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BIOLOGICALLY ACTIVE SUBSTANCES OF SALIX PURPUREA F. GRACILIS (GREN. & GODR.) C.K. SCHNEID. (SALICACEAE)

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Лікарська сировина багатьох представників сімейства Salicaceae здавна широко застосовується як в народній медицині так і в сучасній фармацевтиці. В даний час деякі види роду верба є офіційними в Європейських країнах. У 2014 році «Salicis cortex» набула статусу офіційної лікарської сировини і включена до Державної Фармакопеї України. У зв'язку з цим актуальним є дослідження біологічно активних сполук різних видів, сортів і гібридних форм верб, що дозволить розширити асортимент лікарської рослинної сировини як за рахунок місцевих, так і за рахунок інтродукованих видів верб, поширених в Україні.

Мета. *Вивчення якісного та кількісного складу біологічно активних речовин пагонів Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid., що виростає в умовах України.*

Методи дослідження. *Об'єктом дослідження були сухі пагони Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid. Рослинну сировину збирали в 2016–2017 роках НБС ім. М. М. Гришка НАН України.. Компонентний склад летких речовин визначали за допомогою хроматографа Agilent Technologies 6890 з масспектрометричним детектором 5973. Содержаніе суми фенольних речовин визначали колориметрически за методом Фолина-Чіокальтео. Визначення кількісного вмісту суми флавоноїдів по реакції комплексообразования флавоноїдів з хлоридом алюмінію. Компонентний склад фенольних речовин визначали методом високоефективної рідинної хроматографії (ВЕРХ) за допомогою рідинної хроматографічної системи Prominence LC-20 Shimadzu (Японія).*

Результати дослідження. *Визначено якісний склад і кількісний вміст летких сполук і фенольних речовин пагонів Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid. Встановлено, що сировина містить досить високі концентрації летючих сполук, серед яких переважають ароматичні - зокрема гераниол і евгенол, серед терпеноїдів переважає сквален. Виявлено, що фенольні речовини представлені флавоноїдами і гідроксикоричні кислотами. Серед речовин фенольної природи домінують флавонони. Проведені дослідження підтверджують доцільність подальших досліджень видів верби.*

Висновки. *Визначено якісний склад і кількісний вміст летких сполук і фенольних речовин в пагонах Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid. Проведені дослідження значно розширюють відомості щодо хімічного складу сировини рослин роду Salix L. Отримані дані вивчення пагонів Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid. будуть використані для планування фармакологічних досліджень та розробки МКЯ на сировину та лікарські засоби*

Ключові слова: *Salicaceae, верба, пагони, біологічно активні речовини, летючі сполуки, фенольні речовини*

1. Introduction

Plant based medicines become increasingly popular along with the synthetic drugs. The medicinal raw materials of many representatives of the Salicaceae family have long been widely used both in folk medicine and in modern pharmaceuticals. Currently, some species of willow are official medicinal raw materials in some European countries. Whole or fragmented bark of young branches of different species of willow, including Salix purpurea L., Salix fragilis L., Salix daphnoides Vill. included in British Herbal Pharmacopoeia, French Pharmacopoeia, European Pharmacopoeia (including European Pharmacopoeia 7) [1]. Salix acutifolia Willd. included in the Pharmacopoeia of Russia (2008), the willow bark in 2008 is also included in the Pharmacopoeia in the Republic of Belarus. In 2014, Salicis cortex acquired the status of official medicinal raw material and was included in the State Pharmacopoeia of Ukraine [2]. In this regard, the study of biologically active compounds of different species, varieties and hybrid forms of willow is important, which will allow to expand the range of medicinal plant material both at the expense of local and at the expense of introduced species of willow, common in Ukraine.

Willows – one of the largest genera of wood species in temperate climate. It is believed that in the world there are about 350-370 species. Of these, 23-25 species are naturally growing in Ukraine [3, 4]. In the process of evolution, willows developed a wide range of life forms, physiological features and adapted to a large variety of conditions that allow the willows to grow in a variety of places. Salix purpurea f. gracilis (gren. & godr.) C.K. Schneid (Salix purpurea L., purple willow, purpleosier willow, Yellowstone, Purpleosier willow, Basket willow, Gracilis. This epithet most likely corresponds to S. purpurea f. Gracilis Wimm (1866), syn. S. purpurea var. gracilis Grenier & Godron (1855), S. purpurea var. nana Dieck (1899). Female narrow-leafed cultivar (Spath, 1930) with thin branches (Rehder, 1927), v RHS HD) [5, 6] – shrub height up to 4 m, branch thin, bare, greenish-or yellowish-gray or brown with a reddish tinge and a blue-tailed bloom. The buds are reddish brown, pressed into shoots. The leaves are almost opposite, oblanceolate, thinly exaggerated from above, 3–13 cm in length, blue-

ish-blue or greyish-green. Blossoms in March-April, until the leaf blooms or almost simultaneously with them. Men's cat's tail are thick, cylindrical, densely coloured, large anthers of purple colour. For a bright purple coloring of cat's tail during the flowering of this willow, it was given the name "purple" [3, 4]. The area is South and Western Europe, North Africa, in the Central Europe north of the line: the Holstein, the southern coast of the Baltic Sea, Lithuania, Upper Volga, the Southern Urals, as well as Southern and Central Asia. It often grows in flood plains and forests, on wet slopes and along the banks of rivers, on wet, periodically flooded, fertile, usually limy, clay gravel, sandy and muddy soils. Having the ability to quickly settle on new substrates, willows are very common in various secondary habitats created by human activity. They easily populate potholes, quarries, embankments, sod fields and gardens. Recently, due to the high intensity of growth, some species of willow are widely grown for bioenergy purposes as solid biofuel [4, 7]. All this substantially expands the raw material base of Salicaceae plants in Ukraine and increases the potential for their use.

2. Formulation of the problem in a general way, the relevance of the theme and its connection with important scientific and practical issues

It should be noted that although some species of willow are official in European countries, and from year 2014 also in Ukraine, there are many unresolved issues related to the rational use and chemical composition of plant raw materials of the willow flora in Ukraine, which complicates the further expansion of the raw material base and standardization of new types of medicinal plant material of the Salicaceae family [2, 8, 9]. In this regard, the study of biologically active compounds of different species, varieties and hybrid forms of willow is important, which will allow to expand the range of medicinal plant material both at the expense of local and at the expense of introduced species of willow, common in Ukraine.

3. Analysis of recent studies and publications in which a solution of the problem are described and to which the author refers

According to the literature, plants of the Salicaceae family are one of the perspective groups of plants that exhibit diverse biological properties and are used in medicine for the treatment of many diseases such as anti-inflammatory, diuretic, antipyretic, disinfectant, hemostatic, astringent, sedatives, wound healing, choleretic and antirheumatic agents [10, 11]. The antimicrobial action of drugs based on plant raw materials of Salix L. species was proved [12, 13]. Previous studies have found that they contain different classes of natural compounds – volatile compounds, carbohydrates, amino acids, macro and microelements, lipophilic compounds [14, 15]. To date, there are a large number of works devoted to the study of compounds of phenolic nature (phenol spirometers, hydroxycinnamic and hydroxybenzoic acids, coumarins, flavonoids, tannins) [16, 17]. Previously, the authors of the article studied the volatile compounds of the leaves of Salix caprea L. Salix myrsinifolia Salisb. [18, 19]. A chromatographic mass spectrometric study of Salix alatusica L. raw material and lipophilic extract was performed [20]. With GC / MS methods also were studied the volatile compounds and carboxylic acids of Salix cinerea L. and Salix rosmarinifolia L. [1, 21]. The element composition of some species of the willow flora of Ukraine was determined by the method of atomic emission spectrophotometry [22].

4. The field of research considering the general problem, which is described in the article

The analysis of the current state of the study of plants of the Salicaceae family of Ukrainian flora showed the need for further systematic comprehensive study of biologically active substances of new species of willow to provide a rationale for their use in medical practice.

5. Formulation of goals (tasks) of article

Study of qualitative and quantitative composition of biologically active substances of Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid., that grow in Ukraine.

6. Presentation of the main research material (methods and objects) with the justification of the results

The object of the study was the dry shoots of Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid. Plant raw material was collected in 2016–2017 from the collection of willow of NBS named after M. M. Grishko National Academy of Sciences of Ukraine.

Chromat-mass spectrometric study of volatile components was performed on a Agilent Technology 6890N chromatograph with a 5973N mass spectrometric detector, with a capillary HP-5MS column (quartz, column length 30 m, internal diameter of 0.25 mm); gas carrier-helium (flow rate of 1 ml/min). The volume of the sample is 0.1–0.5 μl with a flow distribution of 1/50. Thermostat temperature 50 °C with programming of 4 °C/min to 220 °C. The temperature of the detector and the evaporator is 250 °C. The weighed material (0.5 g) was placed in 20 ml of vial, and an internal standard was added. As an internal standard, tridecane was used, at a rate of 50 μg per weight, followed by the calculation of the obtained internal standard concentration, which was then used for calculations. 10 ml of water was added to the sample and volatile compounds with water vapour were distilled from it for 2 hours. using an air-cooled reflux condenser. In the process of dispersing the volatile substances adsorbed on the inner surface of the reverse refrigerator. The adsorbed substances after cooling the system were washed off by slow addition of 3 ml of particularly pure pentane in dry vial of 10 ml. The washes were concentrated by purging (100 ml/min.) of especially pure nitrogen to a residual extract volume of 10 μl, which was completely removed by a chromatographic syringe. Further concentration of the test was carried out in the syringe itself to a volume of 2 μl. The introduction of the sample into the chromatographic column was carried out in splitless mode, in other words without separating the flow, allowing the sample to be introduced without loss of division and significantly (10–20 times) increasing the sensitivity of the chromatographic method. The determination of organic acids was also carried out

by chromatographic mass spectrometry on a Agilent Technologies 6890 chromatograph with a 5973N mass spectrometry detector. The sample was injected into a splitless chromatographic column, a rate of 1.2 ml/min injection for 0.2 minutes. The determination was carried out under the following conditions: INNOWAX capillary column, length 30 m, internal diameter 0.25 mm; gas carrier - helium; gas speed – carrying 1.2 ml/min; temperature of the injector heater – 250 °C, temperature of the thermostat with programming 4 °C/min from 50 °C to 250 °C; detector temperature 250 °C. The identification of the substances was carried out by comparing the mass spectra of the compounds with the data from the NIST05 and WILEY 2007 mass spectrum libraries in combination with the AMDIS and NIST identification programs. For the quantitative calculations, the internal standard method was used. The calculation of the content of the components was carried out according to the formula: C=K1*K2, mg/kg, where K1=P1/P2 (P1 – peak area of the investigated substance, P2 – area of the peak of the standard), K2=50/M (50 – mass of internal standard, μg; introduced as a sample; M is a sample weight, g). Quantitative determination of substances was expressed in mg/kg of raw material [1, 19, 21, 23].

34 components identified in the investigated raw material were identified. Results of determining the content of volatile substances in raw willow are presented in Fig. 1 and in Table 1.

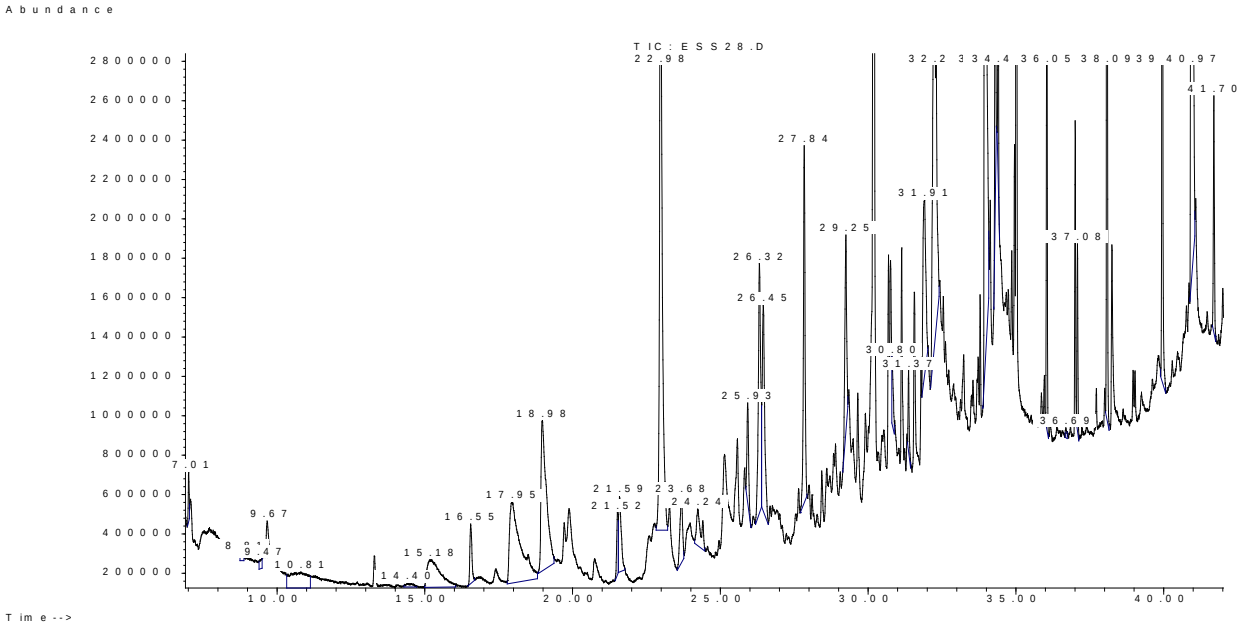

Fig. 1. Chromatogram of volatile compounds of Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid shoots.

Among the identified compounds, aromatic compounds, sesquiterpene compounds, aliphatic alcohols, alkanes, terpenes, hydrocarbons, fatty acids and their derivatives, aldehyde isomers, and oxygen-containing compounds have been identified. Aromatic compounds of raw material of purple willow are represented by biologically active substances, including 2-hydroxybenzaldehyde, phenylethyl alcohol, geraniol, eugenol, 2,4-bis (1,1-dimethylethyl) phenol. Among terpene compounds dominated squalene ((2,6,10,15,19,23-hexamethyltetracoza-2,6,10,14,18,22-hexaene) – acyclic triterpene). Among other compounds, significant amounts of fatty acids and their derivatives, in the total amount of fatty acids, are found in the largest number of palmitic acid.

Component composition of phenolic compounds was determined by the method of high-performance liquid chromatography (HPLC). For extraction of polyphenols, samples of raw materials were pre-triturated in a mortar to a powdered state. To the weighted sample 60 % isopropanol was added in a ratio of 1 g of the sample to 20 ml of isopropanol solution.

Extraction was carried out in sealed containers for 5 days at room temperature with periodic stirring according to the method [14]. Extraction was carried out in the dark to prevent the transformation of extracted substances under the influence of light. The extracts were filtered before analysis using a Supelco Iso-Disc Filters PTFE 25-4 (25mm x 0.45 μm) syringe filter [24–26].

The extract was analyzed by high performance liquid chromatography (HPLC) using the Prominence LC-20 Shimadzu Liquid Chromatographic System (Japan), consisting of the following functional modules: DGU-20A3 degasser, LC-20AD pump module, SIL-20AC auto-sampler refrigerator, photometric Detector SPD-20AV, column of thermostat CTO-20A, column Agilent Technologies Microsorb-MV-150 (turned-phase, silica gel with sewn group C18 (- (CH2) 17CH3), length 150 mm, diameter 4.6 mm, size of sorbent grains 5 μm).

Table 1

Component composition of volatile compounds of Salix purpurea f. Gracilis (Gren. & Godr.) C. K. Schneid shoots

No.	Retention time, min	Component	Content, mg/kg
1	7.012	2-oxybenzaldehyde	9.68
2	8.816	trans-linalool oxide	4.59
3	9.472	cis-linalool oxide	4.51
4	9.664	linalool	18.62
5	10.805	phenylethyl alcohol	59.47
6	14.398	citronellol	5.37
7	15.177	geraniol	77.46
9	17.945	eugenol	187.58
10	18.978	leden oxide	171.34
11	21.515	β-ionone-5,6-epoxide	17.13
12	21.584	β-ionone	42.28
13	22.98	2,4-bis (1,1-dimethylethyl)-phenol	354.57
14	23.682	isoaromadendrene epoxide	37.49
15	24.237	nerolidol	35.16
16	25.925	cubenol	35.47
17	26.318	β-eudesmol	130.07
18	26.45	α-eudesmol	109.27
19	27.837	tetradecanal	92.37
20	29.248	myristic acid	55.23
21	30.806	pentadecanoic acid	13.36
22	31.369	methyl palmitate	13.17
23	31.908	palmitoleic acid	79.14
24	32.248	palmitic acid	240.88
25	33.944	phytol	305.31
26	34.306	ethyl linoleate	62.57
27	34.399	oleic acid	29.04
28	36.049	tricosan	71.60
29	36.689	tetracosan	2.14
30	37.082	pentacosan	25.04
31	38.084	hexacosan	149.23
32	39.958	heptacosan	122.48
33	40.976	squalene	924.98
34	41.7	nonacosane	38.30

HPLC conditions:

1) Composition of the mobile phase (eluent): methanol and 0.9 % solution of phosphoric acid in deionized water (reagents Sigma-Aldrich, Germany).

2) Chromatography mode - gradient. Chromatography - gradient mode, was developed for the qualitative separation of certain phenolic acids and flavonoids in plant extracts [18, 23]. Initial ratio of eluent components: 1:9. The content of methanol in the eluent during the analysis varied according to the following scheme: – the first 13 minutes – increase from 10 to 40 %; from the 13th to the 20th minute – increase from 40 to 53 %; from 20th to 26th minute – increase from 53 to 55 %; from the 26th to the 40th minute – holding 55 %; from the 40th to the 41st minute – down to 10 %; from the 41st to the 56th minute – holding 10 %.

3) The velocity of the eluent – 0.5 ml/min.

4) Column temperature – 400C.

5) The volume of the entered sample – 5 µl.

The identification of the substances in the extract was carried out by comparing the holding time and the spectral characteristics of the test substances with similar characteristics of the standards according to the method of identification of polyphenols [17], for which the chromatography was carried out at wavelengths 225, 255, 286 and 350 nm [25, 27, 28]. For precise identification or identification of the substances belonging to the specific groups of polyphenols, standard samples were used: chlorogenic and coffee acids, catechins, flavonols quercetin and rutin, flavanones naringenin, naringin and hesperetin, hesperidine, luteolin and apigenin flavones, anthocyanin cyanidin (Sigma-Aldrich, Germany). Identification characteristics of the listed standards were obtained under the above described conditions of chromatography. Gauge dependencies "peak area – content of the standard" had a linear appearance with an accuracy of not less than r^2=0.994.

Determination of the content of substances with established affiliation with specific groups of polyphenols, conducted using standards, the degree of similarity with which was the largest, taking into account the chemical form of the substance (aglycone, glycoside). Substances whose degree of similarity to any standard was below 70 % belonged to the group of unidentified substances, and their content was determined by standards, the degree of similarity with which was the largest. The

total content of polyphenols was determined by summing the content of substances found in the range of peaks of flavonoids and phenolic acids on chromatograms [26, 29–31].

In the study of phenolic compounds of Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid found that their total content in the raw material was 32582.98 μg/g. Phenolic compounds of this type of willow are represented by hydroxycinnamic acids and flavonoids. The unidentified substances were 990.14 μg/g. The sum of flavonoids was 27974.77 μg/g, among which the flavanones 21178.17 μg/g are dominant, which is consistent with the results of previous studies. A more detailed representation of the composition of substances in the samples under study gives the results shown in Table 2 and on Fig. 2.

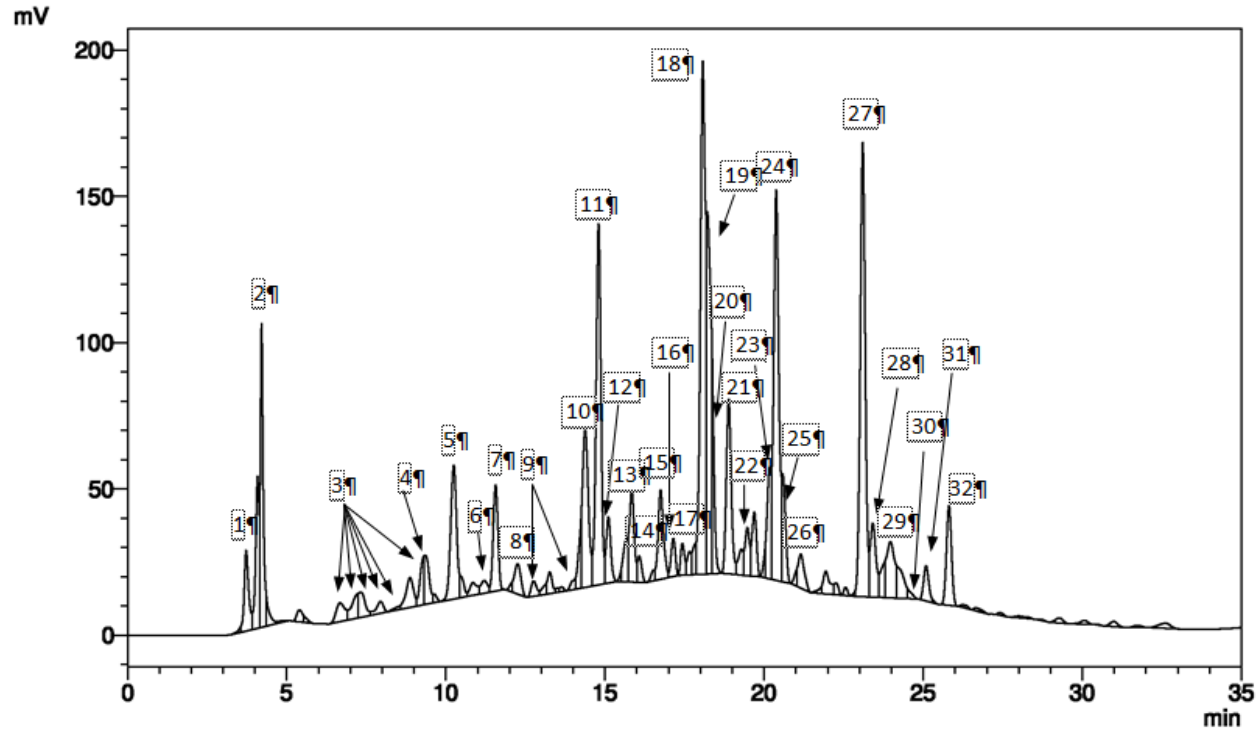

Fig. 2. Chromatogram of compounds of Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid shoots at 255 nm, Axis X – T, min.: Retention time, min., Axis Y – Absorption, mV, 1, 2, 3, 29 – catechin-like substances, 4 – catechin, 5, 7 – naringin glycosides, 6 – chlorogenic acid, 8, 10, 14, 16 – catechins, 9 – phenolic acids, 11, 12, 26 – hesperetin glycosides, 13, 15 – naringenin glycosides, 17 –flavonols glycosides, 18, 20, 24, 25 – luteolin glycosides, 19 – routine, 21 – hesperidin, 22, 32 – apigenin glycosides, 23 – myricetin glycosides, 27 – luteolin, 28 – hesperetin, 30 – flavonols aglycones, 31 – apigenin

Table 2

Component composition of phenolic compounds of Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid shoots

Group of polyphenols	Content, μg/g	Separate compounds	Content, μg/g
Phenolic acids	200.06	chlorogenic acid	125.63
		caffeic acid	-
Catechins	2160.58	catechin	982.96
Catechin-like	3304.94		
Flavonols	1651.57	routine	1279.62
		quercetin	-
		myricetin glycosides	245.69
		myricetin	-
Flavonones	21178.17	naringin	369.34
		naringenin	9.72
		hesperidin	4558.65
		hesperetin	52.79
Flavones	3097.52	luteolin glycosides	1753.86
		luteolin	762.19
		apigenin glycosides	447.89
		apigenin	133.58
Unidentified	990.14		
The sum of polyphenols	32582.98		

Determination of the quantitative content of the amount of phenolic compounds using a Folin's reagent [26].

Previously prepared:

– reagent A – 2 % Na_2CO_3 solution in 0.1 N NaOH solution;

– Folin's reagent "Fluka" diluted with water 1:1;

– control on a reagent containing 0.4 ml of reagent A, 0.4 ml of 70 % ethanol and 0.4 ml of Folin's reagent.

To 0.4 ml of the extract was added 4 ml of Reagent A, and then, after stirring, 0.4 ml of Folin's reagent. At the spectrophotometer, after 30 minutes at a wavelength of 750 nm, the intensity of the coloration against the control of the reagents was determined. Spectrophotometric analysis was performed on a UVmini-1240 Shimadzu spectrophotometer (Japan).

The amount of phenolic compounds in the extract was calculated by the formula:

$$C = \frac{E \cdot n \cdot V}{K \cdot m},$$

where: C – the content of phenolic compounds in μM/g sample; E – extinction; n – breeding; K – coefficient of recalculation of extinction in mg routine; V – amount of extractant in ml; m – mass of raw materials in grams.

Coefficient K was calculated from the calibrated curve, which is constructed by routine solutions at concentrations of 0.25; 0.10; 0.025 and 0.005 mg ml.

Results of quantitative determination of phenolic compounds in Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid is presented in Table 3.

4. Determination of the quantitative content of the amount of flavonoids by the reaction of complexation of flavonoids with aluminium chloride [26, 28].

To 0.5 ml of the extract was added 0.5 ml of a 5 % solution of aluminium chloride in 96 % ethanol and 3 ml of 96 % ethanol and cooled.

After 40 minutes, the optical density of the solution was measured at a wavelength of 410.5 nm on the spectrometer UVmini-1240 Shimadzu (Japan)

The content of flavonoids in terms of routine was calculated according to the formula

$$C = \frac{E_0 \cdot C_{rs} \cdot V_e}{E_{rs} \cdot m_s},$$

where C – content of flavonoids in mg / g of dry sample; E_o is the optical density of the solution of the sample; E_{rs} is the optical density of the solution of the comparison; C_{rs} is the concentration of the comparison solution equal to 0.1 mg/ml; m_s – weighted dry sample in grams; V_e – volume of extract in ml.

Results of quantitative determination of flavonoids in Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid shoots is shown in the Table 3

Table 3

Content of phenolic compounds and flavonoids in Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid shoots

Content of flavonoids, mg/g	Content of phenolic compounds, μMol/g
28.02±0.51	174.59±1.89

Statistical processing was performed according to generally recognized methods [2, 8]. Differences between experimental and control variants were considered significant at $p < 0.05$.

7. Conclusions from the conducted research and prospects for further development of this field

1. Qualitative composition and quantitative content of volatile compounds and phenolic substances in the Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid shoots have been determined.

2. Studies conducted considerably expand the information on the chemical composition of raw materials of plants of the genus Salix L. Obtained data on the study of Salix purpurea f. Gracilis (Gren. & Godr.) C.K. Schneid will be used for the planning of pharmacological research and development of MQC for raw materials and medicines.

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