ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Polyphenols *Calligonum aphyllum* (PALL.) Güerke (*Poligonaceae* Juss.) growing in Kazakhstan.

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ABSTRACT

This report provides information on polyphenolic compounds of *Calligonum aphyllum* collected in the Bolshie Barsuki desert near Shalkar city, Aktobe region of the Republic of Kazakhstan. The tannins are represented by two types: hydrolysable and condensed. The quantitative content of tannins is determined by the methods of titrimetry, spectrophotometry, HPLC with a mass-diode detector. Tannins in the greatest quantity are presented in extracts of stems, then in extracts of roots and leaves, the smallest quantity of tannins in extracts of flowers. By means of HPLC method, a fragment of gallotanins, gallic acid, was determined from hydrolysable tannins, and catechin from condensed polyflavanes. The flavonoids identified by the HPLC method in comparison with the standards are represented by four classes: flavans, flavonones, flavonols, and flavones. The quantitative content of flavonoids is most accurately determined by the method of spectrophotometry, while it was determined that the highest content of flavonoids is noted in extracts of the roots, the smallest - in extracts of stems. Pronounced antiradical activity was revealed. Although the phenolic profile was different in four organs, the results showed that *Calligonum aphyllum* can serve as an excellent source of various natural compounds with a high ability to absorb radicals.

Keywords: *Calligonum aphyllum*, polyphenols, flavonoids, tannins, spectrophotometry, antiradical activity

https://doi.org/10.33887/rjpbcs/2019.10.3.33

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ISSN: 0975-8585

INTRODUCTION

The plant genus *Calligonum L.* (Zhuzgun), belongs to the family *Polygonaceae* Juss.*,* the subfamily of *Polygonoideae*. The Kazakh name of the plant of the genus *Calligonum L.* is Zhuzgun, which emphasizes the similarity of the fruits with the sun. The genus *Calligonum L.* has more than 150 species [1], there are 65 species in Kazakhstan [2].

It is known from the literature that plants of the genus *Calligonum L.* showed the presence of such biologically active substances as flavonoids, tannins, alkaloids, proteins, organic acids [3].

Aqueous extract of *C. polygonoides* Linn. contains flavonoids in all organs except the roots; alkaloids in the roots, kidneys, seeds; tannins, steroids, phenols, carbohydrates and terpenoids are present in all organs [3,4]. Analysis of the methanol extract of *C. polygonoides* showed the content of flavonoids, alkaloids, proteins, tannins, steroids, phenols, carbohydrates and terpenoids, *p*-coumaric and gallic acids [5]. A new substance was isolated from the aqueous-alcoholic extract - kampferol glycoside, as well as 13 known flavonoids [6]. Kempferol-3-O-β-D- (6-O-n-butylglucuronide), kaempferol-3-O-β-D- (6-O-methylglucuronide), quercetin 3-O-D-(6-O-n-butyl) glucuronide), quercetin-3-O-β-D- (6-O-methylglucuronide), quercetin-3-O-glucuronide, kempferol-3-O-glucuronide, quercetin-3-O-rhamnopyranoside, kempferol-3-O-glucopyranoside, quercetin-3-O-glucopyranoside, taxifolin, catechins, dehydrodicatechin A, quercetin, and kaempferol were extracted[6]. Nine different flavonoids were found in this object by HPLC: catechin, delphinidin, fisetin, myricetin, epicatechin, kuromanin, rutin, callistefin and procyanidin A_2 [7].

In methanol and ethyl acetate extracts of *Calligonum comosum* L'Her. established the presence of flavonoids, tannins, alkaloids, steroids, terpenoids, saponin, phenols and flobatanin [5].

The contents of the following flavonoids were discovered in *Calligonum azel* Maire: flavanols - catechin and epicatechin and glycoside forms of flavones (luteolin-7-O-malonyl-glucoside, luteolin-7-O-rutinozide, luteolin-7-O- (2-apiozil-6- malonyl) -glucoside, chrysoerol-7-O-glucoside and apigenin-6,8-di-C-glucoside); phenolic acids: hydroxycinnamic acids; two lignans (7-hydroxyisoisolaricrizinol and secoisolaricisinol) and one stilbene (resveratrol 5-O-glucoside) [8].

The cytotoxic activity of flavonoids from *Calligonum polygonoides* was evaluated in human liver cancer cells and breast cancer cells. The relationship of structure and activity was established by comparing the IC_{50} value of several pairs of flavonoids, differing only in one structural element[7]. Chemoprophylactic effect of *C. comosum* extract on the model of hepatocarcinogenesis in rats was studied [9].

The antioxidant potential of the methanol extract of *C. polygonoides* is due to the presence of phenolic and polyphenolic compounds that reduce free radicals that cause oxidative stress [10]. Stem extract has an effect as an antioxidant (percentage of inhibition - 74.303%), fruit extract (65.74%), root extract (17.9%) [11]. The stem extract with chloroform and ethyl acetate solvents has the best effect on the bacterial strain of *Bacillus subtilis* with the lowest inhibitory concentration [12]. The activity of various *C. comosum* extracts shows high sensitivity for three bacterial strains: two gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and one gram-positive (*Staphylococcus aureus*), except for *E.coli*. The most important antibacterial effect is observed in *S. aureus* for all concentrations of four extracts and three tested antibiotics. *Calligonum comosum* ethanol extract treats infected animals with fasciolosis and improves their health. The anti-inflammatory, antinociceptive, antipyretic and gastroprotective actions of *Calligonum comosum* [13] have been studied.

One of the representatives of this genus is the plant *Calligonum aphyllum* (PALL.) Güerke, widely grown in Kazakhstan. It is distributed in the Caspian, Emba, Turgay, Priaralye, Kyzylorda, Betpak-Dala, Muyunkum, Balkhash and Alakol[2]. *C. aphyllum* is a perennial shrub in height from 1 to 2 meters, with red bark, leaves are linear, annual shoots are green; 2-3 flowers are collected in axillary tufts, perianth white or pink, simple, fruits in outline 4-sided with 4 double, membranous brown wings, usually notched at the edges. Previously, an optimal technology for the extraction of tannins was developed for this object [14], the mineral composition [15] of *Calligonum aphyllum* was determined. Chronic toxicity was studied for the plant stems and wound healing activity was identified [16, 17].

ISSN: 0975-8585

MATERIALS AND APPARATUS

Calligonum aphyllum was collected in the Bolshie Barsuki desert, near the town of Shalkar, Aktobe region. The collection was carried out in June - July 2016 in the flowering phase. Herbarium specimens are stored in the Herbarium Fund (international AA index) of the Institute of Botany and Phytointegration of the Republic of Kazakhstan, the index of the genus *Calligonum* is 2200.

Qualitative methods.

Preparation of extraction from plant raw materials: 1 g of the grinded raw materials is placed in a flask with a capacity of 25 ml and pour 10 ml of ethyl alcohol. The flask is connected to a reflux condenser and heated on a water bath for 10 minutes from the moment alcohol is boiled in the flask. After cooling, the resulting extraction is filtered through a filter paper [18].

For the qualitative and quantitative determination of biologically active substances of various organs of plant raw materials, the paper (PC) and thin layer chromatography (TLC) method were used in the *n*-butanol – glacial acetic acid – water (40: 12.5: 29) and acetic acid (15 %) solvent systems. Specific reagents were used as developers for flavonoids and tannins: 1% solution of ferric ammonium sulfate (FAS), 2% ferric chloride solution, 1% gelatin solution, bromine water, 10% lead acetate solution, 5% aluminum chloride solution, 25% ammonia solution [20]. Quantitative determination of tannins and flavonoids was performed on a Aglient Technologies Cary 60 UV – Vis spectrophotometer, as standards used state standard samples (SSS): tannin, quercetin, gallic acid [19-25]. For the analysis of tannins and flavonoids, an HPLC method was used on an Agilent 1290 Infinity HPLC instrument with an Agilent 1260 Infinity mass - diode detector; gradient separation was performed on a 2.1 × 100 mm, 1.8 µm ZORBAX RRHD SB-C18 column; the mobile phase consisted of A: 0.1% aqueous solution of formic acid and B: acetonitrile, containing formic acid in a concentration of 0.1%; the flow rate is 0.3 ml / min at 30 ° C (samples were kept at 4 ° C in dark glass bottles); registration was carried out with a diode-array detector at 280 nm and 325 nm. A MAC 210 / NH moisture analyzer (Radwag Wagi Elektroniczne, Poland), a Centrifuge 5810 R Eppendorf centrifuge (Hamburg, Germany), a Binder E28 drying oven (Germany), an Ohaus Adventurer analytical laboratory balance (China), reagents and reagents of qualification - chemically pure (CP), extra clean (EC), pure for analysis (PFA), SSS (Sigma -Aldrich) were used.

Experiments to determine the antioxidant activity *in vitro* performed by the method of FRAP. One of the indicators of AOA *in vitro* in spectrophotometric determination by the FRAP method [26] is the value of optical density, which increases proportionally by the example of ascorbic acid. Determination of antiradical activity (DAA) was performed *in vitro* using 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH-method) [27].

RESULTS AND DISCUSION

Quantitative determination of tannins in the extracts of various organs of the plant *Calligonum aphyllum* (Pall.) Güerke. was carried out by the methods of permanganatometry [19], complexonometry [20], spectrophotometry [21-24] and HPLC [25].

The official pharmacopoeial method for determining tannins is the method of permanganatometry (for all types of raw materials containing tannins) [19], which has several disadvantages: overestimated results due to the oxidation of various classes of compounds, there is no clear definition at the equivalent point. The complexometric method for determining tannins is accurate, reproducible, there is a clear color transition at the equivalence point, but the method is cumbersome and time consuming [20]. Currently, the most accurate and efficient, doesn't require a lot of time, is a spectrophotometric method for determining [21-24]. For the validation of spectrophotometric methods, a method was used based on measuring the optical density of the tannins themselves in aqueous-alcoholic solutions, where SSS tannin was used as a standard; as well as colored products of the interaction of tannins with ammonium molybdate, with iron – tartrate reagent. For SSS tannin and for extracts of plant materials from various organs, UV - absorption spectra were taken in the wavelength range of 200 - 800 nm in 30% ethanol. The absorption maximums in the UV spectrum of tannin and extracts of *Calligonum aphyllum* are at a wavelength of 277 nm, which makes it possible to use the absorption maximum to make the calibration curve [24]. The resulting product $C_6H_3(OH)_2OMoO_2$ $(NH_4)_2$ colors the reaction medium yellow, which makes it possible to determine the amount of tannins spectrophotometrically by the intensity of the color [25].

The calibration curve of the relationship between the optical density of the products of the interaction of SSS gallic acid with iron-tartrate reagent in the presence of phosphate buffer and the concentration of gallic acid solutions in the concentration range from 0.005 to 0.04 g / ml is linear. Spectrophotometric monitoring showed that the absorption spectra of the products of the reaction of tannins and gallic acid solution with iron-tartrate reagent in the presence of phosphate buffer practically coincide, the maximum is observed at a wavelength of 545 nm. The reaction is rather specific, non-phenolic impurities do not react with the iron-tartrate complex, and oxidative condensation of tannins, in which there is a loss of pharmacological properties, is accompanied by a decrease in the number of hydroxyl groups. The results of the determination of tannins in table 1.

From table 1 it can be seen that tannins are most abundant in the extracts of the stems, then in the extracts of the roots and leaves, the smallest quantity of tannins in the extracts of flowers.

The HPLC [28] method gives a clear idea of the qualitative and quantitative composition of extracts of various organs of plant raw material.

It was found that tannin was recorded on the chromatogram in the form of multiple peaks (figure 2).

The presence of these peaks on the chromatogram of tannin can be explained by its polymeric structure, which is based on the monomeric subunits of octa- and nonagloylglucose and hexa- and heptagalloylglucose. This is due to the fact that the process in the plant proceeds mainly towards the enlargement of molecules both under the action of the enzyme phenol oxidase and as a result of the formation of semi-quinone radicals from monomeric fragments in redox processes with their spontaneous polymerization with the formation of tannins and is practically irreversible.

Identification of tannins by HPLC showed the presence of monomeric units of flavanes (catechin and epicatechin), characteristic of condensed tannins. From phenolic acids of hydrolyzable tannins, a fragment of gallotanins, gallic acid, was determined by HPLC.

Based on the results of the table, the highest content of gallic acid is contained in extracts of flowers, the smallest - in extracts of leaves.

Methods for the quantitative determination of flavonoids: methods of spectrophotometry at a wavelength of 414 nm, 440 nm [29,30], by HPLC method [30] with external standards [31,32].

The determination was carried out by methods of spectrophotometry in terms of quercetin. The basis for the quantitative determination of flavonoids is the reaction with aluminum chloride, a yellow-green complex appears, which shifts the absorption maximum to the long-wavelength region by 60 nm - 414 nm.

The maximum absorption of the UV spectrum at a wavelength of 440 nm is characteristic of the complex of quercetin with aluminum chloride, where a bathochromic shift of 65 nm occurs (figure 3). The calibration curve in this area for quercetin itself does not quite correspond to the content of flavonoids in plant raw materials. The standard curve for quercetin is shown in figure 4, 5. The results of the quantitative determination of flavonoids are shown in table 3.

Based on the data obtained, it was found that the quantitative content of flavonoids is most accurately determined by spectrophotometry at a wavelength of 414 ± 5 nm. Based on the data of table 3, it is determined that the highest content of flavonoids in extracts of roots, the smallest - in extracts of stems.

The HPLC method gives accurate results for the content of each flavonoid in the extracts of various plant organs, the results in table 4.

The flavonoids identified by the HPLC method in comparison with the SSS standards are represented by four classes: flavans, flavonones, flavonols and flavones. Flavone, identified as apigenin, flavonols - kaempferol, quercetin and its monoglucosides: quercitrin, isoquercetin; flavonones by two naringenin isomers; from flavanes - (+)-catechin, (-)-epicatechin. Based on the data in table 4, it was determined that (+)-catechin is

contained in the extracts of all organs, except for the flowers with the highest content in the extracts of the stems; (-)-epicatechin in the largest quantity is contained in the extracts of the stems, in extracts of the roots and flowers is not represented; (±)-narigenin, apigenin, quercitrin, isoquercetin is found only in flower extracts. In the greatest amount, kaempferol is contained in extracts of flowers, is not contained in extracts of stems and leaves, quercetin is found only in extracts of roots.

The study of antioxidant and anti-radical activity was conducted in the "Medical University of Astana".

Experiments to determine the antioxidant activity *in vitro* performed by the method of FRAP. The obtained data reflected in table 5 and figures 6, 7, which shows the concentration dependences of the optical density for the object under study and the standard ascorbic acid (AA).

As follows from the data in table 5 and figures 6, 7, for solutions of these vegetative organs of *Calligonum aphyllum*, the antioxidant activity is low compared with the effect of ascorbic acid. One of the indicators of *in vitro* AOA in spectrophotometric determination by the FRAP method is the value of optical density, which increases proportionally by the example of ascorbic acid. This dependence indicates a pronounced AOA of the object under study.

Determination of antiradical activity (ARA) was performed *in vitro* using 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH-method) according to the previously described procedure. For the control solution of 2,2-diphenyl-1-picrylhydrazyl, the optical density is 0.7615 ± 0.0067 (table 6 and figure 8, 9).

On the basis of the experiments performed, a low manifestation of the *in vitro* antioxidant activity of the *Calligonum aphyllum* vegetative organs (root, fruits, leaves), which is less pronounced compared with the antioxidant ascorbic acid, as well as a pronounced antiradical activity, comparable to the effect of butylhydroxyanisole with antiradical properties *in vitro*, was established.

Table 1: The results of the determination of tannins

Extracts of various organs	The amount of extractives				
	Permanganometry	Complexometry	Spectrophotometry, 277 nm	Spectrophotometry, 420 nm	Spectrophotometry,545 nm
Flowers	60,82	41,81	41,76	31,6	38,4
Leaves	58,21	48,29	55,81	35,7	49,2
Stems	46,14	55,209	52,18	40,7	47,3
Roots	61,38	50,668	51,08	49,2	61,6

Table 2: Content of gallic acid in extracts of various organs of *Calligonum aphyllum* Pall. Güerke

	Polyphenols	Fruits		Stems		Leaves		Roots	
		mg / g	%	mg / g	%	mg / g	%	mg / g	%
1	Gallic acid	149,3	1,493	48,7	0,487	13,5	0,135	19,2	0,192

Table 3: Flavonoid Results

Extracts	The content of flavanoids,%	
	Spectrophotometry, 414 nm	Spectrophotometry, 440 nm
Flowers	31,61	29,45
Leaves	35,30	34,67
Stems	22,10	22,29
The roots	36,26	28,54

ISSN: 0975-8585

Table 4: The results of the determination of flavonoids in extracts of various organs of the plant Calligonum aphyllum

№	Flavonoids	Extracts of various plant organs							
		flowers		stems		leaves		корни	
		mg / g	%	mg / g	%	mg / g	%	mg / g	%
	flavans								
1	(+)-catechin			198,6	1,986	84,5	0,845	25	0,25
2	(-)-epicatechin			32,5	0,325	15,0	0,15		
	flavonones								
3	(±)-naringenin	0,5	0,005						
	flavones								
4	apigenin	0,3	0,003						
	flavonols								
5	kaempferol	10,2	0,102					0,6	0,006
6	quercetin							0,26	0,0026
7	quercitrin	13,2	0,132						
8	isoquercetrin	63,0	0,630						

Table 5: The dependence of optical density on the concentration of the test object (AOA).

C. aphyllum	0,25 mg / ml	0,5 mg / ml	0,75 mg / ml	1,0 mg / ml
Root	0,0710±0,0035	0,0605±0,0196	0,0544±0,0126	0,0466±0,0016
Fruit	0,0658±0,0031	0,0821±0,0009	0,0668±0,0066	0,0761±0,0084
Leaves	0,0816±0,0013	0,0804±0,0011	0,0828±0,0029	0,068±0,0044
AA	0,949±0,0210	1,269±0,0023	1,423±0,0220	1,808±0,0041

Table 6: The dependence of optical density on the solution concentration of the root of the object under investigation (AOA).

C. aphyllum	0,25 mg / ml	0,5 mg / ml	0,75 mg / ml	1,0 mg / ml
Root	0,2644±0,0081	0,2184±0,0055	0,2046±0,0124	0,1831±0,0933
Fruit	0,0658±0,0031	0,0821±0,0009	0,0668±0,0066	0,0761±0,0084
Leaves	0,2770±0,0108	0,2331±0,0110	0,1826±0,0098	0,1674±0,022
BHA	0,285±0,002	0,240±0,030	0,229±0,018	0,261±0,015

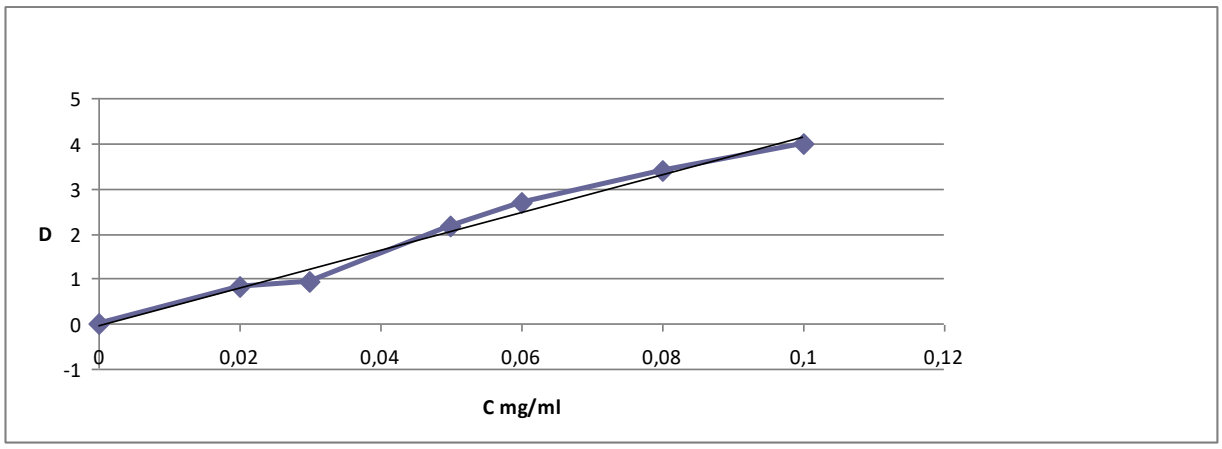

Figure 1: Calibration graph of tannin at a wavelength of 277 nm

ISSN: 0975-8585

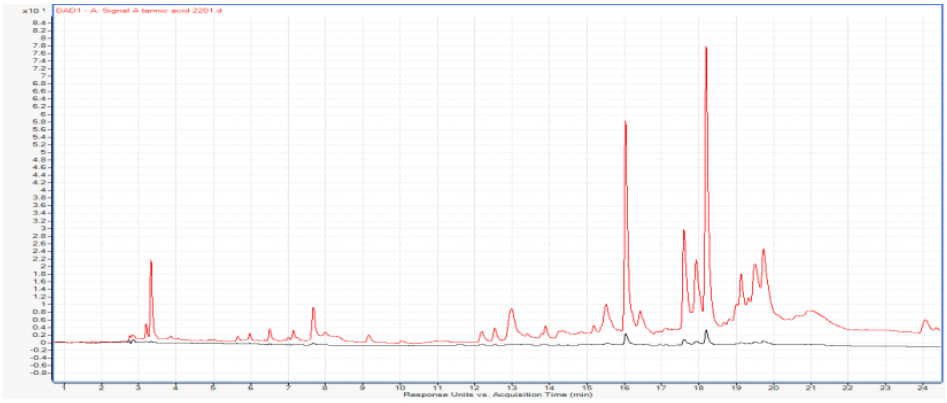

Figure 2: Chromatogram of the SSS of tannin at 280 nm (red line) and 325 nm (black line).

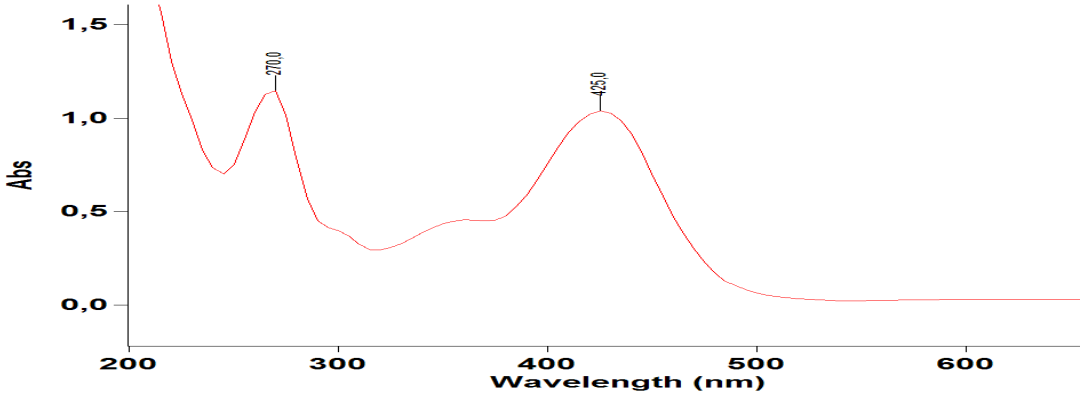

Figure 3: The dependence of optical density on the wavelength for quercetin.

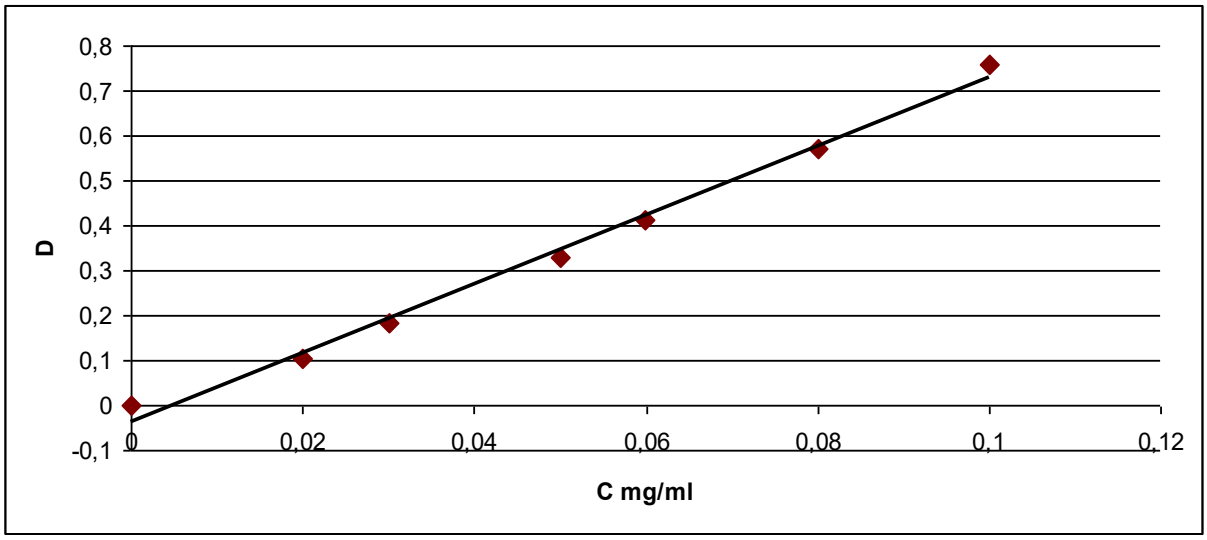

Figure 4: – Quercetin calibration curve at 414 nm

ISSN: 0975-8585

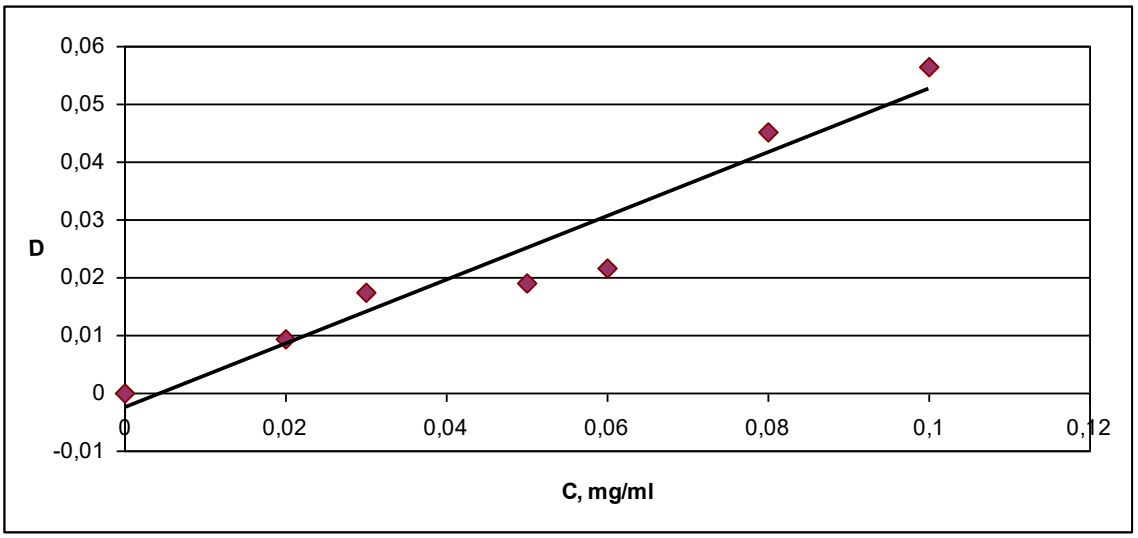

Figure 5: – Quercetin calibration curve at 440 nm

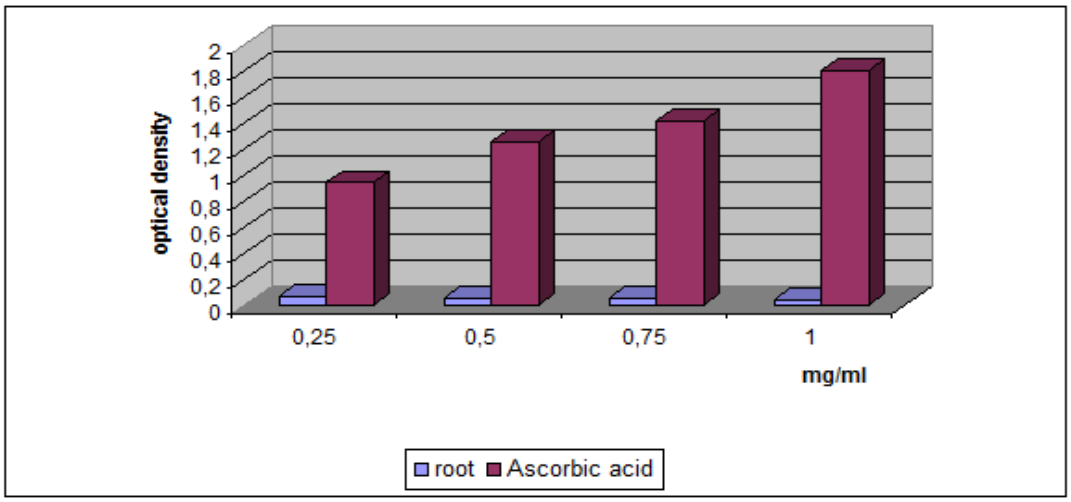

Figure 6: – The dynamics of the dependence of the AOA on the concentration of the solution of the root of *C. aphyllum* (FRAP-method)

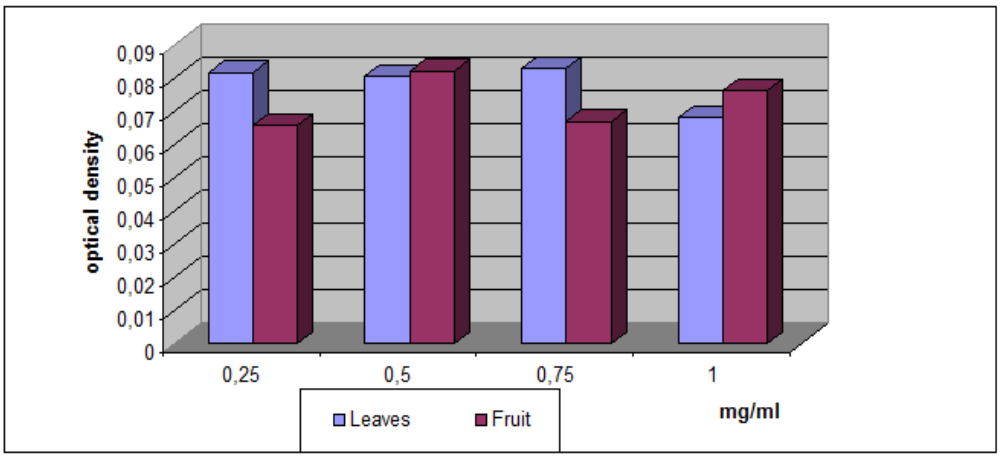

Figure 7: – Dynamics of the AOA dependence on the concentration of the solution of the flower and the leaves of the *C.aphyllum* (FRAP-method)

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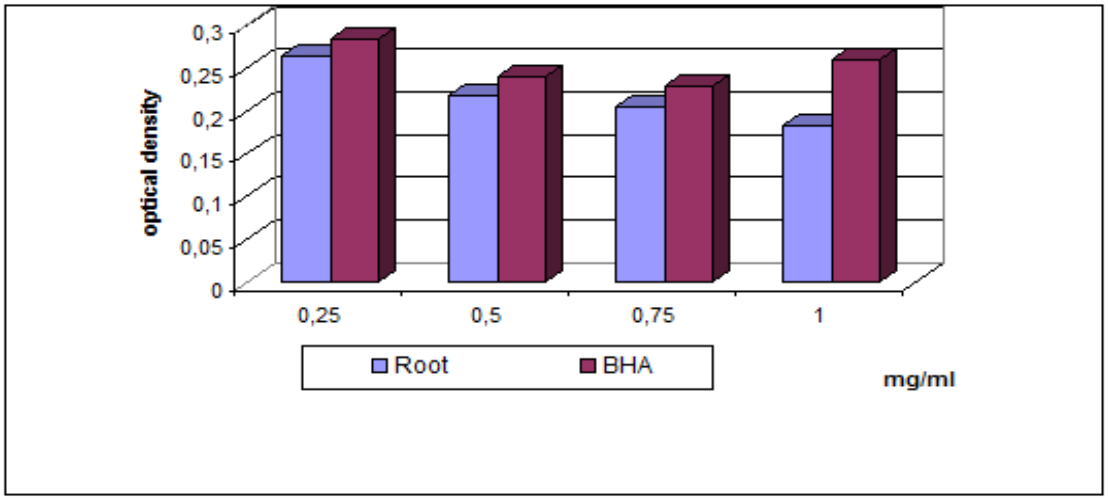

Figure 8: Dynamics of the dependence of ARA on the concentration of the *Calligonum aphyllum* root solution (DPPH method)

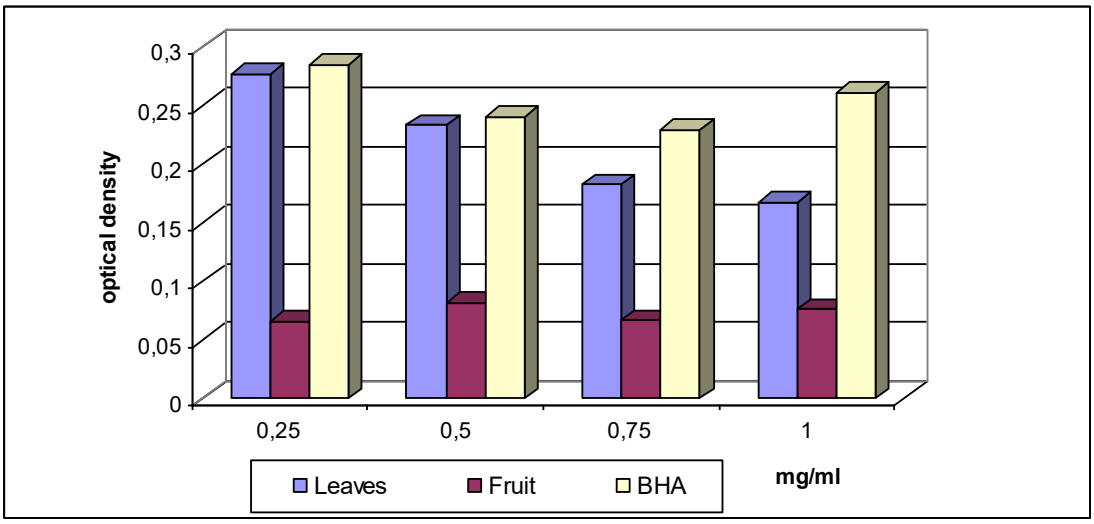

Figure 9: Dynamics of the dependence of ARA on the concentration of the solution of the flower and leaves of *Calligonum aphyllum* (DPPH-method)

CONCLUSION

1. For the first time, for the obtained extracts of various organs: flowers, leaves, stems, roots, the presence of tannins and flavonoids was established on the basis of authenticity reactions.

2. Tannins are represented by two types: hydrolyzable and condensed, giving specific reactions with salts of ferric iron and reaction with vanillin reagent. Identification of tannins by HPLC showed the presence of monomeric units of flavanes (catechin and epicatechin), characteristic of condensed tannins. From the hydrolyzable tannins by HPLC method was determined a gallotanin fragment - gallic acid.

3. Methods of titrimetry, spectrophotometry, HPLC with a mass diode detector with SSS determined the quantitative content of tannins in the extracts of various organs of the plant. Tannins in the greatest quantity are presented in extracts of stems, then in extracts of roots and leaves, the smallest tannins in extracts of flowers.

4. The flavonoids identified by the HPLC method using SSS standards, are represented by four classes: flavans, flavonones, flavonols, flavones. Flavone, identified as apigenin, flavonols - kaempferol, quercetin and its

monoglucosides: quercitrin, isoquercetin, flavonones by two naringenin isomers; flavans - (+)-catechin and (-)-epicatechin.

5. The quantitative content of flavonoids is most accurately determined by spectrophotometry. The highest content of flavonoids in root extracts, the smallest - in the extracts of the stems.

6. A pronounced anti-radical activity in roots, stems and leaves has been identified, while in fruits the anti-radical activity is much less pronounced. The results of the study showed low antioxidant activity in all organs of the object under study.

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