Development of the Chemical Composition of Raspberry Shoot Extract Using Theoretical and Experimental Methods based on Ionization Theory

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ABSTRACT

The aim was to develop chemical composition of raspberry shoot extract using theoretical and experimental methods based on ionization theory. The quantification of phenolic compounds was accomplished through HPLC, the content of organic and phenolcarboxylic acids was determined by GC, molecular docking of the cyclooxygenase-2 (COX-2), phospholipase A2. nuclear factor kB (NF-kB), 5-lypoxygenase (5-LOX), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase, xanthine oxydase enzymes was carried out using the AutoDockTools 1.5.6 software, the anti-inflammatory activity was studied with the carrageenan edema method. The 11 compounds were identified by the HPLC and 36 compounds were detected by GC. The epicatechin (882.00 mg/100 g), (+)-catechin (480.00 mg/100 g), ellagic acid and its derivatives (459.00 mg/100 g), citric acid (49.21 mg/100 g), vanillic acid (2.59 mg/100 g) and levulinic acid (64.67 mg/100 g) were dominated in the obtained extract of raspberry shoots. The free energy of (+)-catechin-anion, epicatechin-anion was higher than (+)-catechin and epicatechin for the active sites of COX-2. phospholipase A2. NF-kB, 5-LOX, NADPH oxidase, myeloperoxidase, xanthine oxidase enzymes. Treatment with arginine-ionized raspberry shoot extract at a dose of 6.5 and 13.0 mg/kg showed a significant reduction of paw edema after 1. 2. 3 and 4 hours by 89.6 and 53.3, 49.4 and 53.3, 40.6 and 45.7, 45.9 and 45.2% compared with the control group, respectively. It has been established that (+)-catechin anion and epicatechin anion have a higher level of affinity than non-ionized (+)-catechin and epicatechin for the active centers of enzymes. The ionized extract showed a significantly higher anti-inflammatory effect than the non-ionized extract. In addition, there was a matching of experimental and theoretical doses in the study of anti-inflammatory activity.

Keywords: Raspberry shoot, HPLC, GC-MS, Molecular docking, Anti-inflammatory activity, Ionization by arginine.

INTRODUCTION

In the present day, the primary means of combating inflammation includes both steroidal (such as prednisolone) and nonsteroidal (like diclofenac and indomethacin) medications, commonly used to address acute and chronic inflammatory conditions such as rheumatoid arthritis and osteoarthritis [1]. However, the use of anti-inflammatory drugs often leads to a plethora of side effects. For example, steroidal medications are associated with conditions like osteoporosis, adrenal atrophy, and suppression of the immune system. Non-steroidal drugs can induce bronchospasm and peptic ulcers, as they inhibit physiological and inflammatory prostaglandins [2]. Hence, there is a current emphasis on seeking out new anti-inflammatory compounds derived from herbal sources.

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There is a growing interest in plant-based medicines rich in flavan-3-ols, as they have shown potential beneficial effects in clinical trials targeting inflammatory-related diseases [3]. The primary source of flavan-3-ols is green tea leaves [6]. However, green tea leaves are not cultivated in Eastern Europe. Consequently, raspberry shoots have been identified as a promising alternative source of flavan-3-ols. Our previous study demonstrated that 80% of all phenolic compounds present in raspberry shoots are flavan-3-ols, with epicatechin and (+)-catechin being the predominant constituents [4]. In addition to flavan-3-ols, raspberry shoots are also rich in derivatives of ellagotannins.

The pharmacological activity of a substance largely depends on its chemical structure and bioavailability. However, it has been noted that certain weak organic acids and bases are less affected by changes in chemical structure and are instead influenced by the degree of ionization. These compounds undergo partial ionization within the physiological pH range. The molecular form of the substance is also significant, as the ionized form, due to its electrical charge, exhibits physical and chemical properties that differ from its uncharged conjugate form. This disparity directly affects distribution, absorption, and binding to target enzymes [5].

The modern approach to determining pharmacological dosages primarily relies on empirical studies. However, this method lacks precision as it disregards the quantity of molecules of the investigated substance and its ionization properties. Therefore, the objective of this study is to theoretically and practically justify the chemical composition and extraction technology required to obtain an anti-inflammatory extract from raspberry shoots.

MATERIALS AND METHODS

Plant material

The *Rubus idaeus (R. idaeus)* shoots were the object of the study, which were collected from places of its native cultivation. The material was collected in 2021 after the fruiting period in the vicinity of the village of Ternova, Kharkiv region (50.193116162220264. 36.66935288403296).

Reagents

Methanol (purchased from "Allchem"), trifluoroacetic acid (purchased from "Allchem"), chloroform (purchased from "Allchem"), sanguiin H-10 isomer 1 (≥98.0%). lambertianin C (≥98.0%), sanguiin H-6(≥98.0%), (+)-catechin (≥98.0%), (-)-epicatechin (≥98.0%), ellagic acid (≥98.0%), cyanidine-3-O-glucoside (≥98.0%), quercetin-3-O-glucurunide (≥98.0%), L-arginine (≥98.0%) from Sigma Aldrich Company.

Extraction procedure

A 250.0 (exact mass) g of *R. idaeus* shoots were grinded in the size 1-2 mm. The extraction was carried out one by 60% ethanol at the ratio of raw material/solvent 1/20 (*m/v*) on water bath at 80º C with a reflux condenser for one hour, the extraction was made two times. Following the cooling process, the solutions were filtered and concentrated to a final volume of 250 mL using a rotary evaporator at 40ºC under vacuum conditions than obtained extract was extracted by a chloroform with volume 125 mL for 15 min two times.

Experimental animals

The study involved 36 male rats of the outbred white strain, weighing between 180 and 220 grams. These rats were sourced from the vivarium of the National University of Pharmacy. Throughout the experiment, the rats were housed in macrolon boxes with five animals in each box. Rats had unrestricted access to water and food, which were provided on a daily basis. The bedding was replaced on a three-day cycle. The rats were maintained under specific conditions, including a temperature of 22±2°C, relative humidity of 60±5%, and a daily light cycle of 12 hours of light and 12 hours of darkness.

All procedures carried out during the study adhered

to the guidelines set by the National Institute of Health for the care and use of laboratory animals, as well as the European Council Directive on 24 November 1986 for the Care and Use of Laboratory Animals (86/609/EEC). The study protocol was approved by the Local Ethics Committee.

HPLC method of analysis

The chromatographic separation was carried out by Agilent Technology model 1100 chromatograph with 150 mm × 2.1 mm ZORBAX-SB C-18 column with granularity at a pore size 3.5 μm. Elution flow rate was 0.25 mL/min. All determinations were undertaken at 45 ºC. The mobile phase binary solvent system consisted of solvent A (0.6% trifluoroacetic acid) and solvent B (70% methanol) . All solvents utilized in the experiment underwent ultrasonic degassing and were subjected to 0.22 μm pore size membrane filtering. The sample injection volume was set at 2 μL, and detection occurred at wavelengths of 254. 280. The mobile phase gradient used was linear and followed the following profile: (Table 1). The concentrations of phenolic compounds in extract were calculated from standard curves using standard of individual compounds.

Table 1. Linear mobile phase gradient

Time, min	0.6% trifluoroacetic acid	70% methnol
0	92	8
8	62	38
24-29	0	100

GC method of analysis

The chromatographic separation of acids was carried out on gas chromatography-mass spectrometer 5973N/6890N MSD/DS «Agilent Technologies» (USA). The mass spectrometer detector is a quadrupole, the ionization method is electron impact (EI), the ionization energy is 70 eV. The full ion current recording mode was used for the analysis. A capillary column was used for distribution HP–INNOWAX (30 m × 250 μm). Stationary phase – INNOWAX; mobile phase – helium, gas flow rate – 1 ml/min; the temperature of the sample introduction heater is 250 °C; the temperature of the thermostat is programmable from 50 to 250 °C. The introduction of a sample of 2 μL into the chromatographic column was performed in the splitless mode (without flow distribution), which allows you to do this without loss of separation and significantly (up to 20 times) increase the sensitivity of the chromatography method. Sample injection speed – 1 mL/min, time – 0.2 min.

Molecular docking

A molecular docking study was conducted using the tool known as AutoDockTools 1.5.6 [6]. The preparation of the protein involved an optimization process, which included the removal of water and other atoms, followed by the addition of a polar hydrogen group. Autogrid was used to configure the grid coordinates (X, Y, and Z) on the binding site. Genetic algorithm parameters were applied for ligand interaction, with 10 runs of this criterion.

COX-2 (PDB ID: 1ddx), phospholipase A2 (PDB ID: 3hsw), 5-LOX (PDB: 2q7m), NF-kB (1svc), myeloperoxidase (PDB: 3f9p), xanthine oxidase (PDB: 1fiq), NADPH oxidase (PDB ID: 5o0X) structures were obtained from PDB database [7]. The resolution of 1ddx was 3.00 Å whereas 5o0X – 2.20 Å, 2q7m – 4.25 Å, 1svc – 2.60 Å, 3f9p – 2.93 Å, 1fiq – 2.50 Å. For docking experiment protein structure is selected if resolution above 2 Å. So, all proteins can be used for the experiment. The ligand structures of (+)-catechin (CID_9064), (-)-epicatechin (CID_72276), were obtained from PubChem database [8]. The ligand structures of (+)-catechin-anion, and (-)-epicatechin-anion were drawn by computer program ACD/ChemSketch. The active site of the docking protein was identified utilizing the Computed Atlas for Surface Topography of Proteins [9].

Anti-inflammatory activity

The anti-exudative activity of extract was studied on 36 white outbred male rats weighing 180-220 g, in which a model of acute inflammation induced by subplantar injection of 0.1 mL of 1% carrageenan (Fluka, Switzerland) into the right hind paw of rats, measurement of paw edema in rats was carried out after 1. 2. 3. 4 hours. [10]:

All animals were divided into 6 groups. The first group was control pathology (animals that were subplantarly administered solution of carrageenan and intragastrically administered with 0.5 ml/kg of distilled water). The second, third and fourth group - animals that were administered carrageenan solution subplantarly and the studied extract was administered intragastrically at a dose of 26.4 mg/kg, 6.5 mg/kg, 13.0 mg/kg, respectively. Animals of group 5 were administered intragastrically drugs of comparison against the background of the introduction of carrageenan: diclofenac sodium at a dose of 8 mg/kg; The 6 group was consisted of intact animals, which were administered 0.1 ml of saline subplantarly.

Results

HPLC and GC analysis

To develop optimal technologies for obtaining an extract with a high level of anti-inflammatory activity, first of all, it is necessary to conduct a qualitative and quantitative analysis of the chemical composition of the native extract of *R. idaeus* shoots. HPLC and GC methods were used for analysis of obtained extract. As a result of our research, we found that the extract of *R. idaeus* shoots contains the following groups of compounds: catechins, ellagitannins, organic (derivatives of mono-, di-, tricarboxylic and fatty acids) and phenolcarboxylic acids. The HPLC method was used to carry out a qualitative and quantitative analysis of phenolic compounds in the obtained extract of *R. idaeus* shoots. According to the results of the study, 11 compounds were identified (Fig. 1.). The total content of phenolic compounds in the obtained extract was 1906.00 mg/100 g of which flavan-3-ols (catechins) – 1362.00 mg/100 g (71.46% out of the total polyphenols), ellagitannins – 85.00 mg/100 g (4.46% out of the total polyphenols), ellagic acid derivatives – 459.00 mg/100 g (24.08% out of the total polyphenols) (Table 2).

Among flavan-3-ols, epicatechin dominates – 882.00±2.00 mg/100 g (46.28% out of the total polyphenols), and (+)-catechin – 480.00±5.00 mg/100 g (25.19% out of the total polyphenols). Among ellagitannins, 3 compounds were identified: sanguine H-10 isomer 1 – 3.00±0.50 mg/100 g (0.16% out of the total polyphenols), sanguine H-10 isomer 2 – 32.00±1.00 mg/100 g (1.69% out of the total polyphenols), lambertianin C – 10.00±1.00 mg/100 g (0.53% out of the total polyphenols) and sanguin H-6 – 40.00±1.00 mg/100 g (2.10% out of the total polyphenols) (Table 2).

As shown in Table 1. sanguine H-6 dominates among all ellagitannins, sanguine H-10 isomer 2 is in second place, and sanguine H-10 isomer 1 is in third place, and the lowest content was lambertianin C. The content of ellagic acid was 181.00±4.00 mg/100 g (0.50% of the total phenolic compounds). As can be seen from the above results, the content of ellagic acid and its derivatives is 81% higher than that of ellagitannins (Table 2).

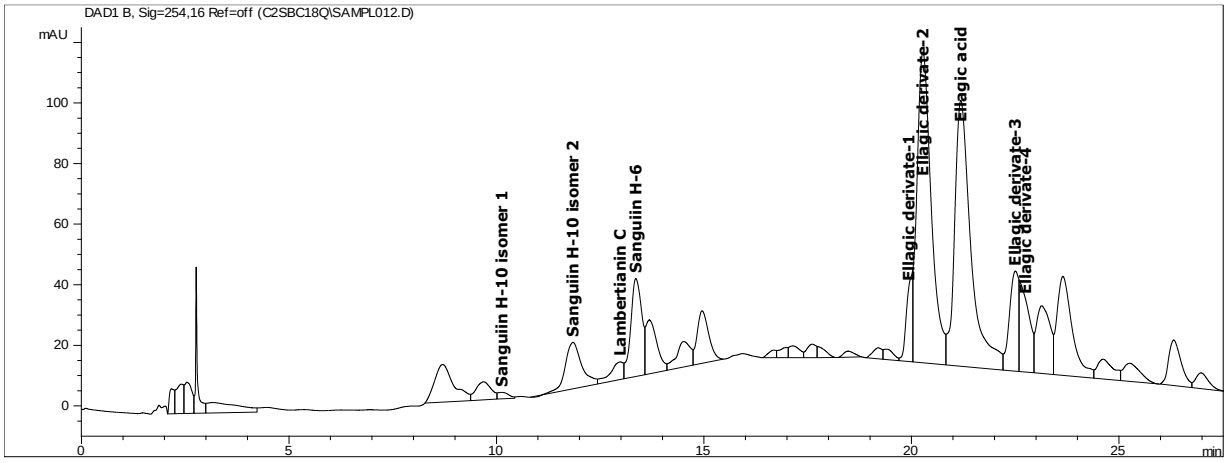

Figure 1. HPLC fingerprint (254 nm) of the *Rubus idaeous* shoots extract

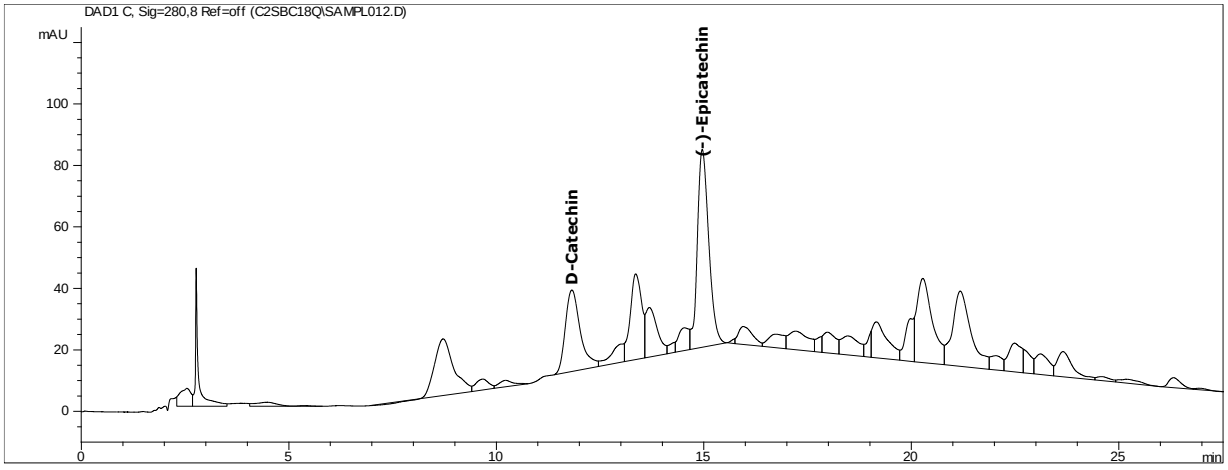

Figure 2. HPLC fingerprint (280 nm) of the *Rubus idaeous* shoots extract

Table 2. Qualitative composition and quantitative content of polyphenols in *Rubus idaeous* shoots extract

	Compound	Rt, min	Quantitative content, mg/100 g ± SD	% out of sum polyphenols
1	Sanguiin H-10 isomer 1	10.08	3.00±0.50	0.16
2	(+)-Catechin	11.89	480.00±5.0	25.19
3	Sanguiin H-10 isomer 2	11.91	32.00±1.0	1.69
4	Lambertianin C	12.91	10.00±1.0	0.53
5	Sanguiin H-6	13.38	40.00±1.0	2.10
6	(-)-Epicatechin	14.96	882.00±2.0	46.28
7	Ellagic acid derivatives 1	19.96	21.00±1.0	1.10
8	Ellagic acid derivatives 2	20.26	184.00±4.0	9.66
9	Ellagic acid	21.20	181.00±4.0	9.50
10	Ellagic acid derivatives 3	22.48	37.00±1.0	1.92
11	Ellagic acid derivatives 4	22.75	36.00±1.0	1.87
	Total content of identified compounds		1906.00	100

n:3, SD: Standard Deviation

Qualitative and quantitative analysis of organic, fatty and phenolcarboxylic acids was carried out using the GC method. Based on the results of the study, 36 compounds were identified. The total content of all acids was 163.61 mg/100 g, of which organic acids - 122.96 mg/100 g (39.62% of the total acids), phenolcarboxylic acids - 13.49 mg/100 g (4.10% of total acids), and fatty acids – 101.93 mg/100 g (56.26% of the total acids).

A total of 10 organic acids were identified, including 2 tricarboxylic acids (citric and iso-citric acid), 7 dicarboxylic acids (oxalic, malic, succinic, adipic, malonic, fumaric, glutaric acid) and 1 monocarboxylic acid (caproic acid). Among organic acids, citric acid dominates - 98.41 mg/100 g (30.02% of the total acids), and the lowest content was in caproic acid (0.41 mg/100 g (0.13% of the total acids)).

Among phenolcarboxylic acids, 8 compounds were identified, namely: vanillic, benzoic, ferulic, p-hydroxybenzoic, gentisic, lilac, salicylic and phenylacetic acid. Vanillic acid prevails among all phenolcarboxylic acids (5.18 mg/100 g (1.57% of the total acids)), in turn, phenylacetic acid is found in raspberry shoots in the smallest amount (0.21 mg/100 g (0 .06% of the amount of acids)).

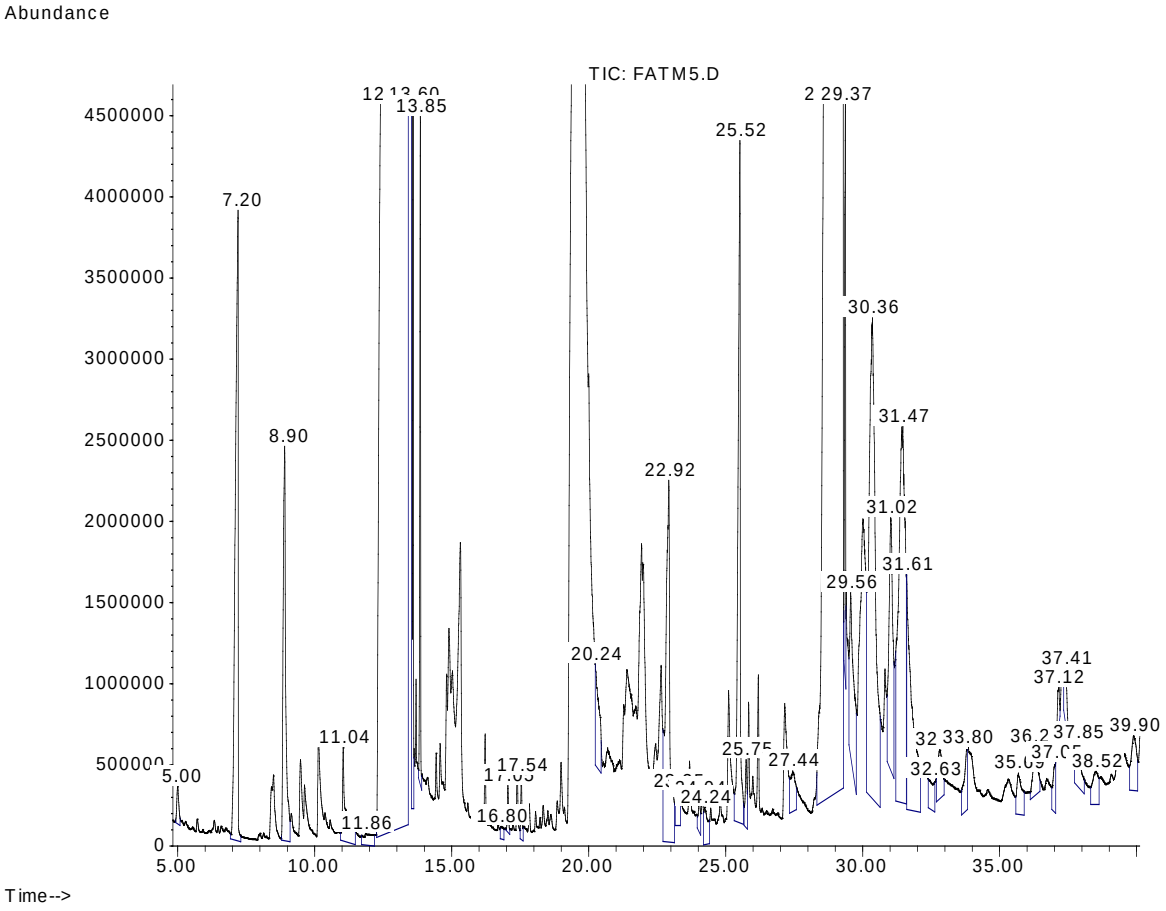

Figure 3. GC fingerprint of *R. idaeus* shoot extract

Table 3. Qualitative composition and quantitative content of organic (mono-, di-, tricarboxylic and fatty acids) and phenolcarboxylic acids in *R. idaeus* shoots extract

	Compound	Rt, min	Quantitative content in extract, mg/100 g ± SD	% out of sum acids
1	Citric acid	28.736	49.21±1.00	30.69
2	Malic acid	21.496	4.07±0.08	2.54
3	Succinic acid	13.812	3.32±0.08	2.07
4	Oxalic acid	8.959	2.76±0.08	1.72
5	Iso-citric acid	31.034	2.59±0.08	1.62
6	Glutaric acid	20.096	0.97±0.04	0.60
7	Malonic acid	11.394	0.95±0.04	0.59
8	Adipic acid	36.274	0.61±0.04	0.38
9	Fumaric acid	12.301	0.31±0.04	0.19
10	Caproic acid	5.056	0.21±0.04	0.13
	Total mono-, di-, tricarboxylic acids		**61.48**	38.34
11	Vanillic acid	31.664	2.59±0.08	1.62
12	Benzoic acid	14.076	1.51±0.08	0.94
13	Ferulic acid	39.815	0.79±0.04	0.49
14	*p*-hydroxybenzoic acid	36.935	0.61±0.04	0.38
15	Syringic acid	37.428	0.42±0.04	0.26
16	Gentisic acid	37.803	0.32±0.04	0.20
17	Salicylic acid	17.154	0.17±0.01	0.11
18	Phenylacetic acid	16.806	0.11±0.01	0.07
	Total phenolcarboxylic acids		**6.75**	4.21
19	Levulinic acid	12.689	64.47±1.00	40.21
20	Linoleic acid	30.294	8.50±0.08	5.30
21	Linolenic acid	31.580	6.80±0.08	4.24
22	Palmitic acid	25.433	4.06±0.08	2.53
23	Oleic acid	30.183	1.91±0.04	1.19
24	Stearic acid	30.061	1.68±0.04	1.05
25	Arachidic acid	32.659	0.64±0.04	0.40
26	Heneicosanoic acid	34.236	0.58±0.04	0.36
27	Behenic acid	35.601	0.58±0.04	0.36
28	Tetracosanoic acid	38.494	0.55±0.04	0.34
29	Heptadecanoic acid	26.410	0.54±0.04	0.34
30	2-hydroxypalmitic acid	32.043	0.43±0.04	0.27
31	Azelaic acid	24.764	0.41±0.04	0.26
32	Palmitoleic acid	25.776	0.34±0.04	0.21
33	Myristic acid	21.738	0.33±0.04	0.21
34	Lauric acid	17.634	0.22±0.01	0.14
35	Tricosanoic acid	37.173	0.21±0.01	0.13
36	Pentadecanoic acid	24.099	0.19±0.01	0.12
	Total Fatty acids		**92.11**	57.45
	TOTAL ACIDS		**160.34**	**100**

n:3, SD: Standard Deviation

Molecular docking

In recent research, it was carried molecular docking study of antioxidant and anti-inflammatory activity of identified phenolic compounds, organic and phenolcarboxylic acids. As a result, it was shown that biologically active compounds as organic acids (mono-, di-, tricarboxylic and fatty acids) and phenolcarboxylic acids are have not potential to suppress inflammatory and oxidative process. Therefore, these groups of compounds must be removed from the native extract. To do this, we used an organic solvent - chloroform, followed by acidification with sulfate acid to pH = 3 to destroy possible salts, extraction was carried out twice within 15 minutes. Thus, the new raspberry shoot extract contains catechin derivatives.

When studying the relationship between "structure and action," it is especially important and relevant to study the dependence of pharmacological activity on the structure of the compound. However, today, according to the available articles indexed in Scopus and Web of Science, there is a negligible amount of work on the study of the relationship between "structure and action" on the degree of ionization of a substance. Consequently, we decided to conduct theoretical and practical studies of the anti-inflammatory activity of the ionizing and non-ionizing forms of (+)-catechin and epicatechin.

The (+)-catechin, epicatechin, (+)-catechin-anion, epicatechin-anion were chosen for molecular modeling of the theoretical anti-inflammatory and antioxidant activity. (Fig. 3). Flavanol-3-ols were selected because their content was 71.46% out all phenolic compounds in the obtained extract.

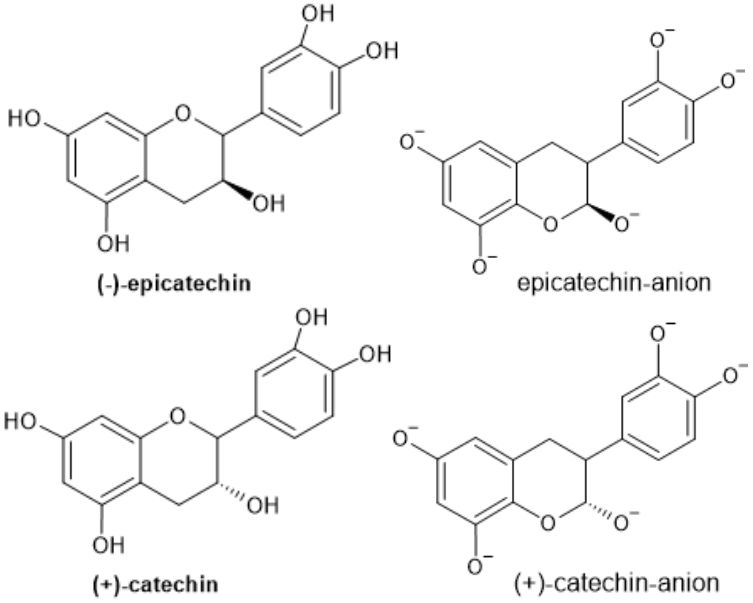

Figure 3. Structure of (+)-catechin, epicatechin, (+)-catechin-anion, epicatechin-anion

All studied compounds have a high level of affinity for the structure of the COX-2 enzyme. (+)-catechin-anion had the highest free energy value (-10.83 kcal/mol), followed by epicatechin-anion (-10.80 kcal/mol). When comparing the obtained results with the diclofenac sodium standard, the affinity of (+)-catechin-anion with the COX-2 active site was 88%, and in the case of epicatechin-anion, 87% more than that of diclofenac sodium, respectively. Comparing compounds with non-ionized form it was observed that the level of free energy of ionized form was

higher 29 and 50% for (+)-catechin and epicatechin, respectively. (Table 4).

All analyzed compounds have a high level of affinity for the structure of the Nf-kB enzyme. The first place was taken by (+)-catechin-anion (-5.92 kcal/mol), the second place epicatechin-anion (-5.99 kcal/mol) and the third one – epicatechin (-5.39 kcal/mol). When comparing the obtained results with the diclofenac sodium standard, the affinity of (+)-catechin-anion with the Nf-kB active site was 52%, and in the case of epicatechin-anion, 54% more than that of diclofenac sodium, respectively. Comparing compounds with non-ionized form it was observed that the level of free energy of ionized form was higher 19 and 10% for (+)-catechin and epicatechin, respectively. (Table 4).

The compounds have a high level of affinity for the structure of the 5-LOX enzyme. (+)-catechin-anion had the highest free energy value (-9.65 kcal/mol), followed by epicatechin-anion (-9.62 kcal/mol). When comparing the obtained results with the diclofenac sodium standard, the affinity of (+)-catechin-anion with the 5-LOX active site was 61%, and epicatechin-anion, 60% more than that of diclofenac sodium, respectively. Comparing compounds with non-ionized form it was observed that the level of free energy of ionized form was higher 53 and 17% for (+)-catechin and epicatechin, respectively. (Table 4).

The compounds have a high level of affinity for the structure of the phospholipase A2 enzyme. Epicatechin-anion (-9.86 kcal/mol) had the highest free energy value (-9.34 kcal/mol), followed by (+)-catechin-anion (-9.43 kcal/mol). When comparing the obtained results with the diclofenac sodium standard, the affinity of (+)-catechin-anion with the phospholipase A2 active site was 23%, and epicatechin-anion, 29% more than that of diclofenac sodium, respectively. Comparing compounds with non-ionized form it was observed that the level of free energy of ionized form was higher 1 and 9% for (+)-catechin and epicatechin, respectively. (Table 4)

Table 4. Results of molecular docking of the compounds identified by the HPLC in the *Rubus idaeous* shoots extract with the COX-2. NF-kB, 5-LOX, phospholipase A2 structures

Ligand	COX-2			NF-kB			5-LOX			Phospholipase A2		
	ΔGbind[a] (kcal/mol)	Ki[b](mmol)	K[c](mg/kg)	ΔGbind[a] (kcal/mol)	Ki[b](mmol)	K[c](mg/kg)	ΔGbind[a] (kcal/mol)	Ki[b](mmol)	K[c](mg/kg)	ΔGbind[a] (kcal/mol)	Ki[b](mmol)	K[c](mg/kg)
Epicatechin	-7.20	0.00526	0.55	-5.39	0.44131	42.64	-8.23	0.00092898	0.09	-9.01	0.0002495	0.02
Epicatechin-anion	-10.80	0.0000122	0.0011	-5.99	0.04054	3.82	-9.62	0.00008936	0.008	-9.86	0.0000596	0.0056
(+)-Catechin	-8.40	0.00070	0.10	-4.82	0.29324	28.0	-4.55	0.46333	44.0	-9.34	0.0001420	0.01
(+)-Catechin-anion	-10.83	0.0000116	0.0011	-5.92	0.04593	4.33	-9.65	0.00008506	0.008	-9.43	0.0001223	0.0054
Diclofenac sodium	-5.76	0.05977	5.85	-3.90	1.38	135.00	-6.00	0.03982	3.90	-7.65	0.00248	0.24

Notes: a – free-binding energy; b – inhibition constant, IC50; c – dose per kg rat weight, for 50% inhibition of the enzyme structure

All analyzed compounds have a high level of affinity for the structure of the NADPH oxidase enzyme. The first place was taken by (+)-epicatechin-anion (-10.72 kcal/mol), the second place (+)-catechin-anion (-7.26 kcal/mol) and the third one – epicatechin (-7.11 kcal/mol). When comparing the obtained results with the epigallocatechin-3-O-gallate standard, the affinity of (+)-catechin-anion with the NADPH oxidase site was 22%,

and in the case of epicatechin-anion, 72% more than that of epigallocatechin-3-O-gallate, respectively. Comparing compounds with non-ionized form it was observed that the level of free energy of ionized form was higher 10 and 44% for (+)-catechin and epicatechin, respectively. (Table 5).

The compounds have a high level of affinity for the structure of the myeloperoxidase enzyme. (+)-catechin-anion (-5.57 kcal/mol) had the highest free energy value, followed by epicatechin-anion (-6.36 kcal/mol). When comparing the obtained results with the epigallocatechin-3-O-gallate standard, the affinity of (+)-catechin-anion with the myeloperoxidase active site was 43%, and epicatechin-anion, 41% more than that of epigallocatechin-3-O-gallate, respectively. Comparing compounds with non-ionized form it was observed that the level of free energy of ionized form was higher 1 and 9% for (+)-catechin and epicatechin, respectively. (Table 5).

All analyzed compounds have a high level of affinity for the structure of the xantine oxidase enzyme. The first place was taken by (+)-catechin-anion (-7.88 kcal/mol), the second place (+)-epicatechin-anion (-7.83 kcal/mol) and the third one – (+)-catechin (-7.43 kcal/mol). When comparing the obtained results with the epigallocatechin-3-O-gallate standard, the affinity of (+)-catechin-anion with the xantine oxidase site was 8%, and in the case of epicatechin-anion, 7% more than that of epigallocatechin-3-O-gallate, respectively. Comparing compounds with non-ionized form it was observed that the level of free energy of ionized form was higher 6 and 9% for (+)-catechin and epicatechin, respectively. (Table 5).

Table 5. Results of molecular docking of the compounds identified by the HPLC in the *Rubus idaeous* shoots extract with the NADPH oxidase, myeloperoxidase, xanthine oxidase structures

Ligand	NADPH oxidase			Myeloperoxidase			Xanthine oxidase		
	ΔGbind[a] (kcal/mol)	Ki[b] (mmol)	K[c] (mg/kg)	ΔGbind[a] (kcal/mol)	Ki[b] (mmol)	K[c] (mg/kg)	ΔGbind[a] (kcal/mol)	Ki[b] (mmol)	K[c] (mg/kg)
Epicatechin	-7.11	0.00616	0.59	-5.04	0.20243	19.42	-7.21	0.00523	0.50
Epicatechin-anion	-10.27	0.00280	0.003	-6.36	0.02171	1.40	-7.83	0.00182	0.17
(+)-Catechin	-6.60	0.01455	1.40	-5.57	0.08306	7.97	-7.43	0.0036	0.35
(+)-Catechin-anion	-7.26	0.00478	0.45	-6.47	0.01797	1.69	-7.88	0.00169	0.16
Epigallocatechin-3-O-gallate	-5.97	0.04237	6.42	-4.52	0.48679	73.75	-7.30	0.00445	0.67

Notes: a – free-binding energy; b – inhibition constant, IC50; c – dose per kg rat weight, for 50% inhibition of the enzyme structure

Table 6 shows theoretical doses of ionized and non-ionized forms of (+)-catechin and epicatechin, that will be inhibit inflammation process 50 and 100%. According to obtain results it can be seen that a dose of ionized forms of catechin lower in 12 times, whereas epicatechin in 11 times than non-ionized forms.

Table 6. Results of calculation the total theoretical dose for inhibition inflammation process for epicatechin, epicatechin-anion, (+)-catechin, (+)-catechin-anion

Ligand	Epicatechin		Epicatechin-anion		(+)-Catechin		(+)-Catechin-anion	
	IC50 (mg/kg)	IC100 (mg/kg)	IC50 (mg/kg)	IC100 (mg/kg)	IC50 (mg/kg)	IC100 (mg/kg)	IC50 (mg/kg)	IC100 (mg/kg)
COX-2	0.50	1.00	0.0011	0.0022	0.10	0.20	0.0011	0.0022
NF-kB	42.64	85.24	3.82	7.64	28.0	56.00	4.33	8.66
5-LOX	0.09	0.18	0.008	0.016	44.0	88.0	0.008	0.016
Phospholipase A2	0.02	0.04	0.0056	0.0112	0.01	0.02	0.0054	0.0108
NADPH oxidase	0.59	1.18	0.003	0.006	1.40	2.80	0.45	0.90
Myeloperoxidase	19.42	38.84	2.05	4.10	7.97	15.94	1.69	3.38
Xanthine oxidase	0.50	1.00	0.17	0.34	0.35	0.70	0.16	0.32
TOTAL	**63.76**	**127.52**	**6.06**	**12.12**	**82.00**	**164.00**	**6.64**	**13.28**

Anti-inflammatory activity

In the mentioned above molecular docking results, we obtained theoretical doses for ionized and non-ionized (+)-catechin and epicatechin for complete or partial suppression of inflammation. To compare theoretical and practical results of the anti-inflammatory effect of ionized and non-ionized forms of flavan-3-ols, we conducted *in vivo* studies at three dose levels: 6.5 mg/kg (dose of ionized (+)-catechin and epicatechin for inflammation suppression at 50% levels), 13.0 mg/kg (dose of ionized (+)-catechin and epicatechin for inflammation suppression at 100% levels), and 26.4 mg/kg (dose of non-ionized catechin and epicatechin from the native *R. idaeus* shoot extract). Complete ionization of (+)-catechin and epicatechin was achieved by adding L-arginine until the pH=9.

The ionized *R. idaeus* shoot extract at a dose 13.0 mg/kg in rats significantly reduces paw edema by 59.6% at the first hour, then after edema decreased by 53.3%, 45.7%, and 45.2% at second, third and fourth hours, respectively. Treatment with *R. idaeus* shoot extract at a dose 6.5 mg/kg showed higher results compared to the treatment at a dose 13.0 mg/kg at the first hour, but in the other hours results were higher at dose 13.0 mg/kg. In the first hour, the paw edema of rat decreased by 89.6%, 49.4%, 40.6%, and 45.9% at the first, second, third and fourth hours, respectively. If compare obtained results with a standard diclofenac sodium at a dose 8 mg/kg we see that the treatment with diclofenac sodium significantly inferior to ionized *R. idaeus* shoot extracts. At a dose 6.5 mg/kg ionized extract suppresses paw edema better at 35%, 30%, 16%, and 24% than diclofenac sodium at the first, second, third and fourth hours, respectively. In the case of ionized extract at a dose 13.0 mg/kg reduced paw edema better at 3%, 40%, 22%, and 2% than diclofenac sodium treatment at the first, second, third, and fourth hours, respectively.

The obtained *R. idaeus* shoot extract at a dose 26.4 mg/kg inhibited inflammation process much lower than ionized *R. idaeus* extracts. The shoot ionized extract with dose 6.5 mg/kg inhibited inflammation was lower 15 and 4% than non-ionized extract at the third and fourth hours, respectively, whereas at the first and second hour suppressed paw edema 258 and 7% higher. In the treatment of paw edema in rats by ionized *R. idaeus* shoot extract at a dose 13.0 mg/kg suppressed edema significantly better than non-ionized extract at 138%, 15% and 84% at first, second, and fourth hours, respectively. But at third hour results was 5% lower compared with *R. idaeus* shoot extract at a dose 26.4 mg/kg. (Table 7)

Table 7. Results of determination of anti-inflammatory activity of the obtained *R. idaeus* shoots extracts ionized and non-ionized

Sample	Dose, mg/kg	% of edema inhibition compared to control ± SD			
		1 hour	2 hours	3 hours	4 hours
60% raspberry extract non-ionized	26.4	25.0±1.3	46.3±2.3	48.0±2.4	48.0±2.4
60% raspberry extract ionized by arginine	6.5	89.6±3.4	49.4±2.0	40.6±2.0	45.9±2.1
60% raspberry extract ionized by arginine	13.0	59.6±2.4	53.3±2.1	45.7±1.8	88.2±3.5
Diclofenac sodium	8.0	58.0±2.9	38.0±1.9	35.0±1.9	37.0±1.9

n:4, SD: Standard Deviation

DISCUSSION

HPLC and GC analysis

Ellagotannins and catechins are considered to be involved in plant defense mechanisms against insects like moths, viruses, bacteria, and herbivores. They achieve this by making the plant tissues unpalatable and non-nutritious, rendering them unsuitable as food sources [11]. A recent study of Krauze-Baranowska M. *et. al*. [12], they have studied the methanolic extract of *R. idaeus* shoots of cultivar "Willamette". They found that sum of polyphenols content was 2.39%, sanguiin H6 – 1.36%, ellagic acid – 0.29%, (+)-catechin – 0.03% and epicatechin – 0.02% in *R. idaeus* shoots extract. Compared to our results, in our research, the sum of polyphenols was in 20.25% lower, the content of sanguiin H6 was lower in 97% and ellagic acid was in 38% less. But, the content of (+)-catechin and epicatechin were higher in 94% and 98%, respectively. We can see that the content of catechins derivatives were dominated in our examined extract, whereas in case of compared extract the content of ellagotannins and ellagic acid were higher. The difference in the chemical composition may be related with the different cultivars and vegetative phase of plant.

Molecular docking

For obtaining the total theoretical dose of ionized and non-ionized (+)-catechin and epicatechin for inhibition the inflammation process at 50 and 100%, we calculated the sum of theoretical doses of each mentioned above enzymes. According to results, the theoretical dose of ionized (+)-catechin and epicatechin significantly lower than for non-ionized forms. As result, it is showed importance of choosing the pH level in the case of calculation the dose for assessing the pharmacological activity of the investigated drug.

Anti-inflammatory activity

In the planning and conduct of the study, we conducted a preliminary literature search for available scientific papers on the study of the anti-inflammatory activity of ionized and non-ionized forms of flavonoid derivatives. The search results did not yield any relevant studies on this topic, indicating that this is an initial exploration in the field of pharmacy and medicine.

The hypothesis of our study is as follows: by ionizing individual compounds through changes in pH using the addition of a weak base, the pharmacological activity of the individual compound increases. We believe this is associated, firstly, with an increase in the affinity for the enzyme's active center, secondly, with improved bioavailability of individual compounds. Additionally, we hypothesize that the theoretical dose of individual compounds obtained through molecular docking is comparable to the experimental dose.

In order to obtain pH=9 of medium we chose as a weak base amino acid – L-arginine. L-Arginine (2-amino-5-guanidinovaleric acid) is a basic, semiessential amino acid that is the substrate for nitric oxide production by vascular endothelial and immune cells. It was reported that L-arginine treats hypertension, angina, atherosclerosis and

MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) syndrome [13]. (Fig. 4) To examine the anti-inflammatory activity, we utilized the carrageenan-induced mouse paw edema model. This model comprises two distinct stages: The initial stage, occurring one hour after administration, involves the formation of edema due to the release of vasoactive amines (histamine and serotonin) and kinins. The subsequent stage begins three hours after edema formation, characterized by increased COX-2 activity leading to the production of a substantial amount of prostaglandins and the release of NO [14, 15, 16].

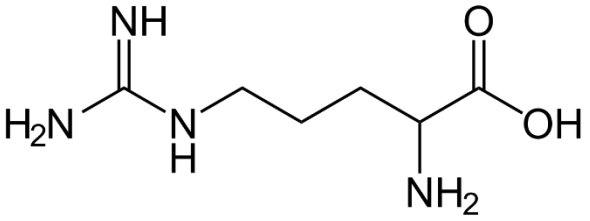

Figure 4. Structure of arginine

According to the results of our study, it was found that the anti-inflammatory activity of the ionized extract is significantly higher than that of the non-ionized extract and the standard comparison, sodium diclofenac. Additionally, we demonstrated that at a dose of 6.5 mg/kg, the ionized extract, corresponding to the theoretical dose for 50% inflammation suppression, inhibited inflammation by 89.6%, 49.4%, 40.6%, and 45.9% at the first, second, third, and fourth hours, respectively. These results indicate a correlation between experimental and theoretical research outcomes. Furthermore, the ionized extract at a dose of 13.0 mg/kg, corresponding to 100% inflammation suppression, reduced paw edema by 59.6%, 53.3%, 45.7%, and 45.2% at the first, second, third, and fourth hours, respectively. Based on these results, it can be established that at a dose of 13.0 mg/kg, the ionized extract inhibited inflammation lower than 100%. At first glance, our hypothesis at the case of 6.5 mg/kg partially correct; however, it is important to note that the bioavailability of flavan-3-ols and the physiology of the experimental animals could have influenced the results of the anti-inflammatory action. This factor influenced at extract with dose 13.0 mg/kg. Therefore, previously hypothesis put forward partially approved by the obtained results of our investigation.

Examples of direct effects of ionization on pharmacological activity in higher animals have also been reported. In a recent study of the action of procaine on the turtle heart *in vitro*, Baird & Hardman [17] deduced that the stimulation threshold and prolongation of conduction time are directly related to the concentration of the cationic form of the drug; negative inotropic activity appeared to be closely correlated with the concentration of unionized procaine. Consistent with these findings, a quaternary derivative of procaine, procaine ethochloride, where lacked the negative inotropic effects entirely.

CONCLUSIONS

R. idaeus shoot extract was dominated by (+)-catechin, epicatechin, levulinic acid, citric acid and vanillic acid. During the study, it was established that (+)-catechin anion and epicatechin anion have a higher level of affinity than non-ionized (+)-catechin and epicatechin for the active centers of phospholipase A2. COX-2. LOX-5. NF-kB, NADPH oxidase, myeloperoxidase and xanthine oxidase. The optimal technology for obtaining an extract with the maximum level of anti-inflammatory effect is the removal of organic and phenolcarboxylic acids, and ionization of the extract to pH>9. The ionized extract showed a significantly higher anti-inflammatory effect than the non-ionized extract. In addition, there was a comparison of experimental and theoretical doses in the study of anti-inflammatory activity. So, the degree of ionization is an important factor influencing the pharmacological activity of individual substances.

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تطوير التركيب الكيميائي لمستخلص براعم توت العليق باستخدام أساليب نظرية وتجريبية قائمة على نظرية التأين

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ملخص

كان الهدف من هذا البحث تطوير التركيب الكيميائي لمستخلص براعم توت العليق باستخدام مناهج نظرية وتجريبية قائمة على نظرية التأين .تم تحديد كمية المركبات الفينولية باستخدام كروماتوغرافيا السائل عالية الأداء (HPLC)، وحُدد محتوى الأحماض العضوية والفينولكاربوكسيلية باستخدام كروماتوغرافيا الغاز (GC)، والالتحام الجزيئي لإنزيمات سيكلوأوكسجيناز-2 (COX-2)، وفوسفوليباز A2، والعامل النووي kB (NF-kB)، و-5ليبوكسيجيناز (5-LOX)، وأوكسيديز نيكوتيناميد أدينين ثنائي النوكليوتيد فوسفات (NADPH)، والميلوبيروكسيديز، وأوكسيديز الزانثين باستخدام برنامج AutoDockToolsالإصدار .1.5.6كما دُرست الفعالية المضادة للالتهابات باستخدام طريقة وذمة الكاراجينان .تم التعرف على المركبات الأحد عشر باستخدام كروماتوغرافيا السائل عالية الأداء (HPLC)، وكشفت كروماتوغرافيا الغاز عن 36مركبًا .سيطر الإبيكاتشين 882.00)ملغ 100/غ(، والكاتشين 480.00) -(+)ملغ 100/غ(، وحمض الإلاجيك ومشتقاته 459.00)ملغ 100/غ(، وحمض الستريك 49.21)ملغ 100/غ(، وحمض الفانيليك 2.59)ملغ 100/غ(، وحمض الليفولينيك 64.67)ملغ 100/غ (على مستخلص براعم التوت .كانت الطاقة الحرة لأنيونات الكاتيكين -(+)، والإبيكاتشين--أنيون أعلى من الكاتيكين والإبيكاتشين -(+)في المواقع النشطة لإنزيمات COX-2، وفوسفوليباز A2، وNF-kB، و5-LOX، وأوكسيديز NADPH، والميلوبيروكسيديز، وأوكسيديز الزانثين .أظهرت المعالجة بمستخلص براعم التوت المتأين بالأرجينين بجرعة 6.5و 13.0ملغم/كغم انخفاضًا ملحوظًا في وذمة القدم بعد 1.2.3و 4ساعات بنسبة 89.6و53.3، و 49.4و53.3، و 40.6و45.7، و 45.9و 45.2%مقارنةً بالمجموعة الضابطة، على التوالي .وقد ثبت أن أنيون -(+)كاتشين وأنيون إبيكاتشين يتمتعان بمستوى أعلى من الألفة مقارنةً بالكاتشين والإبيكاتشين غير المتأينين -(+)كاتشين للمراكز النشطة للإنزيمات .أظهر المستخلص المتأين تأثيرًا مضادًا للالتهابات أعلى بكثير من المستخلص غير المتأين .بالإضافة إلى ذلك، كان هناك توافق بين الجرعات التجريبية والنظرية في دراسة النشاط المضاد للالتهابات.

الكلمات الدالة: براعم التوت، كروماتوغرافيا السائل عالي الأداء (HPLC)، كروماتوغرافيا الغاز-مطياف الكتلة (GC-MS)، الالتحام الجزيئي، النشاط المضاد للالتهابات، التأين بالأرجينين.

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