https://doi.org/10.31925/farmacia.2022.1.23 **ORIGINAL ARTICLE**

STANDARDIZATION OF *ZIZIPHORA CLINOPODIOIDES* LAM. CULTIVATED AND WILD GROWING IN THE SOUTH-CAUCASIAN FLORA

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Abstract

The aim of the current study was the qualitative and quantitative analysis of the extracts obtained from *Ziziphora clinopodioides* by HPLC and spectrophotometric methods. By the HPLC assay there were confirmed the qualitative-quantitative phenolic compositions of the substances in ethanol extracts; apigenin and verbascoside previously identified by thin layer chromatography. The study is using a modified spectrophotometric method for the characterization of the total amount of flavonoids with respect to 7-methyl sudahitin. HPLC analysis showed that the apigenin content ranged from 0.0024 to 0.01 mg/mL and the verbascoside-ranged from 0.114 to 0.504 mg/mL in *Ziziphora* dry extracts. It was shown that the plant *Ziziphora* accumulated a significant quantity of flavonoids of the total amount, 2.57% up to 4.18%. The current study indicated that the raw material of wild growing *Ziziphora* in floras of South-Caucasian region, cultivated in soil and in hydroponics conditions could represent a source of flavonoids, the quantity of which varies not only due to natural climatic conditions, but also due to the growing conditions.

Rezumat

Scopul prezentului studiu a fost analiza calitativă și cantitativă a extractelor obținute din *Ziziphora clinopodioides*, prin HPLC și metode spectrofotometrice. Prin metoda HPLC s-au confirmat compozițiile fenolice calitativ-cantitative din extractele etanolice: apigenină și verbascozidă, identificate anterior prin cromatografie în strat subțire. Articolul prezintă utilizarea unei metodei spectrofotometrice modificate pentru definirea cantității totale de flavonoide în raport cu 7-metil sudahitina. Analiza HPLC a arătat că apigenina a variat de la 0,0024 la 0,01 mg/mL și verbascozida de la 0,114 la 0,504 mg/mL în extractele uscate de *Ziziphora*. S-a demonstrat că specia *Ziziphora* a acumulat o cantitate semnificativă de flavonoide, a căror cantitate totală a fost de 2,57% până la 4,18%. Studiul actual a indicat că materia primă *Ziziphora,* sălbatică în flora sud-caucaziană, cultivată în sol sau în condiții hidroponice, ar putea fi o sursă de flavonoide, a căror cantitate variază nu numai datorită condițiilor climatice naturale, dar și în funcție de condițiile de creștere.

Keywords: *Ziziphora clinopodioides* Lam, HPLC, flavonoids, apigenin, verbascoside, 7-methyl sudahitin

Introduction

Herbal medicines have been widely used for the last decade in the pharmaceutical market. Along with the research for the new medicinal plants, a deep physico-chemical study of the raw materials already applied in traditional medicine becomes relevant.

Nowadays, the interest of the plants phenolic compounds is not random and associated with a wide range of their physiological activities and low toxicity [10, 25].

The scientific literature provides studies of flora from different countries, mainly related to the discovery of phenolic substances in plants and the analysis of their biological activity. Special attention is paid to some endemic plants of different geographical areas, the study of which is currently promising. From this point of view, the study of *Lamiaceae* family species, such as *Ziziphora* found in various floras became relevant.

With the help of the spectral studies, including 1D and 2D NMR spectroscopic data in the chloroform-soluble fraction had been isolated from *Ziziphora tenuior* collected from Ziarat valley of Balochistan province of Pakistan was found Ziziphorins A and B new flavonoids, along with 1-hentetracontanol, β-sito-sterol-3-O-β-D-glucoside, ursolic acid, oleanolic acid and apigenin [12].

The total polyphenolic and flavonoid content, as well as the antioxidant activity of *Ziziphora clinopodioides* Lam. extracts of different polarity, were investigated and revealed ethyl acetate extracts containing a large

number of polyphenolic compounds (19.27%) and flavonoids (65.61%) which have good antioxidant capacity [20]. At the same time were investigated three natural compounds, diosmin, linarin and pulegone, in the raw material *Ziziphora* collected from the different localities in China [21].

From *Ziziphora tenuoir* (*Lamiaceae*) gathered from the west of Iran were investigated flavonoids, anthocyanins, total phenolic compounds for polar and non-polar sub-fraction in different stages of growth (pre-flowering and flowering) [7].

By GC/MS method, nineteen compounds in n-hexane fraction were identified from ethanol (70%) extract from *Ziziphora clinopodioides*, growing widely west and northwest of Iran. The main constituents of the n-hexane fraction were pulegone (24.35%), menthol (14%) and menthone (9.61%) [30].

From the ethanolic extract of the aerial part of *Ziziphora clinopodioides* Lam. (Xinhua) collected from three different origins, it was isolated flavonoid acacetin. Silica gel and pillar layer chromatography were applied for the isolation and purification of acacetin, and the structure of acacetin was confirmed based on ^{1}H and ^{13}C NMR spectroscopy. The content of acacetin in *Z. clinopodioides* was determined by HPLC method [8].

Due to gathered information about four species of Kazakh *Ziziphora*, their traditional utilization confirmed the following compounds: monoterpenes, essential oil, phenolic substances belonging to the flavonoids and phenolic acids and triterpenes, which are identified in extracts obtained from these plants [17]. According to some reports, the essential oil and the extracts obtained from some *Ziziphora* species had significant antioxidant activities. A comprehensive survey revealed that the majority of essential oil from *Ziziphora* plants contains considerable amounts of pulegone [11], and one study showed that the extract of *Ziziphora* species (*Z. tenuior*) and its ethanolic and petroleum ether fractions could also have anti-inflammatory properties [13].

The species *Ziziphora* is one of the most common plants in the floras of South-Caucasian regions. The study of the raw material resources indicated that the wild-growing *Ziziphora clinopodioides* met in South-Caucasian regions has a form of the small scattered semi shrubs in small populations that alternate from the rocky slopes of mountain belts to subalpine elevations [3]. *Ziziphora clinopodioides* Lam. is one of the most promising plants as a valuable raw material for obtaining the essential oils and as a source of flavonoids [3, 5, 9], which is widespread in the South-Caucasian regions floras[22].

Earlier, with the help of the complex physicochemical research methods (UV and IR spectroscopy, NMR ^{1}H and ^{13}C spectroscopy), the flavonoid content of the *Ziziphora* extracts was investigated, and in the result, flavonoid glycosides; chrysin-7-rutinoside, linarin, flavonoid aglycones; diosmin, 7-methyl sudahitin, timonin were revealed [14, 15].

In another study, we determined the chemical constituents of essential oil. We studied the anti-bacterial and antioxidant activities of essential oil and the extracts obtained from the raw material of *Ziziphora clinopodioides* wild growing in the floras of South-Caucasian regions and cultivated in the hydroponic conditions. The study results showed the future prospects of using *Ziziphora clinopodioides* not only as the source of flavonoids and essential oils, but also as antimicrobial and antioxidant agents [22]. But the standardization of extracts of the raw material of *Ziziphora clinopodioides* was not carried out. The aim of this study is the standardization by flavonoids of the raw material *Ziziphora clinopodioides* cultivated and wildly growing in South-Caucasian regions floras for the creation of scientific back-ground for the application in medico-pharmaceutical practice.

Materials and Methods

Herbal material

The samples of *Ziziphora clinopodioides* Lam. were collected in the geographic areas 40°38′39″N 44°28′53″E (sample set 1), 40°10'5"N 44°38'40"E (sample set 2), 39°54′28″N 46°52′14″E (sample set 3), 40°04′07″N 46°54′21″E (sample set 4), 39°39′30″N 46°36′7″E (sample set 5), and the herbs cultivated in soil and hydroponics conditions were collected (from June to July 2018) for scientific research and then identified by the registry for species identification (*Z. clinopodioides* Lam., 1791, Tabl. Encycl. Meth. Bot., Illustr.1:63) according to Takhtajyan and Grossgeym, (GACP, WHO 2003) [8, 18, 26].

The plant's voucher specimen (ERE N194583) was deposited in the Institute of Botany after A.L. Takhtajyan of NAS RA.

Method of cultivation

For the cultivation, nearly 50 plants, of *Ziziphora* bushes collected in the area of the 40°10'5"N 44°38'40"E (sample set 2) and 40°38′39″N 44°28′53″E (sample set 1)region, in mid-April (April 15 2018) were planted on (5 m^2) hydroponic and soil zones. Black slag was used as nutrient filler in a 3 - 15 mm diameter, previously disinfected with 0.05% solution of $KMnO_4$ [3, 4]. In the process of vegetation, the plants were untreated according to Davtyan's method [4] nutrient solution (pH 5.5 - 6.5), 1 - 2 times a day. The first collection of the raw materials was in early July, at the beginning of the flowering phase (GACP, WHO 2003) [26].

Preparation of the extract

Extracts preparation were carried out by extracting the air-dried plant raw material of the *Ziziphora* in 50% ethanol for 30 minutes with further evaporation. The dry extracts were filtered and evaporated to dryness

under the reduced pressure in a rotary evaporator (Quality control methods for herbal materials. WHO, 2011) [27].

Then the total amount of flavonoids in extracts of the *Ziziphora* with respect to 7-methyl sudahitin was defined [2, 6, 24].

HPLC analysis

HPLC analysis was performed using the liquid chromatography "Waters 2695 Separations Module" (USA) and an ultraviolet detector "Waters 2487", as well as the stationary phase of a chromatographic column Nucleosil C18" 5 µm, 250 x 4.6 mm as a reverse phase. Separation of the substances in an isocratic elution mode, and as a mobile phase: A: 0.1% TFA + H_2O (TFA – trifluoroacetic acid), B: MeCN (MeCN – acetonitrile) were used the flow rate of 0.5 mL/min. Detection was provided using a UV detector performed at a wavelength of 350 nm (following the UV maximum absorption), temperature column 25ºC. For each injection, the volume was 10 mL. The standard solutions of the flavonoids apigenin and luteolin, and the phenylpropanoid glycoside verbascoside (10 to 1000 µg/mL) (Sigma-Aldrich, USA) with the purity degree > 99.9% (gradient grade, for HPLC) were used.

Weighted samples were dissolved in 1 mL of ethyl alcohol in the special test tubes for analysis. In order to purify from the mechanical and insoluble small particles which could contaminate the chromatographic columns and decrease their efficiency, the solutions were filtered using the 0.45 µm filter. Then the sample solutions were placed into a special compartment of the chromatograph and then analysed according to the developed method [1, 19].

UV-Vis Spectrophotometric analysis

The modified UV-Vis Spectrophotometric analysis method was used to quantify the total amount of flavonoids in the raw material *Ziziphora*. According to this patented method (Patent №3223A, 03.09.2018RA), approximately 1.0 g (accurately weighed) of raw material was put into a flask of 250 mL, with additions from 250 mL of 50% ethyl alcohol. The flask was connected to the reflux refrigerator and then heated in a boiling water bath for about 30 min. The flask was cooled to room temperature and filtered by filter paper into a volumetric flask of 250 mL, adding the 50% ethanol up to the mark of 250 mL. Then 2 mL of the solution was filled into a 50 mL volumetric flask, bringing to the 50 mL marked scale by adding the 50% ethanol. As a standard, the flavonoid 7-methyl sudahitin (Sigma-Aldrich, USA) was used. The specific absorption coefficient ($E_{1см}^{1\%}$) at an analytical wavelength (207 nm) was determined ($E_{1см}^{1\%}$ = 920).

The optical density of the solution was determined by the UV-Vis spectrophotometer SPECORD UV-Vis (Germany), at a wavelength of 207 nm, with 10 mm cuvette thickness. As a control solution, 50% alcohol was used.

Spectrophotometric method determination of the total amount of flavonoids without prior separation of the components is based on the additivity property for the values optical densities of the mixture components at the light absorption in the same wavelength λ = 207 nm [2].

In the calculation formula, the value of the specific absorption coefficient ($E_{1см}^{1\%}$) for the flavonoid 7-methyl sudahitin was included $E_{1см}^{1\%}$ = 920 [2, 24].

The total amount of flavonoids in the raw material of *Ziziphora* was calculated by the formula (1):

$$x = \frac{D_x \cdot 250 \cdot 50}{920 \cdot 2m}, \qquad (1)$$

where, m – the mass of the sample, Dx – an optical density of the test solution at λ = 207nm; 250 – a solution volume, (mL); 50 – the volume of the aliquot was taken from solution (mL); 920 – is the specific absorption coefficient of flavonoid 7-methyl sudahitin at λ = 207 nm [6, 23].

Statistical analysis

SPSS® for Windows (Version 16.0, Chicago, IL, USA), statistical analysis was done. The results were presented as means ± standard error of the mean (S.E.M) of at least five measurements; $p < 0.05$ was considered as statistically significant. The data were assessed by one-way analysis of variance (ANOVA) followed by Tukey's test.

Results and Discussion

Phenolic content of Ziziphora clinopodioides samples

Using the HPLC method, it was possible to confirm the qualitative phenolic composition and to determine the quantitative content of the substances; flavonoid apigenin and phenylpropanoid glycoside-verbascoside, which previously were identified in the extract by TLC (flavonoid apigenin-chloroform-methanol system 9:1, Rf = 0.65, and phenylpropanoid glycoside-verbascoside-chloroform-methanol-water system 16:2:1, Rf = 0.4).

Series of the prior chromatography experiments confirmed that better separations of the phenolic substances were carried out by an isocratic elution mode. Then identification is implemented by using a UV detector. The identification of substances of the samples by the retention time was carried out, which was previously determined by the standard samples chromatography analysis. The chromatograms of the standard samples showed that the retention times for the standards were the following: for the verbascoside 4.854 min; for luteolin, 6.142 min; for apigenin, 7.134 min, respectively.

The research results are shown in Tables I, II, III and IV) with regard to the ethanolic extracts of raw material of *Ziziphora*, cultivated in the hydroponic

conditions, in the soil, and collected nearby the villages of Voghjaberd and Surenavan.

Comparing the retention times of the analysed sample's chromatogram signals with the standard samples signals presented in the Tables I, II, III and IV we identified the following compounds: flavonoid-apigenin, phenyl-propanoid glycoside-verbascoside.

Table I

Quantitative characteristics of the extract of *Ziziphora* cultivated in the hydroponic conditions

	Name	Retention Time (min)	Area (AU)	%Area	Conc. (mg/mL)
1	Verbascoside	4.995	936114	99.45	0.504
2	Apigenin	7.148	5148	0.55	0.0024

Table II

Quantitative characteristics of the extract of *Ziziphora* cultivated in the soil

	Name	Retention Time (min)	Area (AU)	%Area	Conc. (mg/mL)
1	Verbascoside	4.956	422012	48.26	0.114
2	not identified	5.175	438937	50.20	-
3	Apigenin	7.138	13510	1.54	0.003

Table III

Quantitative characteristics of the extract of *Ziziphora* wild collected 40°10'5"N 44°38'40"E (sample set 2)

	Name	Retention Time (min)	Area (AU)	%Area	Conc. (mg/mL)
1	Verbascoside	4.864	557965	48.91	0.15
2	not identified	5.163	570170	49.98	-
3	Apigenin	7.179	12659	1.11	0.0029

Table IV

Quantitative characteristics of the extract of *Ziziphora* collected 40°04′07″N 46°54′21″E (sample set 4)

	Name	Retention Time (min)	Area (AU)	%Area	Conc. (mg/mL)
1	Verbascoside	4.936	1602009	43.78	0.215
2	not identified	5.079	778519	21.28	-
3	not identified	5.213	1191477	32.56	-
4	Apigenin	7.138	86883	2.37	0.01

It was established that the amount of verbascoside for the samples of the raw material of *Ziziphora* cultivated in the hydroponic conditions was 11.34% (0.504 mg/mL), for the samples of raw material cultivated in the soil – 2.508% (0.114 mg/mL); for the raw materials collected 40°10'5"N 44°38'40"E (sample set 2) – 3.75% (0.15 mg/mL) and 40°04′07″N 46°54′21″E (sample set 4) – 4.053% (0.215 mg/mL). The results presented in Table V showed that the apigenin content for the samples of raw material collected nearby the 40°04′07″N 46°54′21″E (sample set 4) was 0.189% (0.01 mg/mL), for the raw materials collected in the area of the 40°10'5"N 44°38'40"E (sample set 2) – 0.073% (0.0029 mg/mL), for the samples of raw material cultivated in the soil – 0.066% (0.003 mg/ml) and for the samples of raw materials, cultivated in the hydroponic conditions – 0.054% (0.0024 mg/mL).

Luteolin did not reveal in our analytical samples, compared with other *Ziziphora* species, where luteolin was found, according to the scientific literature data [16, 17, 29].

So, the quantitative content presented in Table V showed that the content of apigenin was higher in the raw material of *Ziziphora*, collected from the 40°04′07″N 46°54′21″E (sample set 4) than the amount of apigenin in raw materials from 40°10'5"N 44°38'40"E (sample set 2), and in the samples cultivated in the hydroponic conditions and in soil. The amount of verbascoside for the samples of the raw material of *Ziziphora* cultivated in the hydroponic conditions was higher than that of verbascoside in raw materials from the samples of wild-growing and soil.

Table V

The content of the phenolic compounds in the extracts of the *Ziziphora* cultivated in hydroponics conditions, in soil, and collected 40°10'5"N 44°38'40"E (sample set 2) and 40°04′07″N 46°54′21″E (sample set 4)

The areas of the plants collected	Content of the verbascoside (%)	Content of the apigenin (%)
Hydroponics (black slag)	11.34	0.054
Soil	2.508	0.066
Voghjaberd	3.75	0.073
Surenavan	4.053	0.189

In this study, the presence of two phenolic compounds (flavonoid apigenin and phenylpropanoid glycoside verbascoside) was confirmed in the extracts obtained from the raw material of *Ziziphora* wild growing in South-Caucasian regions and cultivated in hydroponics conditions and soil. It was shown that the growing requirements of *Ziziphora* also had an influence on the dynamics of the accumulation of phenolic compounds.

Total flavonoid content of Ziziphora clinopodioides samples

The results of the developed modified spectrophotometric absorption method presented in the Table VI showed that the total amount of flavonoids was the highest (4.176 ± 0.065%) in the extract from the raw material *Ziziphora* collected from 40°38′39″N 44°28′53″E. (sample set 1), The lowest amount (2.61 ± 0.076%) of flavonoids was in the extract from the raw materials collected from 39°39′30″N 46°36′7″E (sample set 5) ($p < 0.001$). In the extracts from the raw materials of *Ziziphora* collected from 40°04′07″N 46°54′21″E (sample set 4), 40°04′07″N 46°54′21″E (sample set 4), and cultivating in hydroponics condition and the soil, the total amount of flavonoids was similar ($p^* > 0.05$). The results of the study presented in Table VI lead to the conclusion that the plant *Ziziphora* contains a significant quantity of flavonoids ranging from 2.57% to 4.18%. Differences in the quantitative amount of flavonoids are not only due to the climatic factors, but also to the cultivating conditions.

Table VI

The total amount of the flavonoids with respect of 7- methyl sudahitin depending on the raw material collected climatic zones and cultivated conditions

The areas of the plants collected	Absorbance of the test solution, D_x, $\overline{x} \pm E_s$	Amount of flavonoids %, $\overline{x} \pm E_s$	
Hydroponics(black slag)	0.65 ± 0.01	3.404 ± 0.056	p^{b}*, p^{c}, p^{d}, p^{e}*, p^{f}*, p^{g}
Soil	0.60 ± 0.011	3.368 ± 0.063	p^{a}*, P^{c}, p^{d}, p^{e}*, p^{f}*, p^{g}
Sample set 1 (40°38′39″N 44°28′53″E.), altitude 1990 m	0.756 ± 0.012	4.176 ± 0.065	P^{a}, p^{b}, p^{d}, p^{e}, p^{f}, p^{g}
Sample set 2 (40°10'5"N 44°38'40"E.), altitude 1880 m	0.522 ± 0.016	2.954 ± 0.089	p^{a}, p^{b}, p^{c}, p^{e}, p^{f}, p^{g}
Sample set 3 (39°54′28″N 46°52′14″E.), altitude 821 m	0.618 ± 0.013	3.4 ± 0.071	p^{a}*, p^{b}*, p^{c}, p^{d}, p^{f}*, p^{g}
Sample set 4 (40°04′07″N 46°54′21″E.), altitude 1780 m	0.61 ± 0.01	3.51 ± 0.057	p^{a}*, p^{b}*, p^{c}, p^{d}, p^{e}*, p^{g}
Sample set 5 (39°39′30″N 46°36′7″E.), altitude 2650 m	0.528 ± 0.015	2.61 ± 0.076	p^{a}, p^{b}, p^{c}, p^{d}, p^{e}, p^{f}

% in accordance of absolutely dry weight ($n = 5$, $\overline{x}$-mean, E_s- standard error of the mean)

$p^* > 0.05$; $p < 0.001$; p^{a} – each sample *vs*. Hydroponics; p^{b} – each sample *vs*. Soil culture; p^{c} – each sample *vs*. sample set 1; p^{d} – each sample *vs*. sample set 2; p^{e} – each sample *vs*. sample set 3; p^{f} – each sample *vs*. sample set 4; p^{g} – each sample *vs*. sample set 5.

Conclusions

As of our knowledge, the current study is a first report regarding *Ziziphora clinopodioides* Lam. raw material growing and cultivated in South-Caucasian regions. Two phenolic compounds, apigenin and verbascoside, were identified, which could be used as markers for standardization of the *Ziziphora* species raw material.

The modified spectrophotometric method for the quantitative determination of the total amount of flavonoids in the raw material of *Ziziphora* with respect to 7-methyl sudahitin was developed, and the specific absorption coefficient was defined for the flavonoid 7-methyl sudahitin ($E^{1\%}_{1см}$ = 920). This method is effective and can be applied to standardise flavonoids in the plant's raw materials.

The current study indicated that the raw material of *Ziziphora clinopodioides* growing wild, cultivating in soil and hydroponics conditions, is a promising source of flavonoids.

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Conflict of interest

The authors declare no conflict of interest.

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