KAUNAS UNIVERSITY OF TECHNOLOGY

AUŠRA ADOMĖNIENĖ

EVALUATION OF PHYTOCHEMICAL COMPOSITION AND PHYSIOLOGICALLY IMPORTANT ENZYME INHIBITING PROPERTIES OF *DIOSCOREA* L. AND *ASTRAGALUS* L. EXTRACTS PLANT SPECIES

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CONTENT

LIST OF ABBREVIATIONS

ACh – acetylcholine
AChE – acetylcholinesterase
AChEI – acetylcholinesterase inhibitor
AKF, AKF-I – angiotensin converting enzyme
ATChI – acetylthiocholine iodide
CE – catalytic efficiency
[E] – enzyme
EC – enzyme commission
EtOH-Ext. – ethanolic extract
FAPGG – Furylacryloyl-phenylalanyl-glycyl-glycine
GH – glycoside hydrolases
[I] – inhibitor concentration
IC_{50} – half maximal inhibitory concentration of test substance
Ki – inhibition constant
Km – Michaelis-Menten constant
$[M - H]^-$ – negative mode of ionization
m/z – mass-to-charge ratio
MS – mass spectrometry
MS/MS – tandem mass spectrometer
NMR – nuclear magnetic resonance
p-NP – *p*-nitrophenol
p-NPG – *p*-nitrophenyl-α-*D*-glucopyranoside
[S] – substrate concenration
SE – standard error mean
SN – standard deviation mean
t_R – time retention
Vmax – maximum reaction rate
α-GLU – α-Glucosidase

1. SUMMARY

1.1. RELEVANCE OF THE RESEARCH

Studies of phytochemical composition and biological properties of medicinal and other plants accumulate more and more scientific information about new and/or existing herbal preparations that can have a positive physiological effect on various functions of the human body. Such scientific information significantly contributes to the development and production of food products of biological value, pharmaceutical products or ingredients in the production of cosmetic products. Over the past few decades, the antioxidant potential of other plants and the phytochemicals that create it have been particularly widely studied, and research on other properties beneficial to health, i.e. inhibition of cancer cell proliferation, antimicrobial, biological and other activities, has also developed rapidly. Due to these studies, significant data are obtained to prove new health statements, empirical knowledge of traditional medicine is scientifically confirmed, the possibilities of controlling and correcting negative physiological processes and changes in the human body are expanded by applying innovative nutritional solutions.

The importance of controlling the activity of many enzymes has long been proven in medical science. For example, inhibition of certain hydrolase enzymes is recognized to be important in modulating physiological functions in the human body in cases of carbohydrate metabolism disorder, dementia, high blood pressure and some other health problems. It is also acknowledged that the effective use of phytochemicals in medicinal and other plants with inhibitory effects can significantly contribute to the prevention, risk reduction and complex therapy of these negative physiological functions. In order for such plants to be used purposefully for the prevention of various diseases, it is necessary to obtain data on their phytochemical composition and biological properties through reliable and modern scientific research. Therefore, the complex evaluation of the composition and biological properties of plants is a perspective area of research.

Some species of plants of *Dioscorea* spp. and *Astragalus* spp. are well known to traditional Chinese medicine, whose health benefits have recently been positively evaluated by medical specialists in many western countries. Different anatomical parts of plants of this genus are used in the form of dried herbs, powders, extracts and other forms for health enhancement, development of food products and food supplements with increased biological value and treatment.

Some plants of the *Astragalus* L. genus are known as valuable sources of polysaccharides, isoflavonoids and cycloartane-type saponins.

As a result, in some countries most of the research is still focused on the identification of new and/or known saponins in rhizomes with roots of *Dioscorea* (lat. *Rhizoma cum radicibus Dioscoreae*), while the overground parts of the plant have been studied much less. Therefore, there is a lack of scientific information about the composition and biological properties of the biologically active compounds of the overground parts of the plant.

After a detailed study and evaluation of the currently available scientific information, it became clear that the plant species of *Dioscorea caucasica* Lipsky and *Dioscorea nipponica* Makino have been insufficiently and unsystematically studied, especially their overground parts. These species are most common in tropical and subtropical climate zones but are also found in temperate areas. In Lithuania, these species do not grow but they were introduced into the collection of medicinal plants in the Botanical Garden of Kaunas Vytautas Magnus University quite a long time ago. Moreover, the phytochemical composition, antioxidant and biological properties of *Dioscorea* L. plant species grown in Lithuanian botanical gardens have not been studied so far.

Astragalus glycyphyllos L. is naturally growing and widespread in the temperate regions of Europe and usually grows in wet habitats or meadows. There are more scientific studies compared to the published studies of *Dioscorea nipponica* Makino and *Dioscorea caucasica* Lipsky. The antioxidant properties, phytochemical quantitative composition of different plant parts of the self-growing *Astragalus glycyphyllos* L. plant of the Lithuanian flora have been studied, whereas the biological properties have not been researched.

Based on a detailed review and evaluation of scientific, ethnopharmacological and other information, the main hypothesis of this dissertation was formulated: the different anatomical parts of the plants *D. caucasica*, *D. nipponica* and *A. glycyphyllos* selected for the research may contain valuable phytochemical compounds that are little researched, which may be characterised by diverse bioactivity, including targeted effects on the activity of physiologically important enzymes – α-amylase, α-glucosidase, angiotensin-converting enzyme and acetylcholinesterase. Therefore, after carrying out systematic studies of the phytochemical composition and inhibitory properties of the selected plants, it is feasible to expect a wider application of such plants in the creation of new functional food products and nutritional supplements beneficial to health.

The objective of the research is to evaluate the phytochemical composition of the *Dioscorea* and *Astragalus* plants and determine the effect of the extracts isolated from them on physiologically important enzymes with hydrolase activity.

The following tasks were set to achieve the goal of the work:

1. To investigate and evaluate the composition of phytochemical compounds of *Dioscorea nipponica, D. caucasica* and *Astragalus glycyphyllos* L. extracts applying methods of chromatography and mass spectrometry.
2. To apply the known *in vitro* enzyme inhibition methods to the assessment of the effect of the studied plant extracts on enzymes.
3. To evaluate the influence of different concentrations of plant extracts on the activity of α-glucosidase, α-amylase, angiotensin-converting enzyme and acetylcholinesterase.
4. To determine the kinetic parameters of α-glucosidase and acetylcholinesterase inhibition by evaluating the interactions between the

substrate and the inhibitor in model enzyme reaction systems *in vitro* and determine the supposed inhibition type of the extracts for these enzymes.

5. To theoretically substantiate the possible relationships between the bioactive compounds in the extracts and the inhibitory effect of enzymes.

Novelty of the scientific work

1. In this work, systematic studies of the phytochemical composition of different anatomical parts of *D. nipponica* Makino and *D. caucasica* Lipsky plants were carried out by the methods of ultra-efficient liquid chromatography (ESC) and hybrid high-resolution mass spectrometry (UESC-Q- TOF MS/MS) for the first time; the obtained information significantly contributes to the available knowledge about derivatives of different *O*-quercetin glycoside forms, hydroxycinnamic acids (3-*O*-caffeoylquinic, 5-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, caffeoylthreonic, coumaroylquinic, caffeoylshikim, feruloylquinic) and hydroxy fatty acids. Some quercetin derivatives and isomers of hydroxycinnamic acids were detected for the first time in *D. nipponica* and *D. causasica* leaf extracts. For the first time, piscidic acid was detected in the rhizomes of the *D. caucasica*, 3-hydroxydodecanoylcarnitine was found in the rhizomes of both *Dioscoreae*, uridin detected in the leaf of the *A.glycyphyllos.*
2. In this paper, studies on the effects of ethanolic extracts of leaves and rhizomes of *D. nipponica*, *D. caucasica* and leaves of *A. glycyphyllos* on physiologically important enzymes (α-amylase, α-glucosidase, angiotensin-converting enzyme and acetylcholinesterase) were performed for the first time.
3. In this work, applying kinetics model systems of extracts, substrates and enzymes reaction, the inhibition type of extracts for α-glucosidase and acetylcholinesterase enzymes were determined for the first time.
4. Theoretically formulated assumptions about possible relationships between *Dioscorea* spp. differences in the phytochemical composition of leaf and root extracts and their related α-glucosidase inhibitory ability.
5. Systematic studies of phytochemical composition of plants and inhibitory properties of extracts expand scientific knowledge about these plants significantly.

Practical value of the work

The obtained data on the phytochemical composition and inhibitory properties of *D. caucasica*, *D. nipponica* and *A. glycyphyllos* extracts provide important information that is needed for the development of new functional preparations from these plants and the justification of their possible physiological effects. Such preparations as extracts from different anatomical parts and their fractions could be applied to the creation of new food supplements and new functional food products with increased nutritional value. In addition, the received data can serve to predict the benefits of foods for human health containing the studied plants, e.g. as a mild non-

medicinal antidiabetic agent that inhibits the activity of α-glucosidase and α-amylase and reduces the glycemic index after the consumption of starchy food. Since the extracts of the overground plant parts showed a good inhibitory effect on angiotensin-converting enzyme, preparations made from them could be recommended for the control of mild hypertension with food and/or food supplements.

Furthermore, the achieved results encourage the improvement and expansion of research with selected plants. Research in this direction, could be continued by fractionating the extracts, isolating the most effective compounds, studying their activity by various in vitro methods. Further research should continue with animals (*in vivo*) and at the final stage with humans (clinical). Such studies can be expected to produce a sufficiently comprehensive data package for health statements.

Research objects

Species of different genus were selected for the research: *Dioscorea* spp. *caucasica*, *nipponica* and *Astragalus* spp. *glycyphyllos*. These species are grown in the collection of medicinal plants of Kaunas Botanical Garden of Vytautas Magnus University. The studied leaf samples were collected at the beginning of blooming, and the rhizomes were dug up during the vegetation period. The collected raw material i.e. the leaves are dried at room temperature by spreading them in a thin layer in a well-ventilated room protected from direct sunlight. Unpeeled rhizomes with roots (lat. *Rhizoma cum radicibus Dioscorea*) are cut into 10–15 cm long pieces and dried in a dryer (Sencor food dehydtrater, SFD 742 RD, China) at a temperature of 50 ± 0.5 ℃. Dried leaves are light green in colour, with a faint smell, rhizomes are cylindrical pieces with a brown surface, yellowish-white inside, with a slightly sweet taste.

All dried plant parts were ground into a fine powder by a Reatsch ZM200 grinder (Reatch GmbH, Haanas, Germany) with a 0.5 mm mesh sieve.

Research methods

Changing the conditions of the biological methods but following the main aspects of the research methods [1, 2, 3], biological studies were applied to determine the capacity of extracts to inhibit enzyme activity *in vitro*. Enzyme activity was determined using the following biological methods: (1) for determination of α-glucosidase activity – chromogenic method using *p*NPG substrate [1]; (2) for determination of α-amylase activity – a colorimetric method using potato starch as a substrate [2]; (3) to determine the activity of acetylcholinesterase – Ellman's method [3]; (4) to determine the activity of angiotensin-converting enzyme – methodologies described by Vermerissen et al. (2002) [4], Holmquist et al. (1979) [5] and Ronca-Testoni (1983) [6].

In enzyme kinetics, the main parameters are the following: Michaelis-Menten constant (K_m), maximum reaction rate (V_{max}) and inhibition constant (K_i) are estimated according to the double inverse equation of Lineweaver–Burk plot and the Michaelis-Menten equation [7, 8].The type of extract inhibition was determined in accordance with Lineweavers-Burk plot graphs [9,10].

The quality of ethanolic extract of *Dioscorea* spp. *nipponica*, *caucasica* and *Astragalus* spp. *glycyphyllos* was analysed by the method of ultrahight-perfomance liquid chromatography-quadrupole- time of flight – mass spectrometry (UPLC – Q-TOF MS/MS) using a Waters ultra perfomance liquid chromatography (UPLC) (Waters, Milford, USA) and Bruker maXis mass spectrometry (MS/MS) system (Bruker Daltonics, Bremen, Germany) [11].

Compounds are identified by comparing molecular formula and MS/MS fragments with compounds in METLIN, LIPIDMAPS databases and data found in literature sources.

The diagram below shows the process sequence of the research progress

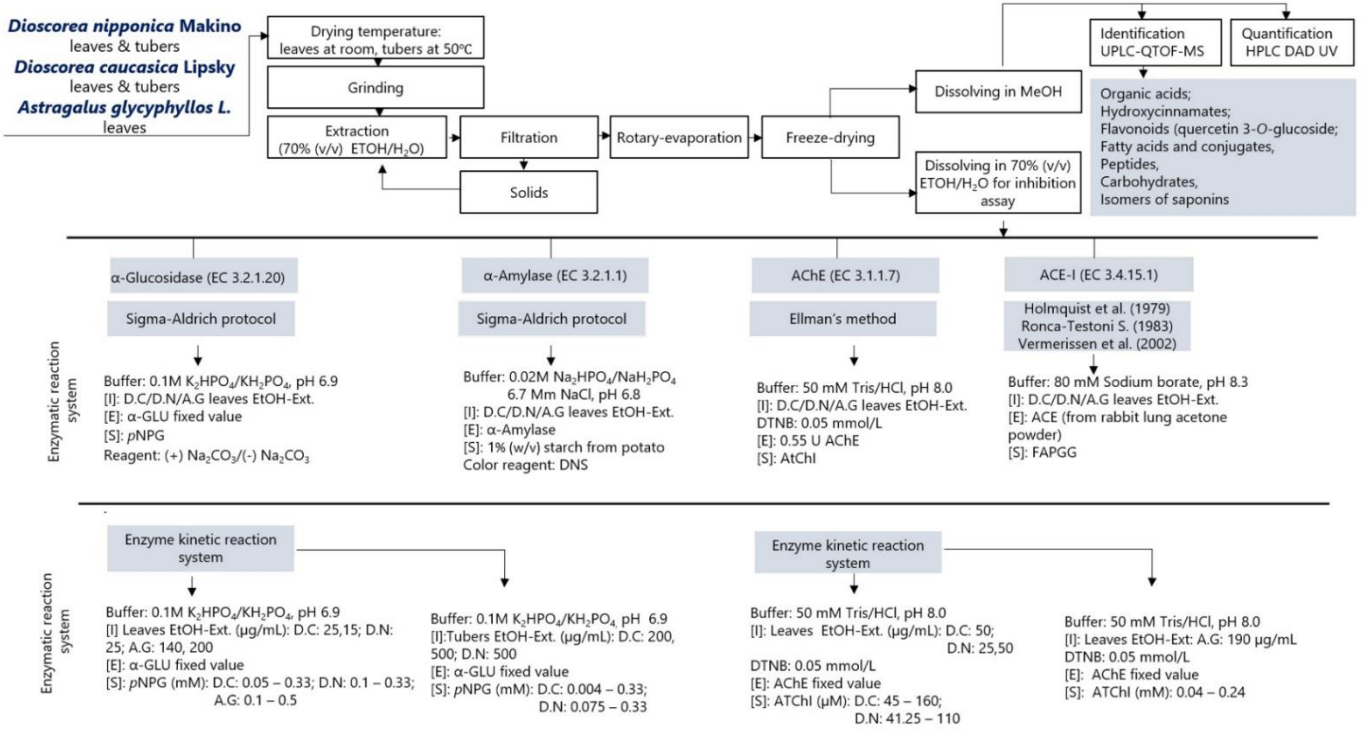

Figure 4.1. The process sequence of the research progress

D.C – *Dioscorea caucasica* Lipsky; D.N – *Dioscorea nipponica* Makino; *Astragalus glycyphyllos* L.; EtOH-Ext, ethanolic extract; UPLC-QTOF-MS – quadrupole time of flight mass spectrometry; HPLC-DAD-UV, Hight-performance Liquid Chromatography coupled to Diode-Array Ultraviolet detector; (+) Na_2CO_3, with reagent; (−) Na_2CO_3, without reagent.

2.1. RESULTS AND THEIR DISCUSSION

2.1.1. Profiles of compounds in leaf and rhizome extracts of *D. caucasica* Lipsky

Compounds were determined in negative mode by ESI(–) by UPLC-QTOF-MS/MS method in *D. caucasica* leaf and rhizome extracts. According to the study of different parts of the plant, compounds and extracts containing them are briefly divided into two groups: (1) leaf ethanolic extract containing four organic acids **(1-4)**, eight hydroxycinnamic acids **(10-17)**, seven flavonoids **(18 -24)**, one carbohydrate **(6)**, two fatty acids **(26-28)** and several compounds of other classes **(31-34)**; (2) rhizome ethanolic extract containing four organic acids **(1-2, 4-5)**, four carbohydrates **(6-9)**, five fatty acids **(25, 27-30)**, six isomers of steroidal glycosides **(35-40)** and several compounds of other classes **(38-39)**. **Table 1** below illustrates the more detailed data for these compounds.

Table 1. Compounds in leaf and rhizome extracts of *D. caucasica* Lipsky are identified by UPLC/Q-TOF-MS/MS method

ID Nr.	t_R, min	m/z [M – H]⁻				Compounds	Leaves	Tubers	Reference
		Found	Calculated	Diagnostic Ion(s)	Error (ppm)				
Organic acids									
1.	0.4	191.0561	191.0556	191[$C_7H_{11}O_6$]⁻	-0.5	(–)-Quinic acid	+		
	0.7[2]	191.0562			-0.6			+	MS
2.	0.5	133.0142	133.0137	133[$C_4H_5O_5$]⁻	-0.5	(*L/D*)-Malic acid/	+		[12,13]
	0.8[2]	133.0142			-0.5	2-Hydroxybutanedioic acid		+	
3.	0.5	173.0454	173.0450	173[$C_7H_9O_5$]⁻	-0.4	Shikimic acid	+		[12, 14]
4.	0.8	191.0196	191.0191	191[$C_6H_7O_7$]⁻	-0.4	2-Hydroxypropane-1,2,3-tricarboxylic acid	+		MS
	1.2[2]	191.0200			-0.8	(Citric/isocitric acid)		+	MS
5.	1.8	255.0512	255.0505	255[$C_{11}H_{11}O_7$]⁻	-0.7	(*p*-hydroxybenzyl)tartaric acid (Piscidic acid)		+	[15]
Carbohydrates									
6.	0.6	179.0561	179.0555	179[$C_6H_{11}O_6$]⁻	-0.6	Hexose	+	+	MS
7.	0.7	341.1089	341.1083	341[$C_{12}H_{21}O_{11}$]⁻	-0.6	Dihexose (Hex_2)		+	[16]
8.	0.7	683.2252	683.2246	683[$C_{24}H_{43}O_{22}$]⁻ [2M – H]⁻	-0.6	Unseparated sugars		+	[17]
9.	0.7	1025.3414	1025.3408	1025[$C_{36}H_{63}O_{33}$]⁻ [3M – H]⁻	-0.6	Unseparated sugars		+	[17]

Table 1 (cont.)

ID Nr.	t_R, min	m/z [M – H]⁻				Compounds	Leaves	Tubers	Reference
		Found	Calculated	Diagnostic Ion(s)	Error (ppm)				
Hydroxycinnamates									
10.		707.1822	707.1823	707[$C_{32}H_{35}O_{18}$]⁻ [2M – H]⁻	-0.1		+		
	1.5	353.0875	353.0872	353[$C_{16}H_{17}O_9$]⁻	-0.3	3-*O*-caffeoylquinic acid[1]			
		707.1822	707.1823	707[$C_{32}H_{35}O_{18}$]⁻ [2M – H]⁻	-0.1		+		
11.		355.0664	355.0665	355[$C_{15}H_{15}O_{10}$]⁻	-0.1	Caffeic acid 3-*O*-glucuronide			[18]
12.	1.6	297.0612	297.0610	297[$C_{13}H_{13}O_8$]⁻	-0.2	Caffeoylthreonic acid	+		
		707.1827	707.1823	707[$C_{32}H_{35}O_{18}$]⁻ [2M – H]⁻	-0.4		+		
13.		353.0876	353.0872	353[$C_{16}H_{17}O_9$]⁻	-0,4	5-*O*-caffeoylquinic acid[1]			
	1.7	191.0559	191.0556	191[$C_7H_{11}O_6$]⁻	-0.5				
14.		337.0930	337.0923	337[$C_{16}H_{17}O_8$]⁻	-0.4	Coumaroylquinic acid	+		[14]
15.		353.0877	353.0872	353[$C_{16}H_{17}O_9$]⁻	-0.5	4-*O*-caffeoylquinic acid	+		[18, 19]
	1.9	191.0564		191[$C_7H_{11}O_6$]⁻	-0.7		+		
16.	2.0	335.0771	335.0766	335[$C_{16}H_{15}O_8$]⁻	-0.5	Caffeoylshikimic acid	+		[14]
17.	2.1	367.1031	367.1029	367[$C_{17}H_{19}O_9$]⁻	-0.2	Feruloylquinic acid	+		[14, 18]

Table 1 (cont.)

ID Nr.	t_R, min	m/z [M – H]⁻				Compounds	Leaves	Tubers	Reference
		Found	Calculated	Diagnostic Ion(s)	Error (ppm)				
Flavonoids									
18.	2.3	609.1463	609.1455	609[$C_{27}H_{29}O_{16}$]⁻	-0.8	Quercetin-3-*O*-rutinoside (rutin)[1]	+		MS
19.	2.4	463.0879	463.0876	463[$C_{21}H_{19}O_{12}$]⁻	-0.3	Quercetin-3-*O*-glucoside (isoquercitrin)[1]	+		MS
20.		505.0995	505.0982	505[$C_{23}H_{21}O_{13}$]⁻	-1.3	Quercetin-3-*O*-acetyl(hexoside)	+		
21.	2.5	549.0883	549.0880	549[$C_{24}H_{21}O_{15}$]⁻	-0,3	Quercetin-3-*O*-malonyl(hexoside)	+		[20]
22.	2.6	447.0930	447.0927	447[$C_{21}H_{19}O_{11}$]⁻	-0,3	Quercetin-3-*O*-rhamnoside (quercitrin)	+		[21]
23.		533.0934	533.0931	533[$C_{24}H_{21}O_{14}$]⁻	-0.27	Quercetin-3-*O*-malonyl(rhamnoside)	+		MS
24.	2.7	489.1036	489.1033	489[$C_{23}H_{21}O_{12}$]⁻	-0.3	Quercetin-3-*O*-acetyl(rhamnoside)	+		
Fatty acids and conjugates									
25.	4.6	329.2335	329.2328	329[$C_{18}H_{33}O_5$]⁻	-0.7	Trihydroxyoctadecenoic acid		+	[22]
26.	5.8	293.2120	293.2117	293[$C_{18}H_{29}O_3$]⁻	-0.3	Hydroxyoctadecatrienoic acid	+		[22], MS
27.	6.8	358.2601	358.2593	358[$C_{19}H_{36}NO_5$]⁻	-0.8	Hydroxydodecanoylcarnitine		+	[3]LMFA 07070032
28.	6.9	295.2280	295.2273	295[$C_{18}H_{31}O_3$]⁻	-0.7	Hydroxyoctadecadienoic acid	+		
	8.0[2]	295.2281			-0.8			+	[22]
29.	8.4	271.2279	271.2273	271[$C_{16}H_{31}O_3$]⁻	-0.6	Hydroxyhexadecanoic		+	[22]
30.	8.8	279.2330	279.2324	279[$C_{18}H_{31}O_2$]⁻	-0.6	Octadecadienoic acid		+	[22]

Table 1 (cont.)

ID Nr.	t_R, min	m/z [M – H]⁻				Compounds	Leaves	Tubers	Reference
		Found	Calculated	Diagnostic Ion(s)	Error (ppm)				
Other compounds									
31.	0.3	225.0617	225.0610	225[$C_7H_{13}O_8$]⁻	-0.7	Glucoheptonic acid	+		[23]
32.	0.9	290.0881	290.0875	290[$C_{11}H_{16}NO_8$]⁻	-0.6	*N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid	+		Metlin
33.		128.0352	128.0347	128[$C_5H_6NO_3$]⁻	-0.5	Pyroglutamic acid	+		[24]
34.	1.4	345.1188	345.1186	345[$C_{15}H_{21}O_9$]⁻	-0.2	Aucubin	+		[25]
35.	3.3	1109.5383	1109.5379	1109[$C_{52}H_{85}O_{25}$]⁻	-0.4	Steroidal glycoside		+	[26]
36.	3.7	1093.5431	1093.5431	1093[$C_{52}H_{85}O_{24}$]⁻	-0.0	Steroidal glycoside		+	[26]
37.	4.3	1075.5327	1075.5325	1075[$C_{52}H_{83}O_{23}$]⁻ [M – H-146]⁻	-0.2	Steroidal glycoside (OP derivative)		+	[26, 27]
38.	6.5	929.4754	929.4746	929[$C_{46}H_{73}O_{19}$]⁻	-0.1	Steroidal glycoside		+	
39.	6.6	913.4798	913.4797	913[$C_{46}H_{73}O_{18}$]⁻	-0.1	Steroidal glycoside		+	
40.	6.8	767.4228	767.4217	767[$C_{40}H_{63}O_{14}$]⁻	-1.1	Steroidal glycoside (pentandroside B)		+	[26, 28]
Unknown compound									
41.	6.1	559.3117	559.3118	559[$C_{28}H_{47}O_{11}$]⁻	-0.1	Unknown compound	+		MS
42.	6.6	483.2727	483.2719	483[$C_{25}H_{35}N_6O_4$]⁻	-0.8	Unknown compound	+		MS
43.	5.6	721.3652	721.3646	721[$C_{34}H_{57}O_{16}$]⁻	-0.6	Unidentified galactolipid	+		MS
44.	6.8	723.3809	723.3803	723[$C_{34}H_{59}O_{16}$]⁻	-0.6	Unidentified galactolipid		+	MS

[1]Identification is supported by the standard. [2]Elution times (t_R) of the same compounds for tuber extracts were slightly higher. [3]LMFA – designates the LIPIDMAPS online database number for the metabolite. [4]OP – ophiopogonin.

The following organic acids were found in extracts of leaves and rhizomes of *D. caucasica*: (-)-quinic acid **(1)** ([M – H]$^-$ m/z 191.0556), (*L/D*)-malic **(2)** ([M – H]$^-$ m/z 133.0137), citric/isocitrine **(4)** ([M – H]$^-$ m/z 191.0191), at different retention times (t_R) of 0.4 and 1.2 min. The latter compounds corresponded to molecular formulas. Shikimic acid **(3)** ([M – H]$^-$ m/z 173.0450) at a retention time (t_R) of 0.5 min and glucoheptanoic acid **(31)** ([M – H]$^-$ m/z 225.0610) at a retention time (t_R) 0.3 min was detected in *D. caucasica* leaf extracts, while piscidic acid **(5)** ([M – H]$^-$ m/z 255.0505) with a retention time (t_R) 1.8 min was detected in rhizome extracts. In previous studies, piscidic acid was identified in rhizomes of *D. nipponica* [15], while shikimic, malic, and threonic acids were identified in *Dioscorea elephantipes, Dioscorea sylvatica*, and other species [12, 14]. Glucoheptonic acid was discovered in the leaves of *D. caucasica* for the first time.

Another compound **(30)** ([M – H]$^-$ ion m/z 290.0875) at a retention time (t_R) of 0.9 min matched the model of derivative fragment of sialic acid (*N*-acetyl-neuraminic acid) that was preliminary identified as *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid (NADNA). In previous studies of *Dioscorea* spp. species, NADNA was not found. At the same retention time as the compound **(30)**, a model of the amino acid fragment **(33)** was detected, which was identified as pyroglutamic acid **(33)** ([M – H]$^-$ m/z 128.0347) [24]. These compounds were found only in the extract of *Dioscorea folium*. Another deprotonated molecular ion **(34)** [M– H]$^-$ at m/z 345.1186 (t_R, 1.4 min) coincided with the spectra provided in the database Metlin and other authors and corresponded to the characteristic of the iridoid glycoside aukubin [25]. The latter iridoid glycoside is mostly found in plant species of the *Plantago* genus.

The isomer of caffeoylquinic acid has the chemical formula $C_{16}H_{18}O_9$ and a monoisotopic ion mass of m/z 354.0950. Similar [M – H]$^-$ precursor ions with m/z value 353.0872 were characteristic of compounds **(10)**, **(13)** and **(15)** at retention times (t_R) of 1.5, 1.7 and 1.9 min, respectively. This pattern of mass spectra indicated the presence of several isomers of caffeoylquinic acid. The first compound **(10)** was identified as 3-*O*-caffeoylquinic acid [18]. The second compound **(13)** was assigned to 5-*O*-caffeoylquinic acid. According to the molecular ion corresponding to the molecular formula and its dimer m/z 707.1823[2M – H]$^-$ (t_R, 1.7 min), it was easy to see the characteristic fragments m/z 191.0556 [M – H]$^-$ (t_R, 1.7 min) specific to 5-*O*-caffeoylquinic acid m/z 353.0872 [M – H]$^-$.The molecular spectra obtained coincided with previous studies [18], and the identity of 5-*O*-caffeoylquinic acid was confirmed by reference standard. Based on the precursor ion m/z 297.0612 [M – H]$^-$ compound **(12)** was preliminary identified as caffeoylthreonic acid. Other compounds **(14)** and **(15)** containing [M – H]$^-$ m/z 337.0923 and m/z 353.0872 molecular ions and fragments m/z 191.0556 [M– H – 146]$^-$ and m /z 191.0556 [M–H–162]$^-$ respectively, were assigned to hydroxycinnamic acids: coumaroylquinic and 4-*O*-caffeoylquinic acids. Compound **(16)** ([M – H]$^-$ ion m/z 335.0766), retention time (t_R) of 2 min. was identified as caffeoylshikimic acid. In earlier studies, it was determined in *D.alata* leaves [14], whereas the compound **(17)** ([M – H]$^-$ ion m/z 367.1029) according to the obtained retention time (t_R) of 2 min was assigned to feruloylquinic acid.

Compounds **18-24** were identified as flavonoids belonging to quercetin derivatives. Quercetin (3,3',4',5,7-pentahydroxylflavone) corresponding to the molecular formula $C_{15}H_{10}O_7$ (monoisotopic mass m/z 302.04265) and having a characteristic fragment ion m/z 301[M – H]$^-$ was definitely associated with the detected quercetin derivatives analysed in leaf extracts of *D. caucasica*. This explains that the molecular spectra of the detected flavonoids are characterised by [M – H (3-*O*-glycoside)]$^-$ compounds. The compounds **(18)** and **(19)** with molecular ions of m/z 609.1455 (t_R, 2.3 min) and m/z 463.0876 (t_R, 2.4 min) corresponded to quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucoside, respectively. The identity of the latter compounds was confirmed by reference standards. The compound **(22)** ([M – H]$^-$, ion m/z 447.0927) ($_{tR}$, 2.6 min) coincided with the spectra of quercitrin reported by other authors [21] and was identified as quercetin-3-*O*-rhamnoside (quercitrin). The detected compounds as t_R, 2.5 min: **(21)** ([M – H]$^-$ ion m/z 549.0880), **(20)** ([M – H]$^-$ ion m/z 505.982); t_R 2.7 min: **(23)**, [M – H]$^-$ ion m/z 533.0931), **(24)** [M – H]$^-$ ion m/z 489.1033), are asociated with quercetin derivatives that may belong to acetylated (malonyl) flavonoids. This is explained by the fact that the loss of the acetyl residue (C_2H_2O) of the malonyl group compounds is due to the loss of CO_2. This is characteristic of both compound **(20)** with m/z 505.0982 [M – H – 44]$^-$ and compound **(24)** with m/z 489.1036 [M – H – 44]$^-$. The presence of the compounds **(20)** and **(21)** was confirmed by the mass spectra distribution path presented in the literature investigating the profiles of phenolic compounds in the fruits and leaves of *Ficus carica* L. by QTOF-MS analysis [20]. Accordingly, acetylated (malonyl) flavonoids were identified as follow: **(20)** quercetin-*O*-acetyl(hexoside), **(21)** quercetin-3-*O*-malonyl(hexoside) **(23)** quercetin-3-*O*-malonyl(rhamnoside) **(24)** quercetin-3-*O*-acetyl(rhamnoside).

Carbohydrates were detected only in samples of *D. caucasica* rhizome extracts, with the exception of a hexose of uncertain structure **(6)**. Sacchrose (**7**) ([M – H]$^-$ m/z 341.1083) was detected at t_R, 0.7 min [16, 17] and at the same retention time, while two other compounds: **(8)** ([2M – H]$^-$, m/z 683.2246) and **(9)** ([3M – H]$^-$ ion, m/z 1025.3408), corresponded to the spectral characteristics of deprotonated dimer and trimer oligosaccharide ions [17]. **Table 1** describes them as non-separated sugars.

The m/z fragment (profile) patterns detected in the obtained mass spectra are associated with fatty acids. Compound **(30)** [M – H]$^-$ m/z 279.2324 (t_R, 8.8 min), corresponded to linoleic acid (C18:2), another compound **(28)** [M – H]$^-$ m/z 295, 2273 (t_R, 6.9 and 8.0 min) corresponded to isomer of hydroxyoctadeca-dienoic acid. Other compounds showed ions of hydroxylated fatty acid molecules: **(29)** ([M – H]$^-$ ion m/z 271.2273) at (t_R) 8.4 min and **(25)** ([M – H]$^-$ m/z 329.2328) at (t_R) 4.6 min. corresponded to hydroxyhexadecanoic acid **(29)** and trihydroxy octadecenoic acid **(25)**, respectively. Hydroxyoctadecatrienoic acid (**26**) [M – H]$^-$ ion m/z 293.2117 was detected only in the leaf extract with a retention time (t_R) of 5.8 min. Furthermore, the [M – H]$^-$ ion of compound **(27)** m/z 358.2593 at (t_R) 6.8 min showed the molecular formula $C_{19}H_{36}NO_5$, which corresponds to a fatty acid ester. Such a fatty acid ester is characteristic of carnitine derivatives. Referring to the literature data and the LIPID MAPS database, this compound is identified as hydroxydodecanoylcarnitine.

In the obtained mass spectra, it was not possible to determine the exact place of hydroxylation, but according to the molecular ion corresponding to the molecular formula, it is easy to see the characteristic fragments specific to fatty acids, and the observed retention time of fatty acids correlates with literature data. It should be noted that conjugates of fatty acids with amino acids are widely found in animal products, but their availability in plants is limited.

The exact structures of the detected compounds **(35)**, **(36)**, **(37)**, **(38)** and **(39)** could not be clarified, but based on their molecular ions $[M - H]^-$ m/z 1109.5379 (t_R, 3.3 min), 1093.5431 (t_R, 3.7 min.), 1075.5325 (t_R, 4.3 min.), 929.4746 (t_R, 6.5 min.), 913.4797 (t_R, 6.6 min.), corresponding to the molecular formulas $C_{52}H_{85}O_{25}$, $C_{52}H_{85}O_{24}$, $C_{52}H_{83}O_{23}$, $C_{46}H_{73}O_{19}$ and $C_{46}H_{73}O_{18}$, they undoubtedly belong to the steroidal glycosides abundant in various species of *Dioscorea*. The spectral data of compound **(40)** $[M - H]^-$ ion m/z 767.4217 (t_R, 6.8 min) also indicate the steroidal saponin pentandroside B [26]. Moreover, compound **(44)** $[M - H]^-$ m/z 723.3803 corresponding to the molecular formula $C_{34}H_{59}O_{16}$ was detected in rhizomes with a retention time (t_R) of 6.8 min, while compound **(43)** $[M - H]^-$ ion m /z 721.3646 was detected in leaves with a retention time of 5.6 min. The latter compounds most likely belong to galactolipids.

2.1.2. Profiles of compounds in *D. nipponica* Makino leaf and rhisome extracts

Referring to the study of different plant parts, the compounds and extracts containing them are briefly divided into two groups: (1) leaf ethanolic extract containing six organic acids (**1-5, 7**), eight hydroxycinnamic acids (**14-21**), ten flavonoids (**22-31**), three carbohydrates (**8-9,11**), one cluster ion (**13**), three isomers of steroidal glycosides (**41, 44, 48**) and several compounds of other classes (**37-40**); (2) rhizome ethanol extract containing five organic acids (**1–2, 4–6**), four carbohydrates (**8, 10–12**), one cluster ion corresponding to the hexose dimer of quinic acid (**13**), four fatty acids (**32-33, 35-36**), eight isomers of steroidal glycosides (**42-49**) and several compounds of other classes (**38-39**). More detailed data for these compounds are shown below (**Table 2.**).

Table 2. Compounds in leaf and rhizome extracts of *D. nipponica* Makino are identified by UPLC/Q-TOF-MS/MS method

ID Nr.	t_R, min	m/z [M – H]⁻				Compounds	Leaves	Tubers	Reference
		Found	Calculated	Diagnostic Ion(s)	Error (ppm)				
Organic acids									
1.	0.4	191.0563	191.0556	191[$C_7H_{11}O_6$]⁻	-0.7	(-)-Quinic acid	+		
	0.7[2]	191.0561			-0.5			+	MS
2.	0.5	133.0142	133.0137	133[$C_4H_5O_5$]⁻	-0.7	2-Hydroxybutanedioic acid (Malic acid)	+		[12]
	0.7[2]	133.0142			-0.6			+	
3.	0.5	173.0454	173.0450	173[$C_7H_9O_5$]⁻	-0.7	Shikimic acid	+		[12]
4.	0.7	191.0198	191.0191	191[$C_6H_7O_7$]⁻	-0.7	2-hydroxypropane-1,2,3-tricarboxylic acid	+		MS
	1.2[2]	191.0198			-0.7	(Citric/isocitric acid)		+	MS
5.	1.0	147.0299	147.0294	147[$C_5H_7O_5$]⁻	-0.5	Citramalic acid	+		MS
	1.4[2]	147.0300			-0.6			+	
6.	1.8	255.0514	255.0505	255[$C_{11}H_{11}O_7$]⁻	-0.9	Piscidic acid		+	[15]
7.	2.8	187.0976	187.0970	187[$C_9H_{15}O_4$]⁻	-0.6	Nonane diacid (Azelaic acid)	+		[29]
Carbohydrates									
8.	0.3	179.0561	179.0556	179[$C_6H_{11}O_6$]⁻	-0.5	Hexose	+		MS
	0.6[2]	179.0563	179.0556	179[$C_6H_{11}O_6$]⁻	-0.7			+	
9.	0.4	149.0456	149.0450	147[$C_5H_9O_5$]⁻	-0.6	Pentose	+		MS
10.	0.4	387.1146	387.1139	387[$C_{13}H_{23}O_{13}$]⁻	-0.7	Unknown disaccharide ([3]DBE= 2)	+		
	0.7	387.1146			-0.8			+	MS

Table 2 (cont.)

ID Nr.	t_R, min	m/z [M – H]⁻				Compounds	Leaves	Tubers	Reference
		Found	Calculated	Diagnostic Ion(s)	Error (ppm)				
11.	0.7	341.1089	341.1083	341[$C_{12}H_{21}O_{11}$]⁻	-0.6	Hex_2		+	[16]
12.	0.7	683.2256	683.2246	683[$C_{24}H_{43}O_{22}$]⁻ [2M – H]⁻	-0.1	Unseparated sugars		+	[17]
Cluster ion									
13.	0.4	533.1722	533.1717	533[$C_{19}H_{33}O_{17}$]⁻	-0.5		+		
	0.7[2]	533.1723			-0.6	Quinic acid + dihexose (Hex_2)		+	MS
Hydroxycinnamates (HCAs)									
14.	1,5	707.1829	707.1823	707[$C_{32}H_{35}O_{18}$]⁻ [2M – H]⁻	-0.6				[18]
		353.0879	353.0873	353[$C_{16}H_{17}O_9$]⁻	-0.6	3-*O*-caffeoylquinic acid[1]	+		MS
15.		355.0672	355.0665	355[$C_{15}H_{15}O_{10}$]⁻	-0.7	Caffeic acid 3-*O*-glucuronide	+		[18]
16.	1.6	297.0621	297.0610	297[$C_{13}H_{13}O_8$]⁻	-1.1	Caffeoylthreonic acid	+		[18]
		707.1828	707.1823	707[$C_{32}H_{35}O_{18}$]⁻ [2M – H]⁻	-0.5				[18]
17.	1.7	353.0880	353.0873	353[$C_{16}H_{17}O_9$]⁻	-0.7	5-*O*-caffeoylquinic acid[1]	+		MS
		191.0562	191.0556	191[$C_7H_{11}O_6$]⁻	-0.6				
18.	1.9	337.0929	337.0923	337[$C_{16}H_{17}O_8$]⁻ [M – H – 146]⁻	-0.6	Coumaroylquinic acid	+		MS

Table 2 (cont.)

ID Nr.	t_R, min	m/z [M – H]⁻				Compounds	Leaves	Tubers	Reference
		Found	Calculated	Diagnostic Ion(s)	Error (ppm)				
19.	1.9	353.0877	353.0873	353[$C_{16}H_{17}O_9$]⁻	-0.4	4-*O*-caffeoylquinic acid	+		MS
		191.0564	191.0556	191[$C_7H_{11}O_6$]⁻	-0.8		+		
20.	2.0	335.0771	335.0766	335[$C_{16}H_{15}O_8$]⁻	-0.5	Caffeoylshikimic acid	+		[30]
21.	2.1	367.1031	367.1029	367[$C_{17}H_{19}O_9$]⁻	-0.2	Feruloylquinic acid	+		[14,31]
Flavonoids									
22.	1.8	577.1354	577.1346	577[$C_{30}H_{25}O_{12}$]⁻	-0.8	Procyanidin dimer (B type)	+		[32,33]
23.	1,9	289.0719	289.0712	289[$C_{15}H_{13}O_6$]⁻	-0,7		+		
	2.1[2]	289.0723		289[$C_{15}H_{13}O_6$]⁻	-1.09	Catechin		+	[34]
24.	2.3	609.1461	609.1456	609[$C_{27}H_{29}O_{16}$]⁻	-0.5	Quercetin-3-*O*-rutinoside (rutin) [1]	+		MS
25.	2.4	477.0676	477.0669	477[$C_{21}H_{17}O_{13}$]⁻	-0.7	Quercetin-3-*O*-glucuronide	+		[35]
26.	2.4	463.0891	463.0876	463[$C_{21}H_{19}O_{12}$]⁻	-1.5	Quercetin-3-*O*-glucoside (isoquercitrin)[1]	+		MS
27.		505.0995	505.0982	505[$C_{23}H_{21}O_{13}$]⁻	-1.3	Quercetin-3-*O*-acetyl(hexoside)	+		[20]
28.	2.6	549.0883	549.0880	549[$C_{24}H_{21}O_{15}$]⁻	-0.3	Quercetin-3-*O*-malonyl(hexoside)	+		[20]
29.	2.7	447.0941	447.0924	447[$C_{21}H_{19}O_{11}$]⁻	-0.3	Quercetin-3-*O*-rhamnoside (quercitrin)	+		[21]
30.	2.8	533.0934	533.0931	533[$C_{24}H_{21}O_{14}$]⁻	-0.27	Quercetin-3-*O*-malonyl(rhamnoside)	+		MS
31.		489.1036	489.1033	489[$C_{23}H_{21}O_{12}$]⁻	-0.3	Quercetin-3-*O*-acetyl(rhamnoside)	+		

Table 2 (cont.)

ID Nr.	t_R, min	m/z [M – H]$^-$				Compounds	Leaves	Tubers	Reference
		Found	Calculated	Diagnostic Ion(s)	Error (ppm)				
Fatty acids and conjugates									
32.	3.8	327.2181	327.2172	327[$C_{18}H_{31}O_5$]$^-$	-0.9	Hydroperoxy-epoxy-octadecenoic acid	+		[22]
	4.3	327.2180			-0.8			+	
33.		329.2337	329.2328	329[$C_{18}H_{33}O_5$]$^-$	-0.9	Trihydroxyoctadecenoic acid		+	[22]
		329.2332			-0.4				
34.	5.8	293.2122	293.2117	293[$C_{18}H_{29}O_3$]$^-$	-0.5	Hydroxyoctadecatrieonic acid	+		[22]
35.	6.3	295.2280	295.2273	295[$C_{18}H_{31}O_3$]$^-$	-0.7	Isomer of hydroxyoctadecadienoic acid	+		
	7.0	295.2281			-0.8			+	[22]
36.	6,8	358,2601	358,2593	358[$C_{19}H_{36}NO_5$]$^-$	-0.8	Hydroxydodecanoylcarnitine		+	[4]LMFA 07070032
Other compounds									
37.	0.3	225.0617	225.0610	225[$C_7H_{13}O_8$]$^-$	-0.7	Glucoheptonic acid	+		[23]
38.	0.9	290.0881	290.0875	290[$C_{11}H_{16}NO_8$]$^-$	-0.6	*N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid	+		
	1.3	290.0882	290.0875	290[$C_{11}H_{16}NO_8$]$^-$	-0.7			+	MS
39.	0.9	128.0354	128.0347	128 [$C_5H_6NO_3$]$^-$	-0.7	Pyroglutamic acid	+		
	1.3	128.0354	128.0347	128 [$C_5H_6NO_3$]$^-$	-0.7			+	
40.	1.4	345.1188	345.1186	345[$C_{15}H_{21}O_9$]$^-$	-0.2	Aucubin	+		[25]

Table 2 (cont.)

ID Nr.	t_R, min	m/z [M – H]⁻				Compounds	Leaves	Tubers	Reference
		Found	Calculated	Diagnostic Ion(s)	Error (ppm)				
Saponin isomer									
41.	3.2	947.4856	947.4852	947[$C_{46}H_{75}O_{20}$]⁻	-0.4	Steroidal glycoside	+		MS
42.	3.3	1125.5336	1125.5329	1125[$C_{52}H_{85}O_{26}$]⁻	-0.7	Steroidal glycoside (OP derivative)		+	[27]
43.		1109.5390	1109.5379	1109[$C_{52}H_{85}O_{25}$]⁻ [M – H – 162]⁻	-1.1	Steroidal glycoside		+	
44.	3.4	945.4693	945.4695	945[$C_{46}H_{73}O_{20}$]⁻	-0.2	Steroidal glycoside	+		[27]
	5.6	945.4701	945.4695	945[$C_{46}H_{73}O_{20}$]⁻	-0.6			+	
45.	6.2	1091.5283	1091.5274	1091[$C_{52}H_{83}O_{24}$]⁻	-0.9	Steroidal glycoside (OP derivative)		+	[26,27]
46.	6.5	929.4751	929.4746	929[$C_{46}H_{73}O_{19}$]⁻	-0.5	Steroidal glycoside		+	[26]
47.	6.8	899.4644	899.4640	899[$C_{45}H_{71}O_{18}$]⁻	-0.4	Steroidal glycoside		+	[26]
48.	5.9	767.4216	767.4218	767[$C_{40}H_{63}O_{14}$]⁻	-0.2	Steroidal glycoside (pentandroside B)	+		[26]
	6.9	767.4227		767[$C_{40}H_{63}O_{14}$]⁻	-0.9			+	[26]
Peptides									
49.	3.3	585.2678	585.2673	585[$C_{28}H_{37}N_6O_8$]⁻	-0.5	Tetrapeptide		+	MS (Metlin)
50.	4.1	570.2792	570.2788	570[$C_{26}H_{36}N_9O_6$]⁻	-0.4	Tetrapeptide		+	MS (Metlin)
51.	3.5	658.2956	658.2949	658[$C_{29}H_{40}N_9O_9$]⁻	-0.7	Pentapeptide		+	MS (Metlin)

Table 2 (cont.)

ID Nr.	t_R, min	m/z [M – H]⁻				Compounds	Leaves	Tubers	Reference
		Found	Calculated	Diagnostic Ion(s)	Error (ppm)				
Unknown compound									
52.	2.8	429.1764	429.1760	429[$C_{20}H_{29}O_{10}$]⁻	-0.4	Unidentified (*O*-glycosyl compounds)	+		MS
53.	3.1	635.2926	635.2915	658[$C_{29}H_{47}O_{15}$]⁻	-1.1	Unidentified (acylsucrose)	+		MS
	3.5	635.2927		658[$C_{29}H_{47}O_{15}$]⁻	-1.2			+	
54.	3.4	495.2341	495.2324	495[$C_{36}H_{31}O_2$]⁻	-1.7	Unidentified	+		MS
55.	4.3	449.2753	449.2751	449[$C_{22}H_{41}O_9$]⁻	-0.2	Unidentified		+	MS
56.	5.5	721.3650	721.3646	721[$C_{34}H_{57}O_{16}$]⁻	-0.4	Unidentified galactolipid	+		MS

[1]Identification is supported by the standard. [2]Elution times (t_R) of the same compounds for tuber extracts were slightly higher.[3]DBE – Double Bond Equivalent. [4]LMFA – designates the LIPIDMAPS online database number for the metabolite.[4]OP – ophiopogonin.

Organic acids found in extracts of leaves and rhizomes of *Dioscorea nipponica* are the following: (-)-quinic acid (**1**) ([M – H]$^-$, m/z 191.0556), (*L/D*)-malic (**2**)([M – H]$^-$, m/ z 133.0137), citric/isocitrine (**4**) [M – H]$^-$, m/z 191.0191) citramalic (**5**) ([M – H]$^-$, m/z 147.0294) at different retention times (t_R) of 0.4 and 1.2 min. Piscidic acid (**6**) ([M – H] $^-$, m/z 255.0505) with a retention time (t_R) of 1.8 min. was only found in rhizome extracts, while shikimic (**3**) ([M – H]$^-$, m/z 173.0450) and azelaic (**7**) [M – H] $^-$, m/z 187.0970) acids with retention times (t_R) 0.5 and 2.8 min were determined only in leaf extracts. In previous studies, shikimic and malic acids were determined in leaves of *D. elephantipes, D. sylvatica, D. mexicana* [12], azelaic acid in rhizomes of *D. alata, D. bulbifera* [29], and piscidic acid in *D. nipponica* rhizomes [15].

Carbohydrate-specific molecular ions were detected in both leaf and rhizome extracts. Sucrose specific molecular ion (**11**) ([M – H]$^-$, m/z 341.1089) [16, 17] with a retention time (t_R) of 0.7 min. and the following ion of the compound (**12**) [2M – H]$^-$, m/z 683.2252) at the same retention time matched well the spectral characteristics of the deprotonated dimer oligosaccharide ion and was assigned to unrefined sugars. A similar carbohydrate-specific compound (**10**) ([M – H]$^-$, m/z 387.1139) according to retention time (t_R) of 0.4 min. was detected in both leaf and rhizome extracts. This unknown disaccharide (**10**) could have formed from various hexoses. It is also known that cluster ion compounds exist in nature as blends of mixed groups (clusters) with other molecules. Namely, the mass spectrum of compound ion (**13**) ([M – H]$^-$, m/z 533.1717) (t_R, 0.4 min. leaves; t_R, 0.7 min. rhizomes) clearly indicated quinic acid ester and a hexose mixture ([quinic acid + Hex_2 – H]$^-$). Also, the MS spectrum of the compound (**37**) ([M – H]$^-$, m/z 225.0610) corresponded well to the formula of glucoheptanoic acid, $C_7H_{13}O_8$ [23], and another MS spectrum (**39**), ([M – H]$^-$, m/z 128.0347) corresponded to the pyroglutamic acid formula $C_5H_6NO_3$; the first compound was detected only in the leaf extract and the second in both extracts (**Table 4.2.2**).

N-acetyl-2,3-dehydro-2-deoxyneuraminic acid **(38)** belonging to sialic acid derivatives [M – H]$^-$, m/z 290.0875) (t_R, 0.9 min: leaves; t_R, 1.3 min: rhizomes) matched the molecular mass spectrum in the Metlin database. The molecular ion (**40**) of another compound ([M–H]$^-$, m/z 345.1186) matched the mass spectrum of the iridoid glycoside well and was preliminary identified as aukubin. This compound belonging to iridoid glycosides is usually found in different parts of plants and is responsible for defensive functions.

The different isomers of caffeoylquinic acid have the chemical formula $C_{16}H_{18}O_9$ and a monoisotopic mass of 354.0950. During the research, the molecular ion [M – H]$^-$ m/z 353.0876 of caffeoylquinic acid precurseor was characteristic of molecular isomers of compounds (**14**), (**17**) and **(19)** formed at different retention times of 1.5, 1.7 and 1.9 min, respectively. From the molecular ion (**14**) ([M – H]$^-$, m/z 353.0873) corresponding to the molecular formula $C_{16}H_{17}O_9$ and the adjacent dimer (m/z 707.1823) (t_R, 1.5 min) it was easy to envision the presence of 3-*O*-caffeoylquinic acid. A similar arrangement of the spectra is characteristic for the retention time of 1.7 min. The most intense m/z peaks of the molecular ion are located

in the chromatogram as follow: 707.1823 [2M – H]⁻; 353.0873, [M – H]⁻ and 191.0556 [M – H]⁻, confirmed the presence of (**17**) 5-*O*-caffeoylquinic acid. The presence of these acids coincided with the spectra of caffeoylquinic acid isomers reported by other authors [18]. Also, the identity is confirmed by an authentic standard. Referring to well-fitting molecular formulas and MS spectra, the ion (**16**) of compound ([M – H]⁻, m/z 297.0610) at a retention time (t_R) of 1.6 min was preliminary identified as caffeoylthreonic acid and ion (**15**) of compound ([M – H]⁻, m/z 355.0665) as the caffeic acid 3-*O*-glucuronid [18]. Another compound (**18**) with a retention time (t_R) of 1.9 min and m/z 337.0923 was identified as coumaroylquinic acid based on the clearly visible (146 Da) separation of the sugar part. Ions (**20**) of compounds ([M – H]⁻, m/z 335.0766) and (**21**) ([M – H]⁻, m/z 367.1029) showed typical mass spectra of caffeoylschimic and feruloylquinic acids, respectively [14, 18].

The various isomers of quercetin have the chemical formula $C_{15}H_{10}O_7$ (3,3',4',5,7-pentahydroxylflavone) and a monoisotopic mass is 302.04265. [M – H (3-*O*-glycoside)]⁻ flavonoid isomers were dominant in *D. nipponica* leaf extracts, as shown in **Table 2.**

Compounds (**24**) and (**26**) at m/z 609.1456 and 463.0876 were identified as quercetin-3-*O*-rutinoside (rutin) and quercetin-3-*O*-glucoside (isoquercitrin), respectively; their identity is confirmed by reference standards. Another quercetin-glycoside derivative (**29**) with a retention time (t_R) of 1.9 min and m/z 447.0924 was identified as quercetin-3-*O*-rhamnoside (quercitrin). The identity of the latter coincided with mass spectral data reported by other scientists [21]. Also, the mass spectra m/z (477.0669) presented in the literature [33] coincide with the obtained spectra of the compound ion (**25**) ([M – H]⁻, m/z 447.0669). The latter compound has been identified as quercetin-3-*O*-glucuronide. Compounds (**27**) and (**28**) showed precursor ions [M – H]⁻ m/z 549.0880 and 505.0982, accordingly. The latter were identified as quercetin-3-*O*-malonyl glucoside and quercetin-3-*O*-acetylglucoside, accordingly. The presence of these compounds is confirmed by the mass spectral data presented in the literature [20]. A similar path in the mass spectrum is characteristic of other detected compounds: molecular ions (**30**) ([M – H]⁻, m/z 533.0931) and (**31**) ([M – H]⁻, m/z 489.1033) corresponding to molecular formulas $C_{24}H_{21}O_{14}$ and $C_{23}H_{21}O_{12}$ respectively, led to the preliminary identification of quercetin-3-*O*-malonylrhamnoside and quercetin-3-*O*-acetylrhamnoside, accordingly. The ion (**22**) of compound ([M – H]⁻, m/z 577.1346) was identified as the procyanide dimer [32]. With respect to the data obtained for this oligomer it is confirmed that the compound ion (**23**) ([M – H]⁻, m/z 289.0712) is associated with catechin. Previous studies have detected catechin in many species of the genus *Dioscorea*: *D. bulbifera*, [16, 35], *D. hirtiflora* [35], *D. hamiltonii* [36], and procyanides have been identified in previous studies in *D. cirrhosa* [37] and *D. alata* [38] species.

Long-chain fatty acids and their hydroxylated derivatives are common in a variety of plant materials, including rhizomes of *Dioscorea* [22, 38]. In the investigated MS spectra, the ion (**35**) of compound ([M – H]⁻, m/z 295.2273)

corresponded to hydroxyoctadecadienoic acid. Other derivatives showed structures of hydroxylated fatty acids: ion (**32**) [M – H]$^-$, m/z 327.2172) corresponded to hydroperoxy-epoxy-octadecenoic acid; molecular ion (**33**) ([M – H]$^-$, m/z 329.2328) corresponded to trihydroxyoctadecenoic acid. Hydroxyoctadecatrienoic acid corresponded to the molecular ion (**34**) ([M – H]$^-$, m/z 293.2117), but was only detected in leaf extracts of *D. nipponica* Makino. It should be noted that MS/MS data do not provide sufficient information about the exact position of the hydroxy groups in the fatty acid chain. Furthermore, the ion (**36**) of the compound ([M – H]$^-$, m/z 358.2593) corresponded to the molecular formula $C_{19}H_{36}NO_5$, which was associated with a fatty acid ester. The latter was assigned to a carnitine derivative, namely as hydroxydodecanoylcarnitine (C12-OH). However, the obtained mass spectra did not allow to determine the exact location of hydroxylation. It is worth mentioning that conjugates of fatty acids with amino acids are widely found in foods of animal origin, but are relatively rare in plants.

Steroidal glycosides (saponins) were detected in rhizome samples of *Dioscorea nipponica*, but only a few were detected in leaf samples. Steroidal saponins identified in previous studies also dominated in the rhizomes of *Dioscorea* [34]. QTOF/MS data are insufficient to elucidate the exact structures of the compounds; their purification and detailed spectral data, including various BMR, FTIR modifications, would be required; however, such work was beyond the scope of our study. Nevertheless, the compounds (**41**), (**45**) and (**47**) with molecular ions [M – H]$^-$ m/z 947.4852, 1091.5274 and 899.4640, respectively, corresponded to the molecular formulas $C_{46}H_{75}O_{20}$, $C_{52}H_{83}O_{24}$ and $C_{45}H_{71}O_{18}$, and definitely belong to the steroidal glycosides abundant in the rhizomes of *Dioscorea* species [34, 39] and some other plants [27, 40]. In the MS spectrum, the compound ion (**48**) ([M – H]$^-$, m/z 767.4218) corresponded to the molecular formula $C_{40}H_{63}O_{14}$, which coincides with the presence of the steroidal saponin pentandroside B [26]. Hamed et al. (2004) [28] detected the ion of the compound – pentandroside B ([M – H]$^-$ m/z 767) in *Tribulus* genus plant – *Tribulus pentadrus*.

The molecular formula $C_{46}H_{73}O_{19}$ of the compound ion (**46**) ([M – H]$^-$, m/z 929.4746) differed by one additional oxygen atom from the compound ion (**44**) ([M – H]$^-$, m/z 945.4695) of the molecular formula $C_{46}H_{73}O_{20}$, suggesting that the identities of both compounds may belong to steroidal saponins. Similar differences in molecular formulas are observed among compounds: molecular ions: (**42**) ([M – H]$^-$, m/z 1125.5329 $C_{52}H_{85}O_{26}$) and (**43**) ([M – H]$^-$, m/z 1109.5379 $C_{52}H_{85}O_{25}$). The latter compounds may also belong to steroidal saponins, i.e. derivatives of ophiopogonin [27]. The compound (**56**) ([M – H]$^-$, m/z 721.3646) found in the leaf extract and corresponding to the formula $C_{34}H_{57}O_{16}$ was identified as an unknown galactolipid.

Compounds (**49**), (**50**) and (**51**) with [M – H]$^-$ m/z 585.2673, 570.2788 and 658.2949 molecular ions accordingly, corresponded to $C_{28}H_{37}N_6O_8$, $C_{26}H_{36}N_9O_6$ and $C_{29}H_{40}N_9O_9$ molecular formulas belonging to peptides. With reference to the Metlin database, compounds (**49)** and (**50**) are identified as tetrapeptides, while (**51**) is identified as a pentapeptide. There is not much evidence about peptides extracted from

plant parts of *D. niponnica*. A well-known peptide so far is cyclo-(Leu-Tyr) found in rhizomes of *D. niponnica* [40] and later in other species of *Dioscorea* [24].

2.1.3. Profiles of compounds in leaf extracts *A. glycyphyllos* L.

A. glycyphyllos leaf extract contains: organic acids (**1-3**); carbohydrates (**4-6**); uridine (**8**); hydroxycinnamic acids (**9-10**); cycloartane glycosides **(11-13**). More detailed data for these compounds are shown below (**Table 3**).

Table 3. Compounds in leaf extracts of *A. glycyphyllos* L. are identified by UPLC/Q-TOF-MS/MS method

ID Nr.	t_R, min	m/z[M– H]$^-$			Error (ppm)	Compound	Reference
		Found	Calculated	Daignostic Ion(s)			
1.	0.5	133.0142	133.0137	133[$C_4H_5O_5$]$^-$	-0.5	(*L/D*)-Malic acid/ malic acid	MS
2.	1.4	117.0191	117.0187	117[$C_4H_5O_4$]$^-$	-0.4	Succinic acid	MS
3.	2.8	187.0978	187.0970	187[$C_9H_{15}O_4$]$^-$	-0.8	Azelaic acid	MS
5.	0.6	149.0456	149.0450	149[$C_5H_9O_5$]$^-$	-0.6	Pentose	
6.		341.1082	341.1083	341[$C_{12}H_{21}O_{11}$]$^-$	-0.1	Sucrose/ isosucrose	[41]
7.		387.1140	387.1139	387[$C_{13}H_{23}O_{13}$]$^-$	-0.1	Unknown disaccharide (DBE= 2)[1]	MS
8.	0.9	243.0623	243.06171	243[$C_9H_{11}N_2O_6$]$^-$	-0.6	Uridin	MS
9.	2.2	337.0929	337.0923	337[$C_{16}H_{17}O_8$]$^-$	-	Coumaroylquinic acid	
10..	2.5	367.1039	367.1029	367[$C_{17}H_{19}O_9$]$^-$	-1.0	Feruloylquinic acid	[18]
11.	4.2	699.4322	699.4319	699[$C_{37}H_{63}O_{12}$]$^-$	-0.3	Cycloartane glycosides	[43]
12..	4.8	695.4008	695.4006	695[$C_{37}H_{59}O_{12}$]$^-$	-0.2	Cycloartane glycosides	[43]
13.	5.4	691.4060	691.4057	691[$C_{38}H_{59}O_{11}$]$^-$	-0.3	Cycloartane glycosides	[43]

[1]DBE – double bond equivalent.

Organic acids identified in the leaves of *A. glycyphyllos* are as follow: (*L/D)*-malic (**1**) ([M – H]$^-$ ion m/z 133.0137), succinic (**2**) ([M – H]$^-$ ion m/z 117.0187), azelaic (**3**), ([M – H] $^-$ ion m/z 187.0970) in retention time (t_R) intervals from 0.5 to 2.8 min. Other molecular ions (**5**) ([M – H]$^-$, m/z 149.0456) (**6**) [M – H]$^-$, m/z 341.1083) and unknown disaccharide (**7**) ([M – H]$^-$, m/z 387.1139), with a retention

time (t_R) of 0.6 min were identified as (**5**) pentose, (**6**) sucrose, and (**7**) an unknown disaccharide, respectively. Knowing that the amounts of many monosaccharides in this species were determined by Lysiuk et al. (2015) [41], it was easy to assign these compounds to the carbohydrate class. Also, the MS spectrum of the compound (**9**) ($[M - H]^-$, m/z 337.0923) corresponded to the coumaroylquinic acid formula $C_{16}H_{17}O_8$, and the other spectrum of the compound (**10**) ($[M - H]^-$, m/z 367.109) – feruloylquine formula $C_{17}H_{19}O_9$. Another ion (**8**) of the compound ($[M - H]^-$, m/z 243.0623) corresponded to the molecular formula $C_9H_{11}N_2O_6$, which matches to the presence of uridine. Previous studies have identified uridine in the ethanolic root extract [42] of the *Astragalus membranaceus*, (Fisch) Bge. var. mongholicus(Bge.) Hsiao.

The exact structures of other compounds (**11**), (**12**) and (**13**) could not be determined but referring to their molecular ions $[M - H]^-$ m/z 699.43195 (t_R, 4.2 min.), 695.4006 (t_R, 4.8 min) and 691.4057 (t_R, 5.4 min), which correspond to the molecular formulas $C_{37}H_{63}O_{12}$, $C_{37}H_{59}O_{12}$ and $C_{38}H_{59}O_{11}$, belong to cycloartane glycosides abundant in plant species of *Astragalus* L. genus [43].

2.1.4. Determination of α-gliucosidase activity

2.1.4.1. Evaluation of inhibition of *D. caucasica* Lipsky extract on α-glucosidase enzyme

The ability of ethanolic extracts of *D. caucasus* to inhibit the enzyme was determined in two modeled enzymatic reaction systems *in vitro* (**Fig. 1**)

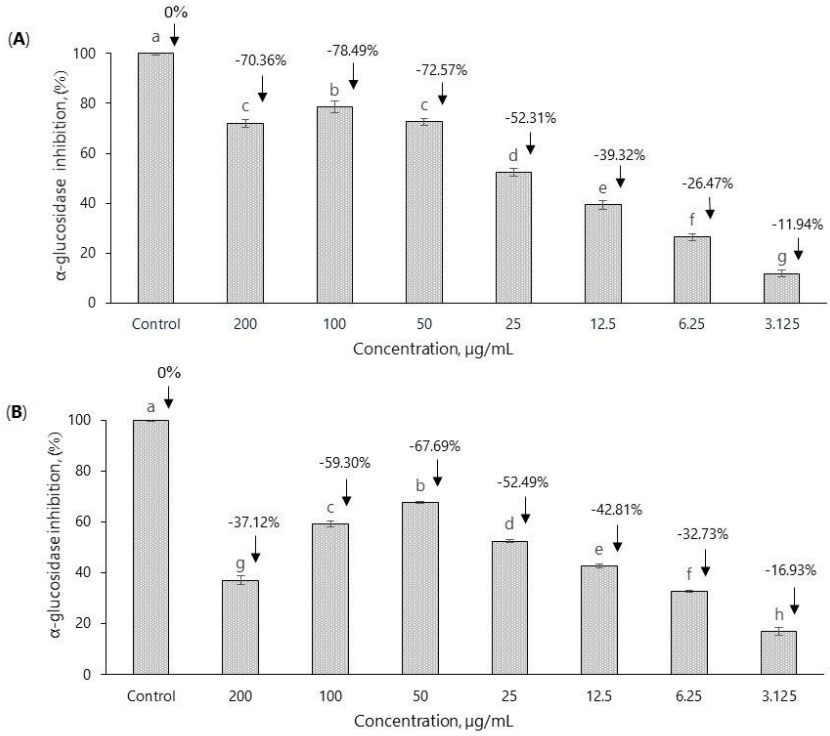

Figure 1. Effect of ethanol extract of *D. caucasica* leaves on α-glucosidase activity in enzymatic reaction systems *in vitro*

Part (**A**) of the figure – the reaction is complete without 0.1 M Na_2CO_3 reagent; part (**B**) of the figure – the reaction is complete with 0.1 M Na_2CO_3 reagent. Significant difference between modeled enzyme reaction systems by Fisher's LSD test ($p < 0.05$) after unifactorial dispersion analysis ANOVA is indicated by different letters above the bars.The inhibitory capacity of the extract is marked with a negative sign in percentage expression above the columns, the enzymatic reaction system without the inhibitor is marked with 0 per cent above the column. Mean values ± SN of three measurements are presented.

Increasing the concentration of the extracts from ≥ 3.125 µg/mL to < 200 µg/mL the activity of the enzyme decreased accordingly: (1) at the end of the reaction without 0.1 M Na_2CO_3: from ≥ 11.94 ± 1.22 per cent to < 70.36 ± 1.52 per cent compared to the control (2) at the end of the reaction with 0.1 M Na_2CO_3: ≥16.93 ± 1.43 to < 37.12 per cent ± 1.8 compared to the control (**Fig.1., (A), (B)**; **Tab. 4**).

When comparing the changes in the reduction of α-glucosidase activity between the two cases of the methods used in the study, a statistically notable difference ($p < 0.05$) in the inhibition ability of different extract concentrations was observed, except for the 25 µg/ml extract concentration, in *in vitro* methods (**Fig. 1**, (**A)**, (**B); Tab. 4**). In the study, using extract concentrations from ≥ 6.25 – 12.5 µg/mL and > 50 µg/mL, the average percent difference in enzyme activity between the applied methods was -6.260 – -3.490 per cent and 4.880 per cent, respectively. When increasing the concentration of the extract from ≥ 100 µg/mL and > 200 µg/mL, a highly statistically significant, average percentage difference in enzyme activity change between the applied methods emerged – 19.19 per cent and 33.24 per cent. As it was mentioned, out of all the extract concentrations selected in the study, only one (25 µg/mL) showed statistically insignificant differences in the average per cent change in enzyme activity, which was -0.180 per cent between the applied methods (P= 0.8639) (**Table 4**).

Table 4. Difference in change in α-glucosidase activity between the two biological surveys applied

***D. caucasica* leaf extracts, µg/mL**	**Enzymatic reaction system**		**Percentage difference**	***p*-value**
	With 0.1M Na_2CO_3	**Without 0.1M Na_2CO_3**		
3.125	16.93 ± 1.43	11.94 ± 1.22	-4.990	P = 0.01
6.25	32.73 ± 0.54	26.47 ± 1.43	-6.260	P = 0.0021
12.5	42.81 ± 0.67	39.32 ± 1.75	-3.490	P = 0.0321
25	52.49 ± 0.47	52.31 ± 1.64	-0.180	P = 0.8639
50	67.69 ± 0.26	72.57 ± 1.52	4.880	P = 0.0054
100	59.30 ± 1.1	78.49 ± 2.39	19.19	P = 0.0002
200	37.12 ± 1.8	70.36 ± 1.52	33.24	P < 0.0001

The values are presented as the means ± standard deviations (SD, n = 3); statistical evaluation of the differences between the values is indicated by paired two sample *t*-test by comparing enzymatic reactions before and after adding 0.1 M Na_2CO_3.

The different response to inhibition could be caused by the compounds and their concentration in the extracts, the relationship between the sodium carbonate response and other reasons, the exact explanations of which could not be found in the scientific literature.

2.1.4.1.1. Kinetic modeling of enzymatic reactions

In order to estimate the kinetic constants (K_m, V_{max}), the model of the interaction between the enzyme (E) and the substrate (S) related to the conversion of the product (P) ($S \xrightarrow{E} P$) was constructed as it was done in the case of the determination of the percentage activity of α-glucosidase, only the composition of the mixture for each enzymatic reaction differed in the amount of substrate.

According to the Lineweaver-Burke line graph, the change in reaction rate 1/V versus 1/[pNPG], expressed by the following equations: (1) in the absence of an inhibitor in the reaction mixture: $y = 55.052x + 0.7009$, $r^2 = 0.9935$; (2) adding 15 µg/mL concentration of leaf ethanolic extract/inhibitor: $y = 99.316x + 2.482$,

r^2 = 0.9852; adding 25 µg/mL concentration of leaf ethanolic extract/inhibitor: y = 193.4x + 2.2253 r^2 = 0.9989 (**Fig. 2, (A) part**).

Similarly, a double reciprocal plot was constructed when the enzymatic reaction was stopped with 0.1M Na_2CO_3((**Fig. 2, (B) part**). The following mathematical equations were obtained: (1) in the absence of an inhibitor in the reaction mixture: y = 134.64x + 1.7083, r^2 = 0.9943;adding 15 µg/mL concentration of leaf ethanolic extract/inhibitor: y = 188.35x + 6.3407, r^2 = 0.9072; adding 25 µg/mL concentration of leaf ethanolic extract/inhibitor: y = 249.6x + 7.4252, r2 = 0.9862.

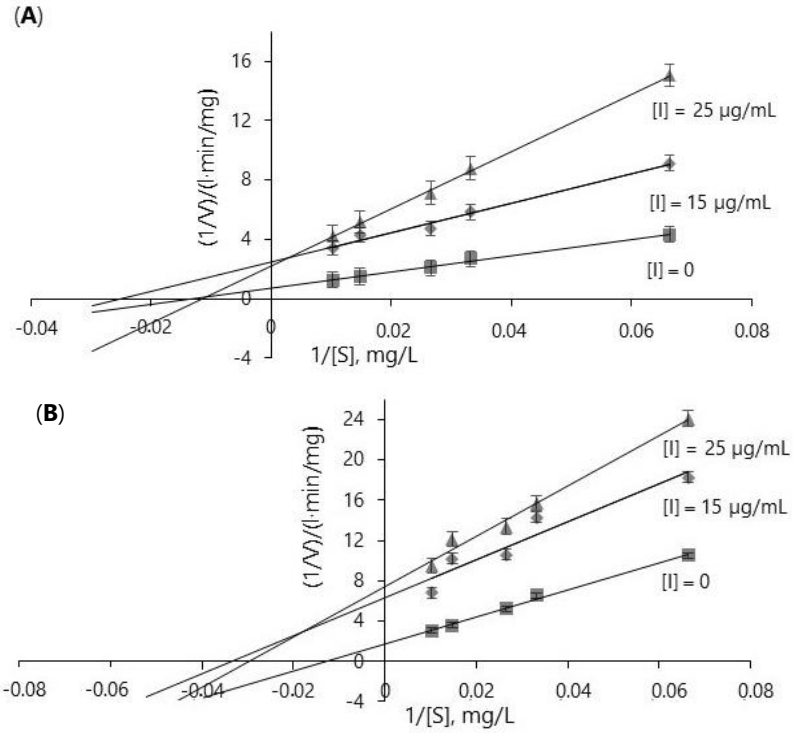

Figure 2. The alleged non-competitive (mixed-type) inhibition on α-glucosidase enzyme caused by ethanolic extract of *D. caucasica* Lipsky leaves

Mathematical model of the Lineweaver-Burke line graph for different concentrations of pNPG (0.05–0.33 mM) (**A, B**). (**A**) – the reaction is complete without 0.1 M Na_2CO_3 reagent; (**B**) – the reaction is complete with 0.1 M Na_2CO_3 reagent; the symbols on the graphic refers: squares (■): enzyme-substrate interaction in a model enzyme reaction system *in vitro*; rhombuses (♦): interaction of enzyme, substrate and inhibitor (15µg/mL) in a model enzyme reaction system *in vitro*; triangles (▲): interaction of enzyme, substrate and inhibitor (25µg/mL) in a model enzyme reaction system *in vitro*. Mean values ± SD of three measurements are presented.

D. caucasica leaf extracts showed the alleged non-competitive (mixed type) inhibition on the α-glucosidase enzyme (**Fig. 2, parts (A)** and **(B)**). During the study, in the system of II enzyme reaction (**Table 5**) under extract concentrations of 15 µg/mL and 25 µg/mL, the $V_{max(app)}$ and $K_{m(app)}$ values decreased by 0.157 ± 0.03

mg/min/mL, 0.134 ± 0.004 mg/min/mL and 29.76 ± 1.54 mg/L, 33.61 ± 0.75 mg/L accordingly. In contrast, in I enzyme reaction system, at 15 µg/mL extract concentration, $V_{max(app)}$, $K_{m(app)}$ values decreased by 0.402 ± 0.041 mg/min/mL and 39.984 mg/L, respectively. But at 25 µg/mL extract concentration, $V_{max(app)}$ value decreased to 0.449 ± 0.026 mg/min/mL and $K_{m(app)}$ value slightly increased to 86.91 mg/L. The obtained values of the kinetic constants showed the alleged non-competitive inhibition on the α-glucosidase enzyme, despite the significant differences in the values of the kinetic constants ($V_{max(app)}$, $K_{m(app)}$) between the enzymatic reaction systems. Non-competitive (mixed type) inhibition was also confirmed by the fact that in the Lineweaver-Burk plot, 1/V versus 1/[S] lines crossing the coordinate axes intersect in the third square [9]

As it can be seen from the results (**Table 5**), the catalytic efficiency (CE) of α-glucosidase in different enzymatic reaction systems decreased as follows: in I enzymatic reaction from 0.007 ([I] = 0) to 0.005 ([I] = 15 µg/mL) and 0.004 ([I] = 25 µg/mL); in II enzymatic reaction from 0.018 ([I] = 0) to 0.01 ([I] = 15 µg/mL) and 0.005 ([I] = 25 µg/mL). The decrease in the rate of catalysis, along with the decrease in the concentration of the formed product, confirmed the ability of the extracts to inhibit α-glucosidase enzyme.

Shown below (**Fig. 3**) is a mathematical model of the Lineweaver-Burk line graph, which indicates the alleged competitive inhibition on the α-glucosidase enzyme caused by the rhizome extract of *D. caucasica*. The Lineweaver-Burk inhibition plots of 1/V versus 1/[pNPG] gave the following equations: (1) in the absence of inhibitor in the reaction mixture y = 30.698x + 0.9772, r^2 = 0.9292; (2) adding 200 µg/mL rhizome ethanolic extract/inhibitor: y = 69.91x + 1.0085, r^2 = 0.9833; adding 500 µg/mL rhizome ethanolic extract/inhibitor: y = 80.37x + 0.967, r^2 = 0.966.

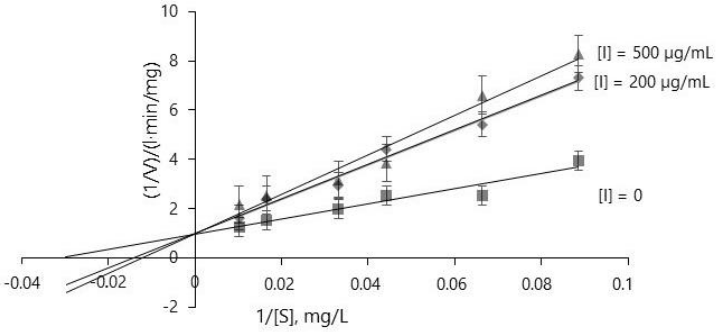

Figure 3 The alleged competitive inhibition on α-glucosidase enzyme caused by *D. caucasica* Lipsky rhizome extract

Mathematical model of the Lineweaver-Burke line graph for different concentrations of *p*NPG (0.004–0.33 mM). The symbols on the graphic refers: squares (■): enzyme-substrate interaction in a model enzyme reaction system *in vitro*; rhombuses (♦): interaction of enzyme, substrate and inhibitor (200 µg/mL) in a model enzyme reaction system *in vitro*; triangles (▲): interaction of enzyme, substrate and inhibitor (500 µg/mL) in a model enzyme reaction system *in vitro*. Mean values ± SD of three measurements are presented.

The rhizome extract of *D. caucasica* showed a competitive type of inhibition with a K_i value of 165.97 to 304.32 µg/mL at 200 µg/mL and 500 µg/mL extract concentrations, respectively. With increasing extract concentration, $K_{m(app)}$ values gradually increased from 69.34 ± 0.31 to 83.33 mg/mL, and $V_{max(app)}$ – 0.99 ± 0.22 and 1.034 ± 0.38 mg/L·min – hardly changed (**Table 5**). An increased value of K_m and unchanged V_{max} indicated a type of competitive inhibition [9]

Table 5 Kinetic parameters of α-glucosidase inhibition kinetics

Interaction pattern of a model enzyme reaction system	Values of kinetic parameters	Kinetic parameters		Mode
		Enzymatic reaction system I[1]	Enzymatic reaction system II[2]	
I	II	III	IV	V
Enzyme/substrate interaction model	K_m, mg/L	78.55 ± 0.11	80.873 ± 0.26	
	V_{max}, mg/L·min	1.43 ± 0.183	0.597 ± 0.07	
	CE	0.018	0.007	
Enzyme/substrate/inhibitor interaction model. D.C leaf extract, 15 µg/mL	$K_{m(app)}$, mg/L	39.984 ±0.31	29.761 ± 1.54	Mixed-type
	$V_{max(app)}$, mg/L·min	0.402 ± 0.041	0.157 ± 0.03	
	K_i, µg/mL	5.86	5.35	
	CE	0.01	0.005	
lEnzyme/substrate/inhibitor interaction model. D.C leaf extract, 25 µg/mL	$K_{m(app)}$, mg/L	86.91 ± 0.16	33.615 ± 0.75	Mixed-type
	$V_{max(app)}$, mg/L·min	0.449 ±0.026	0.134 ± 0.004	
	K_i, µg/mL	11.44	7.23	
	CE	0.005	0.004	
Enzyme/substrate Interaction model	K_m, mg/L	-	31.446 ± 0.28	
	V_{max}, mg/L·min	-	1.023 ± 0.23	
	CE		0.032	
Enzyme/substrate/inhibitor interaction model. D.C tubers extract, 200 µg/mL	$K_{m(app)}$, mg/L	-	69.34 ± 0.31	Competitive
	$V_{max(app)}$, mg/L·min	-	0.991 ± 0.22	
	K_i, µg/mL	-	165.97	
	CE	-	0.014	
Enzyme/substrate/inhibitor interaction model. D.C tubers extract, 500 µg/mL	$K_{m(app)}$, mg/L	-	83.125 ± 0.51	
	$V_{max(app)}$, mg/L·min	-	1.034 ± 0.38	
	K_i, µg/mL	-	304.32	
	CE	-	0.011	

Enzymatic reaction system I[1] – the reaction is completed without 0.1 M Na_2CO_3 reagent; enzymatic reaction system II[2] – the reaction is completed with 0.1 M Na_2CO_3 reagent. D.C – *Dioscorea caucasica* Lipsky.

2.1.4.2. Evaluation of inhibition extracts of *D. nipponica* extracts on α-glucosidase enzyme

Extracts of *D. nipponica* were similarly studied. When investigating the inhibitory ability of the ethanolic extracts of *D. nipponica* (3.125 – 200 μg/mL) on α-glucosidase enzyme, the diagrams below (**Fig. 4**) show a statistically significant difference ($p < 0.05$) in all the modeled enzymatic reaction systems compared to the control.

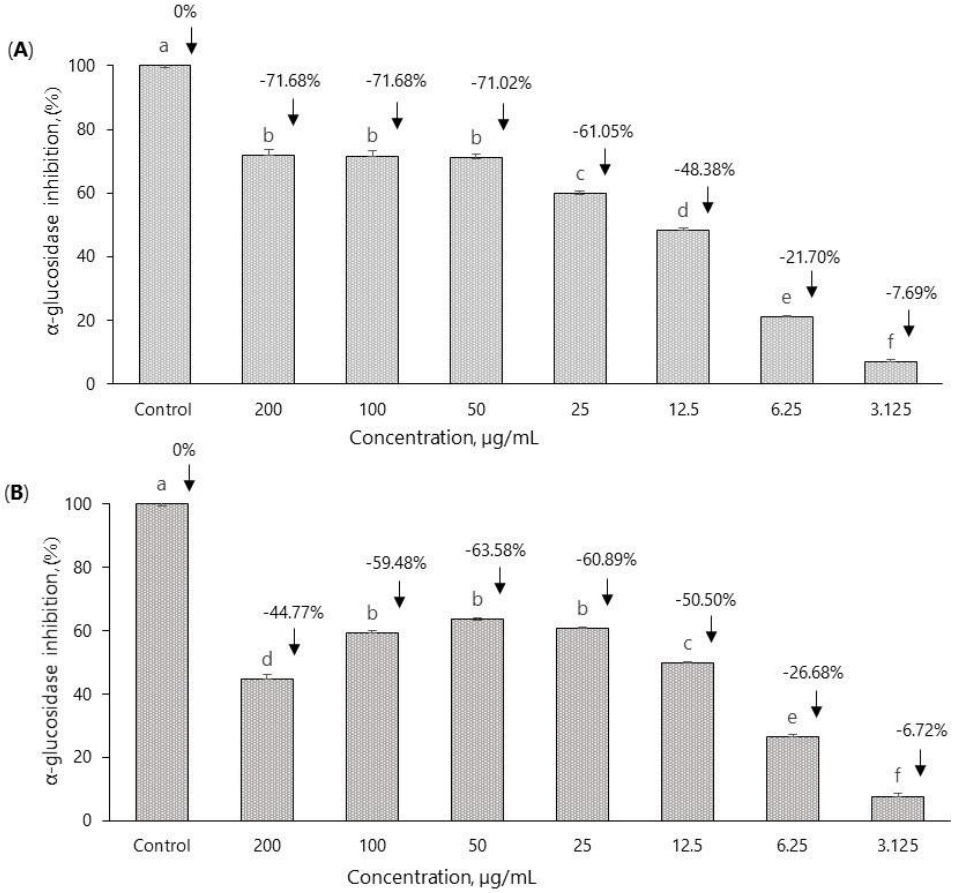

Figure 4. Impact of ethanolic extract of *D. nipponica* leaves on α-glucosidase activity in enzymatic reaction systems *in vitro*

Part (**A**) of the figure – the reaction is complete without 0.1 M Na_2CO_3 reagent; part (**B**) of the figure – the reaction is complete with 0.1 M Na_2CO_3 reagent. Significant difference between modeled enzyme reaction systems by Fisher's LSD test ($p < 0.05$) after unifactorial dispersion analysis ANOVA is indicated by different letters above the bars.The inhibitory capacity of the extract is marked with a negative sign in percentage expression above the columns, the enzymatic reaction system without the inhibitor is marked with 0% per cent above the column. Mean values ± SD of three measurements are presented.

Increasing the extract concentrations from ≥ 3.125 µg/mL to < 200 µg/mL resulted in a corresponding decrease in enzyme activity in all simulated enzymatic reaction systems (**Fig. 4**, parts **(A)** and **(B)**) compared to the control.

Part (**A**) of the diagram (**Fig. 4**) shows a corresponding decrease in α-glucosidase activity from ≥ 7.69 ± 1.55 per cent to < 61.05 ± 1.52 per cent with increasing extract concentrations from ≥ 3.125 to < 25 µg/mL compared to the control. However, increasing the extract concentration from ≥ 50 µg/mL to < 200 µg/mL did not change the enzyme activity and ranged from 71.02 to 71.68 per cent. accordingly. This result showed that enzyme activity was impaced by the extract. A slightly different decrease in enzyme activity than that shown in part (**A**) of the diagram is seen in part (**B**). When increasing the extract concentrations from ≥ 3.125 µg/ml to < 12.5 µg/mL, α-glucosidase activity decreased from ≥ 6.72 ± 1.41 per cent respectively to < 50.50 ± 3.29 per cent compared to the control. Further increasing the extract concentration from ≥ 25 µg/mL to < 100 µg/mL, the decrease in α-glucosidase activity was almost unchanged and ranged from ≥ 60.89 ± 1.88 per cent to < 59.48 ± 0.76 per cent accordingly, compared to the control. However, increasing the extract concentration to ≤ 200 µg/mL decreased the α-glucosidase activity by 44.77 ± 1.98 per cent compared to the control, but not as expected.

When comparing the changes in the reduction of α-glucosidase activity between the two methods applied in the study, a statistically notable percentage difference was found when increasing the extract concentration from ≥ 50 µg/mL to < 200 µg/mL, in *in vitro* methods. The obtained results are presented in Table 4.2.4.3 For the latter concentrations, the average per cent difference in the change in enzyme activity between the applied methods was 7.44 per cent, 12.20 per cent and 26.91 per cent, respectively. Meanwhile, lower extract concentrations were not statistically significantly different:< 3.125 µg/mL: 0.96 per cent (P = 0.4719); 6.25 µg/mL: -4.980 per cent (P = 0.4719); 12,5 µg/mL: -2.12 per cent (P = 0.4054) > 25 µg/mL: -0.16 per cent (P = 0.9143).

Table 6. Differencce in change in α-glucosidase activity between the two biological surveys applied

***D. nipponica* leaf extracts, µg/mL**	**Enzymatic reaction model system**		**Percentage difference**	***p*-value**
	With 0,1M Na_2CO_3	**Without 0,1M Na_2CO_3**		
3.125	6.72 ± 1.41	7.68 ± 1.55	0.960	P = 0.4719
6.25	26.68 ± 2.99	21.70 ± 3.39	-4.980	P = 0.129
12.5	50.50 ± 3.29	48.38 ± 2.19	-2.120	P = 0.4054
25	60.89 ± 1.88	61.05 ± 1.52	-0.16	P = 0.9143
50	63.58 ± 1.96	71.02 ± 1.69	7.44	P = 0.0076
100	59.48 ± 0.76	71.68 ± 1.16	12.20	P= 0.0001
200	44.77 ± 1.98	71.68 ± 0.68	26.91	P < 0.0001

The values are presented as the means ± standard deviations (SD, n = 3); statistical evaluation of the differences between the values is indicated by paired two sample *t*-test by comparing enzymatic reactions before and after adding 0.1 M Na_2CO_3.

After evaluating the inhibition of α-glucosidase caused by the extract, it can be assumed that the 0.1M Na_2CO_3 reagent contained in the enzyme reaction mixtures can significantly change the enzyme activity readings *in vitro*, as in the previous study with *D. caucasica* leaf extracts. It can also be concluded that it is influenced by the compounds present in the extract and their concentrations, since significant differences in inhibition between the reaction systems become particularly pronounced with increasing concentrations of the extract. In the literature, there was no information about possible changes in the enzyme activity readings *in vitro* when studying the inhibition of α-glucosidase caused by herbal preparations by this method. There is also not much research done on completing the reaction without the Na_2CO_3 reagent. Trifonova et al. (2021) [44], Stoilova et al. (2017) [45] investigated the effectiveness of plant extracts in inhibiting α-glucosidase enzyme by classical chromogenic method without using Na_2CO_3 reagent. These insights can be very beneficial for similar studies with plant extracts or plant-derived products.

2.1.4.2.1. Kinetic modeling of enzymatic reactions

By analogy, Lineweaver-Burk line graph was applied to determine the dependence of the enzymatic reaction rate 1/V on the substrate 1/[*p*NPG] calculating the values of the kinetic constants (V_{max}, K_m) while determining the assumed type of inhibition for α-glucosidase inhibition.

According to Lineweaver–Burk mathematical graph, the change in reaction rate 1/V versus 1/[*p*NPG] is expressed by the following enzyme reaction equations: (1) in the absence of an inhibitor in the reaction mixture: $y = 49.172x + 0.5981$, $r^2 = 0.9853$; adding 25 μg/mL concentration of leaf ethanolic extract/inhibitor: $y = 200.95x + 1.0672$, $r^2 = 0.9872$ (**Fig. 5, A**); (2) in the absence of an inhibitor in the reaction mixture: $y = 114.08x + 0.5349$, $r^2 = 0.975$, with the addition of 500 μg/mL rhizome ethanolic extract/inhibitor: $y = 188.05x + 1.0962$, $r^2 = 0.9813$ (**Fig. 6**). Similarly, a double reciprocal graph was formed when the enzymatic reaction was stopped using 0.1 M Na_2CO_3 (**Fig. 5, B**). The following mathematical equations were expressed: (1) in the absence of an inhibitor in the reaction mixture $y = 125.15x + 1.2148$, $r^2 = 0.9887$; adding 25 μg/mL ethanolic leaf extract/inhibitor: $y = 412.31x + 2.328$, $r^2 = 0.9982$.

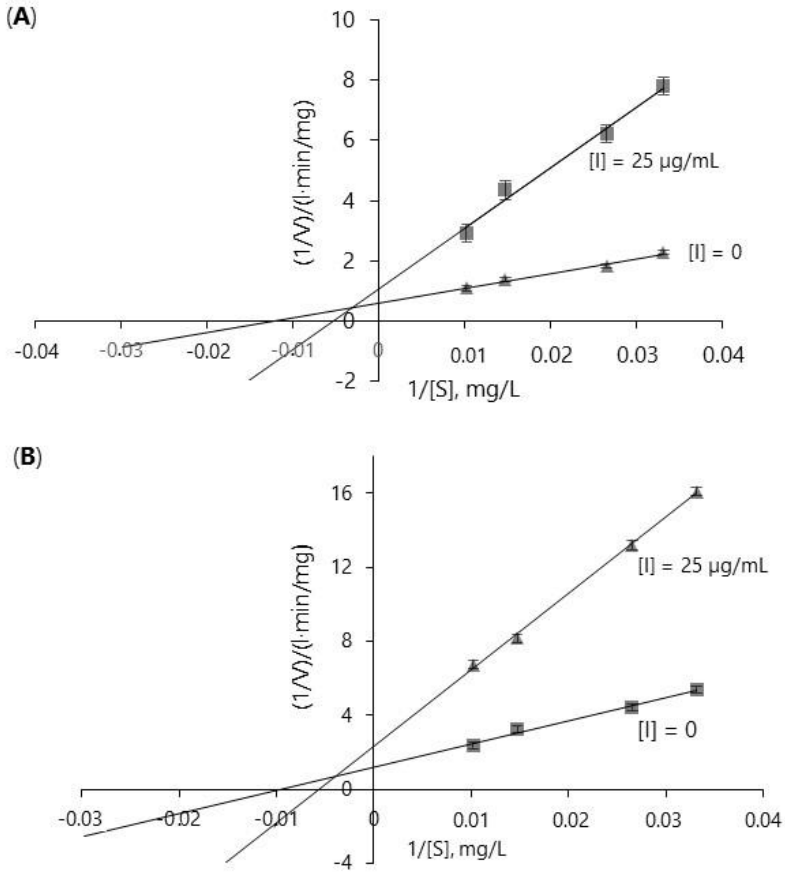

Figure 5. The alleged non-competitive (mixed type) inhibition on α-glucosidase enzyme caused by ethanolic extract of *D. nipponica* Makino leaves

Mathematical model of the Lineweaver-Burk line graph for different concentrations of *p*NPG (0.1–0.33 mM) (**A, B**). (**A) –** the reaction is completed without 0.1 M Na_2CO_3 reagent; the symbols on the graphic refers: triangles (▲): enzyme-substrate interaction in a model enzyme reaction system *in vitro*; squares (■): interaction of enzyme, substrate and inhibitor (25µg/mL) in a model enzyme reaction system *in vitro*. (**B**) – the reaction is complete with 0.1 M Na_2CO_3 reagent; the symbols on the graphic refers: squares (■): enzyme-substrate interaction in a model enzyme reaction system *in vitro*; triangles (▲): interaction of enzyme, substrate and inhibitor (25µg/mL) in a model enzyme reaction system *in vitro*. Mean values ± SD of three measurements are presented.

The extract of *Dioscorea nipponica* leaves (**Fig. 5, parts (A) and (B**) showed the alleged non-competitive (mixed type) inhibition on α-glucosidase enzyme. Enzymatic reactions in different systems (**Table 7**) at 25 µg/mL extract concentration $K_{m(app)*}$, $K_{m(app)}$ values increased by 177.1 ± 0.08 mg/mL, 188.3 ± 2.2 mg/mL, and $V_{max(app)*}$, $V_{max(app)}$ values decrease by 0.43 ± 0.006 mg/min/mL, 0.94 ± 0.13 mg/min/mL, respectively, compared to the control. This inhibition profile indicated

that the inhibitor binds with both the free enzyme (E) and the enzyme-substrate (ES) complex [7]. Similar to leaves, rhizome extract of *D. nipponica* (**Fig. 6**) showed mixed non-competitive inhibition on α-glucosidase enzyme. Non-competitive (mixed type) inhibition was also confirmed by the fact that in the line graph of Lineweaver-Burk 1/V versus 1/[S] the line rotates around the abscissa axis -1/Km [10 p.114].

Table 7. Kinetics constants for α-glucosidase inhibition by *D. nipponica* leaf and tuber extracts

Inhibitor	$K_{m(app)}$, mg/L	$V_{max(app)}$, mg/L·min	K_i, µg/mL	Inhibition type
D.N leaves 25 µg/mL extract	188.29 ± 5.8	0.94 ± 0.35	31.87	
	177.11 ± 0.7*	0.43 ± 0.06*	27.23*	Mix-type
Control[1]	82.21 ± 4.5	1.67 ± 0.28		
	103.02 ± 1.3*	0.82 ± 0.13*		
D.N tubers 500 µg/mL extract	171.54 ± 8.25	0.912 ± 0.45	476.5	Mix-type
Control[1]	213.67 ± 12.92	1.869 ± 0.38		

[1] K_m ir V_{max} – the absence of inhibitor in enzymatic reaction systems
[2] $K_{m(app)}$ ir $V_{max(app)}$ – identical kinetic parameters as K_m and V_{max} only in the presence of inhibitor
*Symbol – enzymatic reaction system with 0.1 M Na_2CO_3
The values are presented as the means ± standard deviations (SD, n = 3).

D. nipponica rhizome extract (**Fig. 6**) inhibited α-glucosidase activity as did the leaf extract in a non-competitive manner. This can be explained by the decreasing values of $K_{m(app)}$ and $V_{max(app)}$ kinetic constants compared to the control. Also, in the line graph of Lineweaver-Burk, the 1/V versus 1/[S] lines crossing the coordinate axes and intersecting in the third square confirmed the mixed type of inhibition [7, 9].

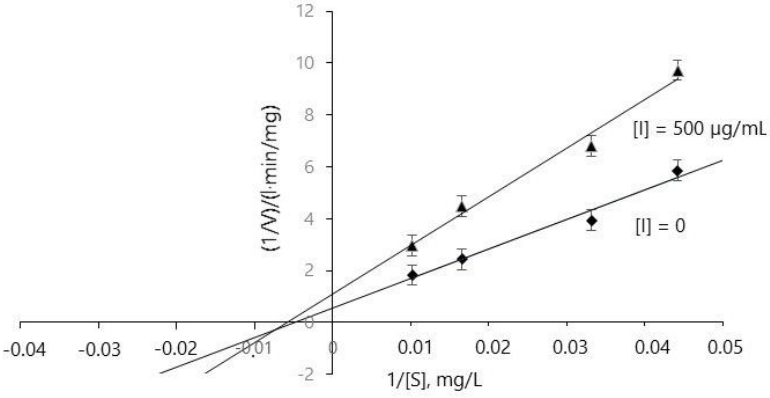

Figure 6. The alleged non-competitive (mixed-type) inhibition on α-glucosidase enzyme caused by ethanol extract of *D. nipponica* Makino) rhizomes

Mathematical model of the Lineweaver-Burk line graph for different concentrations of *p*NPG (0.075 – 0.33 mM). The symbols on the graphic refers: rhombuses (♦): enzyme-substrate interaction in a model enzyme reaction system *in vitro*; triangles (▲): interaction of enzyme, substrate and inhibitor (500µg/mL) in a model enzyme reaction system *in vitro*. Mean values ± SD of three measurements are presented.

Summarizing the results of the study, it can be concluded that the studied *Dioscorea* spp. extracts are capable of inhibiting the α-glucosidase enzyme at very low concentrations. Such inhibitory ability of the extracts can be linked to hydroxycinnamic acids, quercetin derivatives (quercitrin and isoquercitrin), which dominated in the composition of the extracts, and their inhibitory effect has long been scientifically proven [46].

Applying minor changes to the biological research, it was observed that the 0.1M Na_2CO_3 reagent can significantly change the enzyme activity readings *in vitro*, especially at higher extract concentrations. During the study, it was found that leaf extracts of *D. caucasica* and *D. nipponica* (200 µg/mL) inhibited α-glucosidase enzyme > 50 per cent, root extracts (500 µg/mL) inhibited the latter enzyme weakly: 36.79 ± 2.93 per cent by caucasica and 40.79 ± 2.71 per cent by of *Dioscorea nipponica.*

2.1.4.3. Evaluation of inhibition of *A. glycyphyllos* L. extracts on α-glucosidase enzyme

Dry ethanolic extracts of the *Astragalus glycyphyllos* leaves were dissolved in methanol. As it can be seen from **Table 8** below, all selected concentrations of the extract (100 – 400 µg/mL) showed an inhibitory effect on α-glucosidase enzyme.

Table 8. Effect of ethanolic extracts of *A. glycyphyllos* L. leaves on α-glucosidase activity

Solvent	Concentration, µg/mL	Inhibition, per cent
Methanol/water (70/30 v/v)	100	$29.12^{a} \pm 0.39$
	120	$34.21^{b} \pm 0.16$
	160	$38.67^{c} \pm 4.06$
	200	$43.40^{d} \pm 0.17$
	360	$51.04^{e} \pm 0.57$
	400	$54.33^{f} \pm 0.7$

Significant difference between modeled enzyme reaction systems by Fisher's LSD test ($p < 0.05$) after unifactorial dispersion analysis ANOVA is indicated by different letters beside the numbers. Mean values ± SD of three measurements are presented.

During the study, it was found that the activity of α-glucosidase enzyme depended on the concentration of *A. glycyphyllos* extract. When increasing the concentration of the extract from ≥ 100 µg/mL to < 400 µg/mL, the α-glucosidase activity decreased gradually from ≥ 29.12 ± 0.39 to < 54.33 ± 0.7 per cent compared to the control. The data obtained during the *in vitro* study showed that the *A. glycyphyllos* leaf extract at 360 µg/mL inhibited the enzyme by 51.04 ± 3.27 per cent.

2.1.4.3.1. Kinetic modeling of enzymatic reactions

In order to determine the values of the inhibition constants, the dependence of the enzyme reaction rate on the concentration of the substrate was measured in the presence and absence of the inhibitor. *A. glycyphyllos* leaf extract showed alleged non-competitive inhibition on α-glucosidase enzyme. This can be seen in the Lineweaver-Burk line graph (**Fig. 7**), which depicts the enzyme-substrate and enzyme-substrate-extract interaction model.

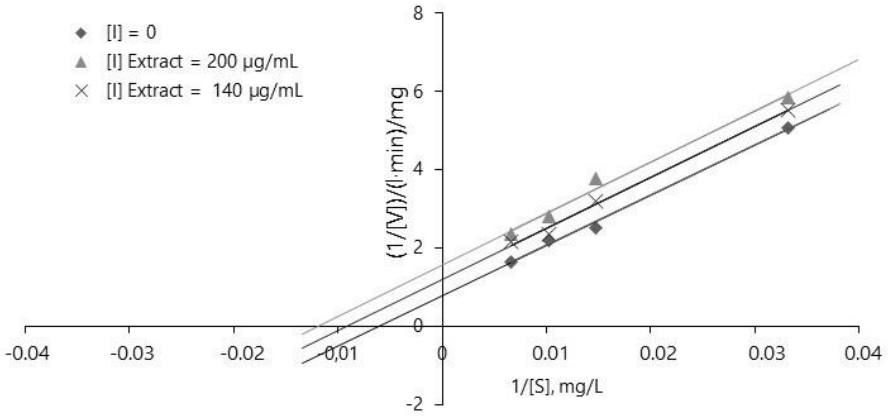

Figure 7. The alleged uncompetitive inhibition on α-glucosidase enzyme caused by ethanol extract of *A. glycyphyllos* leaf

Mathematical model of the Lineweaver-Burke line graph for different concentrations of pNPG (0.1–0.5 mM). The symbols on the graphic refers: squares (♦): enzyme-substrate interaction in a model enzyme reaction system *in vitro*; (X): interaction of enzyme, substrate and inhibitor (140 µg/mL) in a model enzyme reaction system *in vitro*; triangles (▲): interaction of enzyme, substrate and inhibitor (200 µg/mL) in a model enzyme reaction system *in vitro*. Mean values ± SD of three measurements are presented.

During the study, increasing the concentration of the extract, the values of the kinetic parameters decreased accordingly: $K_{m(app)}$: <140 µg/mL: 109.09 ± 22.40; > 200 µg/mL: 84.09 ± 8.75: $V_{max(app)}$: <140 µg/mL: 0.639 ± 0.02 mg/min/mL; > 200 µg/mL: 0.834 ± 0.03 mg/min/mL compared to control. The decrease in the values of the kinetic parameters and the non-intersecting lines on the coordinate axis are characteristic of the alleged uncompetitive inhibition. The latter inhibition profile indicates that the inhibitor binds only with the enzyme-substrate (ES) complex, forming an inactive enzyme-substrate-inhibitor (ESI) complex.

2.1.5. Determination of α-amylase activity

2.1.5.1. Evaluation of inhibition of extracts of *D. caucasica* Lipsky on α-amylase enzyme

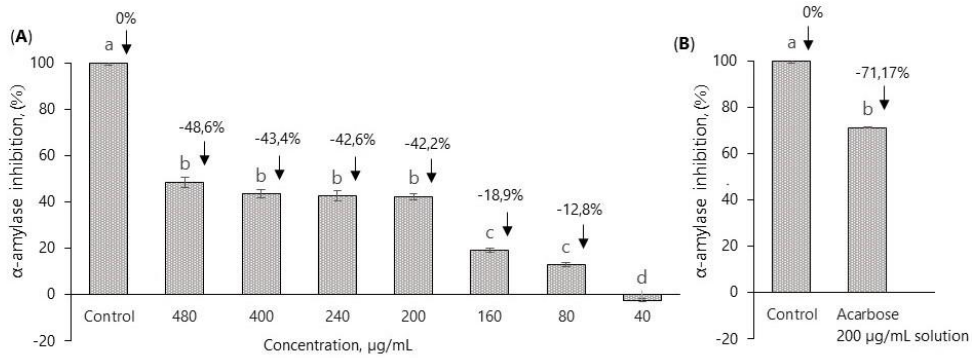

Figure 8. Effect of ethanolic extract of *D. caucasica* Lipsky leaves on α-amylase activity (**A**) and the impact of aqueous acarbose solution on α-amylase activity (**B)** in enzymatic reaction systems

A row of columns in a bar chart (**A**) with the last column below the horizontal line indicate that a concentration of 40 µg/mL leaf extract is unable to inhibit the enzyme activity. Significant difference between modeled enzyme reaction systems by Fisher's LSD test ($p < 0.05$) after unifactorial dispersion analysis ANOVA is indicated by different letters above the bars.The inhibitory capacity of the extract is marked with a negative sign in percentage expression above the columns, the enzymatic reaction system without the inhibitor is marked with 0 per cent above the column. Mean values ± SE of three measurements are presented.

During the action of the enzyme and inhibitor, it is possible to additionally regulate the course of the enzymatic reaction. This can be seen in **Fig. 8** (as well as in previous inhibition studies) inhibitor concentration-dependent enzyme activity. Increasing extract concentrations from ≥ 40 µg/mL to < 480 µg/mL enzyme activity decreased correspondingly in all modeled enzymatic reaction systems (**Fig. 8**) compared to the control, except for 40 µg/mL extract concentration, in *in vitro* studies. However, when comparing the change in α-amylase activity between enzymatic reaction mixtures containing different concentrations of the extract, the percentage of enzyme activity decreased from ≥ 12.8 ± 0.9 per cent to < 42.2 ± 1.3 per cent respectively, increasing the extract concentration from ≥ 80 µg/ml to < 200 µg/mL. When increasing the concentration of the extract from ≥ 200 µg/mL to < 480 µg/mL, α-amylase activity did not change significantly and ranged from 42.6 ± 2.3 per cent to 48.6 ± 2.2 per cent. Such a gradual decrease in enzyme activity *in vitro* indicated sufficient saturation of the inhibitor. The results also showed that the aqueous solution of acarbose reduced the activity of the enzyme by ~71.17 ± 0.4 per cent at a concentration of 200 µg/mL. From these data, it can be concluded that *D. caucasica* leaf extract is effective in inhibiting α-amylase enzyme, but statistically significantly ($p < 0.05$) less than aqueous solution of acarbose.

2.1.5.2. Evaluation of inhibition of extracts of *D. nipponica* Makino on α-amylase enzyme

Similarly as in the studies of *D. caucasica*, a change in α-amylase activity dependent on increasing extract concentration is observed in all simulated enzymatic reaction mixtures.

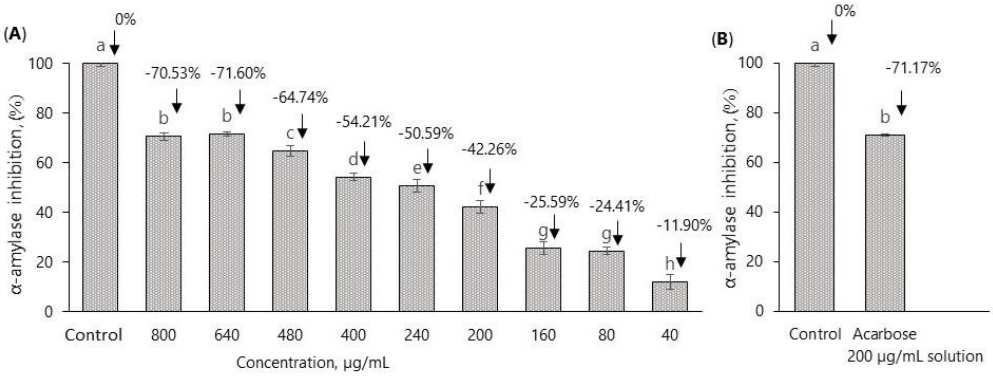

Figure 9. Effect of ethanolic extract of *D. nipponica* Makino leaves on α-amylase activity (**A**) and the impact of aqueous acarbose solution on α-amylase activity (**B)** in enzymatic reaction systems

Significant difference between modeled enzyme reaction systems by Fisher's LSD test ($p < 0.05$) after unifactorial dispersion analysis ANOVA is indicated by different letters above the bars.The inhibitory capacity of the extract is marked with a negative sign in percentage expression above the columns, the enzymatic reaction system without the inhibitor is marked with 0 per cent above the column. Mean values ± SE of three measurements are presented.

Enzyme activity decreased correspondingly by increasing the concentration of the extracts:< 40 µg/mL: 11.9 ± 2.94 per cent; 80 µg/mL: 24.41 ± 1.49 per cent; 160 µg/mL: 25.59 ± 2.73; 200 µg/mL: 42.26 ± 2.72 per cent; 240 µg/mL: 50.59 ± 2.52 per cent; 400 µg/mL: 54.21 ± 1.48 per cent; 480 µg/mL: 64.74 ± 1.0 per cent; 640 µg/mL: 71.6 ± 1.0 per cent; > 800µg/mL: 70.53 ± 1.37 per cent.

The change in enzyme activity in the simulated reaction systems was statistically significantly different ($p < 0.05$) when the extract concentrations were increased: from ≥ 40 µg/mL to < 800 µg/mL, compared to the control; from ≥ 40 – 80 µg/ml and ≥ 160 – 640 µg/mL when comparing between enzyme reaction mixtures. However, the change in enzyme activity reduction was not statistically notably different ($p > 0.05$) between enzyme reaction mixtures with increasing extract concentrations: from ≥ 80 µg/mL to < 160 µg/mL and from ≥ 640 µg/mL to < 800 µg/mL. From an enzymological point of view, the enzyme reaction system gradually became saturated with an inhibitor capable of reducing enzyme activity by > 50 per cent. The data obtained during the *in vitro* study show that the *D. nipponica* leaf extract reduced α-amylase activity with an IC_{50} reached 437.96 µg/mL. From these data, it can be can concluded that *D. nipponica* leaf extract was a potent inhibitor of α-amylase, but not significantly stronger than the aqueous solution of acarbose. The

latter decreased the enzyme activity by ~71.17 ± 0.4 per cent at a concentration of 200 µg/mL.

2.1.5.3. Evaluation of inhibition of extracts of *A. glycyphyllos* L. on α-amylase enzyme

Evaluation of the inhibition of *A. glycyphyllos* extracts on α-amylase enzyme was also investigated by applying the Miller reaction [2].

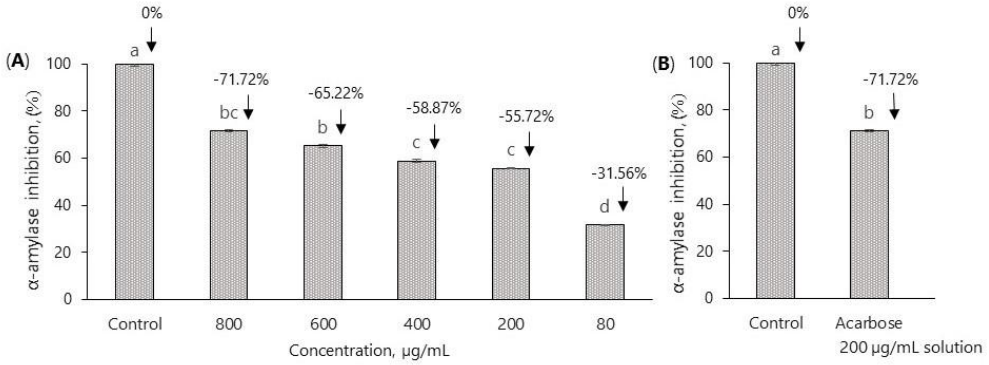

Figure 10. Effect of ethanolic extract of *A. glycyphyllos* L. leaves on α-amylase activity (**A**) and the impact of aqueous acarbose solution on α-amylase activity (**B)** in enzymatic reaction systems *in vitro*

Significant difference between modeled enzyme reaction systems by Fisher's LSD test ($p < 0.05$) after unifactorial dispersion analysis ANOVA is indicated by different letters above the bars.The inhibitory capacity of the extract is marked with a negative sign in percentage expression above the columns, the enzymatic reaction system without the inhibitor is marked with 0 per cent above the column. Mean values ± SE of three measurements are presented.

Enzyme activity decreased correspondingly by increasing extract concentrations < 80 µg/mL: 31.56 ± 0.18 per cent; 200 µg/mL: 55.72 ± 0.78 per cet; 400 µg/mL: 58.87 ± 0.55 per cent; 600 µg/mL: 65.22 ± 0.50 per cent; > 800 µg/mL: 71.72 ± 0.48 per cent. The highest decrease in enzyme activity was determined at 800 µg/mL extract concentration. The change in enzyme activity in the simulated reaction systems was statistically significantly different ($p < 0.05$) when the extract concentrations were increased: from ≥ 80 µg/mL to < 800 µg/mL, compared to the control; from ≥ 80 – 200 µg/mL, from ≥ 400 – 600 µg/mL and between 400 µg/mL and 800 µg/mL extract concentrations when compared between enzymatic reaction mixtures. However, the change in enzyme activity reduction was not statistically notably different ($p > 0.05$) between enzyme reaction mixtures with increasing extract concentrations: from ≥ 200 µg/mL to < 400 µg/mL and from ≥ 600 µg/mL to < 800 µg/mL.

2.1.6. Determination of angiotensin-converting enzyme activity *in vitro*

Angiotensin-converting enzyme (EC 3.4.15.1) is a glycoprotein enzyme mainly produced in renal epithelial cells. Angiotensin I-converting enzyme molecule consists of two homologous domains (N and C domains), each containing an active center [47]. It is proved that drugs intended to inhibit angiotensin-converting enzyme activity bind to the Zn^{2+} ion in the active center of the ACE enzyme [47, 48]. In order for natural molecular compounds and herbal preparations containing them to be able to inhibit the enzyme, they must also bind to the active center of the ACE molecule. However, such interaction with the ionic location depends on the inhibitory properties of the natural compounds and their presence in different parts of the plant.

Considering which molecular compounds were found to dominate *Dioscorea* spp., *Astragalus* spp. species, further studies evaluated the effect of the extracts on AKF activity using spectrophotometric method [49, 50, 2]. Medicine captopril, prepared as a 1mg/ml solution in distilled water [51], was selected as a positive control.

2.1.6.1. Evaluation of inhibition of *D. caucasica* Lipsky extracts on ACE enzyme

When investigating the inhibitory ability of ethanolic extracts of *D. caucasica* (250 – 1250 µg/mL) for ACE enzyme, a statistically significant difference ($p < 0.05$) was obtained in all the modeled enzymatic reaction systems, compared to the control (**Fig. 11**).

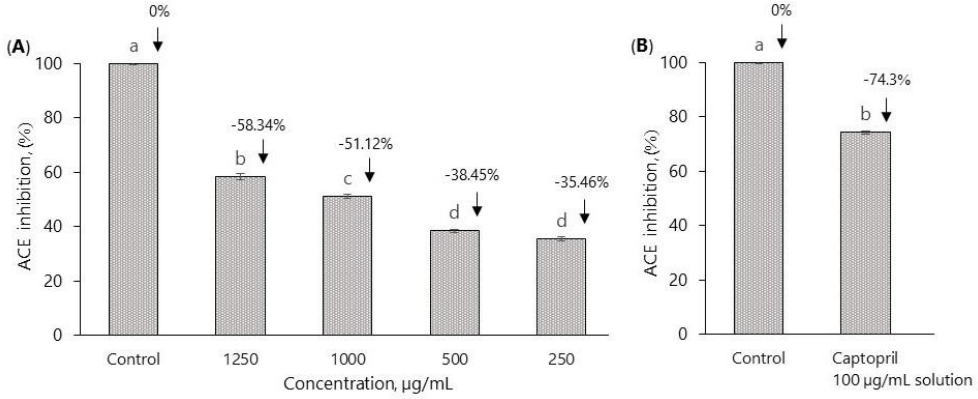

Figure 11. Effect of ethanol extract of *D. caucasica* Lipsky leaves on ACE activity (**A**) and impact of captopril aqueous solution on ACE activity (**B**) in enzymatic reaction systems *in vitro*

Significant difference between modeled enzyme reaction systems by Fisher's LSD test ($p < 0.05$) after unifactorial dispersion analysis ANOVA is indicated by different letters above the bars.The inhibitory capacity of the extract is marked with a negative sign in percentage expression above the columns, the enzymatic reaction system without the inhibitor is marked with 0 per cent above the columns. Mean values ± SE of three measurements are presented.

Enzyme activity decreased correspondingly by increasing extract concentrations: < 250 µg/mL: 35.46 ± 0.76 per cent; 500 µg/mL: 38.45 ± 0.52 per cent; 1000 µg/mL: 51.12 ± 0.81 per cent; > 1250 µg/mL: 58.34 ± 1.01 per cent, enzyme activity decreased respectively compared to the control. However, when comparing the change in ACE activity between enzymatic reaction mixtures containing different extract concentrations, the enzyme activity decreased statistically notably from ≥ 38.45 ± 0.52 per cent to < 58.34 ± 1.01 per cent, increasing the extract concentration from ≥ 500 µg/mL to < 1250 µg/mL, respectively. And there was no statistically significant difference in the decrease of ACE activity when the reaction mixtures were exposed to 250 and 500 µg/mL extract concentrations.

2.1.6.2. Evaluation of the inhibition of *D. nipponica* Makino extracts on ACE enzyme

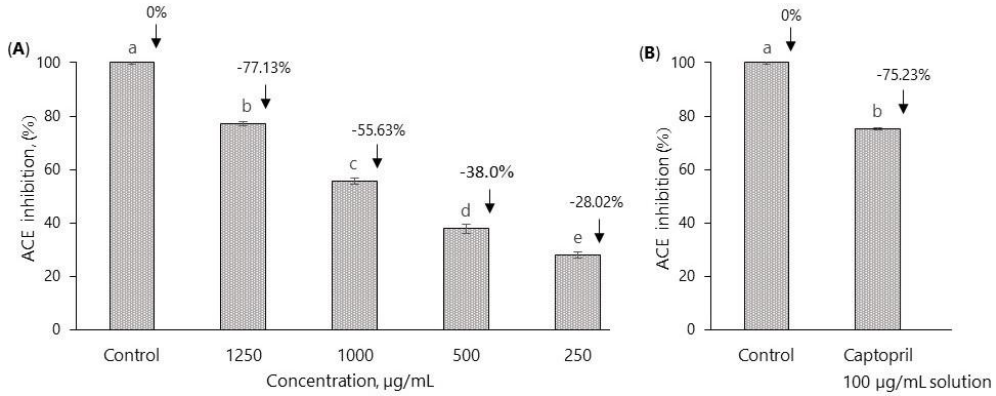

Figure 12. Effect of ethanol extract of *D. nipponica* Makino leaves on ACE activity (**A**) and impact of captopril aqueous solution on ACE activity (**B**) in enzymatic reaction systems *in vitro*

Significant difference between modeled enzyme reaction systems by Fisher's LSD test ($p < 0.05$) after unifactorial dispersion analysis ANOVA is indicated by different letters above the bars.The inhibitory capacity of the extract is marked with a negative sign in percentage expression above the columns, the enzymatic reaction system without the inhibitor is marked with 0 per cent above the columns. Mean values ± SE of three measurements are presented.

The obtained results showed that increasing the extract concentrations: < 250 µg/ml: 28.02 ± 1.17 per cent; 500 µg/mL: 38.0 ± 1.74 per cent; 1000 µg/mL: 55.63 ± 1.3 per cent; > 1250 µg/mL: 77.13 ± 0.78 per cent enzyme activity decreased accordingly. The change in the reduction of ACE activity was statistically significantly different ($p < 0.05$) compared to the control and increasing extract concentration: from ≥ 250 µg/ml to < to < 1250 µg/mL between the enzymatic reaction mixtures.

It has been proven that quercetin derivatives, tannins, peptides are capable of inhibiting ACE activity [52, 53, 54]. Considering the fact that compounds of these

classes were detected in plant preparations, it can be concluded that they had an effect on the reduction of ACE activity *in vitro*.

2.1.6.3. Evaluation of inhibition of *A. glycyphyllos* L. extracts on ACE enzyme

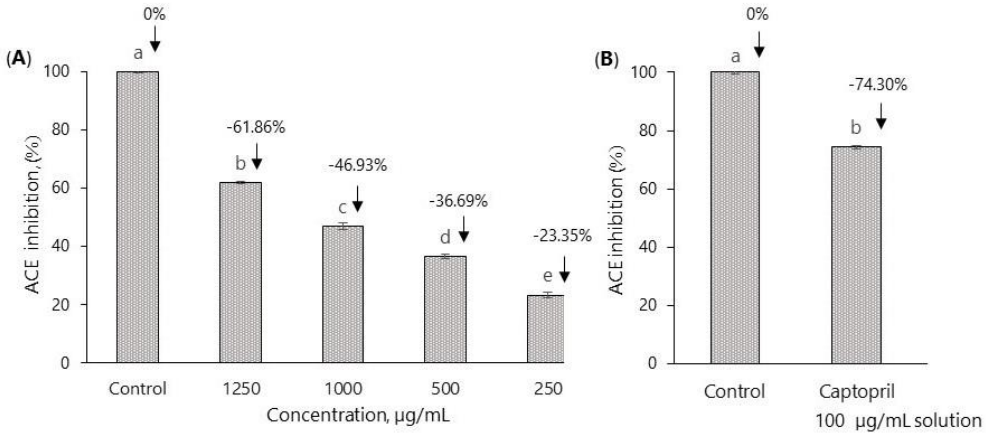

Figure 13. Effect of ethanol extract of *A. glycyphyllos* L. leaves on ACE activity (**A**) and impact of captopril aqueous solution on ACE activity (**B**) in enzymatic reaction systems *in vitro*

Significant difference between modeled enzyme reaction systems by Fisher's LSD test ($p < 0.05$) after unifactorial dispersion analysis ANOVA is indicated by different letters above the bars.The inhibitory capacity of the extract is marked with a negative sign in percentage expression above the columns, the enzymatic reaction system without the inhibitor is marked with 0 per cent above the columns. Mean values ± SE of three measurements are presented.

The obtained results showed that by increasing the extract concentrations: < 250 µg/ml: 23.35 ± 0.35 per cent; 500 µg/mL: 36.69 ±1.18 per cent; 1000 µg/mL: 46.93 ± 0.72; > 1250 µg/mL: 61.86 ± 1.08 per cent. enzyme activity decreased accordingly. The activity of the enzyme in the modeled enzyme reaction systems was statistically significantly different (**Fig. 13, (A))** when the extract concentration was ≥ 250 per cent to < 1250 µg/mL compared to control and among themselves.

2.1.7. Determination of acetylcholinesterase activity *in vitro*

2.1.7.1. Evaluation of inhibition of *D. caucasica* Lipsky extracts on AChE enzyme

To investigate the potential inhibitory effect of *D. caucasica* leaf extracts on AChE catalysis, dry powdered leaves of *Dioscorea caucasica* were dissolved in ethanol (70 per cent, C_2H_5OH). Under the influence of inhibitors (25–100 µg/mL), all enzymatic reaction mixtures showed a decrease in AChE activity. The obtained results are shown in **Table 9.**

Table 9. Effect of ethanolic extracts of *D. caucasica* Lipsky leaf on AChE activity

Conc. of extract, sol., µg/ml	Specific activity, µM/min/mg protein	AChE inhibition, per cent
25	0.1 ± 0.002^{b}	27.55
40	0.082 ± 0.002^{c}	40.58
50	0.078 ± 0.002^{d}	43.48
80	0.076 ± 0.002^{e}	45.31
100	0.074 ± 0.002^{e}	45.85
Control	0.138 ± 0.001^{a}	-

Significant difference between modeled enzyme reaction systems by Fisher LSD test ($p < 0.05$) after unifactorial dispersive analysis ANOVA is indicated by different letters. Mean values ± SE of three measurements are presented.

It can be observed that the inhibitor at the concentration of 25 µg/mL slightly (>30 per cent) but statistically significantly reduced the activity of the enzyme compared to the control. Increasing the inhibitor concentration to 40 µg/mL resulted in a 40.58 per cent decrease in enzyme activity compared to the control. However, increasing the inhibitor concentration from ≥ 50 µg/mL to <100 µg/mL resulted in an insignificant decrease in AChE activity of ≥ 43.48 to < 45.85 per cent compared to the control, respectively. It should be noted that increasing the extract concentration from ≥ 80 µg/mL to < 100 µg/mL did not change the enzyme activity and ranged from 45.31 to 45.85 per cent respectively. The maximum decrease in enzyme activity was achieved at 80 µg/mL extract concentration.

The inhibitory effect of *D. caucasica* leaf extracts on AChE enzyme was compared with 16 µg/mL donepezil hydrochloride solution. The positive control revealed a stronger inhibition of AChE (>50 per cent) than *D. caucasica* leaf extracts.

2.1.7.1.1. Kinetic modeling of enzymatic reactions

In this way, the amount of substrate for the enzymatic reaction system was selected in the range from 45 to 160 µM. According to the Lineweaver-Burk line graph, the change in reaction rate 1/V versus 1/[ATChI] is expressed by the following equations: (1) in the absence of an inhibitor in the reaction mixture: $y = 569.49 + 2.3524$, $r^2 = 0.9874$; (2) adding leaf ethanolic extract/inhibitor of 50 µg/mL concentration: $y = 483.75 + 6.7466$, $r^2 = 0.9755$.

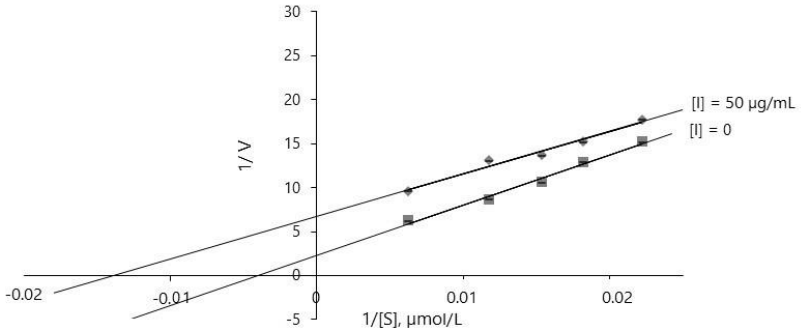

Figure 14. The alleged uncompetitive inhibition of AChE enzyme caused by *D. caucasica* Lipsky leaf extract

Mathematical model of the Lineweaver-Burk line graph for different concentrations of ATChI (45 – 160 µmol/L ATChI). The symbols on the graphic refers: squares (■): enzyme-substrate interaction in a model enzyme reaction system *in vitro*; rhombuses (♦): interaction of enzyme, substrate and inhibitor (50 µg/mL) in a model enzyme reaction system *in vitro*. Mean values ± SD of three measurements are presented.

D. caucasica leaf extract demonstrated the suggested uncompetitive inhibition on AChE (**Fig. 14**) enzyme. In accordance with Lineweaver-Burk line graph, in the absence of inhibitor V_{max} in the enzymatic reaction system, K_m values were 0.425 ± 0.11 µM/mg protein/min and 242.08 ± 15.76 µmol/L, respectively; whereas, in the presence of an inhibitor of 50 µg/mL concentration in the reaction system, $V_{max(app)}$, $K_{m(app)}$ values were significantly lower: 0.148 ± 0.015 µM/min/mg protein and 71.56 ± 6.55 µmol/L, accordingly. This model of inhibition suggests that the inhibitor binds only to the enzyme-substrate complex [7]. The inhibitory effect of *D. caucasica* extract on AChE enzyme was compared with donepezil hydrochloride aqueous solution. The positive control reduced the enzyme activity by ~70.62 ± 2 per cent at 16 µg/ml.

2.1.7.2. Evaluation of inhibition of *D. nipponica* Makino extracts on AChE enzyme

Taking into account the identified compounds in *D. caucasica* extracts and the obtained inhibitory capacity, inhibitor concentrations from ≥25 µg/mL to 80 µg/mL were chosen for AChE activity determination studies.

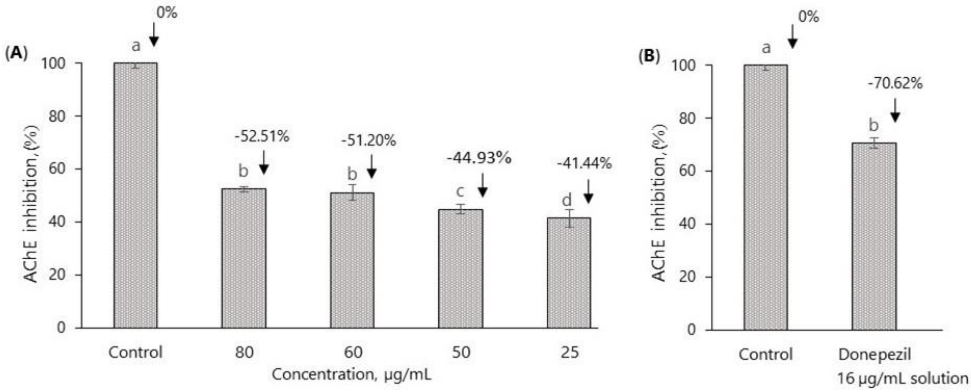

Figure 15. Effect of ethanolic extact of *D. nipponica* Makino leaves on AChE activity (**A**) and the impact of donepezil hydrochloride aqueous solution on AChE activity in enzymatic reaction systems *in vitro* (**B**)

Significant difference between modeled enzyme reaction systems by Fisher's LSD test ($p < 0.05$) after unifactorial dispersion analysis ANOVA is indicated by different letters above the bars.The inhibitory capacity of the extract is marked with a negative sign in percentage expression above the columns, the enzymatic reaction system without the inhibitor is marked with 0 per cent above the columns. Mean values ± SE of three measurements are presented.

Under the influence of inhibitors of different concentrations on the reaction mixtures of the enzymatic system, it is possible to regulate the course of the enzymatic reaction. Increasing the extract concentration decreased the activity of the enzyme accordingly: < 25 µg/mL: 41.44 ± 3.2 per cent; 50 µg/mL: 44.93 ± 1.9 per cent; > 60 µg/mL: 51.20 ± 2.9 per cent compared to the control and between reaction systems.

However, when increasing the extract concentration to 80 µg/mL, the change in enzyme reduction from the previous 60 µg/mL extract concentration was not statistically significantly different ($p > 0.05$) and exhibited 52.51 ± 0.9 per cent. decrease in enzyme activity. At the same time, it is understood that the specific activity of the enzyme decreased consistently in the following sequence: 25 µg/mL: 0.078 ± 0.019 µmol/mg protein/min >50 µg/mL: 0.074 ± 0.023 µmol/mg protein/min > 60 µg/ml: 0.066 ± 0.044 µmol/mg protein/min > 80 µg/mL: 0.064 ± 0.014 µmol/mg protein/min. From an enzymological point of view, the enzyme reaction system gradually became saturated with an inhibitor capable of reducing the activity of the enzyme.

Different concentrations of *D. nipponica* leaf extract inhibited AChE activity less than donepezil hydrochloride solution. The latter reduced the enzyme activity by ~70.62 ± 2.0 per cent at a concentration of 16 µg/mL. This data can imply that the AChE inhibitory effect of *D. nipponica* leaf extract was proved but not significantly lower than Donepezil HCl 70.62 ± 2.0 per cent.

Taking into account the ability of the studied dioscorea leaf extracts to inhibit the enzyme, it is observed that the inhibition caused by the *D. nipponica* leaf extract was slightly superior to that of the *D. caucasica* leaf extract. Although in the literature,

more attention is paid to compounds belonging to the class of alkaloids capable of reducing AChE activity, it has been proven that the compounds found in the extracts could influence the reduction of enzyme activity. This study demonstrates that the predominant compounds in dioscorea leaves are still undiscovered or insufficiently studied and can reduce AChE activity.

Data on the inhibitory properties of *Dioscorea* species and their plant preparations for the AChE enzyme could not be found. However, Tenfen et al. (2019) [55] in the performed research found that the inhibition of *Eugenia* genus leaf extracts on AChE was related to the compounds and amounts of isoquercitrin, quercetin, catechin, epicatechin and myricitrin. Several of these compounds were also detected in *Dioscorea caucasica* and *nipponica* leaf extracts, so it can be concluded that these compounds present in the extract had a significant effect on the reduction of enzyme activity.

2.1.7.2.1. Kinetic modeling of enzymatic reactions

The kinetics of the AChE inhibition reaction were modeled in the substrate concentration range from 41.25 to 110 µmol/L. According to the Lineweaver-Burk graph, the change in reaction rate 1/V versus 1/[ATChI] was expressed by the following equations: (1) in the absence of an inhibitor in the reaction mixture: $y = 447.92x + 2.577$, $r^2 = 0.9671$; (2) adding leaf ethanolic extract/inhibitor of 25 µg/mL concentration: $y = 451.863 + 7.5815$, $r^2 = 0.97955$; adding leaf ethanolic extract/inhibitor of 50 µg/mL concentration: $y = 447.02 + 9.4292$, $r^2 = 0.9541$.

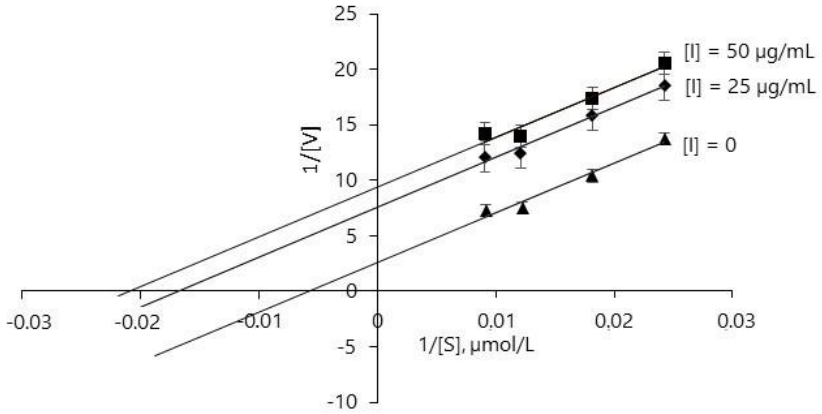

Figure 16. The alleged uncompetitive inhibition of AChE enzyme caused by *D. nipponica* Makino leaf extract

Mathematical model of the Lineweaver-Burk line graph for different concentrations of ATChI (41.25–110 µmol/L ATChI). The symbols on the graphic refers: triangles (▲): enzyme-substrate interaction in a model enzyme reaction system *in vitro*; rhombuses (♦): interaction of enzyme, substrate and inhibitor (25 µg/mL) in a model enzyme reaction system *in vitro*; squares (■): interaction of enzyme, substrate and inhibitor (50 µg/mL) in a model enzyme reaction system *in vitro*. Mean values ± SD of three measurements are presented.

$V_{max(app)}$ value after increasing extract concentration from 25 to and 50 µg/mL decreased from 0.132 ± 0.01 to 0.106 ± 0.01 µmol/mg protein/min. The $K_{m(app)}$ value also decreased, from 59.60 ± 0.06–47.407 µmol/L. These findings suggest, that the inhibitor can only bind to the enzyme-substrate complex [7, 56]. This behaviour can be explained by the *Le Chatlier's* principle stating that due the inhibitor effect, the equilibrium shifts towards forming more enzyme-substrate complexes and leads to a lower K_m, which indicates a higher binding affinity [56].

2.1.7.3. Kinetic modeling of enzymatic reaction investigating inhibition capacity of *A. glycyphyllos L.* leaf extract on AChE

To determine the effect of inhibitor, AChE activity was measured in the enzymatic system reaction mixture in the presence or absence of the stated concentration of inhibitor at varyng concentrations of substrate (**Fig. 17**)

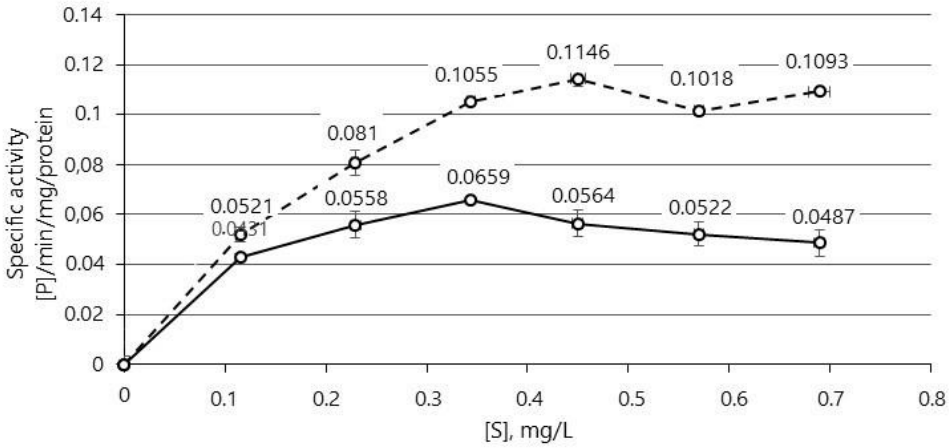

Figure 17. Effect of ethanolic extract of *A. glycyphyllos* L. leaves on AChE activity in enzymatic reaction systems *in vitro* with different concetrations of substrates (0,12 mM – 0,24 mM). Mean values ± SD of three measurements are presented.

As shown in Figure 4.2.4.17, the AChE gradually reached a plateau of maximal activity at 0.12 mM – 24 mM concentration of the substrat, which gradually decreased with the addition of *A. glycyphyllos* extract (190 µg/mL). The inhibitory activity of the samples obtained varied as follows: 55.41 ± 0.82 per cent (for substrate 24 mM) > 48.65 ± 0.6 per cent (for substrate 0.2 mM) ≈ 50.73 ± 0.73 per cent (for substrate 0.16 mM) > 36.1 ± 0.2 per cent (for substrate 0.12 mM) > 31.15 ± 0.65 per cent (for substrate 0.08 mM), 17.24 ± 0.2 per cent (for substrate 0.04 mM). The optimal model of the interaction between the enzyme and the substrate used during hydrolysis, followed by the model of the interaction between the enzyme, the substrate and the extract, showed that a substrate concentration of 0.16 mM was sufficient for 50 per cent inhibition to achieve.

As expected, in the kinetic modeling, A. *glycyphyllos* extract caused an decrease in the $V_{max(app)}$ and $K_{m(app)}$ values (0.0589 ± 0.001 μmoL/mg protein/min and 0.009470 ± 0.003 μmol/L) compared to V_{max} and K_m values in the absence of *A. glycyphyllos* extract (0.1436 ± 0.001 μmoL/mg protein/min and 0.05817 ± 0.002 μmol/L). Decreasing valuse of $V_{max(app)}$, $K_{m(app)}$ allow suggested that possible, that the inhibitor profil can only binds to the enzyme-substrate (ES) complex. (7, 56].

The results of the biological activity studies confirmed the data published in the scientific literature, that herbal preparations made from many species of plants of the genus of *Astragalus L.* and *Dioscorea L*. are capable of inhibiting physiologically important enzymes. The same tendency was revealed in this dissertation research paper, which investigated the inhibitory ability of extracts from the leaves and rhizomes of dioscorea caucasica and nipponica (*Dioscorea nipponica Makino* et *Dioscorea caucasica* Lipsky) and the extract from the leaves of Astragalus glycyphyllos (*Astragalus glycyphyllos L*.). After evaluating the inhibitory properties, it was determined that natural inhibitors reduced enzyme activity in model *in vitro* enzyme systems depending on the chosen concentration. Inhibitory capacity of *Dioscorea* spp. *nipponica*, *caucasica* leaf extracts is statistically notably stronger than rhizome extracts. The main difference between the composition of compounds in the leaves and rhizomes is the abundance of flavonoids, quercetin derivatives. A large variety of different forms of *O*-quercetin glycoside was found in the leaf extracts, and not in the rhizome extracts. The quantitative composition also differed. Accumulated amounts of hydroxycinnamic acids (chlorogenic and neochlorogenic) in *Dioscorea* spp. leaf extracts were significantly higher than in rhizome extracts.

In order to clarify the supposed type of inhibition of the extracts more precisely, kinetic measurements were performed. It was found that *Dioscorea* spp. *caucasica, nipponica*, leaf and *D. nipponica* rhizome extracts characterized mixed-type inhibition of α-glucosidase, *D. caucasica* exract of rhizome characterized competitive inhibition, *Astragalus glycyphyllos* leaf extract is a uncompetitive α-glucosidase inhibitor. Leaf extracts of all studied plant species characterized uncompetitive inhibiton on acetylcholinesterase enzyme.

CONCLUSIONS

1. The phytochemical composition of extracts from leaves and rhizomes of *D. nipponica*, *D. caucasica* and extracts from leaves of *A. glycyphyllos* has been investigated and it is shown that:
   1.1 Great diversity forms of *O*-quercetin glycoside have been identified in both leaves and rhizomes of *Dioscorea* spp. – quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-malonylhexoside, quercetin-3-*O*-acetylhexoside, quercetin-3-*O*-malonylrhamnoside, and quercetin-3-*O*-acetylrhamnoside.
   1.2 Isomers of hydroxycinnamic acids and hydroxy fatty acids have been identified in both leaves and rhizomes of *Dioscorea* spp.
   1.3 Steroidal saponins and their isomers have been identified in both rhizome and leaeves of *Dioscorea* spp.
   1.4 Higher amounts of hydroxycinnamic acids and their isomers were determined in the leaf extracts of *D. nipponica*, while the amount of quercetin-3-*O*-glucoside determined in the leaf extracts of both species was similar.
   1.5 The lowest amounts of chlorogenic acid and neochlorogenic acid were determined in *D. nipponica, caucasica* spp. in root extracts.
   1.6 In *A.glycyphyllos* leaf extract the dominant compounds were: succinic acid, azelaic acid, feruloylquinic acid, cumaroylquinic acid, uridine, and cycloartane-type isomers.
   1.7 For the first-time, uridine has been determined in *A.glycyphyllos* leaf extracts, hydroxydodecanoylcarnitine – in *D.caucasica, nipponica* root extracts, and in leaf extracts – quercetin-3-*O*-malonylhexoside, quercetin-3-*O*-acetylhexoside, quercetin-3-*O*-malonylrhamnoside, and quercetin-3-*O*-acetylrhamnoside.
2. A suitable enzyme/substrate/inhibitor interaction model was selected for the applied methodologies and the inhibitory capacity of the extracts were investigated. The applied mathematical modeling of the enzymatic reaction system is suitable for creating Lineweaver-Burk plot, determining kinetic parameters, and evaluating the type of inhibition.
3. The inhibition capacities of extracts were determined and after evaluating them, it was shown that all extracts inhibited enzymes activity in a dose–response manner. By using applied methodologies, it has been determined, that:
   3.1 The greatest decrease α-GLU activity was achieved at the concentration of 50 μg/mL (71.02 ± 1.69 per cent) of D.N leaf extract concentration; 100 μg/mL (78.49 ± 2.39 per cent) of D.C leaf extract concentration and 400 μg/mL (54.33 ± 0.7 per cent) of A.G leaf extract concentration.
   3.2 The greatest decrease in α-amylase activity was achieved at the concentration of 640 μg/mL (71.6 ± 1.0 per cent) of D.N leaf extract,

480 µg/mL (48.6 ± 2.2 per cent) of D.C leaf extract; and 800 µg/mL (71.72 ± 0.48 per cent) of A.G leaf extract concentration.

3.3 More than half of the ACE inhibition capacity was achieved with 1,000 µg/mL D.C / D.N leaf extracts content, A.G – with 1,250 µg/mL leaf extracts content.

3.4 The greatest in AChE reduction were achieved for 45.85 per cent with 100 µg/mL D.C leaf extract concentration and 52.51 ± 0.9 per cent with 80 µg/mL D.N leaf extract concentration.

3.5 Optimal model of enzyme-substrate interaction used during hydrolysis, then – enzyme-substrate-extract (*A.glycyphyllos* L.) interaction model showed, that 0.16 mM substrate concentration was sufficient to achieve 50 per cent inhibition.

3.6 The inhibition capacity of D.C and D.N rhizomes extract with 500 µg/mL concentration did not reach 50 per cent of α-GLU activity.

4. The values of the kinetic parameters were determined and after evaluating them, it was shown that:

4.1 *Dioscorea* spp. *caucasica, nipponica* leaf and *Dioscorea nipponica* rhizome extracts characterized mixed-type inhibition of α-glucosidase with *Ki* values of 5.35–11.44 µg/mL, 27.23–31.87 µg/mL, and 476.65 µg/mL, respectively.

4.2 *Dioscorea caucasica* extract of rhizoma characterized competitive of α-glucosidase inhibition with a K_i value of 165.97–304.32 µg/mL.

4.3 *Astragalus glycyphyllos* leaf extract characterized an uncompetitive of α-glucosidase inhibition with a K_i value of 0.195–0.25 µg/mL.

4.4 All of the species extracts of leaf characterized uncompetitive inhibition of acetylcholinesterase.

5. After evaluating the inhibitory properties of extracts of *Dioscorea* L., *Astragalus* L. plant raw materials, theoretically formulated assumption that extracts contain natural compounds capable of binding with enzymes exhibiting hydrolase activity and thus reduce their activity. It is theoretically reasonable that this is influenced by:

5.1 Hydroxycinnamic acids and their isomers, *O*-quercetin glycoside forms were determined in extracts of *D. nipponica*, *D. caucasica* leaves.

5.2 Peptides, catechin were determined in extracts of *D. nipponica*.

5.3 Hydroxycinnamic acids and their isomers were determined in extract of *A. glycyphyllos* leaves.

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PADĖKA

Nuoširdžiai dėkoju darbo vadovui prof. dr. Petrui Rimantui Venskutoniui už suteiktą galimybę atlikti darbą ir tobulėti, už vertingus patarimus darbo eigoje, už pastabumą ir kantrybę. Dėkoju Cheminės technologijos fakulteto, Maisto mokslo ir technologijos kateros doc. dr. R. Degutytei ir Vytauto Didžiojo universiteto, instrumentinės analizės atviros prieigos centro doc. dr. M. Stankevičiui už kruopštų šio darbo peržiūrėjimą, už tikslias pastabas ir vertingus pasiūlymus. Ačiū artimiesiems už kantrybę.

Aušra Adomėnienė

DIOSCOREA L. IR *ASTRAGALUS* L. AUGALŲ RŪŠIŲ EKSTRAKTŲ FITOCHEMINĖS SUDĖTIES IR FIZIOLOGIŠKAI SVARBIŲ FERMENTŲ INHIBAVIMO ĮVERTINIMAS

SANTRAUKA

TEMOS AKTUALUMAS

Tiriant vaistinių ir kitų augalų fitocheminę sudėtį bei biologines savybes sukaupiama vis daugiau mokslinės informacijos apie naujus ir/ar žinomus augalinius ruošinius[1], galinčius turėti teigiamą fiziologinį poveikį įvairioms žmogaus organizmo funkcijoms. Tokia mokslinė informacija ženkliai prisideda kuriant ir gaminant ingredientus aukštesnės biologinės vertės maisto produktams, farmacijos preparatams ir kosmetikos gaminiams. Per pastaruosius kelis dešimtmečius ypač plačiai buvo tiriamas antioksidantinis įvairių augalų potencialas ir jį sukuriančios fitocheminės medžiagos, sparčiai vystėsi ir kitų sveikatai naudingų savybių tyrimai – vėžinių ląstelių proliferacijos slopinimas, antimikrobinis, priešuždegiminis ir kitoks aktyvumas. Šių tyrimų dėka gaunami svarbūs duomenys naujiems maistinių medžiagų sveikumo teiginiams pagrįsti, moksliškai patvirtinamos dažnai empirinės tradicinės medicinos žinios, praplečiamos neigiamų fiziologinių procesų bei pokyčių kontrolės ir pakoregavimo galimybės žmogaus organizme taikant inovatyvius mitybos sprendimus.

Medicinos moksle seniai įrodyta daugelio fermentų aktyvumo kontrolės svarba. Pavyzdžiui, pripažįstama, kad angliavandenių apykaitos sutrikimo, demencijos, padidėjusio kraujospūdžio ir kai kurių kitų sveikatos problemų atvejais tam tikrų hidrolazės fermentų slopinimas[2] yra svarbus moduliuojant fiziologines funkcijas žmogaus organizme. Pripažįstama ir tai, kad prie šių neigiamų fiziologinių funkcijų prevencijos, rizikos sumažinimo bei kompleksinės terapijos gali reikšmingai prisidėti ir efektyvus vaistiniuose ir kituose augaluose esančių fitocheminių medžiagų, pasižyminčių inhibitoriniu poveikiu, panaudojimas. Tam, kad tokie augalai galėtų būti tikslingai pritaikomi įvairių ligų prevencijai, reikia patikimais ir šiuolaikiškais moksliniais tyrimais gauti duomenų apie jų fitocheminę sudėtį ir biologines savybes. Todėl kompleksinis įvairių augalų sudėties bei biologinių savybių įvertinimas yra perspektyvi tyrimo sritis.

Tradicinei kinų medicinai gerai žinomos kai kurios Dioskorėjos (*Dioscorea* spp.) ir Kulkšnės (*Astragalus* spp.) genčių augalų rūšys, kurių naudą sveikatai pastaruoju metu teigiamai įvertino ir daugelio vakarų šalių medicinos specialistai.

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[1] Augaliniai ruošiniai – tai ruošiniai, išgauti ekstrahuojant, distiliuojant, spaudžiant, frakcionuojant, gryninant, koncentruojant arba fermentuojant augalines medžiagas: sutrintos ar susmulkintos į miltelius augalinės medžiagos, tinktūros, ekstraktai, eteriniai aliejai, išspaustos sultys ir perdirbti eksudatai [52]

[2] Žodžiai *inhibitorius, slopiklis* yra sinonimai (*aut.past.*)

Sveikatos stiprinimo, padidintos biologinės vertės maisto produktų ir maisto papildų kūrimo ir gydymo tikslais skirtingos šių genčių augalų anatominės dalys naudojamos išdžiovintų vaistažolių, miltelių, ekstraktų ir kitais pavidalais.

Kai kurie *Astragalus* L. genties augalai yra žinomi kaip vertingas polisacharidų, izoflavonoidų ir cikloartano tipo saponinų šaltinis. *Dioscorea* L. genties augalų šakniastiebiai su šaknimis[3] vertinami dėl juose esančių steroidinių saponinų, ypač diosgenino, kuris farmacijos pramonėje buvo panaudotas kaip cheminis modelis kuriant hormoninius vaistus. Dėl to, kai kuriose šalyse dauguma tyrimų iki šiol tebėra sutelkti į naujų ir/ar žinomų saponinų nustatymą dioskorėjų šakniastiebiuose su šaknimis (lot. *Rhizoma cum radicibus Dioscoreae*), tuo tarpu antžeminės augalų dalys tirtos daug mažiau. Todėl trūksta mokslinės informacijos apie antžeminių augalo dalių biologiškai aktyvių junginių sudėtį ir biologines savybes.

Išsamiai susipažinus ir įvertinus šiuo metu turimą mokslinę informaciją, paaiškėjo, kad nepakankamai ir nesistemingai ištirtos kaukazinės dioskorėjos (*D. caucasica* Lipsky) ir niponinės dioskorėjos (*Dioscorea nipponica* Makino) augalų rūšys, ypač jų antžeminės dalys. Šios rūšys yra daugiausiai paplitę tropinio ir subtropinio klimato juostose, tačiau aptinkamos ir vidutinio klimato juostos srityse. Lietuvoje šios rūšys savaime neauga, tačiau jau gana seniai auginamos Vytauto Didžiojo universiteto Kauno botanikos sodo vaistinių augalų kolekcijoje. Taip pat iki šiol nebuvo tirta Lietuvos botanikos soduose auginamų *Dioscorea* L. genties augalų rūšių fitocheminė sudėtis, antioksidantinės ir biologinės savybės.

Saldžialapė kulkšnė (*Astragalus glycyphyllos* L.) yra natūraliai auganti ir plačiai paplitusi Europos vidutinio klimato juostos regionuose ir dažniausiai auganti drėgnose buveinėse arba pievose. Mokslinių tyrimų rezultatų apie šį augalą, lyginant su niponine ir kaukazine dioskorėja, paskelbtais daugiau. Tirtos, Lietuvoje savaime augančios saldžialapės kulkšnės skirtingų augalo dalių antioksidantinės savybės ir fitocheminė sudėtis, tačiau biologinės savybės, netirtos.

Remiantis išsamiais mokslinės, etnofarmakologinės ir kitokios informacijos apžvalga bei įvertinimu buvo suformuluota pagrindinė šio disertacinio darbo hipotezė: tyrimams pasirinktų augalų *D. caucasica*, *D. nipponica* ir *A. glycyphyllos* skirtingų anatominių dalių sudėtyje gali būti vertingų ir šiuose augaluose mažai iširtų fitocheminių junginių, kurie gali pasižymėti įvairiu bioaktyvumu, tarp jų ir kryptingu poveikiu fiziologiškai svarbių fermentų – α-amilazės, α-gliukozidazės, angiotenziną konvertuojančio fermento ir acetilcholinesterazės aktyvumui. Todėl atlikus sisteminius pasirinktų augalų fitocheminės sudėties ir inhibitorinių savybių tyrimus, galima tikėtis platesnio tokių augalų pritaikymo naujų sveikatai naudingų funkcinio maisto produktų ir maisto papildų sukūrimui.

Darbo tikslas – įvertinti *Dioscorea* L. ir *Astragalus* L. augalų rūšių fitocheminę sudėtį bei nustatyti iš jų išskirtų ekstraktų poveikį fiziologiškai svarbiems hidrolaziniu aktyvumu pasižymintiems fermentams.

[3] Žodžiai *šakniastiebiai su šaknimis, šakniastiebiai*, šaknys vartojami sinonimiškai (*aut.past.*)

Darbo tikslui pasiekti buvo iškelti šie uždaviniai:

1. Ištirti ir įvertinti *Dioscorea nipponica*, *D. caucasica* ir *Astragalus glycyphyllos* L. ekstraktų fitocheminių junginių sudėtį, taikant chromatografijos ir masių spektrometrijos metodus.
2. Pritaikyti žinomus *in vitro* fermentų inhibicijos metodus tiriamų augalų ekstraktų poveikio fermentams įvertinimui.
3. Įvertinti skirtingų augalų ekstraktų koncentracijų įtaką α-gliukozidazės, α-amilazės, angiotenziną konvertuojančio fermento ir acetilcholinesterazės aktyvumui.
4. Nustatyti α-gliukozidazės ir acetilcholinesterazės inhibicijos kinetinius parametrus, įvertinant substrato ir inhibitoriaus sąveikas modelinėse fermentinės reakcijos sistemose *in vitro* ir nustatyti ekstraktų tariamąjį inhibicijos tipą šiems fermentams.
5. Teoretiškai pagrįsti galimus ryšius tarp ekstraktuose esančių bioaktyvių junginių ir fermentų inhibitorinio poveikio.

Mokslinio darbo naujumas

1. Šiame darbe pirmą kartą atlikti sisteminiai *D. nipponica* Makino ir *D. caucasica* Lipsky augalų rūšių, skirtingų anatominių dalių, fitocheminės sudėties tyrimai efektyviosios skysčių chromatografijos (ESC) ir hibridinės didelės raiškos masių spektrometrijos (UESC-Q-TOF MS/MS) metodais; gauta informacija ženkliai papildo turimas žinias apie šiuose augaluose esančius skirtingus *O*-kvercetino glikozidų darinius, hidroksicinamono rūgštis (3-*O*-kafeoilchino, 5-*O*-kafeoilchino, 4-*O*-kafeolichino, kafeoiltreono, kumaroilchino, kafeoilšikimo ir feruloilchino) ir hidroksiriebalų rūgštis. Pirmą kartą *D. nipponica* ir *D. causasica* lapų ekstraktuose nustatyti kai kurie kvercetino dariniai ir hidroksicinamono rūgščių izomerai. Pirmą kartą kaukazinės dioskorėjos šakniastiebiuose nustatyta piscido rūgštis, abiejų dioskorėjų šakniastiebiuose nustatytas itin retai aptinkamas augaluose acetilkarnitino ir lauro rūgšties esteris – hidroksidodekanoilkarnitinas, o saldžialapės kulkšnės lapų ekstrakte – uridinas.
2. Pirmą kartą atlikti *D. nipponica* ir *D. caucasica* lapų ir šakniastiebių, o taip pat *A. glycyphyllos* lapų ekstraktų inhibicinio poveikio, fiziologiškai svarbiems fermentams (α-amilazės, α-gliukozidazės, angiotenziną konvertuojančio fermento ir acetilcholinesterazės), tyrimai.
3. Taikant ekstraktų, substratų ir fermentų reakcijos kinetikos modelines sistemas, pirmą kartą nustatytas ekstraktų slopinimo tipas α-gliukozidazės ir acetilcholinesterazės fermentams.
4. Teoriškai suformuluotos prielaidos apie galimus ryšius tarp *Dioscorea* spp. lapų ir šaknų ekstraktų fitocheminės sudėties ypatumų ir su jais susijusių α-gliukozidazės slopinimo gebos skirtumų.

5. Atlikti sisteminiai augalų fitocheminės sudėties ir ekstraktų inhibavimo savybių tyrimai reikšmingai praplečia mokslines žinias apie šiuos augalus ir sudaro papildomas prielaidas platesniam jų praktiniam pritaikymui.

Darbo praktinė vertė

Gauti duomenys apie *D. caucasica, D. nipponica* ir *A. glycyphyllos* fitocheminę sudėtį ir ekstraktų inhibitorines savybes suteikia svarbią informaciją, kuri reikalinga naujų funkcinių preparatų iš šių augalų sukūrimui ir galimo jų fiziologinio poveikio pagrindimui. Tokie preparatai – iš skirtingų anatominių dalių išskirti ekstraktai ir jų frakcijos galėtų būti pritaikomi naujų maisto papildų ir naujų padidintos mitybinės vertės funkcinio maisto produktų sukūrimui. Be to, gauti duomenys gali pasitarnauti prognozuojant maisto produktų, kurių sudėtyje yra tirti augalai, naudą žmonių sveikatai, pvz. kaip švelni nevaistinė antidiabetinė priemonė inhibuojanti α-gliukozidazės bei α-amilazės aktyvumą ir mažinanti glikeminį indeksą po krakmolingo maisto vartojimo. Kadangi antžeminių augalų dalių ekstraktai pasižymėjo neblogu angiotenziną konvertuojančio fermento inhibitoriniu poveikiu, iš jų pagaminti preparatai galėtų būti rekomenduojami nedidelės hipertenzijos kontrolei maisto ir/ar maisto papildais.

Be to, gauti rezultatai skatina tobulinti ir plėsti tyrimus su pasirinktais augalais. Šios krypties tyrimai galėtų būti tęsiami frakcionuojant ekstraktus, išskiriant efektyviausius junginius, ištiriant jų aktyvumą įvairiais glaudžiau susietais su biologinėmis sistemomis *in vitro* metodais. Toliau tyrimai turėtų būti tęsiami su gyvūnais (*in vivo*) ir baigiamojoje stadijoje su žmonėmis (klinikiniai). Atlikus tokius tyrimus galima tikėtis sudaryti pakankamai išsamų duomenų paketą sveikumo teiginiams.

Darbo apimtis ir struktūra

Disertaciją sudaro įvadas, trys skyriai (literatūros apžvalga, tyrimų objektas ir metodai, tyrimų rezultatai ir jų aptarimas ir bendrosios išvados). Bendra apimtis – 186 puslapiai. Darbe pateikti 44 paveikslai ir 28 lentelės. Rašant disertaciją naudotasi 187 literatūros šaltiniais. Disertacija parašyta lietuvių kalba.

Ginamieji disertacijos teiginiai

1. Tyrimams pasirinktų augalų *D. caucasica, D. nipponica* ir *A. glycyphyllos* skirtingų anatominių dalių sudėtyje yra vertingų ir šiuose augaluose mažai ištirtų fitocheminių junginių, kurie pasižymi įvairiu bioaktyvumu, tarp jų ir kryptingu poveikiu fiziologiškai svarbių fermentų – α-amilazės, α-gliukozidazės, angiotenziną konvertuojančio fermento ir acetilcholinesterazės aktyvumui.
2. Atlikus sisteminius pasirinktų augalų fitocheminės sudėties ir inhibitorinių savybių tyrimus, galima tikėtis platesnio tokių augalų pritaikymo naujų sveikatai naudingų funkcinio maisto produktų ir maisto papildų sukūrimui.

IŠVADOS

1. Ištirta *D. nipponica*, D.*caucasica* ekstraktų iš lapų ir šaknų bei A. *glycyphyllos* ekstraktų iš lapų fitocheminė sudėtis ir parodyta, kad:
   1.1. *D. nipponica, D.caucasica* lapų ekstraktuose dominuoja didelė įvairovė *O*-kvercetino glikozido formų – kvercetin-3-*O*-rutinozidas, kvercetin-3-*O*-gliukozidas, kvercetin-3-*O*-malonilheksozidas, kvercetin-3-*O*-acetilheksozidas, kvercetin-3-*O*-malonilramnozidas ir kvercetin-3-*O*-acetilramnozidas;
   1.2. *Dioscorea* spp. lapų ekstraktuose dominuoja hidroksicinamono rūgštys ir jų izomerai bei hidroksiriebalų rūgštys;
   1.3. tiek *Dioscorea* spp. šakniastiebių, tiek *Dioscorea* spp. lapų ekstraktuose nustatyti steroidiniai saponinai ir jų izomerai;
   1.4. didesni hidroksicinamono rūgščių ir jų izomerų kiekiai nustatyti niponinės dioskorėjos lapų ekstraktuose, o kvercetin-3-*O*-gliukozido nustatytas kiekis abiejų rūšių lapų ekstraktuose panašus;
   1.5. *D. nipponica, caucasica* spp. šaknų ekstraktuose nustatytas mažiausias chlorogeno rūgšties ir neochlorogeno rūgšties kiekis;
   1.6. saldžialapės kulkšnės, *A.glycyphyllos* L., lapų ekstrakte nustatyta gintaro, azelaino rūgštys; kumaroilchino, feruloilchino rūgštys bei cikloartano tipo izomerai;
   1.7. saldžialapės kulkšnės, *A.glycyphyllos* L., lapų ekstrakte pirmą kartą nustatytas uridinas, *D.caucasica, nipponica* šakniastiebių ekstraktuose – hidroksidodekanoilkarnitinas, lapų ekstraktuose – kvercetin-3-*O*-malonilheksozidas, kvercetin-3-*O*-acetilheksozidas, kvercetin-3-*O*-malonilramnozidas ir kvercetin-3-*O*-acetilramnozidas.
2. Taikytinom metodikom parinktas tinkamas fermento/substrato/slopiklio sąveikos modelis ir ištirtos ekstraktų slopinimo gebos savybės. Taikytas matematinis fermentinės reakcijos sistemos modeliavimas tinkamas Lainuiver'io-Berk'o tiesių grafikų sudarymui, kinetinių parametrų nustatymui bei inhibavimo tipo įvertinimui.
3. Nustatyta ekstraktų inhibicinė geba ir ją įvertinus parodyta, kad inhibicinės gebos savybes, slopinant hidrolazės fermentus, turi visi ekstraktai. Taikytinom metodikom nustatyta, kad:
   3.1. didžiausias α-GLU aktyvumo sumažėjimas pasiektas esant 50 µg/ml (71,02 ± 1,69 proc.) D.N lapų ekstrakto koncentracijai; 100 µg/ml (78,49 ± 2,39 proc.) D.C lapų ekstrakto koncentracijai ir 400 µg/ml (54,33 ± 0,7 proc.) A.G lapų ekstrakto koncentracijai;
   3.2. didžiausias α-amilazės aktyvumo sumažėjimas pasiektas esant 640 µg/ml (71,6 ± 1,0 proc.) D.N lapų ekstrakto koncentracijai, 480 µg/ml (48,6 ± 2,2 proc.) D.C lapų koncentracijai; ir 800 µg/ml (71,72 ± 0,48 proc.) A.G lapų ekstrakto koncentracijai;
   3.3. didesnė nei 50 proc. inhibicinė geba pasiekta su D.C / D.N lapų ekstraktais esant 1000 µg/ml koncentracijoms, o A.G lapų ekstrakto – 1250 µg/ml koncentracijai;

3.4. didžiausias AChE aktyvumo sumažėjimas pasiektas esant 100 µg/ml (45,85 proc.) D.C lapų koncentracijai ir 80 µg/ml (52,51 ± 0,9 proc) D.N lapų koncentracijai;
3.5. optimalus hidrolizės metu naudojamo fermento ir substrato sąveikos modelis, po to – fermento, substrato ir ekstrakto sąveikos modelis parodė, kad 0,16 mM substrato koncentracijos užteko 50 proc. inhibicijai pasiekti;
3.6. D.C ir D.N šakniastiebių ekstraktų (500 µg/ml) inhibicinė geba nesiekė 50 procentų.

4. Nustatytos kinetinių parametrų vertės ir jas įvertinus parodyta, kad:
4.1 *Dioscorea* spp. *caucasica, nipponica,* lapų ir *Dioscorea nipponica* šaknų ekstraktamas būdingas tariamasis mišrus inhibavimo tipas α-gliukozidazės fermentui, kurių *Ki* vertė 5,35 – 11,44 µg/ml, 27,23 – 31,87 µg/ml ir 476,65 µg/ml, atitinkamai;
4.2 *Dioscorea caucasica* šaknų ekstraktui būdingas tariamasis konkurencinis inhibavimo tipas α-gliukozidazės fermentui, kurio *Ki* vertė 165,97 – 304,32 µg/ml;
4.3 *Astragalus glycyphyllos* lapų ekstraktui būdingas tariamasis bekonkurencinis inhibavimo tipas α-gliukozidazės fermentui, kurio K_i vertė yra 0,195 – 0,25 µg/ml;
4.4 visų tirtų rūšių lapų ekstraktams būdingas tariamasis bekonkurencinis inhibavimo tipas acetilcholinestreazės fermentui.

5. Įvertinus *Dioscorea* L., *Astragalus* L. augalinių žaliavų ekstraktų inhibicinės gebos savybes, teoriškai suformuluota prielaida, jog ekstraktų sudėtyje yra gamtinių junginių gebančių jungtis su hidrolaziniu aktyvumu pasižyminčiais fermentais ir taip mažinti jų aktyvumą. Teoriškai pagrįsta, kad tam įtakos turi:
5.1 hidroksicinamono rūgštys ir jų izomerai, *O*-kvercetino glikozido formos nustatytos *D. nipponica, D.caucasica* lapų ekstraktuose;
5.2 peptidai, katechinai nustatyti *D. nipponica* ekstraktuose;
5.3 hidroksicinamono rūgštys ir jų izomerai nustatyti *A. glycyphyllos* lapų ekstraktuose.