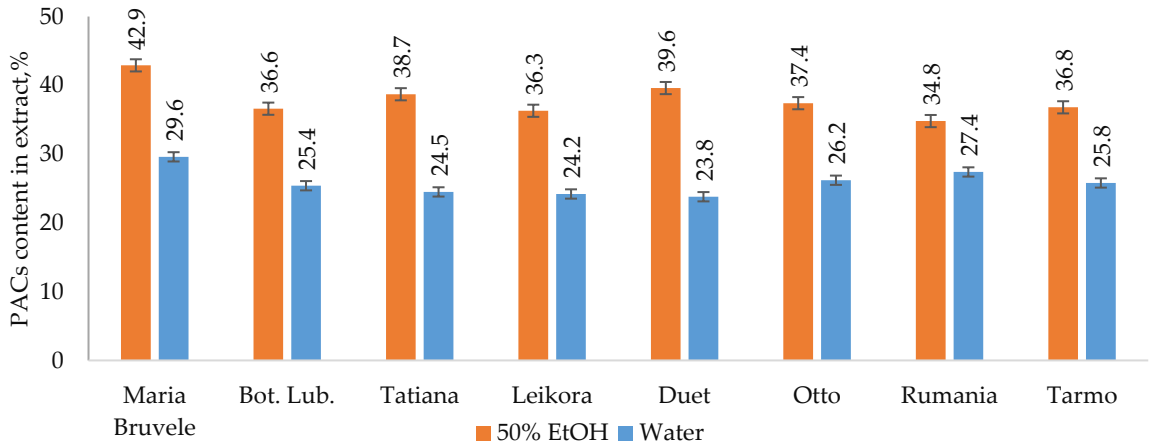

Figure 1. PAC content in extract isolated by 50% EtOH or water from eight SBT cultivars' twigs.

For the extracts obtained using the same solvent, the PAC values were close to each other for all SBT cultivars growing on the same plantation, which indicates the similarity of their ability to synthesize secondary metabolites. SBT extracts contain not only oligomeric PACs, but also other polyphenolic compounds such as quercetin (16), quinic acid (2), and gallocatechin or its isomer epigallocatechin (4), which have excellent documented antibacterial activity against *Staphylococcus aureus, Bacillus subtilis*, and *Escherichia coli* [35,36]. Polar triterpenoids are also present in the composition of the extracts, which for the extracts from the other plants, have shown strong anti-inflammatory effects [37]. The list of identified components is shown in Table 1, and the structures of some of the identified components in Figure 2.

Table 1. Tentatively identified components in tested SBT extracts.

Peak No.	t_R (min)	$[M-H]^-$ (*m*/*z*)	Fragments	Identification
1	0.41	341.1124	179; 161; 143; 119; 113; 101	Sucrose, fructose, glucose
2	0.47	191.0239	111; 173; 127; 85	Quinic acid
3	0.98	175.0778	159; 147	Serotonin
4	1.84	305.0706	179; 125	Gallocatechin or its isomer epigallocatechin
5	1.89	593.1289	407; 425; 305; 467; 289	(epi)catechin-(epi)gallocatechin
6	1.97	1185.2393	881; 593; 305; 289; 245	Procyanidin tetramer
7	2.06	1055.2609	881; 593; 305; 289	Procyanidin tetramer
8	2.30	865.1929	577; 289; 245	Procyanidin trimer
9	2.38	289.0754	245; 125	Catechin/Epicatechin
10	2.50	1153.2501	865; 577; 289; 245	Procyanidin tetramer
11	3.28	609.4297	301; 271	Quercetin-3-O-rutinoside
12	3.33	301.0027	286; 109	Quercetin
13	7.14	487.3439	293; 117	Triterpenoid
14	7.79	471.3486	452; 265; 117	Triterpenoid
15	7.86	471.3490	265; 117	Triterpenoid
16	8.07	455.3535	277; 117	Triterpenoid
17	8.01	617.3828	255; 117	Acylated triterpenoid

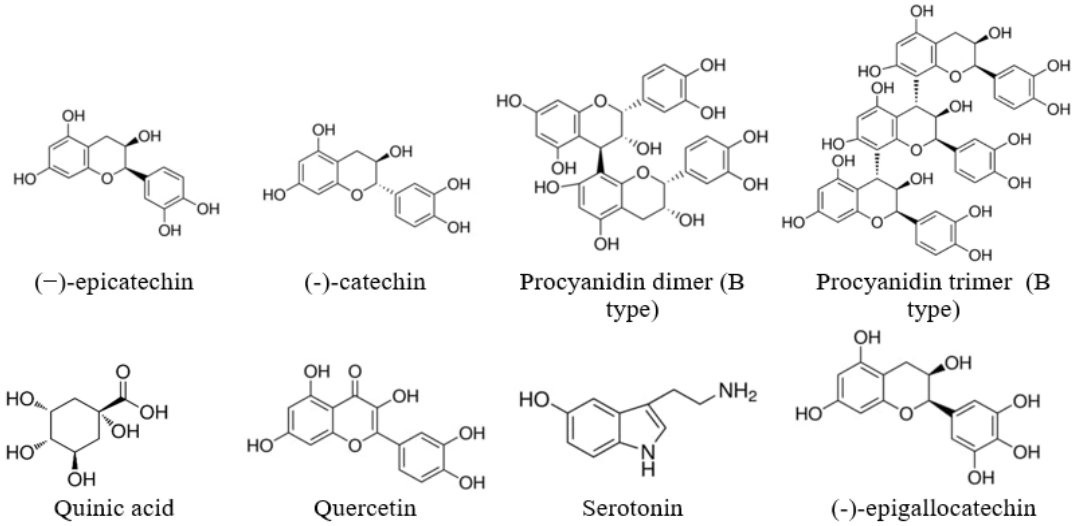

Figure 2. Some of the identified components of the extracts.

When comparing the chemical composition of 50% EtOH and water extracts of eight varieties of SBT, the following changes were noted: the decrease in the content of monomeric and oligomeric flavonoids (Figures 1, 3, and 4) and triterpenoids in water extracts (Figure 4), as well as the increase in the content of carbohydrates (in free and glycosidic form), which can adversely affect biological activity. The most abundant monosaccharides in the water extract of all cultivars were sucrose, fructose, and glucose.

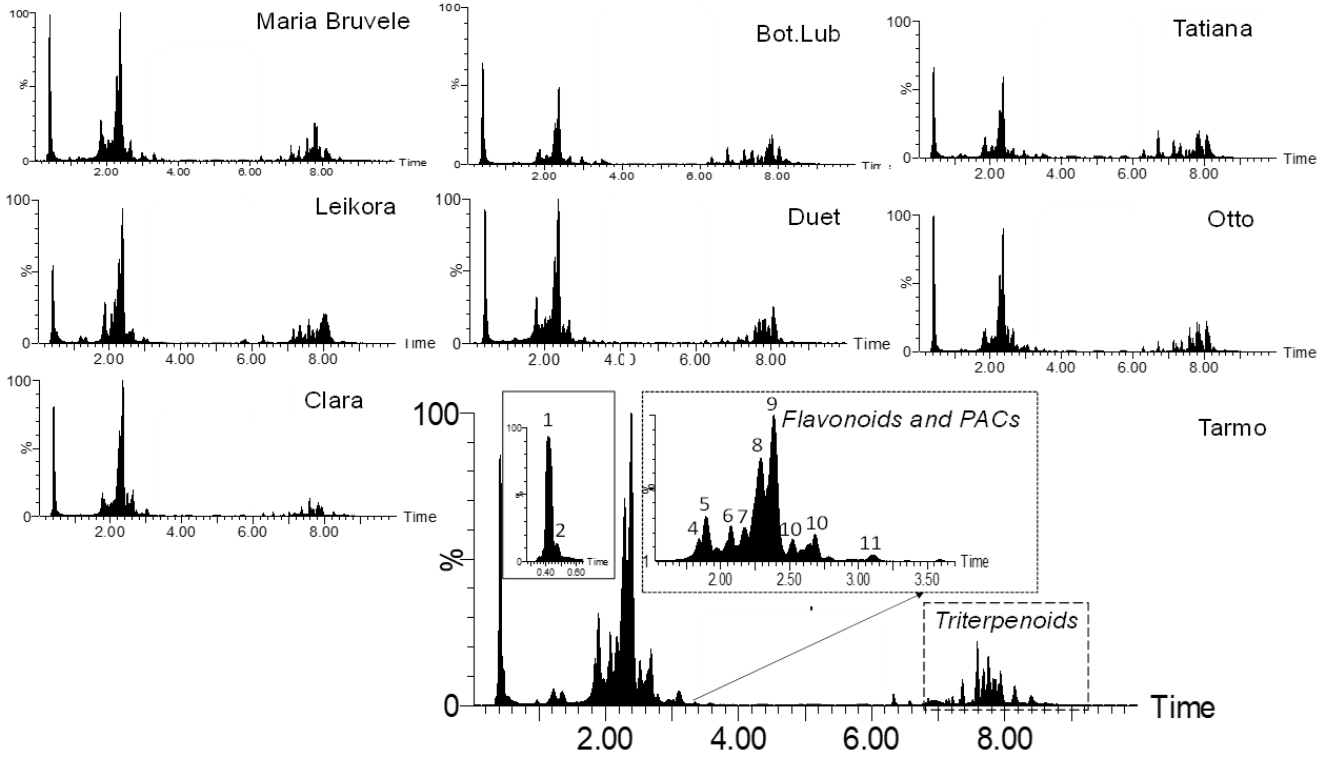

Figure 3. Comparison of the chemical composition of the sea buckthorn 50% EtOH extracts by UHPLC-TOF/MS chromatograms.

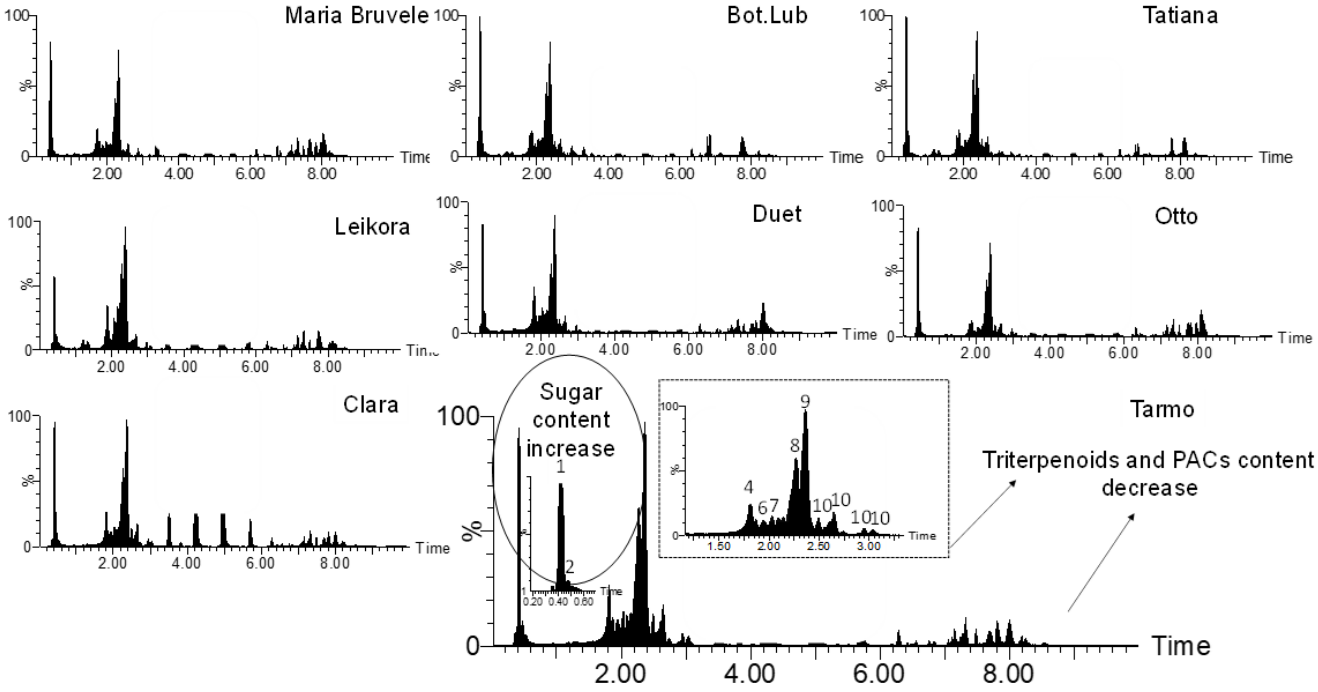

Figure 4. Comparison of the chemical composition of the sea buckthorn water extracts by UHPLC-TOF/MS chromatograms.

2.2. Anti-Bacterial and Anti-Fungal Activity

The anti-microbial activity of extracts obtained by extraction with water and 50% EtOH from eight SBT cultivars' twigs is shown in Table 2.

Table 2. Anti-bacterial and ant—fungal activity of the extracts from SBT samples.

SBT Cultivars	*E.coli* MIC/MBC, mg/mL	*P. aeruginosa* MIC/MBC, mg/mL	*S. aureus* MIC/MBC, mg/mL	*B. cereus* MIC/MBC, mg/mL	*C. albicans* MIC/MFC, mg/mL
			50% EtOH extracts		
Maria Bruvele	0.2/0.2	0.39/0.78	0.2/0.39	0.39/50	0.2/>50
Bot. Lub.	0.39/0.39	0.78/1.56	0.39/0.78	0.78/50	0.2/>50
Tatiana	0.39/0.39	3.13/3.13	0.2/0.78	0.78/50	0.39/>50
Leikora	0.39/0.39	0.78/1.56	0.39/0.78	0.39/12.5	12.5/25
Duet	0.2/0.2	0.78/0.78	0.39/0.78	0.39/12.5	12.5/25
Otto	0.2/0.2	0.78 /1.56	0.39/0.78	0.39/12.5	6.25/25
Clara	0.2/0.2	0.78/1.56	1.56/3.13	0.39/12.5	12.5/25
Tarmo	0.78/0.78	0.78/1.56	0.78/0.78	0.39/12.5	6.25/12.5
			Water extracts		
Maria Bruvele	0.39/0.39	0.39/3.13	0.39/0.78	0.78/>50	0.39/>50
Bot. Lub.	0.78/50	0.78/50	0.39/12.2	0.78/>50	0.39/>50
Tatiana	0.39/0.39	0.78/1.56	0.39/0.78	0.78/>50	0.39/>50
Leikora	0.39/0.39	1.56/1.56	1.56/1.56	0.78/25	12.5/12.5
Duet	0.39/>50	1.56/>50	12.5/12.5	1.56/25	12.5/25
Otto	0.78/>50	6.25/50	6.25/12.5	0.78/25	12.5/25
Clara	0.39/0.39	1.56/1.56	0.78/0.78	0.78/25	12.5/25
Tarmo	0.39/0.39	0.78/1.56	0.78/1.56	0.78/25	12.5/12.5

The results showed that all the extracts inhibit the growth of Gram-positive and Gram-negative bacteria as well as pathogenic fungus. Moreover, the inhibitory activity of almost all the extracts against *S. aureus* and *E. coli* is higher than the data found in the literature for

well-known natural anti-bacterial extracts of *Echinacea purpurea* and *Arctium lappa* (MIC: 2.93 mg/mL, MBC: 5.86 mg/mL, for both plants), and for some of the extracts ('Otto' and 'Tarmo'), the anti-fungal activity against *Candida albicans* is similar to the mentioned plant extracts (MIC: 5.86 mg/mL, MFC: 11.72 mg/mL, for both *Echinacea purpurea* and *Arctium lappa*) [38]. The MIC of the extracts under study was also lower than that of garlic (MIC of hybrid garlic against *S. aureus* started at a concentration of 5.0 mg/mL for water extracts and at 10 mg/mL for ethanol extract) [39].

For most of the extracts, MICs/MBCs against *E. coli*, *P. aeruginosa*, and *S. aureus* are less than 2 mg/mL, which accounts for a high anti-bacterial activity for the natural compounds in the literature [40]. This allows the consideration of the SBT extracts' prospective for natural anti-bacterial preparations.

The extracts isolated with 50% EtOH were more effective than water extracts; this could be due to the higher content of PACs in the extracts (Figure 4). In relation to *E. coli*, extracts isolated from SBT twigs of cultivars 'Duet', 'Otto', 'Clara', and 'Maria Bruvele' were more active than those from other cultivars. However, 50% EtOH extract from 'Clara' twigs showed weaker activity against *S. aureus* bacteria (MIC: 1.56 mg/mL). The MIC concentration in relation to *P. aeruginosa* was the same for all extracts (0.78 mg/mL), except for the extract from 'Tatyana' twigs (4 times weaker—3.13 mg/mL) and 'Maria Bruvele' twigs (2 times more effective—0.39 mg/mL). In the inhibition of *B. cereus*, the extract from 'Bot. Lub.' twigs by 50% EtOH was two times weaker (0.78 mg/mL) than other 50% EtOH extracts. In relation to *C. albicans*, extracts from 'Maria Bruvele', 'Bot. Lub.', and 'Tatiana' were more effective compared to other SBT cultivars.

The lowest MIC values were found for water extracts isolated from the twigs of 'Duet' (12.5 mg/mL against *E. coli*; 12.5 mg/mL against *S. aureus*), 'Leikora' (12.5 mg/mL to *C. albicans*), and 'Otto' (6.25 mg/mL against *P. aeruginosa* and *S. aureus*). 'Clara' and 'Tarmo' water extracts were also less effective in inhibiting *C.albicans*. Among all extracts, 50% EtOH extract isolated from 'Maria Bruvele' twigs showed the highest biological effect on all types of pathogenic bacteria (MIC: 0.2–0.39 mg/mL). The following tendency of microbial sensitivity was observed: *E. coli* = *S. aureus* > *P. aeruginosa* = *B. cereus*. For a water extract, the tendency of bacteria sensitivity was very similar: *E. coli* = *S. aureus* = *P. aeruginosa* > *B. cereus* (Table 2).

The minimum bactericidal/fungicidal concentration (MBC/MFC) is the lowest concentration of an anti-bacterial/anti-fungal sample required to kill bacteria/fungi over a fixed period under a specific set of conditions. The MFC determination could be useful in severe fungal infections in immunocompromised patients. According to the summarized results presented in Table 2, the extracts isolated by 50% EtOH from all SBT cultivars were the most active against such strains as *E. coli*, *P. aeruginosa*, and *S. aureus*.

Comparing the MBC value of 50% EtOH extracts with water extracts, water extracts were weaker, especially those of cultivars 'Bot.Lub.', 'Duet', and 'Otto'. Quantitative analysis of water extracts showed the highest content of sugars (sucrose, glucose, fructose) and polyphenolic glycosides (quercetin-3-O-rutinoside), which could be the reason for the higher MBC of the aforementioned cultivars. The MBC of water extract from the 'Maria Bruvele' cultivar in relation to *E. coli* was 2 times higher, in relation to *P. aeruginosa* it was 4 times higher, and in relation to *S. aureus* it was 2 times higher than for 50% EtOH extract; the MBC against *B. cereus* and MFC against *C. albicans* were $\geq$50 mg/mL for both water and 50% EtOH extract, which indicates that a higher concentration is probably needed.

As is shown in Figure 1, PACs are the dominant polyphenolic compounds in all the extracts. PACs were isolated and further tested on pathogenic bacteria under study (*E. coli*, *P. aeruginosa*, *S. aureus*, and *B. cereus*). The purity of PACs from the 'Maria Bruvele' 50% EtOH extract was 92% (determined by the butanol-acid method). In relation to *E. coli*, the isolated PACs were 5 times more effective than the 50% EtOH extract and nearly 10 times more effective than the aqueous extract (Figure 5).

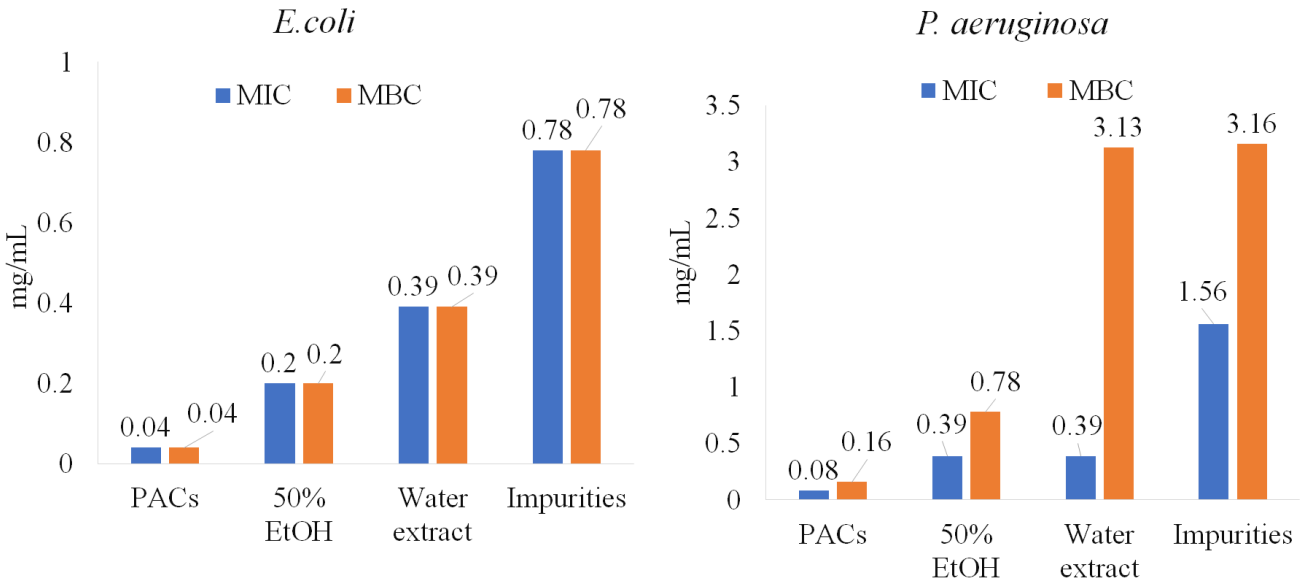

Figure 5. PACs' role in anti-bacterial activity against *E. coli* and *P. aeruginosa*.

Similar results were observed for *P. aeruginosa*, *S. aureus*, and *B. cereus* (Figure 6). Impurities were also tested and showed lower anti-bacterial activity potency, suggesting that PACs have a key role in inhibiting pathogenic bacteria.

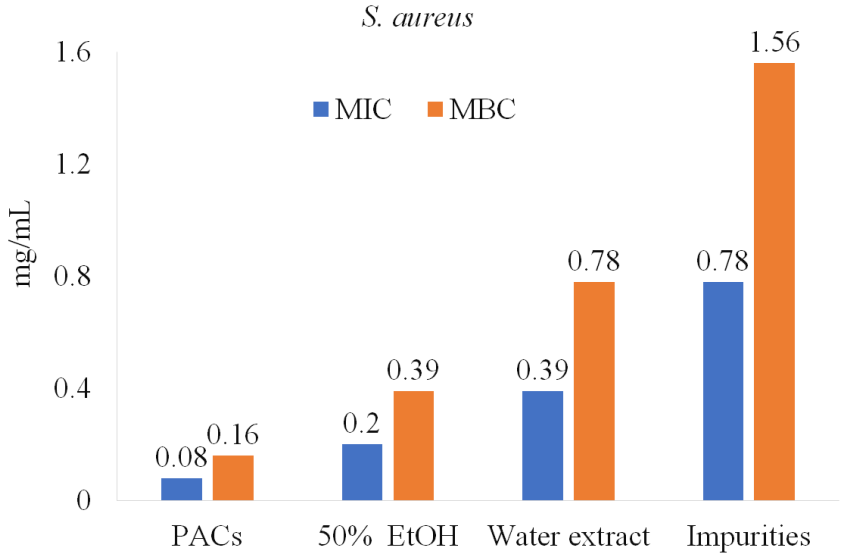

Figure 6. PACs' role in anti-bacterial activity against *S. aureus*.

Oral candidiasis is a common fungal disease caused mainly by *C. albicans*. When comparing the anti-fungal activity of three samples (50% EtOH, water extract, and PACs of 'Maria Bruvele'), isolated PACs were weaker at the initial stage. The following MIC tendency was observed: 50% ethanol extract (0.20 mg/mL) > water extract (0.39 mg/mL) > PACs (1.25 mg/mL) (Table 3).

Table 3. PACs' role in anti-microbial activity against *B. cereus* and *C.albicans*.

Samples	*B. cereus* MIC/MBC, mg/mL	*C.albicans* MIC/MFC, mg/mL
Maria Bruvele 50% EtOH extract	0.39/50	0.20/>50
Maria Bruvele water extract	0.78/>50	0.39/>50
PACs	0.63/1.25	1.25/>2.5
Impurities	1.56/>50	12.5/>50

It is assumed that at the initial stage of anti-fungal activity, the low-molecular-weight polyphenolic compounds listed in Table 1 have a synergistic effect with PACs, thereby improving the MIC value. However, only purified PACs have fungicidal activity (MBC = 1.25 mg/mL). The complex chemical structure of PACs and their composition, which includes a dimer, a trimer, a tetramer, etc., have a synergistic anti-microbial effect and will not allow the fungus to develop resistance to them.

2.3. Cytotoxicity Assessment

The cytotoxicity was evaluated to determine the toxic concentration of the extracts and PACs and compared with the anti-microbial concentration observed (MIC) for further analysis of their application in anti-microbial therapy [28,41]. Cytotoxicity of all SBT extracts was tested at a concentration range of 0.0313–4.0 mg/mL. Water extracts were slightly more cytotoxic than ethanol extracts. An extract at a specific concentration was considered to be cytotoxic if the cell viability was reduced by more than 20%. Cytotoxic concentrations of 50% EtOH and water extracts from 'Maria Bruvele', as well as isolated PACs from 'Maria Bruvele' extract, did not exceed the concentrations needed to inhibit the growth of the tested microorganisms. Samples tested in the concentration range of 0.0313–4.0 mg/mL did not have cytotoxicity except the PAC sample that, at a concentration of 1 mg/mL, reduced cell viability by 29.56% (Figure 7).

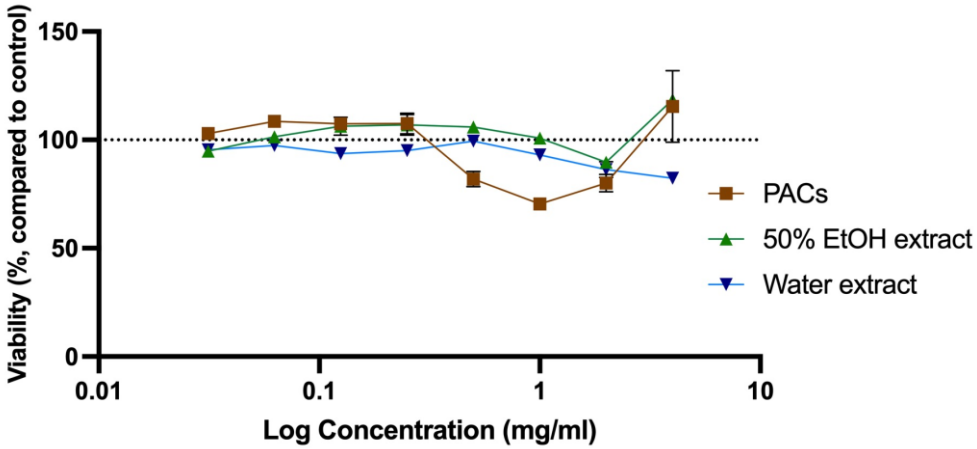

Figure 7. Cytotoxicity of SBT extracts evaluated as viability changes in neutral red uptake test in Balb/c 3T3 cell culture (n = 3). The dotted line corresponds to the control level.

2.4. Hemolysis

All extracts were tested for their hemolytic activity at a concentration of 0.5 mg/mL. None of the extracts induced hemolysis after 1 h or 8 h incubation, indicating the high biocompatibility and safety of the extracts (Figures 8 and 9).

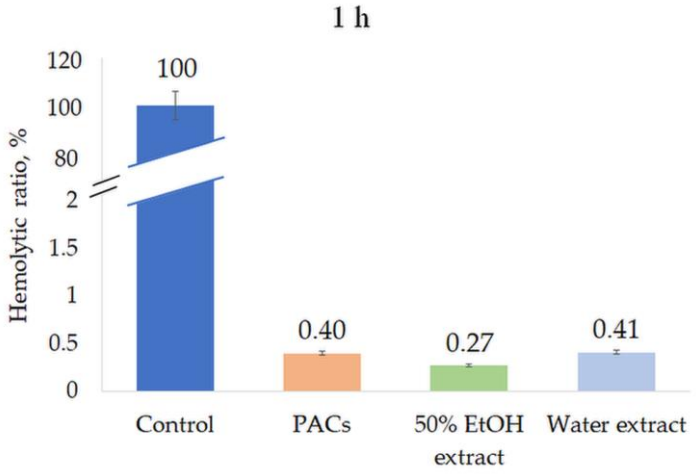

Figure 8. Hemolytic ratio (%) of 'Maria Bruvele' twigs' samples in fresh human blood hemolysis test after 1 h incubation. Control—deionized water, n = 3.

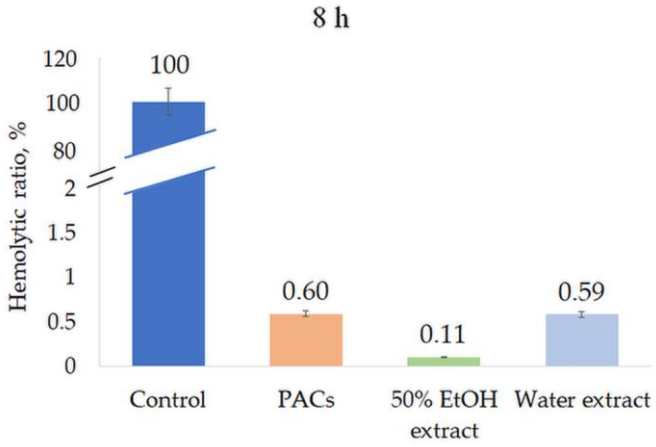

Figure 9. Hemolytic ratio (%) of 'Maria Bruvele' twigs' samples in fresh human blood hemolysis test after 8 h incubation. Control—deionized water, n = 3.

2.5. Immunomodulating Activity

IL-6 and IL-8 secretion in human peripheral blood mononuclear cells (PBMNCs) after 24 h incubation with SBT samples was investigated. Although the mouse blood macrophage RAW264.7 cell model is popular for screening immunomodulating activities, we chose human PBMNCs as a more relevant model. There have been studies with known immunomodulators that showed differences in how RAW264.7 and human PBMNCs respond. Compounds and extracts that might have immunomodulatory activity in humans might be missed in rodent cell models as they may not always mimic the responses of human immune cells [42]. It should be noted that PBMNCs are a heterogenous cell population that, in the case of immunomodulation studies, provides additional benefits in assessing overall effects on immune cells. According to the data obtained, 50% EtOH and water extracts from 'Maria Bruvele' increased the IL-8 secretion at both tested concentrations. PACs at a concentration of 0.5 mg/mL reduced IL-8 secretion in unstimulated PBMNCs and significantly reduced IL-8 secretion in poly-I:C-stimulated PBMNCs (Figure 10).

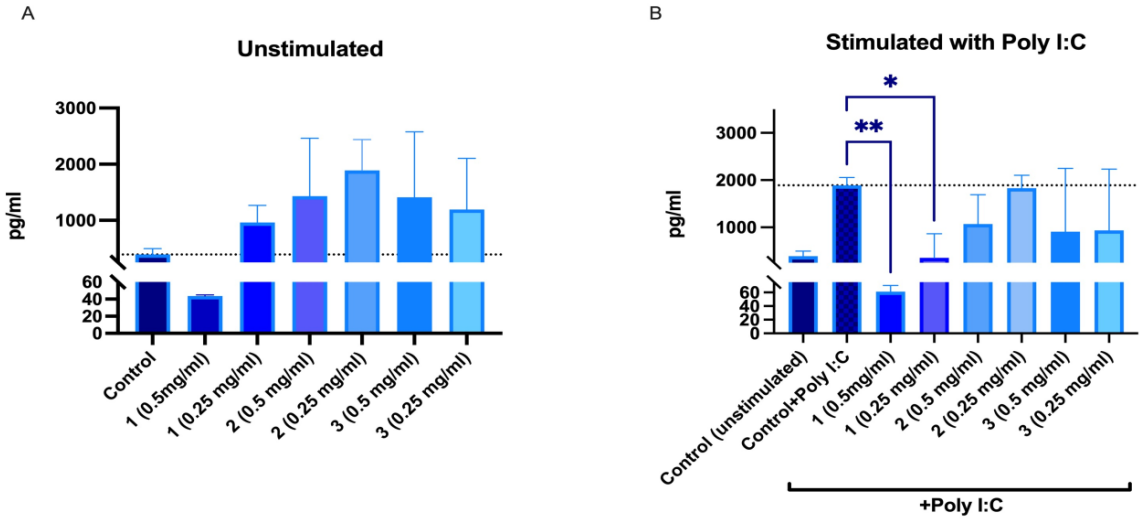

Figure 10. Changes in IL-8 secretion from unstimulated (**A**) and poly-I:C-stimulated (**B**) human peripheral blood mononuclear cells after 24 h incubation with SBT samples: 1—PACs, 2—50% EtOH extract, 3—water extract. * $p < 0.05$, ** $p < 0.01$ one-way ANOVA, n = 3. The dotted line corresponds to the control level.

Polyinosinic:polycytidylic acid (poly I:C) mimics viral double-stranded RNA and binds TRL3 receptors of human cells, thus mimicking inflammation related to viral infections. All 'Maria Bruvele' samples (PACs, 50% EtOH extract, and water extract) reduced secretion of IL-8 in the presence of poly I:C. Results indicate the ability of plant PACs and PACs containing extracts to reduce inflammation related to viral infections (Figure 10). The best results were observed for isolated PACs from 'Maria Bruvele' 50% EtOH extract.

Without poly I:C stimulation, no increase in IL-6 secretion was observed after incubation with the samples. When samples and poly I:C were added to PBMNCs simultaneously, it was observed that PACs and ethanolic extracts significantly reduced secretion of IL-6 (Figure 11).

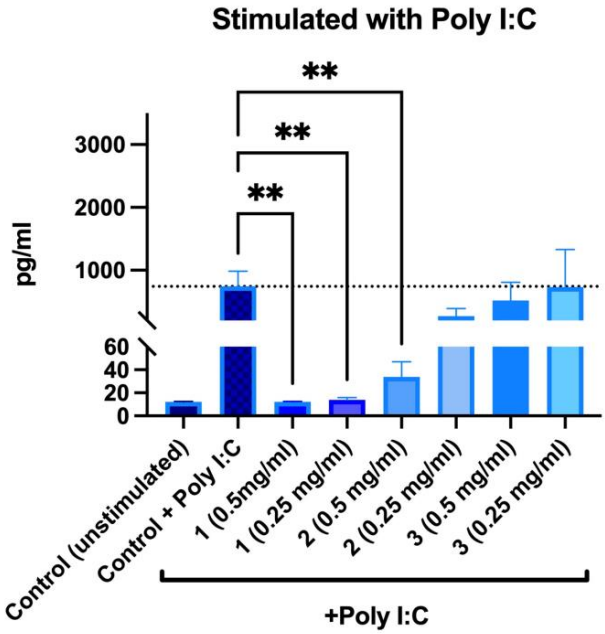

Figure 11. Changes in IL-6 secretion in stimulated peripheral blood mononuclear cells after 24 h incubations: 1—PACs, 2—50% EtOH extract, 3—water extract. ** $p < 0.01$ one-way ANOVA, n = 3. The dotted line corresponds to the control level.

In presence of PACs, IL-6 secretion was reduced to an unstimulated control level; furthermore, no differences were observed between both tested concentrations. For 50% EtOH extracts, the inhibitory effect was concentration-dependent—0.5 mg/mL reduced IL-6 secretion by 95.43%, whereas 0.25 mg/mL reduced it by 63.75%. Water extracts did not reduce poly-I:C-induced IL-6 secretion. Overall, our findings are consistent with other biomass studies where the effects of PACs on IL-6 and IL-8 secretion have been described in inflammation models [43–45].

To our knowledge, there are just a few studies describing the effects of PACs on poly-I:C-induced inflammation in vitro and no previous studies on PACs specifically isolated from SBT.

3. Materials and Methods

3.1. Materials

3.1.1. SBT Biomass

The twigs of eight sea buckthorn cultivars (*Hippopae rhamnoides* 'Leikora', 'Otto', 'Clara', 'Duet', 'Tamo', 'Tatiana', 'Maria Bruvele', and 'Botanisheskaya Lubitelskaya') and leaves of 'Maria Bruvere' were collected from the sea buckthorn (SBT) plantation area in Latvia in the late summer of 2020. The twigs and leaves were dried at room temperature and ground with a knife mill (Cutting Mill SM100, Retsch, Haan, Germany). The particle size of the grounded SBT twigs was between 1 and 4 mm, and the leaves were between 0.5 and 1 mm.

3.1.2. Chemicals

Procyanidin B2 (≥90% HPLC) analytical standards were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), LC-MS hyper grade acetonitrile from Merck (LiChrosolv®, Merck KGaA, Darmstadt, Germany), and formic acid (HiperSOLV Chromanorm) from VWR Chemicals (Radnor, PA, USA). Milli-Q Type 1 ultrapure water (suitable for chromatography and other advanced analytical techniques) was used for sample preparation as well as the mobile phase.

Reagents including $FeNH_4(SO_4)_2 \cdot 12\ H_2O$, *n*-butanol (purity ≥ 99.4%), and crosslinked dextran-based resin Sephadex LH-20 were purchased from Aldrich Sigma (Merck KGaA, Darmstadt, Germany).

3.2. Methods

3.2.1. PAC-Rich Extract Isolation from SBT Biomass

Extracts were isolated by the convective extraction of SBT biomass at 60 °C for 30 min using the following solvents: distilled water or aqueous ethanol (1:1, *v*/*v*). The extracts were freeze-dried to yield a brown solid. The yield of the extracts is presented as a percentage based on the oven-dried (o.d.) biomass.

3.2.2. Determination of PAC Content in the Extract

PAC content in the extracts was measured by the butanol–HCl method [46] using procyanidin dimer B2 as a reference compound. Amounts of 6 mL of acid butanol (5% (*v*/*v*) concentrated HCl in n-butanol) and 0.2 mL of iron reagent (*w*/*v*) ($FeNH_4(SO_4)_2 \cdot 12\ H_2O$ in 2 N HCl) were added to 1 mL of the extract aliquots whilst stirring the tube without heating and allowing it to be heated in a water bath at 80 °C for 50 min. After 50 min, the absorbance of the mixture was measured against a blank solution at 550 nm using a UV/VIS spectrometer Lambda 650 (Perkin Elmer, Inc., Waltham, MA, USA). Each extract was analyzed in triplicate, and assay results were expressed as a percentage per oven-dried (o.d.) extract. The confidence interval (CI) for the results did not exceed 3% at $\alpha = 0.05$.

3.2.3. UHPLC-ESI-MS/MS Qualitative Analysis

The identification of compounds was performed by an Acquity UPLC system (Waters Corp., Milford, MA, USA) coupled with a quadrupole-time of flight (Q-TOF) MS instrument

(UPLC/Synapt Q-TOF MS, Waters, Milford, MA, USA) with an electrospray ionization (ESI) source. The UHPLC separation was carried out using a Waters Acquity BEHC18 (2.1 mm × 50 mm i.d., 1.7 µm). The mobile phase consisted of 0.1% formic acid, water (A), and acetonitrile (B), with a flow rate of 0.35 mL/min under the gradient program of 5–20% (B) for an initial 1 min, 20–25% (B); 5–6 min, 25–75% (B), 6–7 min, 75–80% (B), 7–8 min, 80–5% (B), 8–10 min, 5% (B); the injection volume was 2.0 µL.

Mass spectrometric analysis was conducted in negative and positive ion mode, and the full scan mass spectral data were collected over a range from m/z 50 to 1200. The optimum source parameters were as follows: capillary voltage, 2.5 kV (−); cone voltage, 60 V; cone gas flow, 50 L/h; collision energy, 6 eV; source temperature, 120 °C; desolvation temperature, 350 °C; collision gas, argon; desolvation gas, nitrogen; flow rate, 500 L/h.

3.2.4. Purification of PACs

The purification of PACs from non-tannin and sugar was carried out using a solvent-resistant (SR) column packed with Sephadex LH-20 with 96% EtOH and 70% (v/v) acetone as the respective purification solvents. In the purification process, low-molecular-weight phenolics were eluted with 96% EtOH until the absorbance at 280 nm started to approach zero, and the PACs were eluted with 70% (v/v) acetone. Purified CTs were evaporated using a rotary evaporator (Heidolph Instruments, Schwabach, Germany) prior to being freeze-dried and stored at −8 °C.

3.2.5. Determination of the Anti-Microbial Activity

The anti-microbial activity tests of the extracts from the twigs of 8 cultivars of SBT, purified PACs from 50% EtOH extract of 'Maria Bruvele' twigs, and admixture after PAC purification were performed at the Faculty of Biology, University of Latvia. To determine anti-microbial activity, several reference microbial strains, received from the Microbial Strain Collection of Latvia (MSCL), University of Latvia, were used: *Pseudomonas aeruginosa* MSCL 334, *Staphylococcus aureus* MSCL 330, *Escherichia coli* MSCL 332, *Bacillus cereus* MSCL 330, and *Candida albicans* MSCL 378. The evaluation of the anti-microbial activity of the samples against the test cultures of microorganisms was carried out according to the method for determining the sensitivity of microorganisms to anti-microbial drugs. Anti-microbial activity was studied in 96-well plates by the two-fold serial broth microdilution method, which allowed the determination of the minimum inhibitory (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC) (Figure 12).

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

Figure 12. (**a**) Minimum inhibitory concentration (MIC) of 'Maria Bruvele' extract against *C. albicans* in 96-well plates by the two-fold serial broth microdilution method; (**b**) Minimum bactericidal concentration (MBC) of 'Maria Bruvele' extract against *P. aeruginosa* and minimum fungicidal concentration (MFC) against *C. albicans*.

The MIC was determined as the lowest concentration of the studied material, which showed no visible growth.

3.2.6. Cell Lines and Cultivation

The BALB/c 3T3 murine fibroblast cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were propagated in DMEM medium (Sigma, Irvine, UK) supplemented with 1% penicillin (100 U/mL)–streptomycin (100 µg/mL) and 10% calf serum (Sigma, St. Louis, MO, USA). All cultivations were performed in a humidified 5% CO_2 atmosphere at 37 °C.

3.2.7. Hemolysis Assay

A hemolysis test was performed to assess the hemocompatibility of the extracts. Blood from healthy donors was collected in Monovette vacutainers containing Ethylenediamine tetra-acetic acid (EDTA). Blood was diluted with 0.9% sodium chloride solution (4:5 ratio by volume). Extracts were added to 15 mL tubes containing fresh 9.8 mL PBS and incubated at 37 °C and 5% CO_2 for 30 min, and 0.2 mL of diluted blood was added to each tube and incubated at 37 °C and 5% CO_2 for 1 h and 8 h. PBS was used as a negative control and deionized water as a positive control. After incubation tubes were centrifuged at 2000 rpm for 5 min, the supernatants were collected, and the absorbance was measured at a wavelength of 545 nm in a microplate reader Tecan Infinite® 200 PRO (Tecan Group Ltd., Mannedorf, Switzerland).

The hemolytic ratio (HR) was calculated by the following equation:

$$\text{HR } (\%) = \frac{(\text{Abs(sample)} - \text{Abs(negative control)})}{(\text{Abs (positive control)} - \text{Abs (negative control)})} \times 100$$

3.2.8. Cytotoxicity Assay

The cytotoxicity of the extracts was tested for the BALB/c3T3 cell line by the neutral red (NR) uptake assay. Cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After 24 h of incubation, extracts in a concentration range of 0.125 to 4 mg/mL were added. Dilutions were made in a cell cultivation medium. Cultivation in the presence of extracts was performed for 48 h. Afterward, the plates were washed with phosphate-buffered saline (PBS) (Sigma, D8537, Irvine, UK), and a 25 µg/mL NR solution (Sigma, N2889, Irvine, UK) diluted in 5%-fetal-calf-serum-containing-media was added. After 3 h incubation in a humidified 5% CO_2 atmosphere at 37 °C, the plate was washed with PBS, and the NR taken up by viable cells was extracted using desorbing fixative (50% ethanol/1% acetic acid/49% water). Absorbance at 540 nm was measured using a microplate reader Tecan Infinite® 200 PRO (Tecan Group Ltd., Mannedorf, Switzerland). Cytotoxicity was expressed as a concentration-dependent reduction in the uptake of NR, compared to the untreated controls.

3.2.9. Quantification of IL-8 and IL-6 Release from Human Peripheral Blood Mononuclear Cells (PBMNCs)

The effect of the extracts was evaluated in human peripheral blood mononuclear cells (PBMNCs). Blood from healthy donors was collected in Monovette vacutainers containing EDTA. Blood was collected in accordance with the approval of the Committee of Research Ethics of the Institute of Cardiology and Regenerative Medicine, University of Latvia. Blood was diluted (1:2 ratio by volume) with 0.9% sodium chloride solution supplemented with 10 U/mL heparin and mononuclear cell fraction isolated by gradient centrifugation. Diluted blood samples were layered on Ficoll-Paque solution (GE Healthcare, Chicago, IL, USA), and density gradient centrifugation was performed at 800× *g* for 20 min at room temperature in a swing-out centrifuge. Mononuclear cells containing buffy coats were aspirated and washed twice with phosphate-buffered saline and centrifuged at 600× *g* for 20 min at room temperature. The cell pellet was suspended in DMEM medium (Sigma, D6046, Irvine, UK) supplemented with 1% penicillin (100 U/mL)–streptomycin (100 µg/mL) and

10% fetal bovine serum (Sigma, St. Louis, MO, USA), and cells were seeded on 24-well plates at a density of 3×10^5 cells per well and incubated at 37 °C, 5% CO_2. Cells were allowed to adhere overnight prior to the addition of extracts at concentrations 0.5 and 0.25 mg/mL, 10 μg/mL poly I:C (Sigma, St. Louis, MO, USA), or a combination of both. Cells were incubated for 4 or 24 h at 37 °C, 5% CO_2, and incubation media were collected and stored at −80 °C for further analysis.

Concentrations of IL-8 or IL-6 secreted in cultivation media by PBMNCs were determined using enzyme-linked immunosorbent assay (ELISA). Human IL-8 DuoSet ELISA kits (RnD Systems®, Minneapolis, MN, USA) were used according to the manufacturer's recommendations.

3.3. Statistical Analysis

All measurements were conducted in triplicate, and the results are presented as the mean value ± standard deviation (SD). Statistical analyses were performed using Microsoft Excel 2016. Confidence intervals for a mean using Student's T distribution were calculated at a significance level $\alpha = 0.05$. A significance level of $p < 0.05$ was used.

For quantification of IL-8 and IL-6 release from human PBMNCs, the data were analyzed and graphs were generated using GraphPad Prism 5.0 software (San Diego, CA, USA). One-way ANOVA test was used. Differences were considered statistically significant if $p < 0.01$ and $p < 0.05$ (corresponding level was marked on the Figures).

4. Conclusions

The study showed that lignocellulosic biomass after harvesting, particularly, SBT twigs, could be a potential source of anti-inflammatory, anti-bacterial, and anti-fungal treatments. The 50% EtOH extracts have higher anti-bacterial and anti-fungal properties and between all the cultivar extracts, 'Maria Bruvele' is one of the most prospective sources for anti-bacterial treatments, while the extracts from 'Tarmo' are the most prospective for anti-fungal treatments. PACs isolated from SBT twigs have much higher anti-bacterial and anti-fungal properties, and in addition, they have high anti-inflammatory activity. Therefore, PACs having all these valuable properties in complex, not only inhibiting the pathogen by itself but also suppressing the inflammation that it provokes, are show very good prospects as a new therapeutic agent in prophylaxis and treatment of bacterial and fungal infections. Due to the low cytotoxicity in the bacteria/fungi inhibition diapason, PACs could also be studied for the treatment of internal organ infections. It should be noted that to align with 3R principles (replacement, reductions, refinement), in vitro testing is crucial—it allows the selection of the most prospective samples and provides information about the starting doses, resulting in a reduced number of animals needed for such studies. This study has produced a valuable volume of data for the later follow-up with in vivo testing.

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Institutional Review Board Statement: The hemocompatibility studies and peripheral blood mononuclear cell isolations were performed in accordance with the approval of the Committee of Research Ethics of the Institute of Cardiology and Regenerative Medicine, University of Latvia (Approval No. 3/2021, date 7.01.2021).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

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Conflicts of Interest: The authors declare no conflict of interest. Ekokompozit Ltd. declares no conflict of interest with this paper.

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LIGNIN AND LIGNOCELLULOSE-BASED ORGANOMINERAL COMPLEX FOR ORGANIC AGRICULTURE

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ABSTRACT

The aim of this work was to evaluate the effect of low rates (20-40 kg ha^{-1}) of silicon-containing organo-mineral complex (LignoCel-Si) application on potatoes "*Imanta*" and summer wheat "*Vinjet*" productivity and quality, under conditions of organic farming. The LignoCel-Si organo-mineral complex was obtained on the basis of the residues of hydrolysis lignin and sea buckthorn (*Hippophae rhamnoides L.*) agro-waste lignocellulosic biomass after water-ethanol extraction, and enriched with silicon (Si)-containing inorganic oligomer. The field experiments were carried out at a certified biological field intended for scientific purposes. It was shown that LignoCel-Si has a favorable influence on the harvest volume and product quality at low application rates of 20-40 kg ha^{-1}. In comparison with the control, the additional yield of wheat achieved on the background of LignoCel-Si (20 and 40 kg ha^{-1}) was from 10 to 27 %, and for potatoes from 8 to 21%, correspondingly. The application of 40 kg ha^{-1} of LignoCel-Si complex also contributed to an increase in the quality of potatoes, which was reflected as an increase in the yield of ware potatoes (potato tubers > 75 mm in diameter). The results confirm the value of the LignoCel-Si complex as a soil biologically active additive and the possibility of its application in biological agriculture.

Keywords: sea buckthorn, lignin, silicon, lignocellulosic biomass, organomineral fertilizer

INTRODUCTION

The soil is a living organism and in recent decades it has been experiencing huge stress due to intensive agriculture. If previously only mechanical stress was considered the main problem, now experts note the mineralization of humus, which inevitably leads to a decrease in soil fertility. Soil also loses its ability to absorb and store nutrients; the general physical properties of the soil are getting worse as well. Humid climates typical also for the Baltic States favors the podzolization and lessivation of automorphic soils, and the content of humus, in this case, is 2.5 – 4% which is less than the optimal 6%

and much less than for black earth type of soil. Humus content in sandy soils is even less – around 0.5 – 1.5 [1-4]. Sandy soil occupies a big part of the Baltic States and Northern Europe and occurs extensively in the USA, in Florida, Nebraska, Michigan, Texas, Georgia, Wisconsin, and Minnesota [5].

One of the ways to increase soil fertility is to increase the amount of organic matter by introducing organic fertilizers into it. Unlike inorganic fertilizers, natural organic fertilizers nourish the soil, the soil becomes loose and saturated with air, absorbs more moisture and nutrients, and promotes the growth of soil microorganisms, at the same time it nourishes the plants, contributing to the development of a healthy plant root system. The humus accumulation process is slow. It is estimated that to increase its content by 1%, it will be necessary to apply 5–6 kg/m² of humus or compost within 5 years [6, 7].

Traditional organic fertilizers, such as manure, have certain limitations in their use, such as heating during decomposition, high nitrate content in fresh feces, the presence of pathogens, and others.

Lignin and lignocellulosic biomass are valuable sources of organic matter. The development of organo-mineral fertilizers based on lignin and lignocellulosic biomass makes it possible to return to nature the organic part taken from it, which is necessary for the normal operation of the soil-biotic complex. It was shown in authors' previous studies [8] that organomineral fertilizer on the basis of lignin and silicon (Si) has a prolonged influence on plants' growth and development; and after a year, soil was enriched with organic substances and nitrogen. SBT lignocellulose biomass contains macro and microelements (nitrogen, calcium, magnesium, iron, boron, etc.) [9] which are necessary for successful plant growth and development. A combination of previously studied lignin and SBT biomass could be an effective soil additive. Its effectivity can be further improved by adding inorganic components, particularly, Si.

It is known that lignocellulosic biomass, including sea buckthorn (SBT), contains a fairly large amount of valuable water-soluble polyphenolic compounds [9,10], but they negatively affect plant growth when introduced to the soil. Therefore, it is advisable to carry out the preliminary removal of polyphenols from the SBT biomass before its application as a soil additive. In addition, preliminary biomass extraction removes spores of fungi, bacteria, and other harmful plant growth residues from it. It has been shown that extracted polyphenolic compounds, including proanthocyanidins, can be successfully used as food additives, antioxidants, and anti-inflammatory agents in health care, food, and cosmetic industries [10-12]. The biomass residues after extraction are characterized by high porosity and moisture content, which ensures their efficiency when used as structuring additives and can be tested for obtaining fertilizers. This approach is promising from a circular bioeconomy point of view, for the cascading use of biomass, obtaining the maximum number of added-value products from the production cycle.

Under natural conditions, lignin in lignocellulosic biomass serves as a soil precursor in situ and is able to perform the functions of all humic substances (accumulative, transport, regulatory, protective, and physiological) due to the structural and functional properties of lignin. Due to the presence of lignin functional groups and high particle surface activity, it has an excellent ability to absorb minerals and organic components, reducing nutrient leaching and prolonging the action of fertilizers.

Si is one of the most abundant macronutrients (the eighth most abundant element in nature and the second most abundant element in the soil after oxygen), which plays an important role in plant resistance to environmental stress, diseases, and pathogens. In addition, Si can improve the health of soils that contain toxic levels of heavy metals. It is mentioned that Si minimizes the toxicity of Fe, Al, and Mn, increases the availability of P, and increases plant resistance to drought, salt, high temperatures, and frost due to the formation of sililated tissues in plants [13–15]. The role of Si can be compared to the role of organic secondary metabolites, which play a protective role in plants. Deficiency in Si causes growth, developmental and reproductive disorders in many plants.

Currently, the search for ecologically friendly products that promote plant productivity, quality, and resistance to biological and abiotic diseases without harming the environment is a very actual task. More care is needed to grow organic products, but the investment pays off with healthier and better-quality produce.

The previous studies showed that Si-containing lignin-based fertilizers promote a strong root system with a large number of root buds and side roots, increasing plant productivity, quality, and disease resistance [8, 16]. Si-containing organic-mineral complex on the basis of combination of hydrolysis lignin and the residue of lignocellulosic biomass after green extraction should be of scientific interest and have not been studied yet.

The aim of this work was to evaluate the application effect of low rates of silicon-containing organo-mineral complex on the basis of lignin and SBT biomass residues after extraction (LignoCel-Si) on potatoes and summer wheat productivity and quality, under conditions of organic farming.

MATERIALS AND METHODS

The LignoCel-Si complex was obtained on the basis of mixture of lignin and SBT residues after extraction (SBT:hydrolysis lignin, 1:1 (w/w)) with Si. Acid hydrolysis lignin that was previously tested with Si fertilizers [8] was obtained from the residues of Kedainai hydrolysis factory (Kedainiai, Lithunaia). SBT twigs were collected from the SBT plantation area near Engure, Latvia, in autumn 2020. SBT biomass after water-ethanol extraction was mixed with lignin and enriched with Si. The Si content in the organo-mineral complex was 5% on the dry lignocellulosic biomass. The Si content was chosen on the basis of authors' experience with lignin-based organomineral fertilizers [16]. Testing was carried out with various LignoCel-Si application rates in soil.

The chemical characterization of the LignoCel-Si complex was carried out according to the following European standards specified in Table 1. The content of lignin was determined by analytical pyrolysis Py-GC/MS/FID method [17].

Table 1. Methods according to the standards.

Characteristics	Testing method standard
Humidity, %	LVS EN 13040:2008
Dry matter, %	LVS EN 13040:2008

Organic matter content, %	LVS EN 13039:2012
Humic acids, %	T-261-33:2014 P.3.1
Total nitrogen (N), %	LVS EN 13654-1:2003/NAC:2004
Total phosphorus (P_2O_5), %	LVS 398:2002
Total potassium (K_2O), %	LVS ISO 11466:1995
Mercury, mg/kg	LVS 346:2005
Cadmium, mg/kg	LVS ISO 11047:1998A
Arsenic, mg/kg	LVS ISO 110466:1995
Soil pH	LVS ISO 10390:2006

The field experiments were carried out from 2021 to 2022 at a certified biological field intended for scientific purposes. The test crops were the wheat "*Vinjet*" and potatoes "*Imanta*" from self-grown organic seeds. The potato variety "*Imanta*" was bred by the Priekuli Plant Breeding Institute and has grown in Latvia since 2008. The variety "*Imanta*" is suitable for organic farming. The variety is moderately late, suitable for food and processing into starch, resistant to potato crayfish (*Synchytrium endobioticum*) and nematodes (*Globodera rostochiensis*), moderately resistant to leaf rot (*Phytophthora infestans*) [16]. The wheat variety "*Vinjet*" was bred by the Research Institute of Agronomy. The "*Vinjet*" is a medium-intensive type variety, high yielding, and shows good resistance to diseases.

Preliminary experiments in laboratory conditions (vegetation tests in pots, WinRHIZO root scanner after 20 days of vegetation) were performed with the addition of LignoCel-Si to the soil at a rate from 10 to 40 kg ha^{-1}. Further field experiments with LignoCel-Si were carried out in 3 options in 4 repetitions:

1. Reference plot (without LignoCel-Si);
2. LignoCel-Si – 20 kg ha^{-1};
3. LignoCel-Si – 40 kg ha^{-1}.

The agrochemical parameters of the soil were as follows: pH 6.0, organic matter content 2.7 %, amount of phosphorus available to plants 51 mg kg^{-1} (low), and potassium 67 mg kg^{-1} (low). The precursor was red clover for seed production.

The field trials were arranged in four replicates. The size of the field was 25.2 m^2 (2.8 m x 9 m). Four furrows with a distance of 70 cm were made in the field, the feeding area of one plant was 0.21 m^2. In 2021, the potatoes were planted on May 14, and harvested on September 17. In 2022, the potatoes were planted on May 17 and harvested on September 29.

In 2021, the wheat sowing was carried out on April 29, and wheat threshing was carried out on September 8. In 2022, wheat sowing was carried out on April 20, wheat threshing was carried out on September 8. The seeding rate was 240 kg ha^{-1} (self-grown seeds).

For summer wheat, the following characteristics were determined: yield difference between test variants; yield quality indicators: crude protein, gluten, and starch content, Zeleny index, and bulk density. Grain quality analyses (formation of crop structural elements (number of productive stalks, weight of one spike, number of grains per spike, a mass of 1000 grains) were performed using the analyser Infratec™ NOVA (Foss, Hilleroed, Denmark). Samples were taken in each iteration with a 0.1 m^2 frame. In turn, the mass of 1000 grains was determined by the standard method (LVS EN ISO 520).

For potatoes, the following characteristics were determined: yield difference between variants; yield quality (starch content); yield of the products, distribution of tubers by fractions, and weight of one tuber.

The least significant difference (LSD) method at the probability level of 0.05 was used to separate the mean differences in crop yield.

RESULTS

The chemical characterization of the LignoCel-Si complex was shown in Table 2.

Table 2. Chemical characterization of LignoCel-Si complex

Indicator of sample	Value	Indicator of sample	Value
Humidity, %	6.4±0.1	Total phosphorus, %	0.06±0.01
Dry matter, %	94.0±0.1	Total potassium, %	0.04±0.01
Organic matter content, %	97.8±1.4	Mercury, mg/kg	<0.2
Lignin, %	38.5±0.5	Cadmium, mg/kg	1.13 ±0.09
Humic acids, %	4.3±0.1	Arsenic, mg/kg	0.17±0.02
Total nitrogen (N), %	1.35±0.02	pH	8.7±0.1

Appropriate potato growth conditions during the vegetation period, as well as the necessary agronomic works performed, ensured a good potato yield in 2021 (11.2 – 13.8% at 20 kg ha^{-1} and 40 kg ha^{-1} correspondingly). In 2022, the growth and development of potatoes were significantly affected by the dry and hot weather during the summer months. Nevertheless, a significant increase in yield has been observed with the LignoCel-Si application. Using LignoCel-Si in the rate of 20 kg ha^{-1}, the increase of potato yield was 1.3 t ha^{-1} or 8.4 % compared to the control variant. By increasing the LignoCel-Si application rate to 40 kg ha^{-1}, the yield increase was 3.3 t ha^{-1} or 21.4 % in comparison with the control (Table 3).

Table 3. Influence of the LignoCel-Si on the food crop yield and its structure for the potato variety “Imanta”

Rate of LignoCel-	Total yield, t	Yield structure

Si (kg ha^{-1})	ha^{-1}	Tubers, <35 mm in diameter	Tubers, 35-75 mm in diameter	Tubers, > 75 mm in diameter
Field experiment in 2021				
0 (reference sample)	38.5	1.3	6.6	30.6
20	42.8	1.8	4.1	37.0
40	43.8	1.8	4.6	37.4
$LSD_{0.05}$	4.8	1.2	1.7	5.5
Field experiment in 2022				
0 (reference sample)	15.4	3.4	6.7	5.2
20	16.7	3.4	7.5	5.8
40	18.7	3.6	8.3	6.9
$LSD_{0.05}$	0.8	0.4	0.6	0.7

The analysis of the summer wheat yield in 2021 has shown a 9.5 % and 11.7% increase at LignoCel-Si rates of 20 kg ha^{-1} and 40 kg ha^{-1}, correspondingly (Table 4). In 2022, the yield of the summer wheat was slightly lower (2.49-3.15 t ha^{-1}) than in 2021, but a comparative analysis showed a more significant increase in wheat yield (16.5 – 26.5 %) in the presence of LignoCel-Si.

Table 4. Influence of the LignoCel-Si on the yield and crop quality of the summer wheat variety "*Vinjet*"

Amount of LignoCel-Si (kg ha^{-1})	Yield, t ha^{-1}	Protein content, %	Gluten content, %	Starch content, %	Zeleny index, mL	Bulk density, g L^{-1}
Field experiment in 2021						
0 (reference sample)	2.83	12.53	23.63	69.34	41.68	729.7
20	3.10	12.57	24.39	67.73	41.96	726.6
40	3.16	12.67	25.17	66.70	42.03	719.2
LSD $_{0.05}$	0.31	x	x	x	x	x
Field experiment in 2022						
0 (reference sample)	2.49	13.15	27.84	66.17	40.57	679.6
20	2.90	13.39	27.93	65.72	40.66	682.6
40	3.15	13.35	27.61	65.89	41.26	689.7
LSD $_{0.05}$	0.46	x	x	x	x	x

The yield of grain crops is a complex indicator of all conditions prevailing during the period of growth and development of plants. First of all, it mainly depends on the number of productive stalks and the number of grains in the ear, 1000 grain mass, and

the grain mass of one ear. The analysis of the harvest constituent elements in 2021 and 2022 (number of productive stalks; number of grains per ear of wheat; 1000 grain mass; grain mass of one ear of wheat of the wheat), showed a slight increase or no significant differences between the options (Table 5). The grains obtained in the experiment had a low bulk density (<700 g L^{-1}) and a weight of 1000 grains (27.2 - 28.2 g).

Table 5. Harvest constituent elements

Amount of LignoCel-Si (kg ha^{-1})	Number of productive stalks, pcs./m^2	Number of grains per ear of wheat, pcs.	1000 grain mass, g	Grain mass of one ear of wheat, g
Field experiment in 2021				
0 (reference sample)	565	24.7	36.3	0.67
20	567	25.1	36.5	0.69
40	573	24.8	36.4	0.67
Field experiment in 2022				
0 (reference sample)	560	20.4	27.2	0.52
20	561	20.3	27.6	0.54
40	567	21.2	28.2	0.56

When evaluating grain quality according to the baking quality requirements, they meet the requirements of group 3. Also, in 2022, dry and hot weather significantly affected the grain quality.

DISCUSSION

The porous structure and the presence of active functional groups of different nature make lignin and lignocellulosic biomass processing residues a valuable material for creating organo-mineral complex with prolonged action. The chemical characterization of LignoCel-Si complex shows that it is a great source of organic matter. Based on the results of analytical pyrolysis, the content of lignin in the lignocellulosic complex was 28.5 %. LignoCel-Si in small quantities contains all the elements (N, K, P) necessary for the successful growth and development of plants, as well as substances that stimulate the processes of plant growth. The content of humic acids was 4.3% on the oven-dry sample. The introduction of 5% Si into the lignocellulosic biomass compensates for the low content of elements responsible for plant growth and development. The obtained product characteristics comply with the Cabinet of Ministers Regulation No. 506 “Regulations for the Identification, Conformity Assessment and Marketing of Fertilizers” [17], the maximum permissible concentration of undesirable impurities in the fertilizer is not exceeded. No Pb content was detected. LignoCel-Si complex has a basic environment (pH 8.7) and can serve as a neutralizer of soil acidity.

For the summer wheat "*Vinjet*", a significant increase in harvesting amount was observed at LignoCel-Si application in the dosage of 40 kg ha^{-1} (average yield increase per 2 years: +19,1%). Unfortunately, dry and hot weather influenced grain ripening. They formed of small size (weight of 1000 grains 27.2 – 28.2 g, and bulk density <700 g L^{-1}) and didn't correspond to the quality requirements of food wheat. Under organic farming conditions, it can be considered relatively high. It should be noted that in the first two decades of August, when the grains ripened, there was very little rainfall (16.0% of the long-term norm), and this could be the reason for the low weight and volume weight of 1000 grains (36.3-36.5 g L^{-1}).

A noticeable increase in potato "*Imanta*" yield was obtained already at the LignoCel-Si dose of 20 kg ha^{-1} (average yield increase per 2 years: 9.8%), while at increasing of the LignoCel-Si dose to 40 t ha^{-1}, significant yield increase was observed (average yield increase per 2 years: 17.6 %) comparing to the control. The application of LignoCel-Si reduced the proportion of seed potatoes (tubers, < 35 mm in diameter) and increased the proportion of ware potatoes (tubers, > 75 mm in diameter) in the crop.

CONCLUSION

Despite unfavorable weather conditions, a noticeable increase in yield both for the summer wheat variety "*Vinjet*" and potatoes variety "*Imanta*" was observed with the application of lignocellulosic organomineral complex LignoCel-Si. At the same time, the quality parameters of both crops improved insignificantly. In spite of this, the efficiency rate of LignoCel-Si organomineral complex comply with the Cabinet of Ministers Regulation No. 506 "Regulations for the Identification, Conformity Assessment and Marketing of Fertilizers" (based on Annex I to European Commission Regulation (EC) No 889/2008 of 5 September 2008) for registration and application of his preparation as a plant growth promoter for biological farming, since one of the parameters (crops yield or their quality parameters) has to be at least 10%. However, taking into account organic farming's need for possibly less amount of different additives, we consider further testing of the influence of LignoCel-Si with a bigger than 5% amount of Si in the complex.

Since the literature and our previous research showed that the quality of soil can be changed not earlier than after 5 years of the organomineral complex application, such additional studies will allow also investigating the soil quality parameters after LignoCel-Si complex continuous application.

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plants

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Article

Sea Buckthorn (*Hippophae rhamnoides*) Waste Biomass after Harvesting as a Source of Valuable Biologically Active Compounds with Nutraceutical and Antibacterial Potential

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† Sadly, Galina Telysheva passed away in November 2021. As she was the head of the project, and took part in the conceptualization, writing, choice of methodology, and investigation, the rest of the authors decided to submit the paper with her name as co-author. This is our tribute to our dear master. Anna Andersone (2nd author) as a daughter of Galina Telysheva confirms that Galina Telysheva approved the publication, and there is no conflict of interest.

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Abstract: For sustainable sea buckthorn *(Hippophae rhamnoides)* berry production, the task at hand is to find an application for the large amount of biomass waste arising at harvesting. Sea buckthorn (SBT) vegetation is currently poorly studied. The purpose of this research was to assess the composition and potential of SBT twigs as a source of valuable biologically active substances. Water and 50% EtOH extracts of twigs of three Latvian SBT cultivars with a high berry yield and quality, popular for cultivation in many countries (*H. rhamnoides* 'Maria Bruvele', 'Tatiana', 'Botanicheskaya Lubitelskaya'), were investigated for the first time. The phytochemical composition (UHPLC-ESI-MS/MS analysis) and biological activity of the obtained hydrophilic extracts were determined. The highest yield of polyphenolic compounds and serotonin was observed for 'Maria Bruvele'. Hydrophilic extracts were investigated for radical scavenging activity (DPPH˙ test), antibacterial/antifungal activity against five pathogenic bacteria/yeast, cytotoxicity, and the enzymatic activity of *alpha*-amylase (via in vitro testing), which is extremely important for the treatment of people with underweight, wasting, and malabsorption. The results showed a high potential of sea buckthorn biomass as a source of valuable biologically active compounds for the creation of preparations for the food industry, nutraceuticals, and cosmetics.

Keywords: sea buckthorn twigs; plant secondary metabolites; polyphenols; proanthocyanidins; serotonin; biological activity; antioxidant; antibacterial activity; cytotoxicity; *alpha*-amylase

1. Introduction

SBT (*Hippophae rhamnoides L.*) is a nitrogen-fixing and pest-resistant deciduous shrub tree which grows widely in Europe and high-altitude cold regions of Asia, and North and South America. Surviving the extreme temperatures (from −40 to +40 °C) [1,2] forces the plant to develop adaptogenic qualities. The plant has evolved a diversified chemical portfolio, and every part of the plant is nutritious, including its leaves, twigs, and even bark [3,4].

The global SBT berries export market is valued at USD 2 billion. The top exporter in 2020 was Canada, with USD 0.417 billion and a yearly increase of 13.9% [5]. On an industrial scale, SBT is cultivated in Russia, China, Canada, Finland, Germany, Latvia, Romania, and Estonia [6]. The overall market of SBT products is ~17 times bigger than just for berries and is constantly growing [7]. The industry uses fruits, but there are almost no applications for the green part of SBT [5], which comprises ~12–15% of the harvested mass [8,9].

Varietal characteristics have a significant impact on the quality indicators of raw material [10–12]. The advantages of Latvian SBT varieties are frost resistance, high-quality (large and sweet) fruits, and winter hardiness. Among the varieties exported to different countries, the largest-fruited (0.5–1 g) are *H. rhamnoides* 'Botanicheskaya Lubitelskaja', 'Prozrachnaya', 'Maria Bruvele', 'Tatiana', 'Avgustinka', 'Perchik', and 'Trofimovskaya' [13–15] (henceforth to be identified as the cultivars 'Botanicheskaya Lubitelskaja', 'Prozrachnaya', 'Maria Bruvele', 'Tatiana', 'Avgustinka', 'Perchik', and 'Trofimovskaya').

SBT fruits are quite well studied in terms of phytochemical composition and application [16–20]. The application of SBT fruit oil and leaf has no side effects [21]. The demand for natural biologically active substances, including antioxidant, antibacterial, and biostimulating substances, replacing harmful chemically synthesized ones, is constantly growing. Plant secondary metabolites possess enormous potential for further uses [22].

Upon harvesting at the production scale, a large amount of SBT biomass waste is produced, as the berries are collected by cutting the whole branch, freezing it, and shaking off the frozen berries. When the plant rests from growing berries, pruning is carried out to rejuvenate the bushes. Finding applications for the SBT biomass waste is necessary for the sustainable use of resources, which is the task of the European Green Deal [1], and for the creation of additional income for SBT growers and workers in rural area.

Sea buckthorn (SBT) is one of the most ancient plants on Earth (older than 2 billion years), and its fruits are mentioned among the most valuable in the world [8,23,24]. In recent studies, it was found that the biomass of all parts of the SBT tree also contains practically all biologically active groups of organic compounds currently known and 18 important microelements [1,25]. This is why in Latvia SBT is called the Latvian Gold. This makes the biomass of SBT a promising raw material for different branches of the economy [26].

SBT accumulates significant amounts of polyphenolic substances, including flavonols, flavones, phenolic acids, proanthocyanidins (PACs), and hydrolysable tannins, which are reported as the major contributors to antioxidant activities of SBT berries and leaves [17,27], and could be used both for the creation of pharmaceuticals and in the food industry to slow down oxidative processes in raw materials and finished products.

SBT bark contains another valuable secondary metabolite, serotonin [28], which is one of the most interesting and expensive components of SBT extractives. The research on obtaining serotonin from SBT is very limited worldwide.

The chemical structures of serotonin and B-type procyanidin are shown in Figure 1.

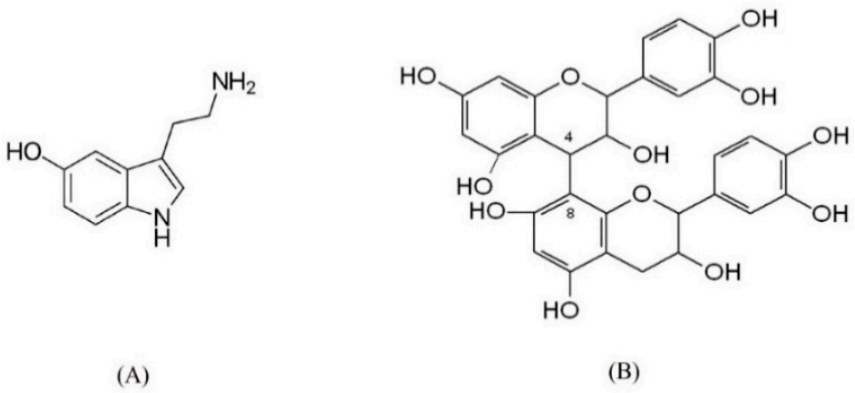

Figure 1. Chemical structure of serotonin (**A**) and B-type procyanidin (**B**), represented by 4–8 epicatechin dimer.

The levels of serotonin vary in different plant parts [29]. Serotonin has been implicated in diverse physiological functions in plants such as growth regulation, flowering, xylem sap exudation, ion permeability, plant morphogenesis, and the regulation of abiotic stress tolerance [30]. It also defends plants against fungi [31]. In mammals, serotonin acts as a neurotransmitter in the central nervous system and affects motor activity and the functioning of the gastrointestinal tract. It can be beneficial for treating cancer, HIV, Parkinson-like symptoms, obesity, depression, insomnia, alcohol abuse, schizophrenia, and several chronic diseases [28,32]. The content of serotonin in the bark of SBT is one-thousand times higher than in bananas or chocolate [28]. The antioxidant activity of serotonin far exceeds that of tryptophan, tryptamine, and serotonin derivatives [33]. Furthermore, 95% of serotonin is produced in the peripheral organs, and serotonin in the digestive system may work independently of serotonin in the brain [34]. In addition to its application in human diets, serotonin could be used as a natural biostimulant for plant rooting [30].

Another important urgent task is the search for natural substances with antibacterial properties and low toxicity to the human body since the resistance to synthetic antibiotics among Gram-positive and Gram-negative bacterial pathogens is growing tremendously. Each year, approximately 25,000 patients in the EU die from infections due to multidrug-resistant bacteria [35]. *Escherichia coli* and *Staphylococcus aureus* are pathogens that are responsible for the most primary and secondary skin and blood infections. *S. aureus* also constitutes 30% of burn wounds. Non-healing wounds are a huge problem for diabetic and older patients [36]. Extracts of the vegetative part of SBT have not yet been studied as antimicrobial agents, although studies on the effect of PACs and quinic acid on the activity of *E. coli* in cells indicate the possibility of creating antimicrobial agents based on them [37]. Quinic acid inhibits the growth of most microorganisms [38].

An equally important problem is metabolic disorders in the body. There is evidence of the effect of PACs on the activity of the hydrolytic cleavage of carbohydrates to monosaccharides, under the action of α-amylase in saliva [39,40]. It has been proven that the initial processes of human macro-metabolism are extremely important, and saliva plays a major physiologic role in food digestion [41]. Studies have linked higher amylase levels to better glucose tolerance after eating starch-rich meals [42,43]. PAC-containing extracts, based on the concentration of the active substance, can both activate and inhibit the activity of amylase in the initial breakdown of carbohydrates. Therefore, the possibility of using SBT twig extracts for the normalization of the enzymatic activity of saliva can be studied.

Taking into account the relevance of the topic of the complex and rational use of plant raw materials, and the insufficient amount of knowledge about SBT biomass, the aim of this study was to assess the potential of pruned SBT twigs, as waste after SBT berries harvesting, of three the most prospective cultivars of SBT ('Maria Bruvele', 'Tatiana', and 'Botanicheskaya Lubitelskaya') as a source of valuable biologically active substances, mainly polyphenols and serotonin, with the establishment of the phytochemical composition and biological activity (antioxidant, antibacterial, and enzymatic) of the obtained hydrophilic extracts to determine their practical significance.

2. Results and Discussion

2.1. Yield and Chemical Composition of Hydrophilic Extracts

A review of the published literature data [44] and our preliminary experiments [45,46] showed that ethanol (EtOH) and its aqueous solutions are the most suitable extraction solvents for the isolation of biologically active polyphenolic compounds from plant biomass, including SBT biomass. For the study of three SBT cultivars ('Maria Bruvele'; 'Tatiana'; and 'Botanicheskaya Lubitelskaya'—'Bot. Lub.'), 50% EtOH and distilled water were used as extractants for hydrophilic extract isolation, based on their selectivity for polyphenolic compounds, chemical inertness, low toxicity, and low cost. The quantitative analysis of the total polyphenolic compounds was performed for the obtained extracts, determining the effect of extractant concentration on the efficiency of polyphenolic compound release (Figure 1). The yield of hydrophilic extracts was determined by the gravimetric method

after the freeze-drying of the samples. The yield of hydrophilic extracts (hereinafter extracts) from twigs of three SBT cultivars varied from 15% to 30% in terms of dry SBT biomass (Figure 1). The obtained extracts were rich in polyphenolic compounds that are known to be responsible for free radical scavenging activity. The 'Maria Bruvele' biomass had the highest yield of 50% EtOH extract (30% from o.d. biomass) and the highest content of phenolic compounds in the extracts (48.1 GAE g/100 g extract or 14.4% on o.d. biomass with confidence interval CI = 0.2 at $\alpha = 0.05$). Despite the high content of the total polyphenols in 50% EtOH and water extracts of 'Tatiana' (41.3 and 35.1 GAE g/100 g extract, CI = 0.2 at $\alpha = 0.05$), the yield of the extracts themselves, compared to 'Maria Bruvele', was 2 times less (15 and 16% on o.d. extract, with CI = 0.4 at $\alpha = 0.05$), which reduced the yield of polyphenols from SBT biomass (6.2% and 5.6% on o.d. biomass). Based on these observations, 'Maria Bruvele' is the raw material with the most potential between the 3 investigated popular cultivars for obtaining polyphenol-rich extracts, which has also been confirmed by other authors by examining the chemical composition of sea buckthorn twigs grown in Poland [16]. The contents of total polyphenolic compounds of all the extracts are given in Figure 2.

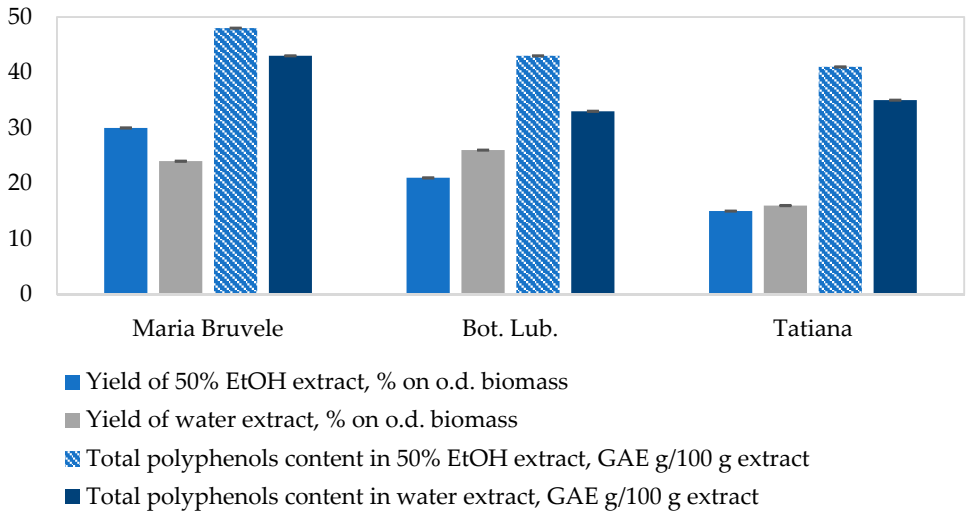

Figure 2. Effect of the extractants on the extract yield from twigs of SBT and selectivity for polyphenolic compounds (single-step extraction, 30 min, 60 °C, biomass and extractant weight ratio 1:8). Data represented as mean ± SD ($n = 3$).

The freeze-dried 50% EtOH and water extracts were analyzed by UHPLC-ESI-MS/MS for the identification of biologically active compounds, including polyphenolic compounds, representing a significant part of the extract. UHPLC-ELS chromatograms of extracts are shown in Figures 3 and 4.

The compounds identified are listed in Table 1, with the most abundant ones being quinic acid, catechin/epicatechin, gallocatechin, procyanidin trimer, procyanidin tetramer, (epi)catechin-(epi)gallocatechin, quercetin, quercetin-3-O-rutinoside, triterpenoids, and acylated triterpenoids. Part of the polyphenolic compounds in the extracts is in the form of O-glycosides, which consist of a residue of aglycone and carbohydrates consisting mainly of glucose. At the same time, the compositional similarity of the composition of the 50% EtOH and water extracts of the branches of all three varieties of sea buckthorn was found. All these compounds are biologically active natural antioxidants and antimicrobial agents, which can be used as ingredients in the formulation of different medications.

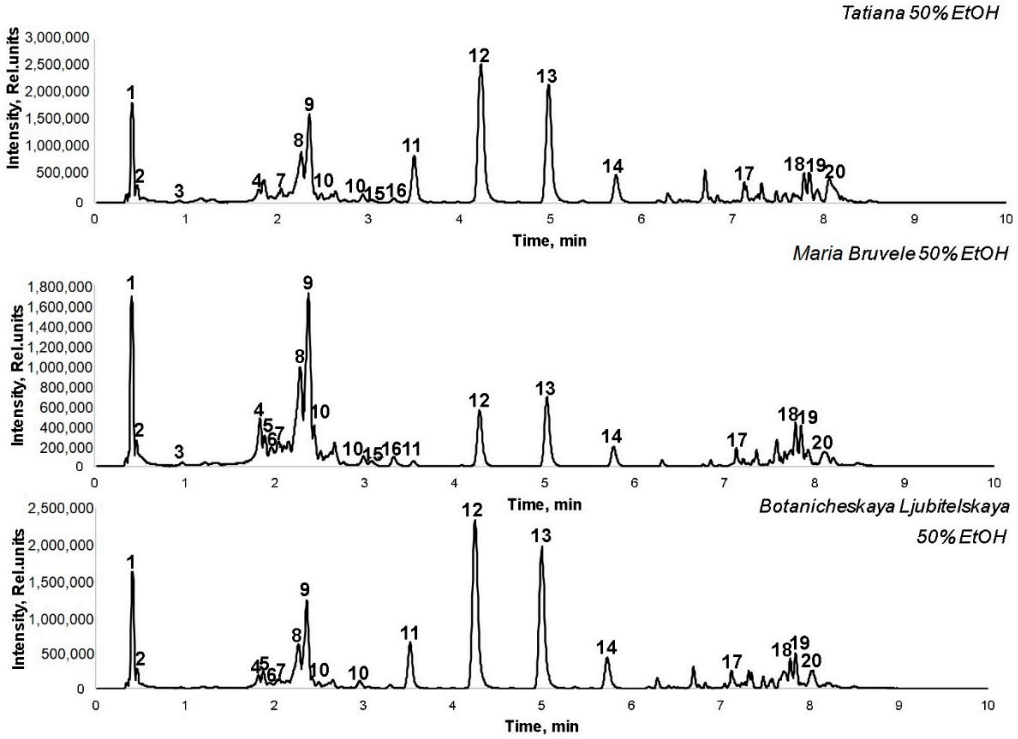

Figure 3. UHPLC-TOF/MS chromatograms of 50% EtOH extracts of SBT samples.

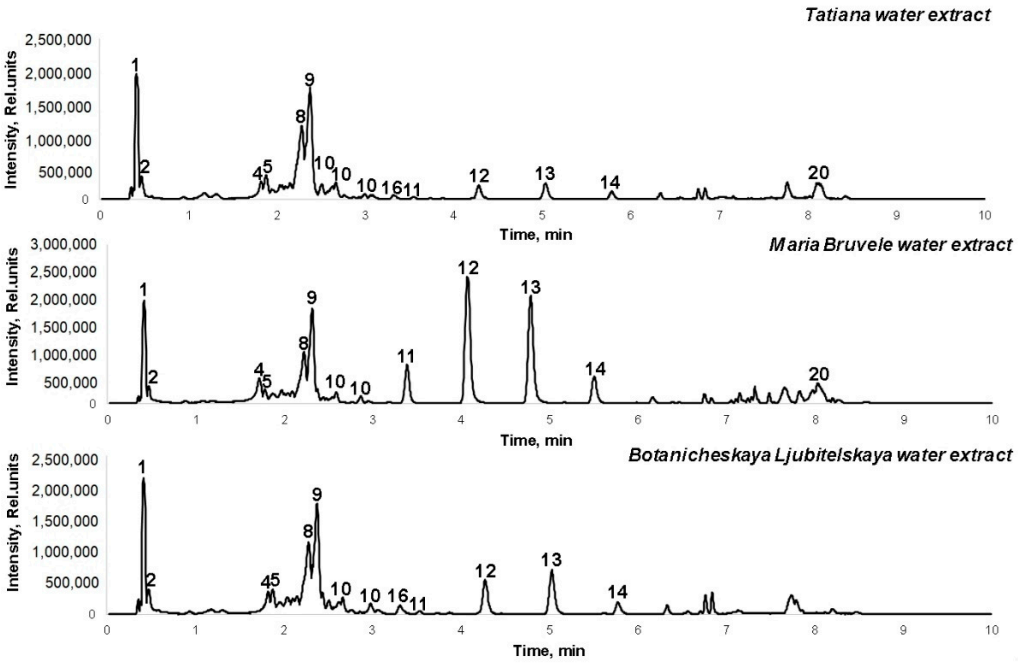

Figure 4. UHPLC-TOF/MS chromatograms of the water extracts of SBT samples.

Among the identified polyphenolics compounds, quinic acid, quercetin, and triterpenoids have documented excellent antibacterial activity against *Staphylococcus aureus*, which is a leading Gram-positive pathogen associated with a number of diseases, including osteomyelitis, pneumonia, endocarditis, and septicemia. This bacterium is also frequently found in many food products such as dairy, eggs, seafood, and meat, and can cause food poisoning, which is a major concern for the international community and the food industry. Additionally, they show antibacterial activity toward Staphylococcus epidermidis, Bacillus subtilis, and Escherichia coli [37].

Table 1. Dominant compounds in the chromatograms of SBT twigs.

Peak No.	t^R (min)	$[M-H]^-$ (m/z)	Fragments	Identification [1]	Relative Abundance, %					
					Tatiana		Maria Bruvele		Bot. Lub.	
					Water	50% EtOH	Water	50% EtOH	Water	50% EtOH
1	0.41	341	179; 161; 143; 119; 113; 101	Sucrose, fructose, glucose	9.7	9.0	10.7	8.2	10.9	9.3
2	0.47	191	111; 173; 127; 85	Quinic acid	5.7	4.4	6.1	5.0	5.6	4.2
3	0.98	175	159; 147	Serotonin [2]	0.1	0.1	0.1	0.1	0.1	0.1
4	1.84	305	179; 125	Gallocatechin or its isomer epigallocatechin	0.7	1.8	2.6	1.7	3.2	1.5
5	1.89	593	407; 425; 305; 467; 289	(epi)catechin-(epi)gallocatechin	2.2	0.5	0.9	1.1	1.3	0.4
6	1.97	1185	881; 593; 305; 289; 245	Procyanidin tetramer	4.1	2.2	2.6	1.0	2.8	1.7
7	2.06	1055	881; 593; 305; 289	Procyanidin tetramer	4.4	1.8	4.6	2.7	4.4	2.3
8	2.30	865	577; 289; 245	Procyanidin trimer	4.1	4.5	5.2	4.9	4.2	3.4
9	2.38	289	245; 125	Catechin/Epicatechin	6.9	5.3	6.7	8.1	7.0	4.2
10	2.50	1153	865; 577; 289; 245	Procyanidin tetramer	13.5	13.0	9.6	12.2	17.2	7.0
11	3.51	610	-	Contaminant from solvent—ethanol, nylon filter	0.1	1.4	1.7	0.7	0.1	1.1
12	4.28	723	-	Contaminant from solvent—ethanol, nylon filter	0.6	5.2	7.2	1.3	1.0	5.6
13	5.04	836	-	Contaminant from solvent—ethanol, nylon filter	0.6	4.6	6.5	1.8	1.4	5.2
14	5.77	949	-	Contaminant from solvent—ethanol, nylon filter	0.3	1.1	1.8	0.6	0.4	1.3
15	3.28	609	301; 271	Quercetin-3-O-rutinoside	-	-	-	0.01	-	-
16	3.33	301	286; 109	Quercetin	0.1	0.1	0.2	0.4	0.1	0.1
17	7.14	487	293; 117	Triterpenoid	-	2.8	3.0	2.5	-	3.0
18	7.79	471	452; 265; 117	Triterpenoid	-	1.8	-	3.1	-	2.1
19	7.86	471	265; 117	Triterpenoid	-	1.9	-	1.8	-	2.4
20	8.07	455	277; 117	Triterpenoid	3.2	3.4	7.2	4.3	-	-
21	8.01	617	255; 117	Acylated triterpenoid	-	-	-	-	-	2.8

[1] Serotonin showed weak signal in negative ion ESI LC-MS, which was quantified in MRM positive ionization mode. [2] Compounds were tentatively identified compared with those reported in the literature and confirmed through databases, specifically the Dictionary of Natural Products and ChemSpider, focusing on MS/MS fragmentation patterns and accurate mass.

A comparison of the relative peak areas of the dominant biologically active compounds calculated for mg/mg extract is shown in Table 2.

Table 2. The comparison of relative peak areas of dominant biologically active compounds calculated on mg/mg extract.

No.	Tentative Identification	Relative Peak Area (Relative Units)		Cultivar
		50% EtOH	H_2O	
1	Quinic acid	25,954	31,176	Tatiana
		43,608	53,949	Maria Bruvele
		38,504	44,680	Bot. Lub.
2	Gallocatechin or its isomer epigallocatechin	14,917	11,890	Maria Bruvele
		2369	4476	Tatiana
		5291	8424	Bot. Lub.
3	(epi)catechin-(epi)gallocatechin	6283	2855	Maria Bruvele
		4782	5539	Tatiana
		5314	6759	Bot. Lub.
4	Procyanidin tetramer	8054	3230	Maria Bruvele
		1674	4395	Tatiana
		5335	5841	Bot. Lub.
5	Procyanidin tetramer	8879	2397	Maria Bruvele
		-	4665	Tatiana
		-	7803	Bot. Lub.
6	Procyanidin trimer	37,952	31,036	Maria Bruvele
		15,879	26,104	Tatiana
		19,967	33,381	Bot. Lub.
7	Catechin/Epicatehin	51,282	56,089	Maria Bruvele
		24,115	34,058	Tatiana
		35,098	46,065	Bot. Lub.
8	Procyanidin tetramer	3004	1739	Maria Bruvele
		916	3500	Tatiana
		1864	3656	Bot. Lub.
9	Procyanidin tetramer	9768	5576	Maria Bruvele
		2897	6506	Tatiana
		4632	7877	Bot. Lub.
10	Procyanidin tetramer	635	2850	Maria Bruvele
		236	619	Tatiana
		382	751	Bot. Lub.
11	Procyanidin tetramer	1348	973	Maria Bruvele
		1893	1023	Tatiana
		2946	3040	Bot. Lub.
12	Quercetin-3-O-rutinoside	334	-	Maria Bruvele
		-	-	Tatiana
		-	-	Bot. Lub.
13	Quercetin	2759	677	Maria Bruvele
		933	1003	Tatiana
		1287	3105	Bot. Lub.
14	Triterpenoid	3218	-	Maria Bruvele
		3750	-	Tatiana
		4561	-	Bot. Lub.
15	Triterpenoid	7631	-	Maria Bruvele
		5000	-	Tatiana
		6950	-	Bot. Lub.
16	Triterpenoid	7273	-	Maria Bruvele
		5860	-	Tatiana
		8651	-	Bot. Lub.
17	Triterpenoid	4923	14,713	Maria Bruvele
		8978	6910	Tatiana
		-	-	Bot. Lub.
18	Acylated triterpenoid	-	-	Maria Bruvele
		-	-	Tatiana
		6808	-	Bot. Lub.

2.2. Evaluation of the Antimicrobial Activity of SBT Extracts

The evaluation of the antimicrobial activity of the plant extracts against test cultures of microorganisms was carried out according to the method for determining the sensitivity of microorganisms to antimicrobial drugs. Antimicrobial activity was studied in 96-well plates by the two-fold serial broth microdilution method, which allowed the determination of the minimum inhibitory (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC) [47].

The comparison of the results of the chemical composition and antimicrobial activity of 50% EtOH extracts showed that among the three varieties of SBT, 'Maria Bruvele' was the most active in suppressing pathogenic bacteria. This could be explained by the increased content of such biologically active compounds as quinic acid, gallocatechin, isomer epigallocatechin, (epi)catechin-(epi)gallocatechin, and procyanidins.

The results show that all extracts have high antimicrobial activity against both Gram-positive and Gram-negative bacteria (see Table 3).

Table 3. Antimicrobial activity of the extracts from SBT samples.

	Maria Bruvele	**Bot. Lub.**	**Tatiana**	**Maria Bruvele**	**Bot. Lub.**	**Tatiana**
	50% EtOH Extract, mg/mL			**Water Extract, mg/mL**		
E. coli MIC/MBC	0.20/0.20	0.39/0.39	0.39/0.39	0.39/0.39	0.78/50	0.39/0.39
P. aeruginosa MIC/MBC	0.39/0.78	0.78/1.56	3.13/3.13	0.39/3.13	0.78/50	0.78/1.56
S. aureus MIC/MBC	0.20/0.39	0.39/0.78	0.20/0.78	0.39/0.78	0.39/12.2	0.39/0.78
B. cereus MIC/MBC	0.39/50	0.78/>50	0.78/50	0.78/>50	0.78/>50	0.78/50
C. albicans MIC/MFC	0.20/>50	0.20/>50	0.39/>50	0.20/>50	0.39/>50	0.39/>50
	Antibiotics					
	Gentamicin (Reference), μg/mL		**Fluconazole (Reference), μg/mL**		**Tetracycline Hydrochloride [32], mg/mL**	
E. coli MIC/MBC	1.00/4.00		ND		0.76	
P. aeruginosa MIC/MBC	0.25/4.00		ND		ND	
S. aureus MIC/MBC	0.25/4.00		ND		1.52	
B. cereus MIC/MBC	ND		ND		0.76	
C. albicans MIC/MFC	ND		32/>256		ND	

MIC—minimum inhibitory concentration; MBC—minimum bactericidal concentration. MFC—minimum fungicidal concentration; ND—not determined. MIC tests were performed in triplicate for each strain and antimicrobial compound. Confidence interval is ±0.01 at $\alpha = 0.05$.

When comparing the 50% EtOH and water extract of SBT 'Maria Bruvele', we found that despite the fact that the content of quinic acid in the water extract was 1.24 times higher, the 50% EtOH extract was more active; this could be attributed to the synergetic activity with the other antimicrobial agents, namely, an increased amount of such polyphenolic compounds as gallocatechin or isomer epigallocatechin (1.25 times higher in the 50% EtOH extract), (epi)catechin-(epi)gallocatechin (1.13 times higher) and procyanidins (2.5 times higher).

The minimum bactericidal concentration of the extracts showed that the extracts were able to completely neutralize the bacteria under study, and their MICs were even comparable with those of weaker antibiotics.

2.3. Evaluation of the Cytotoxicity of Extracts

The graphs below show the results of the cytotoxicity tests, by changes in cell viability and IC_{50} values. It was reasonable to evaluate the cytotoxicity around the range of the antimicrobial activity of the extracts. The testing started from lower concentrations of 0.078 mg/mL up to 10 mg/mL (Figure 5). The use of higher concentrations made it difficult to read the results objectively as the intensive coloring influenced the absorbance.

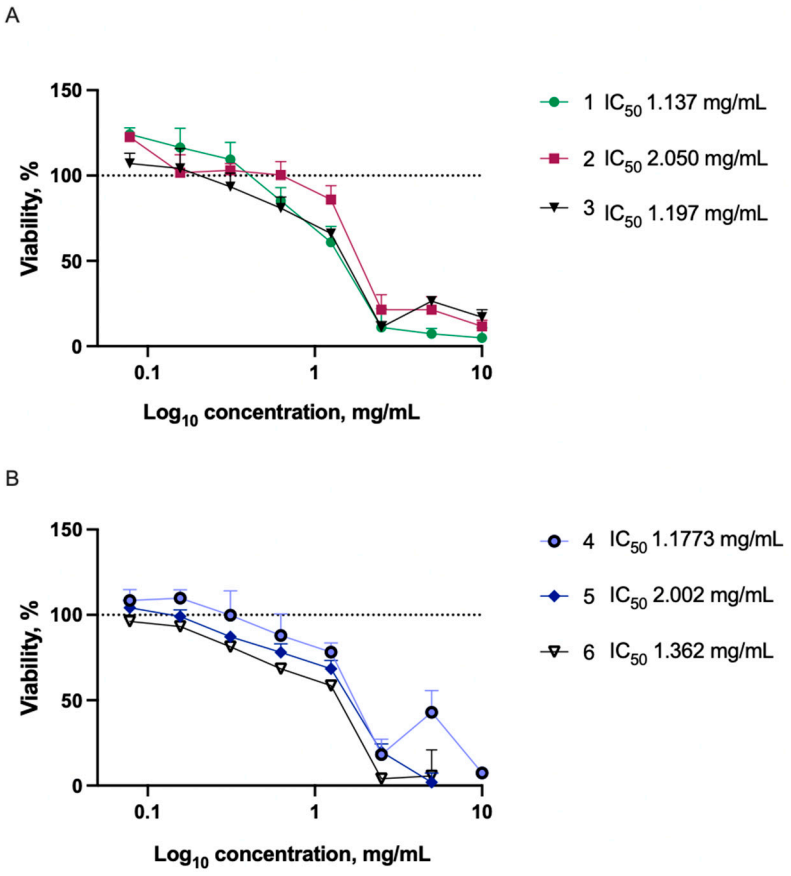

Figure 5. Cytotoxicity of extracts in Balb/c 3T3 cell line. Results expressed as a relative change compared to untreated control. (**A**)—50% EtOH extracts: 1—Maria Bruvele; 2—Tatiana; 3—Bot. Lub.; (**B**)—water extracts: 4—Tatiana; 5—Bot. Lub.; 6—Maria Bruvele. Data represented as mean ± SD (n = 3). Dotted line represents the control level (100%).

In most cases, at concentrations similar to the MIC values observed in antimicrobial activity tests, no toxic effect was observed. Water extracts were slightly more cytotoxic than ethanol extracts. An extract at a specific concentration was considered to be cytotoxic if the cell viability was reduced by more than 20%. Cytotoxic concentrations of ethanol extracts ('Maria Bruvele', 'Tatiana') did not exceed the concentrations needed to inhibit the growth of the tested microorganisms. Some variations in the effects of water extracts on cell viability were observed, with the 'Tatiana' extract being less cytotoxic than the other two water extracts. Compared to other studies, extracts tested here have low cytotoxicity. Triterpenoid-rich SBT extracts have been reported to be cytotoxic to cancerous and normal human cell lines at lower concentrations than in our study, with IC_{50} values ranging between 14.58–74.58 µg/mL [48]. SBT extract within concentration range 0.62–62 µg/mL was shown to have no negative effects on NIH 3T3 cell line but reduced viability of glioma

cells in vitro. This emphasizes the variations of the effects in different cell lines [49]. At the same time, in a study by Rozalska et al., IC_{50} values above 1 mg/mL for phenolic SBT fractions have been reported [50]. The effects on cell lines depend on the extract production methods and chemical composition. Varying cytotoxicity data among studies might be explained mainly by the variations in chemical composition on SBT extracts.

The low concentrations needed to inhibit the growth of specific microorganisms, especially *C. albicans* and *S. aureus*, along with the absence of cytotoxicity at low concentrations, indicate on the potential of the tested extracts to be further developed for various antimicrobial applications. The low concentrations needed to inhibit the growth of specific microorganisms, especially *C. albicans* and *S. aureus*, along with the absence of cytotoxicity at low concentrations, indicate to the potential of the tested extracts to be further developed for various antimicrobial applications.

Interestingly, at low concentrations, ethanol extracts of 'Maria Bruvele' and 'Tatiana' slightly increased cell viability and proliferation (increases of 24.13 and 22.59%, respectively). This phenomenon could be further researched in future studies.

2.4. The Radical Scavenging Activity of SBT Extracts

The radical scavenging activity of SBT extracts was evaluated by DPPH· tests, expressed as the IC_{50} value, the concentration required for the 50% inhibition of free radicals [51]. The results of the radical scavenging activity assays are shown in Figure 6. Among the SBT samples under study, the 50% EtOH extracts of 'Maria Bruvele' and 'Bot. Lub.' twigs manifested the highest radical scavenging activity (IC_{50} = 6.8 mg/L and 6.6 mg/L) compared to the other extracts.

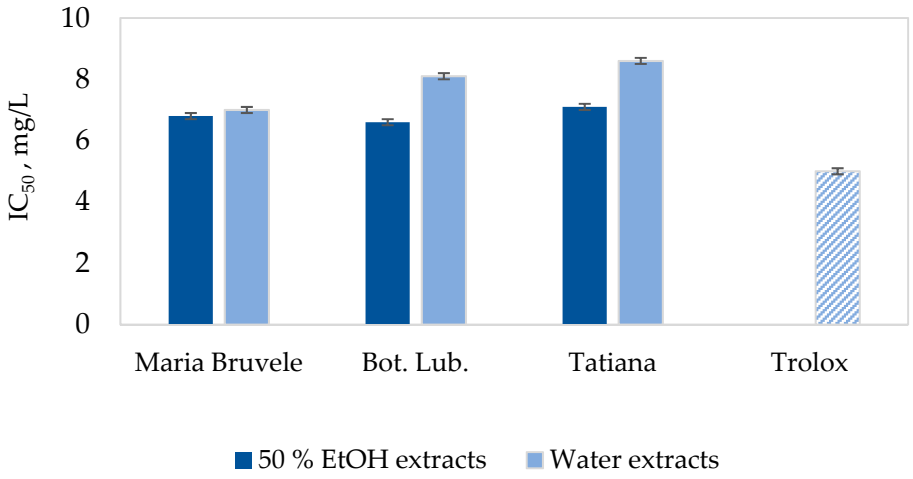

Figure 6. Radical scavenging activity of SBT biomass extracts by DPPH· test. Data represented as mean ± SD (n = 3).

As shown in Figure 7, such high activity of these extracts is associated with a high content of polyphenolic compounds (48 and 43 GAE g/100 g extract), including PACs (13 and 11% on the o.d. extract, CI = 0.2 at α = 0.05).

When comparing the radical scavenging activity of the extracts from SBT twigs, a correlation between the content of carbohydrates as unwanted compounds in the extracts and their radical scavenging activity could be observed (Figure 8). With the increase in carbohydrate content in the extracts, their radical scavenging activity decreased in the DPPH· test. The water extract of Tatjana twigs had a weaker ability to deactivate radicals due to the high content of carbohydrate impurities (23% on o.d. extract, CI = 0.03 at α = 0.05) and low content of PACs (7% on o.d. extract) in the extract. The polar groups of carbohydrate impurities can form hydrogen bonds with the hydroxyl groups of polyphenolic compounds, thus reducing their radical scavenging activity. The carbohydrate content in the 50% EtOH and water extracts of SBT biomass is shown in Figure 8.

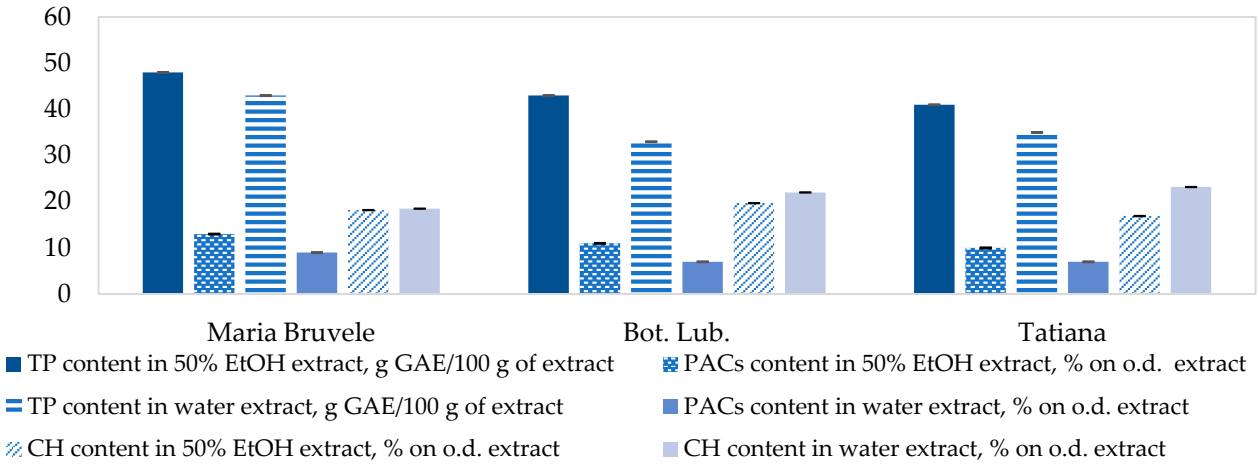

Figure 7. Chemical composition of extracts from SBT biomass. Data represented as mean ± SD (n = 3).

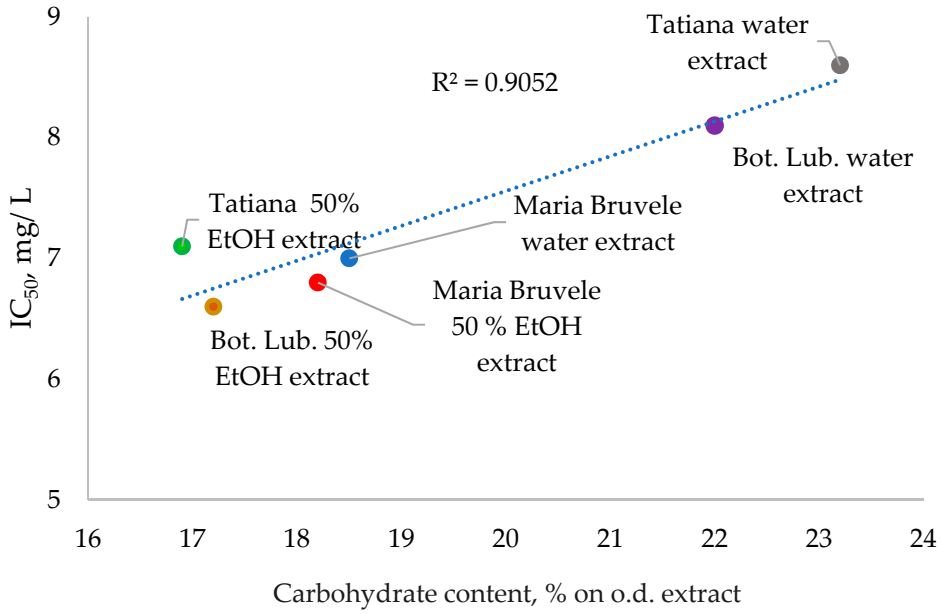

Figure 8. Effect of carbohydrate content on radical scavenging activity. Confidence intervals for antioxidant activity: CI ≤ 0.1 at α = 0.05, for carbohydrate content: CI ≤ 0.03 at α = 0.05.

One way to increase antioxidant activity is to remove impurities from the dominant components with strong antioxidant properties. Our preliminary research indicates that proanthocyanidins are powerful antioxidants. Based on this, it was decided to purify proanthocyanidins by Sephadex LH-20. As a result, two fractions were obtained, which were also characterized by UHPLC-ELS (Figure 9).

In comparison to the synthetic antioxidant Trolox as a reference, which is a water-soluble derivative of vitamin E (IC_{50} = 5 mg/L in DPPH˙ test), the 50% EtOH extract of 'Bot. Lub.' and 'Maria Bruvele' showed the most promising results (IC_{50} = 7.1 mg/L in DPPH˙ test, CI = 0.1 at α = 0.05). The antioxidant activity of purified procyanidin was significantly higher (IC_{50} = 2.6 mg/L in DPPH˙ test, CI = 0.1 at α = 0.05).

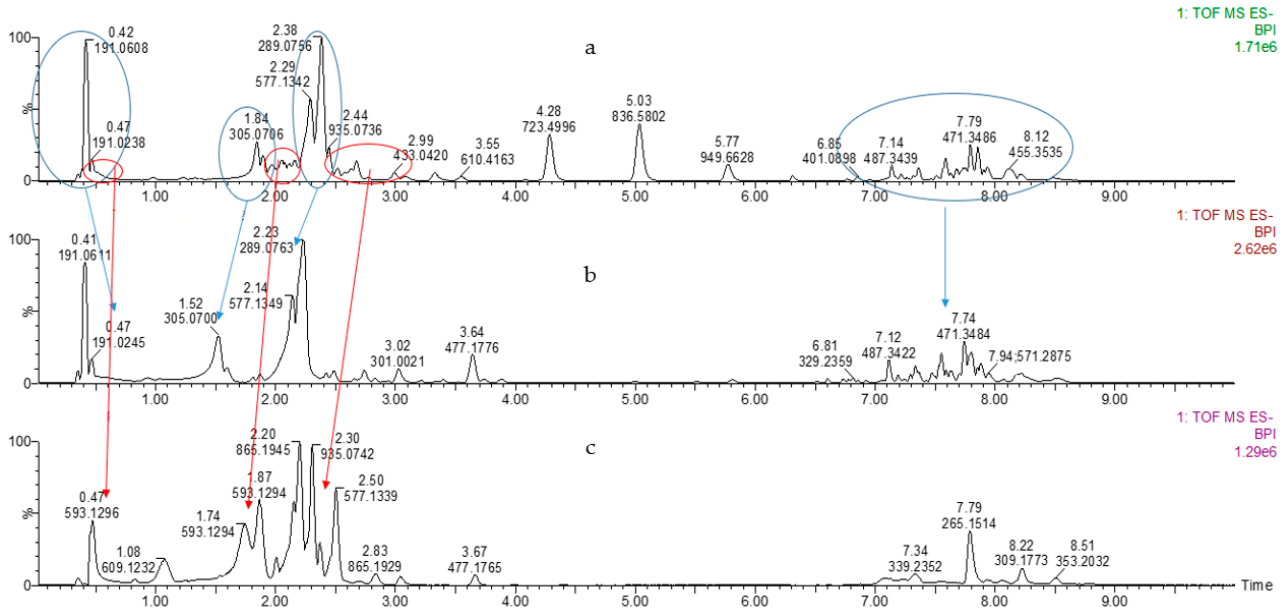

Figure 9. UHPLC-ELS chromatograms: (**a**)—50% EtOH extract of 'Maria Bruvele' biomass; (**b**)—low molecular compound rich fraction; (**c**)—procyanidin trimer rich fraction.

2.5. The Content of Serotonin in SBT Extracts

Of the alkaloid compounds, SBT lignocellulosic biomass contains serotonin, which is a powerful antidepressant and stimulant of psychological and physical activity. The analysis of liquid chromatography (UHPLC-ELS) proved its presence in the composition of hydrophilic extracts. The serotonin content in the SBT extracts (Figure 10) varied from 3.6 to 7.5% per dry extract, and, starting from the highest content, decreased in the following order: water extracts of 'Maria Bruvele' and 'Tatiana' >50% EtOH extract of 'Bot. Lub.' >50% EtOH extract of 'Maria Bruvele' > water extract of 'Bot. Lub.'.

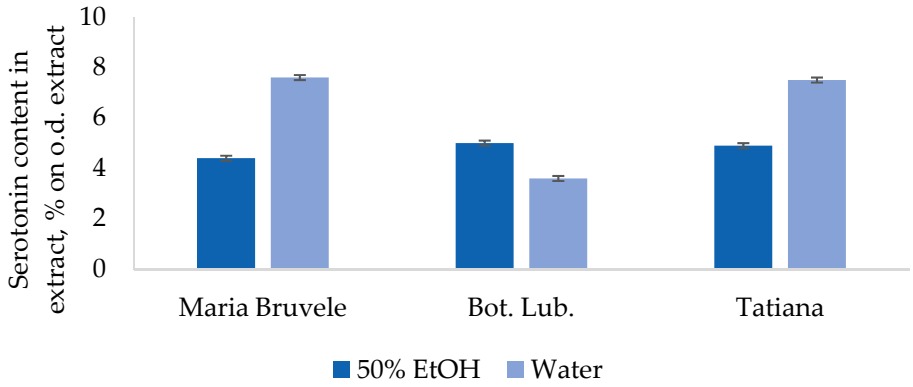

Figure 10. Content of serotonin in extracts from SBT biomass. Data presented as mean ± SD (n = 3). CI ≤ 0.04 at α = 0.05.

When using extracts as biologically active food additives or as an additional component of activators of food enzymes, serotonin will only increase the value of this biologically active product.

To prove the potential of the extract's biological activity, in the following stage, in vitro experiments were performed.

2.6. Evaluation of the Influence of the Extracts on Amylase Activity

In vitro tests were also carried out for the evaluation of the influence of the extracts on the initial processes of human macro-metabolism, which is relevant for the extract usage for health care and disease prevention. Earlier tests in collaboration with Riga Stradinš University in this regard have revealed the beneficial effects of PACs-containing extracts on amylase activity, resulting in the acceleration of starch degradation to glucose, which could be useful for the treatment of persons with underweight, malnutrition, and malabsorption [52,53]. Under normal physiological conditions, all extracts at dosages of 100 µL, 500 µL, and 1000 µL at an extract concentration of 2 mg/L showed a significant activation (two times) of amyloclastic force (AF). With the increase in the extract concentration from 2 mg/L to 20 mg/L, at dosages of 100 µL and 500 µL of the extracts, the same activation was observed. Increased α-amylase activation can accelerate the degradation of starch to glucose, which may be useful in the treatment of people with underweight, malnutrition, and malabsorption.

3. Materials and Methods

3.1. Materials

The twigs (without leaves) of three sea buckthorn cultivars—*H. rhamnoides* 'Maria Bruvele', 'Botanicheskaya Lubitelskaya', and 'Tatiana'—were collected from the sea buckthorn plantation area in Latvia under the same growing conditions in summer of 2020. Varieties suitable for commercial growth in the NE of Europe and Canada, larger and more juicy fruits with better taste, and fruits with significantly less troublesome stellate hairs were chosen. The twigs were dried at room temperature, ground with a knife mill (Cutting Mill SM100, Retsch, Haan, Germany) and sieved to select the particles between 1 and 4 mm. These fractions were stored at −8 °C.

3.2. Isolation of the Hydrophilic Extracts from Twigs of Sea Buckthorn Biomass

Hydrophilic extracts were isolated by the convective extraction of SBT biomass at 60 °C for 30 min using the following solvents: distilled water or aqueous ethanol (1:1, *v/v*). The extracts were freeze-dried to yield a brown solid. The yield of the extracts is presented as a percentage based on the oven-dried (o.d.) biomass.

3.3. Total Polyphenols Content in the Extracts

The total polyphenols (TP) content of the extracts was determined using the Folin–Chicolteu method using gallic acid as the standard. Amounts of 5 mL of 10% Folin–Ciocalteu reagent and 4 mL of 7.5% sodium carbonate solution were added to 1 mL of the extract. Distilled water was used instead of gallic acid as a reference solution. After 30 min, the absorbance of the mixture was measured against a blank solution at 765 nm using a UV/VIS spectrometer Lambda 650 (Perkin Elmer, Shelton, CT, USA). Gallic acid was used to calibrate the standard curve. Each extract was analyzed in triplicate, and the results were expressed in grams of gallic acid per 100 g of extract sample (g GAE/100 g extract) [54].

3.4. Proanthocyanidins Content in the Extracts

The PACs content of the extracts was determined by oxidative depolymerization to anthocyanidins in acid butanol [55] using procyanidin dimer B2 as a reference compound. Amounts of 6 mL of acid butanol (5% (*v/v*) concentrated HCl in n-butanol) and 0.2 mL of iron reagent (*w/v*) ($FeNH_4(SO_4)_2 \cdot 12\ H_2O$ in 2 M HCl) were added to 1 mL of the extract aliquots while stirring the tube without heating and allowing it to be heated in a water bath at 80 °C for 50 min. After 50 min, the absorbance of the mixture was measured against a blank solution at 550 nm using UV/VIS spectrometer Lambda 650 (Perkin Elmer, Shelton, CT, USA). Each extract was analyzed in triplicate, and assay results were expressed as a percentage per o.d. extract.

3.5. Purification of PACs

The purification of PACs from non-tannin phenolics and sugar was carried out using a Sephadex LH-20 with 96% EtOH and 70% (*v/v*) acetone as the respective purification solvents. In the purification process, low-molecular-weight phenolics were eluted with 96% EtOH, and the PACs were eluted with 70% (*v/v*) acetone. Purified PACs were evaporated using a rotary evaporator prior to being freeze-dried and stored at −8 °C.

3.6. Carbohydrate Content in the Extract

The total amounts of the carbohydrate in the extracts were determined using GC analysis after hydrolysis, reduction, and acetylation [56]. Extract hydrolysis: 0.125 mL of sulfuric acid was added to 10 mg of the extract; after 45 min, it was diluted with 3.5 mL of water and placed into a thermostat for 1 h at 121 °C. After hydrolysis, the sample was neutralized with 0.32 mL of ammonium hydroxide solution and 0.1 mL of analytical standard methyl α-D-glucose. Reduction and acetylation: 1 mL of borohydride solution was added to 0.2 mL of neutralized solution and heated for 90 min at 40 °C. The excess reagent was partitioned with 0.1 mL of concentrated acetic acid. After reduction, 2 mL of acetic anhydride and 0.3 mL of 1-methylimidazole were added. After 10 min (30 °C), the excess acetic anhydride was partitioned with 5 mL of distilled water. The cooled solution was extracted once with 1 mL of CH_2Cl_2. The lower layer was transferred to the chromatography flask with a Pasteur pipette and stored at −20 °C until gas chromatographic analysis. Gas chromatographic analysis was performed using an Agilent 6850 Series GS System (Agilent Technologies, Santa Clara, CA, USA): column—DB-1701; length—30 m; internal diameter—0.25 mm; layer thickness—0.25 μm.

3.7. UHPLC-ESI-MS/MS Analysis

Extract analysis was performed on an Acquity UPLC system (Waters Corp., Singapore) coupled with a quadrupole-time of flight (Q-TOF) MS instrument (UPLC/SYNAPT G2Si HDMS Q-TOF Mass Spectrometer, Waters, Milford, MA, USA) with an electrospray ionization (ESI) source.

The separation was carried out on a U-HPLC column (2.1 mm × 50 mm i.d., 1.7 μm, BEHC18) (Waters Acquity) at a flow rate 0.35 mL/min. The eluent was 0.1% formic acid, water (A), and acetonitrile (B). A gradient solvent system was used: 0–1 min, 5–20% (B); 1–5 min, 20–25% (B); 5–6 min, 25–75% (B), 6–7 min, 75–80% (B), 7–8 min, 80–5% (B), 8–10 min, 5% (B). The injection volume was 2.0 μL.

The major operating parameters for the Q-TOF MS were set as follows: capillary voltage, 2.5 kV (-); cone voltage, 60 V; cone gas flow, 50 L/h; collision energy, 6 eV; source temperature, 120 °C; desolvation temperature, 350 °C; collision gas, argon; desolvation gas, nitrogen; flow rate, 500 L/h; data acquisition range, m/z 50–1.200 Da; ionization mode negative.

3.8. Determination of the Antimicrobial Activity

The antimicrobial activity tests of the hydrophilic extracts from the sea buckthorn twigs were performed at the Faculty of Biology, University of Latvia. To determine antimicrobial activity, several reference microbial strains, received from the Microbial Strain Collection of Latvia (MSCL), University of Latvia, were used: *Pseudomonas aeruginosa* MSCL 334, *Staphylococcus aureus* MSCL 330, *Escherichia coli* MSCL 332, *Bacillus cereus* MSCL 330, and *Candida albicans* MSCL 378. The evaluation of the antimicrobial activity of the plant extracts against the test cultures of microorganisms was carried out according to the method for determining the sensitivity of microorganisms to antimicrobial drugs. Antimicrobial activity was studied in 96-well plates by the two-fold serial broth microdilution method [49], which allowed the determination of the minimum inhibitory (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC). The MIC was determined as the lowest concentration of the studied material, which showed no visible growth. From wells where

growth was not detected, 4 µL of medium was seeded on an appropriate solidified medium for MBC/MFC determination. The test was performed in triplicate.

3.9. Cell Line and Cultivation

The BALB/c 3T3 murine fibroblast cell line was obtained from ATCC (American Type Culture Collection). Cells were propagated in DMEM medium (Sigma, D6046, Irvine, UK) supplemented with 1% penicillin (100 U/mL)–streptomycin (100 µg/mL) and 10% calf serum (Sigma, C8056, St Louis, MO, USA). All cultivations were performed in a humidified 5% CO_2 atmosphere at 37 °C.

3.10. Cytotoxicity Assay

The cytotoxicity of the extracts was tested for the BALB/c3T3 cell line by the neutral red (NR) uptake assay. Cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After 24 h of incubation, extracts in a concentration range of 0.078 to 10 mg/mL were added. Dilutions were made in a cell cultivation medium. Cultivation in the presence of extracts was performed for 48 h. Afterwards, the plates were washed with phosphate-buffered saline (PBS) (Sigma, D8537, Irvine, UK), and 25 µg/mL NR solution (Sigma, N2889, Irvine, UK) diluted in 5% fetal calf serum containing media was added. After 3 h incubation in a humidified 5% CO_2 atmosphere at 37 °C, the plate was washed with PBS, and the NR taken up by viable cells was extracted using desorbing fixative (50% ethanol/1% acetic acid/49% water). Absorbance at 540 nm was measured using a Tecan M200 Infinite Pro microplate reader (Tecan, Switzerland). Cytotoxicity was expressed as a concentration-dependent reduction in the uptake of NR, compared to the untreated controls, and the IC_{50} value for each compound was calculated using GraphPad 9 software. The cell line and test method complied with OECD guidelines [57].

3.11. Determination of the Radical Scavenging Activity

Hydrophilic extracts were tested for their radical scavenging activity against the $DPPH^{\cdot}$ assay [51] using UV/VIS spectrometer Lambda 650 (Perkin Elmer, Shelton, CT, USA). The free radical scavenging activity is expressed as the concentration of antioxidant, mg/L, required for a 50% inhibition of the free radicals (IC_{50}). The lower the IC_{50} value, the higher the radical scavenging activity of the compounds.

3.12. In Vitro Test of the Alpha-Amylase Activity

In vitro tests of the hydrophilic extracts from sea buckthorn samples were performed at the Department of Human Physiology and Biochemistry of Riga Stradinš University based on the determination of amyloclastic force by starch-iodine color assay [53,54]. The saliva used for research was donated on a volunteer basis by a group of students with no record of chronic or acute illnesses. The students were non-smokers, as smoking can increase amylase activity [58]. Any sporting or other serious physical activity was stopped 48 h before the experiment according to the protocol [59]. No chewing gum was allowed. No alcohol or caffeine was taken 18 h before the experiment, and the last meals and soft drinks with low pH were 2 h before the examination to obtain clean results.

The extracts were tested in the doses from 100 µL to 500 µL at a concentration of extracts from 2 mg/L to 20 mg/L. The influence of the extracts on salivary amylase was measured by the breakdown of polysaccharides containing linear α-1,4 glucose bonds in starch. The amylase activity was characterized by the amyloclastic force (AF), that is, the volume of the 0.1% starch solution in milliliters that is hydrolyzed by 1 mL of saliva in the test tubes at 38 °C for 30 min. Then, 1% iodine solution was added (as a marker for the presence of starch by color changes). The amyloclastic force is denoted as $D_{30/38^\circ}$. The average range of AF for a healthy person is from 320 to 1280. Saliva without extract was used as a reference. The amyloclastic force of the reference sample was $D_{30/38^\circ}$ 640.

3.13. Statistical Analysis

All measurements were conducted in triplicate, and the results are presented as the mean value ± standard deviation (SD). Statistical analyses were performed using Microsoft Excel 2016. Confidence intervals for a mean using a Student's T distribution were calculated at a significance level $\alpha = 0.05$. The correlation is presented by the Pearson coefficient. A significance level of $p < 0.05$ was used.

4. Conclusions

The chemical composition of the water and ethanol extracts from the SBT biomass of three prospective cultivars, *H. rhamnoides* 'Maria Bruvele', 'Tatiana', and 'Botanicheskaya Lubitelskaya', was determined for the first time. The results of UHPLC-ELS chromatograms showed that the extracts of SBT are rich in polyphenolic compounds, such as quinic acid, PAC monomers and oligomers, which predetermines their radical scavenging activity. Maria Bruvele twigs biomass has the highest content of phenolic compounds in the 50% EtOH extract (48 GAE g/100 g extract).

The 50% EtOH extracts of 'Maria Bruvele' and 'Botanicheskaya Lubitelskaya' twigs showed the most promising results for radical scavenging activity (IC_{50} = 6.8 mg/L and 6.6 mg/L, in the $DPPH^{\cdot}$ test) compared to the other extracts. This could be connected to the higher content of polyphenolic compounds (48 and 43 GAE g/100 g extract), including PACs (13 and 11% on the o.d. extract). These extract properties can be valuable not only in the creation of pharmaceuticals on the basis of plant secondary metabolites, but also in the food and cosmetics industries to slow down the oxidative processes occurring in raw materials and finished products at different stages of the technological process during storage.

Among the three varieties of SBT, 'Maria Bruvele' 50% EtOH extract's antimicrobial activity in suppressing the pathogenic bacteria and yeast is the highest, and its effectiveness against bacteria is comparable with that of some of the synthetic antibiotics. This could be explained by the high content of quinic acid, gallocatechin, isomer epigallocatechin, (epi)catechin-(epi)gallocatechin, and proanthocyanidins in the 'Maria Bruvele' extract and their possible synergetic activity. These results provide the scope of further research on the SBT extracts for applications in the prevention and treatment of infectious diseases. At concentrations similar to the MIC values observed in antimicrobial activity tests, no toxic effect was observed, thus confirming the potential for practical use of the extracts.

The serotonin content in the SBT extracts varied from 3.6 to 7.5% per dry extract, and, starting from the highest content, decreased in the following order: water extracts of 'Maria Bruvele' and 'Tatiana' >50% EtOH extract of 'Bot. Lub.' >50% EtOH extract of 'Maria Bruvele' > water extract of 'Bot. Lub.'. As the serotonin content of 4–6% per dry extract is already considered in the literature as high, these results are very promising for obtaining serotonin preparations on the basis of SBT. The discovery of mammalian neurohormones in plants provides new avenues for the investigation of medicinally active compounds. There is a lack of research on the effects of serotonin. As SBT is a very stress-resistant plant, it would be very interesting to explore if serotonin additives would help to improve similar characteristics in the human body. Currently, the concept of the influence of the gut microbiota on the central nervous system is gaining ground and building evidence. In this case, serotonin in the digestive system could affect mood and brain health. Further research on serotonin effects is planned.

In vitro tests carried out for the evaluation of the influence of the extracts on the initial processes of human macro-metabolism, presented by saliva amylase activity, showed that under normal physiological conditions, all extracts have a significant activation (two times higher than the reference) of amyloclastic force (AF). These results are very promising for the treatment of people with underweight, malnutrition, and malabsorption. The effect of combined SBT extracts on amylase activation and serotonin anti-depression and physical activity improvement could also be beneficial.

The results of this research showed that the biomass of the cultivars under study has high biological activity and nutraceutical and pharmaceutical potential, and the creation of a wasteless biorefinery processing scheme for SBT sustainable production including both berries and biomass applications is possible and prospective. SBT acreage is evaluated as 3 mln ha in 2017 [8], and average crop productivity is ~4–5 t/ha [1,8,9], that is, potentially 13,5 mln t annually. Considering that biomass volume, as mentioned above, comprises ~12–15% of berries, an average volume of biomass could be ~2 mln t yearly. The potential contribution of twigs to the market values will depend on the ways of application: as a raw biomass, as an extract, as a group of compounds, or as purified compounds. On the example of PACs, considering the average for all cultivars yield of extracts of 23%, average yield of PACs 10% on o.d. biomass, and the very approximate price for raw material 10 EUR/kg [60], the market value for PACs could be very roughly estimated as EUR 480 million annually. However, if to calculate on the basis of the final product packed as biologically active additives and sold, e.g., on iHerb [61] then the price could reach 5700 EUR/kg, and the market value theoretically could increase up to EUR 260 billion annually. That would be a hardly achievable figure considering the worldwide market value, the global health and wellness market is forecast to be worth one-trillion dollars by 2026 [62]. The theoretical figures would increase even more if we take, for example, analytical standard serotonin as a basis for calculation. However, the demand for the product, possible market share, possibilities of compounds purification, and other important market and technological factors have to be considered. In addition, today not all wild plantations are used for industrial berries collection, and the amount of SBT biomass could be approximately 4 times less [63], correspondingly. For more precise market value data, a separate, more profound research is necessary which is not the aim of this article. However, even such rough calculations show that SBT biomass contribution to the market is comparable with berries market value.

The evaluation of the best harvesting time and possibilities for the use of SBT biomass for the pruning of shrubs, as well as the comparison of seasonal changes in the phytochemical profile and biological activity of hydrophilic extracts, will be performed in further research.

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