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Five Flavonoids from Lucerne Varieties

Olena Grechana[1], Anatoly Serbin[2], Anna Rudnik[1] and Olena Saliy[3]

1. Zaporizhzhia State Medical University, Mayakovskyi Av., 26, Zaporizhzhia, 69035 Ukraine
2. National University of Pharmacy, Pushkinska St., 53, Kharkiv, 61000 Ukraine
3. Kyiv National University of Technologies and Design, Nemyrovych-Danchenko St., 2, Kyiv, 01011 Ukraine

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*Corresponding author
Olena Grechana

Email:
1310grechanaya@ukr.net

ABSTRACT

Alfalfa (Fabaceae) is known as perennial herbaceous leguminous plant species that originated in southwestern Asia and is used as a folk medicine for the treatment of various ailments. The upper ground part of Lucerne contains phenolic compound such as flavonoids and others, which exhibit biological activities. This study aimed to determine five widely known flavonoids in extracts 20 alfalfa varieties herb at the Ukrainian steppe growing. 50 seeds of the same size were selected from twenty varieties of alfalfa from different countries, and cultivated in controlled areas of the southern part of the Left Bank of Ukraine at the border of forest-steppe and steppe zones (Zaporizhzhya, Ukraine) from April to June, with 15 °C/ 07 °C (day/night), 14 h/10 h (light/dark) and 60–65% relative humidity. The content of flavonoids was found unequable in ethanol extracts. The chemical compositions and their content were assessed by ultrahigh-performance liquid chromatography. The content of five flavonoids was different in the 20 alfalfa varieties raw materials. Umbelliferone was found high in ethanol extract of Mongolian colorful hybrid (Mongolia, 0.23 mg/g). Four sorts have not contained umbelliferone: Kisvardai (Hungary), Nizona (Cuba), Tanhuato (Mexico), and Mesopotamian (Iraq). The leader from cinaroside content was sort Commercial 2-52-75 of UK origin. Routine has been found in the highest quantities in WL 50 from the USA. Ferganska 700 from Uzbekistan was the leader in luteolin content and Kisvardai, Hungary was the leader in an average of kaempferol content (0.030 mg/g). The present article comprises the hierarchical cluster analyses from the data flavonoid assay. In fact, a real method has been obtained for the targeted production of valuable biologically active components with a high content from plant sources.

Key words: Lucerne, Cultivar, Flavonoids, Hierarchical Clustering

INTRODUCTION

The medicine remedies derived from plants are of great importance in the therapy of various types of diseases and are widely used in medical practice (Sadowska *et al*., 2014). They are included in more than 30 pharmacotherapeutic groups of medicine remedies and practically do not have equivalent substitutes (Trease *et al*., 2009). Ukrainian domestic medical practice of new types of herbal substances and their derivative products, require the expansion of the range of herbal formulations (Grechana *et al*., 2021).

A. Decandol first grounded the theory of plant introduction, later was developed by M. Vavilov as a complex biological process, which requires knowledge of the limits of endurance of the introduced and its features – the temperature reaction, soil and air humidity, light, phylogenetic features and geographical origin (Malysheva *et al*., 2018). Because only the investigation of the whole complex of factors: thermal, bioecological, geographic and chemical, revealing among them the integral and functional dependence can give an opportunity to predict the effect of introduction (Chen and Chen, 2013; Janska *et al*., 2010; Major *et al*., 1991; Xu *et al*., 2020 a).

Cultivated medicinal herbs and raw materials gathered from it have several advantages over wild-growing thickets. Growing can use mechanized processing techniques, improving agronomy and plant breeding, etc. (Alter, 1919; Trischuk *et al*., 2014). Cultivated medicinal herbs

and raw materials gathered from it have several advantages over compounding wild-growing thickets, where can use mechanized processing techniques, improving agronomy and plant breeding, etc.(Alter, 1919; Trischuk *et al*., 2014).

As we conduct the introduction of medicinal plants, a special place is given to the most important features of the chemical composition in view of its possible variability at the new conditions of existence (Bertrand *et al*., 2017; Malysheva *et al*., 2018). The countries population with a high standard of living today consumes from 23 mg to 1-2 g of flavonoids daily with food.

Flavonoid preparations are powerful antioxidants, immunomodulators, and anti-inflammatory pharmacologically active compounds. They have attracted the attention of phytochemical researchers due to their wide range of pharmacodynamics and lowly toxicity. Dietary plants containing flavonoids are reported to be functional foods that provide a wide range of protection against different organ-induced oxidative damage and protects from various lethal disorders by increasing antioxidants (Cieśla *et al*. 2013; Khaleel *et al*., 2005) and suppressing inflammation and apoptosis in various tissues including the brain, liver, kidney and the heart (Chen and Chen, 2013; Chitturi *et al*., 2019). It vides prescribe as a potential inhibitor of COVID 19 (Khaerunnisa *et al*., 2020) and other viruses (Lalani and Poh, 2020).

Lucerne (*Medicago sativa* subspecies) is arguably the one world's most important and drought-tolerant temperate perennial legume (Fabaceae L.). It is commonly known as the "father of all foods" (al-fal-fa), originated in Asia. M. sativa has been grown for a variety of purposes such as medicinal uses. One common plant that has been used as traditional medicine is *Medicago sativa* L., known as Lucerne or alfalfa and it belongs to the family Fabaceae L. (Trease and Evans, 2009).

The purpose was to 1) investigate the acclimation of Medicago sativa L. (20 varieties from different countries) as the medicinal herbs into Ukrainian territories where they have not met until now and assay the five their flavonoids; (2) clarify the relationship between flavonoids (percentage) in Zaporizhzhya (Ukraine) climate of alfalfa sorts by hierarchical clustering.

MATERIAL AND METHODS

Plant Material

This experiment was conducted in controlled areas of the southern part of the Ukrainian left-bank (Primorske village, Vasyliv district, Zaporizhzhya region, 47 ° 37′28 (N, 35 ° 17′39 (S), at the border of forest-steppe and steppe zones (Zaporizhzhya, Ukraine) from April to June, with 15 °C/07 °C (day/night), 14 h/10 h (light/dark) and 60–65% relative humidity. We selected 50 seeds of the same size from twenty alfalfa cultivars (provided by Feed and Agriculture Institute of Podillya, Ukrainian National Academy of Agrarian Sciences) without any processing and seeded them into the soil (previously was analyzed) (Table I). D-r Yury G. Gamulya identified the plants, and the voucher specimens were stored in Karazin Kharkiv National University, Kharkiv, Ukraine.

Soil - medium loamy, low-humus. Agricultural research techniques are common for the steppe zone. The sowing of alfalfa seeds in the experiment was carried out according to the soil ready for the optimum terms for the culture (April). The plots were placed without repetitions. The sowing method was with spacing 70 cm (4 rows). The cultivation technology is generally accepted for alfalfa (Bertrand *et al*., 2017; Grechana and Serbin., 2021; Xu *et al*., 2020a).

Accounts for the main features and phenological observations of plant development were conducted following the Methodological Instructions and the Technique of field experiments.

Weeds were removed regularly. The cultivation continued until the time of mass flowering when medicinal plant raw materials were harvested (June-July). Moreover, other of them were continually growing. Immediately plants were used to determine morphology-physiological indicators. We divided the sample into two parts, one part was used for the determination of flavonoid, and the other part was used for the determination of morphological indicators and calculating biomass. After measuring, the samples were weighed after being dried in place without sunlight. Ethanol, acetonitrile, trifluoroaceti c acid were obtained from Merck (Darmstadt, Germany). Umbelliferon, cinaroside, routine, luteolin and kaempferol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analysis

The experimental data were done in triplicate measurements and reported as Me. Statistical comparisons were performed using a one-way analysis of variance (ANOVA).

Table I. Seed material from alfalfa sorts

No.	Sort	Origin country	No National Catalog
1	Commercial 2-52-75	United Kingdom	UJ0700195
2	Sevany-1	Russia	UJ0700189
3	Kisvardai	Hungary	UJ0700190
4	Vertibenda	Germany	UJ0700390
5	Mega	Sweden	UJ0700365
6	JJ Paso	Argentina	UJ0700364
7	Peruvian pubescent	Peru	UJ0700414
8	Boreale	France	UJ0700406
9	Saladina sintetica la Banda	Argentina	UJ0700354
10	Ferganska 700	Uzbekistan	UJ0700380
11	Vakhshska 233	Tajikistan	UJ0700379
12	Krasnovodopadska 8	Kazakhstan	UJ0700329
13	Nizona	Cuba	UJ0700368
14	WL 50	USA	UJ0700397
15	Moremmona	Italy	UJ0700344
16	Liguen	Chile	UJ0700429
17	Tanhuato	Mexico	UJ0700339
18	Mesopotamian	Iraq	UJ0700428
19	Mongolian colorful hybrid	Mongolia	UJ0700188
20	Sinucha	Ukraine	UJ0700134

A significant difference was defined at the 95% confidence level ($p<0.05$). Clustering of the flavonoid content of the M. sativa sorts extracts was performed using principal component analysis (PCA). Summary of linear equations, quadratic correlation coefficient, the limit of determination, and limit of quantification of the five flavonoids need to be presented.

Phytochemical Analysis

HPLC was performed according to the method previously described by Golembiovska O. M. sativa herbs were dried, pulverized into a powder, and then sieved with 100 mesh particle size. The powdered Medicago samples (0.100 g) were extracted by intermittent shaking with 1 mL of 70% (v/v) ethanol at 70 °C, and the mixture was centrifuged at 12,000 rpm for 10 min. The supernatant was then diluted at 1:10 (sample: 70% ethanol) and filtered through a 0.45-μm syringe filter. The diluted samples were analyzed with a Shimadzu LC20 Prominence HPLC system in a modular system equipped with a four-channel pump LC20AD, column thermostat CTO20A, automatic sampler SIL20A, diode-matrix detectors SPDM20A and ChemStation LC20. We used column Phenomenex Luna C18 (2) with the size 250 mm x 4.6 mm (particle size 5 μm), with a olumn temperature of 35 °C. The absorbance of the supernatant was measured at 330 nm (flavonoid glycosides), 350 and 370 nm (flavonoid aglycones).

The HPLC conditions were as follows: solvent A, 0.1% water solution of trifluoroacetic acid; solvent B, 0.1% acetonitrile solution of trifluoroacetic acid, flow rate, 1 mL/min. We had injected the sample volume 5 μl. The eluent balance has varied following proportions during chromatography; 95 %: 5 % at the first 5 minutes and the finishing (Table II). We were identified components by the retention time and by standard substances UV-spectra compliance. The absorbance of the umbelliferon (U) was measured at 232 nm (OD232), cinaroside (C) (luteolin-7-glucoside) at 348 nm (OD348) and 254 nm (OD254); routine (R) at 256 nm (OD256); luteolin (L) at 254 nm (OD254); kaempferol (K) at 265 nm (OD265) and 366 nm (OD366 (Figure 1). The flavonoids content X (%) was measured according to eq. 1, where P (%) is the standard activity; V_{pr} (mL) is the total volume of the supernatant, V_{st} (mL) is the volume of the standard; m_{st} (mg) and m_{pr} (mg) are the weights of the standard and dried sample; A_{pr} *and* A_{st} are the peak areas of the substance and standard respectively.

$$X = \frac{A_{pr} \times m_{st} \times V_{pr} \times P}{A_{st} \times V_{st} \times m_{pr}} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Table II. The chromatography eluent balance

Chromatography time (min)	eluent A (%)	eluent B (%)
0–5	95	5
5–35	95 → 75	5 → 25
35–40	75	25
40–60	75 → 50	25 → 50
60–65	50 → 20	50 → 80
65–70	20	80
70–85	95	5

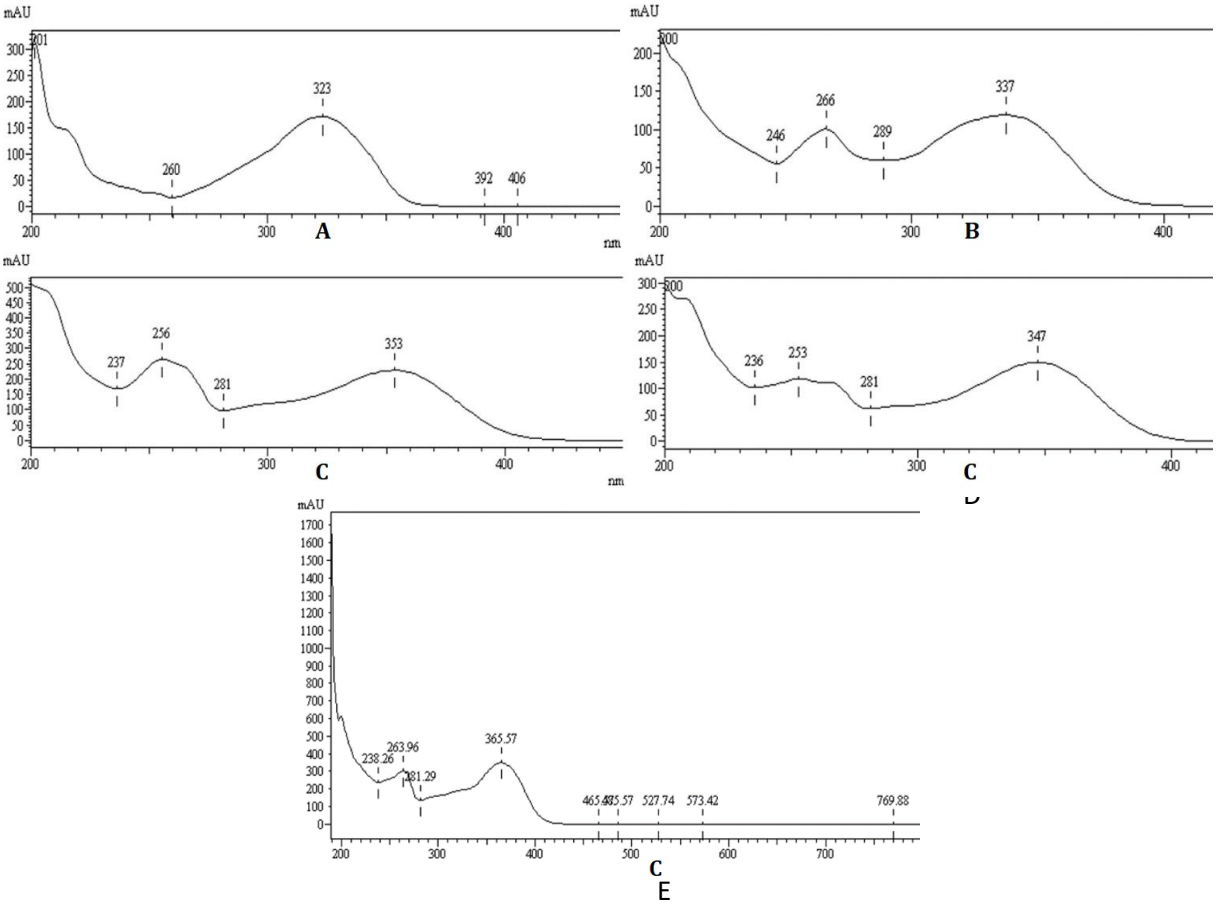

Figure 1. Standard spectras (A – Umbelliferon; B - Cinaroside (luteolin-7-glucoside); C - Routine; D – Luteolin; E – Kaempferol)

Simultaneously were noted the calendar terms of the beginning and mass passage of the main phases of development for 20 Medicago varieties (Altemimi *et al*., 2017; Esmaili *et al*., 2015). All the data was collected from three replicate experiments. Shapiro–Wilk test and Levene test showed that all data in this experiment obeyed a normal distribution and satisfied the homogeneity of variance. Data in this study were subjected to a two-way analysis of variance between treatments using SPSS (SPSS Inc., Chicago, USA). The differences were considered significant at $p < 0.05$ and $p < 0.01$. Hierarchical clustering was performed using the R statistical software. The distance matrixwas used to construct a dendrogram via the Single communication method, Euclidean distance.

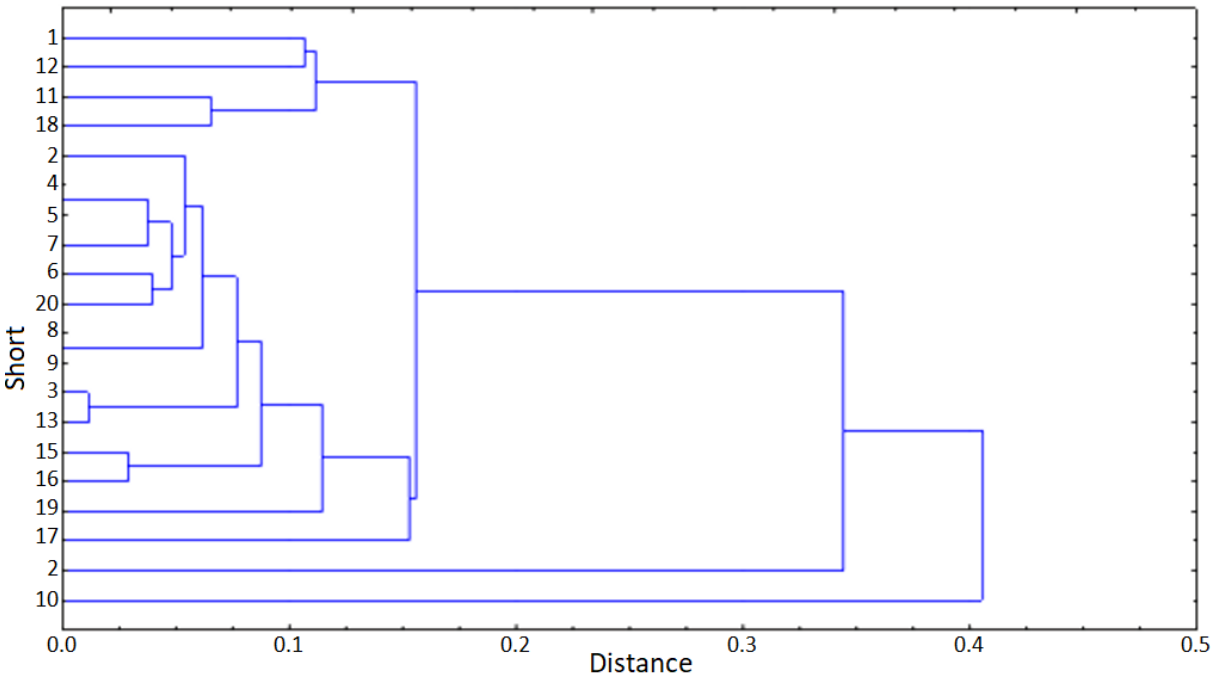

Figure II Cluster dendrogram for 20 alfalfas sorts flavonoids.

RESULT AND DISCUSSION

Growing is the first step to the extraction of the bioactive phytochemical compounds from plant materials (Supplementary Data). Among the characteristics in Medicago descriptors, morphological traits and phytochemical content tend to be the most affected by environmental factors (temperature, water regime, sunlight amount). In addition, the origin and growing conditions of the Medicago plant affect the content of Medicago's phytochemicals, which change bioactivity.

Identification of compounds was achieved by comparing their spectra (Figure 2) and retention times with those of standards and literature data. The distribution of the two flavonoid glycosides (rutin and luteolin-7-O-glucoside) and three flavonoid aglycones (umbellyferone, rutin, kaempferol) content in the 20 Medicago accessions (Table III).

The average of U content varying from 0.234 mg|g in Mongolian colorful hybrid (No 19, Mongolia) to 0.025 mg/g in WL 50 (No 14, USA). The average of U content decreased in the following order: 19 > 15 > 16 > 10 > 1 > 6 > 8 = 9 > 4 = 5 > 12 > 20 > 7 > 11 > 2 > 14. Noteworthy there are four sorts that have not contained U: Kisvardai (No 3, Hungary), Nizona (No 13, Cuba), Tanhuato (No 17, Mexico) and Mesopotamian (No 18, Iraq). There are works with describing studies of differences in the morphology of organs, the composition and quantity of chemical components of plants of different ecotypes (Lei Z., 2019). The *M. sativa* sorts analyzed in this work represented 20 geographical origins, i.e., United Kingdom, Russia, Hungary, Germany, Sweden, Argentina, Uzbekistan, Kazakhstan, Tajikistan, Cuba, USA, Italy, Chile, Mexico, Iraq, Mongolia, and Ukraine (Table I). Probably the origin of the variety has a certain effect on the chemical composition and in particular on the presence and amount of U or due to the different growth rates of the different sorts. Like the Medicago truncatulla ecotipes (Lei Z., 2019) data, our objects were harvested on the same date. Moreover, apparently these varieties have not yet accumulated ingredients for the biosynthesis of U. C was abundant flavonoid (from 0.480 mg|g in No 1, Commercial 2-52-75, United Kingdom and 0.404 mg/g (No 12, Krasnovodopadska 8, Kazakhstan) to 0.066 mg|g in No 8 (Boreale, France) and No 9 (Saladina sintetica la Banda, Argentina). C content decreased in the following order: 1 > 12 > 18 > 10 = 11 > 14 > 19 > 6 > 20 > 15 > 13 > 3 > 17 > 7 > 16 > 4 = 5 > 2 > 8 = 9. R, one from the most known and are used flavonoids, in the largest quantities was in No 14, WL 50 from USA (0.509 mg|g). R was find less in No 17 Tanhuato from Mexico in four time (0.197 mg/g).

Table III. Amount of flavonoid in 20 sorts Medicago, (%) on dry matters (n=3) and Standard Error of Mean (SEM)

No	U	C	R	L	K
1	0.12773±0.00003	0.48507±0.0037	0.0199±0	0.00874±0	0.00677±0
2	0.05078±0	0.07753±0	0.0071±0	0.00335±0	0.00065±0
3	n.d.	0.13157±0.0002	0.042±50	0.01304±0	0.03027±0
4	0.09775±0	0.12743±0.00003	0.0323±0	0.01461±0	0.00343±0
5	0.09775±0	0.12743±0.00003	0.0323±0	0.01461±0	0.00343±0
6	0.12137±0.00002	0.18277±0.00003	0.0182±0	0.01556±0	0.01320±0
7	0.06580±0	0.12816±0.00002	0.0133±0	0.00793±0	0.00424±0
8	0.11148±0.00002	0.06683±0	0.0101±0	0.00337±0	0.00078±0
9	0.11148±0.00002	0.06683±0	0.0101±0	0.00337±0	0.00078±0
10	0.17098±0.0003	0.32441±0.0004	0.0399±0	0.405410±	0.00868±0
11	0.05801±0	0.32441±0.0004	0.00771±0	0.00764±0	0.00868±0
12	0.09085±0	0.40432±0.0057	0.07504±0	0.01393±0	0.02989±0
13	n.d.	0.14126±0.00008	0.04548±0	0.01307±0	0.02514±0
14	0.02453±0	0.27302±0.0006	0.50920±0.004	0.01627±0	0.00620±0
15	0.20465±0.0002	0.15587±0.0001	0.01249±0	0.01304±0	0.01106±0
16	0.20957±0.0002	0.12872±0.00008	0.01078±0	0.00735±0	0.00654±0
17	n.d.	0.12884±0.0001	0.19732±0.0003	0.02441±0	0.01448±0
18	n.d.	0.32730±0.0006	0.03649±0	0.00594±0	0.00082±0
19	0.23423±0.0002	0.26636±0.0003	0.01616±0	0.01024±0	0.01787±0
20	0.08611±0	0.16968±0.00007	0.01505±0	0.02200±0	0.00364±0

PS: n.d. – not fetected

Some Medicago varieties (No 2, Sevany-1, Russia and Vakhshska 233, Tajikistan) have been marked with trace R amount. The sorts line of decreasing R content was following: 14 > 17 > 12 > 13 > 3 > 10 > 18 > 5 = 4 > 1 > 6 > 19 > 20 > 7 > 15 > 16 > 8 = 9 > 11 >2. No 10 Ferganska 700 from Uzbekistan has leadered from luteolin (L) content. The average of L content was 0.405410 mg|g here. Other varieties contained L an order of magnitude less and are arranged in this order: 10 > 17 > 20 > 14 > 6 > 5 = 4 > 12 > 13 > 3 > 15 > 19 > 1 > 7 > 16 > 11 > 18 > 9 =8 > 2. The flavonol kaempferol (K) has been founded in varieties across too and they decrease in the following order: 3 > 12 > 13 > 19 > 17 > 6 > 15 > 10=11 > 1 > 16 > 14 > 7 > 20 > 4 = 5 > 18 > 8 = 9 > 2. The average of K content varying from 0.03027 mg|g in Kisvardai (No 3, Hungary) to 0.00065 mg|g in Sevany-1, Russia, No 2. The K amounts from No 12, Krasnovodopadska 8, Kazakhstan and Nizona, Cuba have were close (0.02989 and 0.02514 mg|g respectively).

These results were successfully shown that different origin samples in the same climate and conditions show distinct opportunities for the formation of structures of interest to us with promising pharmacological action. The hierarchical clustering analysis has been helped to find the link of sorts origin with their flavonoids composition in Ukrainian growing. We performed a tree structure containing a k-block set partition for each value of k between 1 and n, where n is the number of data points to cluster. Note have been connectioned the sorts origin only to the five flavonoids composition in clustering analysis (Figure II).

The dendrogram on the x-axis indicates the degree of similarity between the flavonoids, the closer the amont of flavonoid the higher the level of similarity in them and the flavonoid have been clustered using hierarchical clustering. Similarly, the dendrogram on the y-axis indicates the degree of similarity between the different samples, the closer the samples the higher the level of similarity in them and they have been clustered using hierarchical clustering (Single communication method, Euclidean distance).

Hierarchical clustering analysis is the process of organizing data into a tree structure that is based on similarity of these data. It led to the identification of the Medicago sativa variety clusters based on their chemical profiles. The main idea is to create a set of elements in the tree with many branches, if the elements are similar to each other, short branches join them, and vice versa, if their similarity decreases, then the branches

increase. In the largest sorts cluster of the Medicago have been aggregated at the bottom level (i.e., at the height of zero) in the dendrogram and are grouped together at low levels (No 3, 13, 15, 16). However, some sorts are far apart (No 5, 7, 6, 20, 1, 12, 17). Between the some pairs of sorts showed significantly different patterns.

The greatest distances in the hierarchical tree are formed by cultivars 2 (Sevany-1, Russia) and 10 (Ferganska 700, Uzbekistan). They are members of different clusters.

CONCLUSION

In this study, we tried to unravel the complex interaction between chemicals (flavonoids) and plants origin using the computational prediction model relationship between the plants and their chemicals (Leung *et al*., 2013). In chemotaxonomy (by hierarchical cluster analysis) metabolites can be used as specialized markers to distinguish among plants. However, us we saw not all secondary metabolites (in our casual) are produced in the same taxon (Kumar *et al*., 2013, Wink, 2003). The approach is based on the different sorts plant chemicals that could be used us closely interact with each other and could be suggested to acclimatization and growing in Ukraine. For our mind, this is in fact a real way to obtain high-content valuable biologically active components from plant sources purposefully.

We hope that the clustering information by the flavonoids profiles between the plants sorts can be utilized more widely.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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