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herba polonica
From Botanical to Medical Research
Vol. 61 No. 3 2015
DOI: 10.1515/hepo-2015-0020

EXPERIMENTAL PAPER

Variability of southern sweet-grass (*Hierochloë australis* /Schrad./ Roem. & Schult.) wild growing populations occurring in eastern Poland

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S u m m a r y

Southern sweet-grass is a perennial grass distributed through north-eastern Europe. In Poland, this rare, partially protected plant grows wild in well-lit coniferous or mixed forests. Its leaves, rich in coumarin compounds, are used for aromatization of alcohols. Taking into account high demand on this raw material and decrease in the number of southern sweet-grass populations it is recommended to introduce the plant into cultivation systems. In this study 12 populations of southern sweet-grass were selected and assessed in terms of their cultivation usefulness. The presence of associated plant species growing on its natural sites was investigated as well as light irradiance on those stands. The species was found in 3 types of forest communities. Air dry mass of leaves per plant varied in analyzed populations from 1.73 to 11.07 g. They were significantly diversified in the total content of coumarins (0.36–0.96%), flavonoids (0.09–0.26%) and polyphenolic acids (0.13–0.29%). The diversity concerning the content of coumarin, a quality indicator of leaves, was even higher. The content of this compound varied from 52.03 to 275.74 mg/100 g d. m. Among the analyzed populations, the most interesting seem to be population No. 12 (Jedwabno) and population No. 10 (Ruciane) characterized with relatively high mass of leaves and the highest content of the identified active compounds, including coumarin.

Key words*: southern sweet-grass, populations, light, coumarins, flavonoids, polyphenolic acids*

INTRODUCTION

Southern sweet-grass (*Hierochloë australis* /Schrad./ Roem. & Schult.) is a perennial grass from *Poaceae* family, distributed throughout the north-eastern Europe. In Poland, the species occurs in minor, dispersed populations, almost exclusively at the area of Podlasie, Warmia and Mazury regions. It grows at the edges of mixed and coniferous forests, in slightly shaded sites, especially forest fellings [1-3]. Southern sweet-grass, partially protected species, is also included into the Red List of Vascular Plants in Poland [4].

The raw material obtained from this plant are leaves characterized by sweet, cherry-flower like aroma, used for extracts production, utilized in food industry for flavouring alcoholic beverages and tobacco products [5, 6]. Main group of compounds present in this raw material are coumarins, with coumarin as a dominant and indicator of dried leaves quality [1, 6]. Coumarin reveals antifungicidal and antitumor activity, it also increases blood-thinning and flow in the veins, however when used at high doses it can be toxic [7-9].

Southern sweet-grass leaves are collected exclusively from the wild in Poland [5, 6]. Despite the legal protection of the species, up to now the harvest of raw material has been uncontrolled and extensive, which causes significant decrease in the number of populations [10]. Due to decline of this plant on natural sites and high demand for the raw material, it is advisable to introduce the southern sweet-grass into cultivation. Thus, firstly it is crucial to determine the range of variability of wild growing populations of this species, being a reserve of its genetic resources.

The aim of the present work was to determine the variability of selected southern sweet-grass populations located in eastern Poland, in terms of some morphological features as well as content of coumarin, coumarins, phenolic acids and flavonoids in leaves.

MATERIAL AND METHODS

Plant material

Objects of the study were 12 wild growing southern sweet-grass populations located in natural sites in eastern Poland. Their geographical coordinates are provided in table 1. In June 2012, at each population, morphological observations were carried out on six randomly chosen plants. The number of leaves per plant, fresh and dry mass of leaves were determined. Plant material was collected from several (min. 5) plants, and then mixed to obtain a representative sample. Collected raw materials were dried at 35°C, powdered and subjected to chemical analysis. Voucher specimens were deposited at herbarium of Department of Vegetable and Medicinal Plants WULS-SGGW.

Table 1.

Geographical coordinates of natural sites of southern sweet-grass populations and light intensity on these sites

Population No./site		Coordinates		Light intensity [μmol photons/m^2/s]
1	Wiercień	N 52°29.903'	E 022°52.205'	774
2	Siemiatycze	N 52°26.952'	E 022°51.380'	1256
3	Hrabska Droga	N 52°38.705'	E 022°46.600'	1592
4	Sieśki	N 52°51.069'	E 022°82.284'	1410
5	Pietkowo-Wilkowo	N 52°53.242'	E 022°49.905'	848
6	Wyliny-Ruś	N 52°47.036'	E 022°39.957'	1481
7	Wiercień Duży	N 52°30.103'	E 022°51.175'	1233
8	Koryciny	N 52°37.944'	E 022°45.718'	1130
9	Strabla	N 52°53.678'	E 022°04.989'	1273
10	Ruciane	N 52°40.169'	E 021°34.290'	1667
11	Wejsuny	N 52°39.158'	E 021°31.632'	889
12	Jedwabno	N 52°32.425'	E 020°41.928'	1785

Phytosociological observations

At each natural site of southern sweet-grass, the basic phytosociological observations, concerning the composition and abundance of associated plant species were performed, according to Braun-Blanquet approach [11]. Phytosociological relevés were done on each site on the area of 100–200 m^2. Syntaxonomic classification of identified species was performed according to Matuszkiewicz [12]. In order to assign homogeneous communities, systemized phytosociological records were entered into SPSS software. The hierarchical cluster analysis was employed to determine the relationships among populations from different communities. The obtained results were presented graphically, i.e. the dendrogram was constructed on the grounds of agglomerative grouping by the method of average linkage. The linkage between the groups was measured using the squared Euclidean distances according to Ward method.

Light intensity

Light intensity on all the investigated natural sites was determined by using a phytophotometer (RF-100). On each site the measurements were done in 10 replications, about 40 cm above ground level. The data provided in table 1 are mean values form these measurements.

Determination of total content of coumarins, flavonoids, and phenolic acids

Total content of coumarins was determined spectrophotomertically according to Wierzchowska-Renke and Stecka [13]. Total content of flavonoids (expressed as quercetin equivalents, %) and total content of phenolic acids (expressed as caffeic acid equivalents, %) were determined spectrophotometrically, according to Polish Pharmacopoeia 8th [14]. The presented results are mean values from three replications.

HPLC analysis of coumarin

Commercially available standard of coumarin (ChromaDex®) was dissolved in 10 ml volumetric flask with MeOH according to the ChromaDex's Tech Tip 0003: Reference Standard Recovery and Dilution and used as standard stock solution. Further calibration levels were prepared by diluting this solution with methanol in 10 ml volumetric flasks (injected volumes ranges: 10, 50, 100, 200, 500 and 1000 μl). The working solutions were injected (1 μl) on a column in six replicates (n=6) using SIL-20A to generate a seven-point calibration curve, using CLASS VP™ 7.3 chromatography software. The peak table and spectra library (190-450 nm) of coumarin were created. Standard curve parameters were calculated with statistical service e-stat (http://www.chem.uw.edu.pl/stat/e-stat/). Signal-to-noise ratio approach was used to determine LOD (S/N of 3:1) and LOQ (S/N of 10:1).

Air-dry, finely powdered and homogenized raw material (1.000 g of leaves) was extracted with 100 ml of methanol in Büchi Labortechnik AG Extraction System B-811. Soxhlet hot extraction with twenty-five extraction cycles, flushing and drying was used. After evaporation of solvent, the residue was dissolved in 10 ml of methanol. The obtained extracts were filtered with Supelco Iso-Disc™ Syringe Tip Filter Unit, PTFE membrane, diameter 25 mm, pore size 0.20 μm and subjected to HPLC.

The analyses were performed using a Shimadzu chromatograph equipped with auto sampler SIL-20A, photodiode array detector SPD-M10A VP PDA and CLASS VP™ 7.3 chromatography software. A modern C-18 reversed-phase column with core-shell technology (Phenomenex Kinetex® 2.6 μm, C18, 100 Å, 100×4.60 mm i.d.) was used as a solid phase. Binary gradient of mobile phase A (deionised water adjusted to pH 3 with phosphoric acid) and B (ACN adjusted to pH 3 with phosphoric acid) was used as follows: 0 min – 17% B; 0.5 min – 17% B; 2.0 min – 50% B; 2.5 min – 50% B; 2.51 min – 17% B; 10.00 min – STOP. The following conditions were applied: flow rate 1.1 ml/min, oven temperature 35°C, total time of analysis 10 min, injection volume: 1 μl.

UV-spectra were recorded between 190 and 450 nm. Peak identification was conducted by comparison of retention time and UV-spectra recorded between 190 and 450 nm of standard. Detection wave applied: 276 nm. The content of the determined compound was calculated in mg per 100 g of dry matter. Validation parameters of the analysis are provided in Table 2.

Table 2.

Validation parameters of the HPLC-DAD analysis (n=6)

Compound	t_R	Precision (CV)	Regression equation	Linearity (r^2)	Range [μg/ml]	LOD [μg/ml]	LOQ [μg/ml]
Coumarin	6.10	0.70	y=14086.0x–7812.5	0.999	0.40–133.20	0.003	0.010

Statistical analysis

Data were subjected to statistical analysis using Statistica® software. The results were analysed with one-way ANOVA and Tukey's HSD test at α=0.05 significance level. In addition, the coefficient of variation (CV) was determined.

RESULTS AND DOSCUSSION

Most of medicinal and aromatic plants, even today, are still collected from the wild. It is estimated that in Europe about 2000 taxa are used on a commercial basis. Such exploitation has resulted in receding populations of many species in their natural habitat [15, 16]. Raw materials obtained from such plants are used in phytopharmaceutical, cosmetic and food industries [15, 17]. *In situ* conservation of their resources alone, restricted only to low protection, is not effective enough taking into account high and increasing demand for some valuable raw materials. Thus, there is a vivid and inevitable need to develop cultivation programmes aimed not only to prevent overexploitation, but also to create a stable source of standardized plant material for industry. Moreover, medicinal plant production through cultivation can reduce the extend to which wild populations are collected from the wild and in consequence it is the best method to preserve plants in their natural habitat [15-17]. In order to initiate cultivation programmes the crucial thing is the selection of high yielding populations, clones or other forms of superior planting material that provide uniformity and desired quality of obtained produce [15].

In Poland, one of rare and endangered species is southern sweet-grass. Although the plant has been partly protected for many years, its leaves are still commercially collected for alcohol industry only from the wild [6, 10]. The present investigation, aimed to assess the variability of southern sweet grass populations, is the first step to select forms useful in cultivation or breeding programmes. Taking into account the number of individual plants per population on a natural site, for further works only 12 (out of 20 identified) most abundant populations were selected. The populations naturally occurred in three types of forest communities (fig. 1), i.e. in forest communities with majority of Scot's pine with herbaceous undergrowth from *Cladonio-Vaccinietalia* KIELL.-LUND 1967 order among *Vaccinio-Piceetea* BR.-BL. 1939 class – populations No. 1, 2, 5, 6, 9, 10 (tab. 3); in nitrophilous felling communities with the majority of birch (*Betula pendula*) among *Epilobietea angustifolii* R.TX. et PRSG 1950 class – population No. 4 (tab. 4); and in mixed forests communities

with majority of oak (*Quercus robur*) and Scot's pine (*Pinus silvestris*) among *Vaccinio-Piceetea* BR.-BL. 1939 class – populations No. 3, 7, 8, 11, 12 (tab. 5).

Table 3.

Floristic composition of southern sweet-grass natural sites classified into *Cladonio-Vaccinietalia* order among *Vaccinio-Piceetea* class

Layer	Identified species	Site (population No.)/cover-abundance scale					
		Wierceń (1)	Siemiatycze (2)	Pietkowo-Wilkowo (5)	Wyliny-Ruś (6)	Strabla (9)	Ruciane Nida (10)
		Cl. *Vaccinio-Piceetea*					
a	*Pinus sylvestris*	1	4	3	2	3	1
a	*Quercus robur*	.	.	1	1	.	1
b	*Quercus robur*	.	.	.	.	2	+
b	*Picea abies*	.	.	.	.	1	1
b	*Juniperus communis*	.	+	.	1	.	+
b	*Frangula alnus*	+	+	1	+	1	r
b	*Sorbus aucuparia*	.	1	+	.	.	.
c	*Sorbus aucuparia*	.	.	.	.	+	+
c	*Quercus robur*	.	.	+	+	.	.
c	*Hierochloë australis*	1	1	1	2	1	2
c	*Vaccinium myrtillus*	4	1	2	4	3	3
c	*Maianthemum bifolium*	+	.	.	.	.	.
c	*Convallaria majalis*	.	3	1	2	1	+
c	*Melampyrum nemorosum*	+	.	1	r	r	r
		Cl. *Querco-Fagetea*					
a	*Carpinus betulus*	3	.	.	.	.	+
b	*Carpinus betulus*	1	.	.	.	.	.
b	*Corylus avellana*	2	3	2	.	.	1
c	*Stellaria holostea*	r	+	+	+	.	.
c	*Pulmonaria obscura*	+	.	.	.	.	.
c	*Oxalis acetosella*	+	.	.	+	.	.
		Cl. *Epilobietea angustifolii*					
a	*Betula pendula*	+	.	.	.	1	.
b	*Rubus idaeus*	+	.	.	.	.	1
c	*Fragaria vesca*	+	.	.	.	+	+
		Cl. *Artemisietea vulgaris*					
c	*Urtica dioica*	+	.	.	.	.	.
c	*Anthriscus sylvestris*	+	.	.	.	.	.
		Cl. *Nardo-Callunetea*					
c	*Veronica officinalis*	+	.	.	.	+	.
c	*Viola canina*	.	+	.	.	r	.

Table. 4.

Floristic composition of southern sweet-grass natural site classified into *Epilobietea angustifolii* class

Layer	Species	Site (population No.)/cover-abundance scale
		Sieński (4)
	Cl. *Epilobietea angustifolii*	
a	*Betula pendula*	4
c	*fragaria vesca*	+
	Cl. *Vaccinio-Piceetea*	
b	*Quercus robur*	1
b	*Juniperus communis*	+
b	*Frangula alnus*	4
b	*Sorbus aucuparia*	+
c	*Hierochloë australis*	2
c	*Convallaria majalis*	3
	Other species	
c	*Urtica dioica*	+
c	*Veronica officinalis*	1
b	*Corylus avellana*	1

Investigated natural sites differed not only in the floristic composition of associated plant species but also in light conditions (tab. 1, 3-5). Southern sweet-grass, identified at those sites, varied distinctly regarding the number of leaves

Table 5.

Floristic composition of southern sweet-grass natural sites classified into *Vaccinio-Piceetea* class

Layer	Species	Site (population No.)/cover-abundance scale				
		Hrabska Droga (3)	Wierceń Duży (7)	Koryciny (8)	Wejsuny (11)	Jedwabno (12)
	Cl. Vaccinio-Piceetea					
a	*Pinus sylvestris*	1	.	1	3	+
a	*Quercus robur*	2	3	2	2	3
b	*Quercus robur*	+	1	2	.	+
b	*Picea abies*	+	.	.	.	.
b	*Juniperus communis*	.	1	.	.	.
b	*Frangula alnus*	.	+	+	.	1
c	*Sorbus aucuparia*	.	.	.	.	+
c	*Quercus robur*	+	+	r	1	+
c	*Hierochloë australis*	1	1	1	1	2

c	*Vaccinium myrtillus*	.	3	2		2
c	*Maianthemum bifolium*	.	1	.	+	+
c	*Convallaria majalis*	1	.	2	.	.
c	*Melampyrum nemorosum*	r	+	.	.	.
		Cl. Querco-Fagetea				
a	*Carpinus betulus*	+	.	+	.	.
b	*Carpinus betulus*	+	.	+	+	.
b	*Corylus avellana*	2	1	r	1	+
c	*Stellaria holostea*	+	.	.	.	.
c	*Oxalis acetosella*	+	.	.	+	+
		Cl. Epilobietea angustifolii				
a	*Betula pendula*	.	+	.	.	.
c	*Fragaria vesca*	+	.	.	.	.
		Cl. Artemisietea vulgaris				
c	*Urtica dioica*	+	.	.	.	.
c	*Anthriscus sylvestris*	+	.	.	.	.
		Cl. Nardo-Callunetea				
c	*Veronica officinalis*	+	.	.	+	+
c	*Viola canina*	.	.	.	+	.

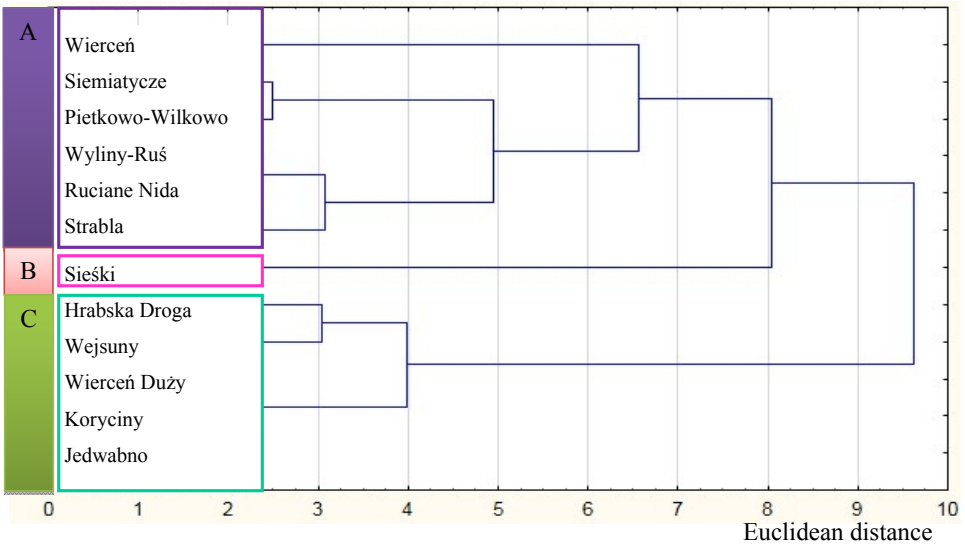


Figure 1.

Analysis of floristic composition of southern sweet-grass natural sites similarities

per plant (39.3-109.6) and in consequence – in their weight (5.3-32.4 g d.m./plant). The highest mass of raw material was obtained from populations No. 3, 4, 6, 10 and 12 which grew on semi-shaded sites characterized with relatively high light intensity, situated mainly on forests fellings in comparison to those that occurred inside forests, in deep shadow (tab. 1 and 6). According to other authors southern sweet-grass occurs mostly at the edges of mixed and coniferous forest or on forest fellings, on semi-shaded stands in dispersed populations [1-3].

It was confirmed that one of the most important environmental factor influencing southern sweet-grass development is light intensity [6]. Ryser and Eek [18] suggest that the adaptive light plasticity among species contribute to their abilities to occupy diverse habitats in nature. Thus, plants with narrow range of light requirements, like southern sweet-grass, are more vulnerable to environmental changes and more endangered.

The quality of southern sweet-grass leaves depends mainly on the content of coumarins with coumarin as a dominant [1, 6]. This compound is also distributed in other plant species among *Fabaceae*, *Lamiaceae* and *Lauraceae* families [19]. It is used mainly in food, tobacco and cosmetic industries [5-9]. Coumarin reveals also some medicinal properties, like anticoagulant, anti-inflammatory, antioxidant, antifungal and antitumor but is well-known and used mostly due to its pleasant odor [7-9, 19, 20].

It was found that coumarin content in plants is related not only to light intensity but also to day lenght and stage of plant development [21, 22]. Previous studies on southern sweet-grass confirm that its content in leaves depends on plant age and may be related to genetic diversity as well [6, 23, 24]. The raw materials collected from the investigated populations varied significantly in the total content of coumarins (0.36–0.96%), flavonoids (0.09–0.26%), phenolic acids (0.13–0.29%), and coumarin (52.03–275.74 mg/100 g d. m.). The coefficient of variation concerning total content of coumarins (31.4%) was similar to that for flavonoids (31.3%) and phenolic acids (28.1%) in comparison to coefficient of variation concerning coumarin content, which was much higher (40.3%) (tab. 7, fig. 2). The most interesting population, characterized with the highest content of all determined groups of compounds and the highest content of coumarin, seemed to be the population No. 12 (Jedwabno) – fig. 3. This population was very abundant and the relatively high irradiance, noted on its natural site, might have affected the accumulation of secondary metabolites in raw material. Interesting raw material, characterized with high content of coumarin, was also obtained from population No. 10 (Ruciane). Further works on this populations should be undertaken in uniform *ex situ* conditions to elucidate the effect of diverse environmental factors influencing plant development and accumulation of biologically active compounds.

Table 6.

Selected morphological traits of investigated southern sweet-grass populations

Population No./site		Number of leaves per plant	Fresh mass of leaves [g/plant]	Dry mass of leaves [g/plant]
1	Wiercień	56.2bc	8.21c	2.37d
2	Siemiatycze	73.6b	17.31b	4.92c
3	Hrabska Droga	99.0a	21.73ab	6.34b
4	Sieński	67.0b	23.13ab	6.95b
5	Pietkowo-Wilkowo	41.2c	5.07c	1.73d
6	Wyliny-Ruś	109.6a	30.16a	7.73b
7	Wiercień Duży	44.5c	5.31c	1.54d
8	Koryciny	92.7a	20.16b	5.48c
9	Strabla	51.7bc	11.05bc	3.01d
10	Ruciane	105.6a	32.42a	11.07a
11	Wejsuny	39.30c	16.43b	4.02cd
12	Jedwabno	104.0a	24.91ab	6.37b
	mean	73.69	17.99	5.12
	CV (%)	36.9	50.9	54.6

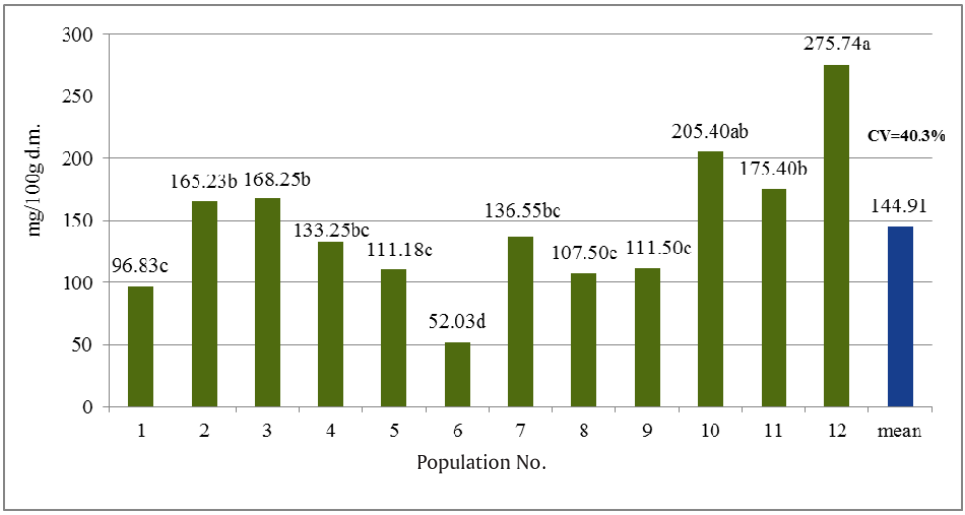
Values in columns followed by the same letter do not differ significantly at $\alpha=0.05$; Tukey's test

Table 7.

Total content of biologically active compounds in leaves of southern sweet-grass populations (%)

Population No./site		Coumarins	Flavonoids	Phenolic acids
1	Wiercień	0.39d	0.10c	0.13d
2	Siemiatycze	0.82b	0.17bc	0.15c
3	Hrabska Droga	0.86ab	0.16bc	0.16c
4	Sieński	0.58c	0.17bc	0.14cd
5	Pietkowo-Wilkowo	0.57c	0.09c	0.14cd
6	Wyliny-Ruś	0.36d	0.14c	0.16c
7	Wiercień Duży	0.48cd	0.18b	0.14cd
8	Koryciny	0.58c	0.20b	0.15c
9	Strabla	0.51cd	0.24a	0.24ab
10	Ruciane	0.53c	0.18b	0.21b
11	Wejsuny	0.51cd	0.11c	0.17c
12	Jedwabno	0.96a	0.26a	0.29a
	mean	0.60	0.17	0.17
	CV (%)	31.4	31.3	28.1

Values in columns followed by the same letter do not differ significantly at $\alpha=0.05$; Tukey's test



Values marked with the same letter do not differ significantly at $\alpha=0.05$; Tukey's test

Figure 2.

The content of coumarin in leaves of southern sweet-grass populations

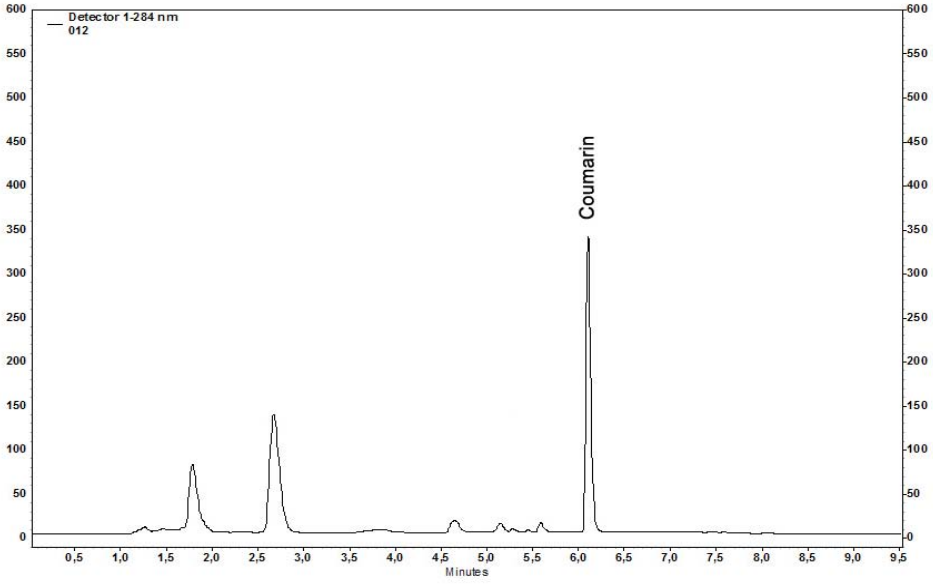


Figure 3.

HPLC chromatogram of southern sweet-grass leaves extract (population No.12)

CONCLUSIONS

- In eastern Poland, southern sweet-grass populations occurred in three types of forest communities differing in respect to the composition of accompanying flora and light irradiance.
- The mass of raw materials of wild growing southern sweet-grass populations seemed to depend on light conditions.
- The investigated populations differed significantly in total contents of coumarins, flavonoids and polyphenolic acids. The variation in the content of coumarin, responsible for the quality of leaves, was the greatest.
- Most interesting, in terms of potential introduction into cultivation, were the population No. 12 (Jedwabno) and population No. 10 (Ruciane), characterized by relatively high mass of leaves and content of analyzed compounds.

ACKNOWLEDGEMENTS

This work was supported by the National Science Centre, Poland, project No. N N310 728440 and Ministry of Agriculture and Rural Development.

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ZRÓŻNICOWANIE DZIKO ROSNĄCYCH POPULACJI TURÓWKI LEŚNEJ (*HIEROCHLOË AUSTRALIS* /SCHRAD./ ROEM. & SCHULT.) WYSTĘPUJĄCYCH WE WSCHODNIEJ POLSCE

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Streszczenie

Turówka leśna to wieloletnia trawa kępkowa występująca w północno-wschodniej Europie. W Polsce jest to roślina rzadka, objęta częściową ochroną prawną, rosnąca w widnych lasach mieszanych i liściastych. Jej liście, bogate w zwiazki kumarynowe, wykorzystywane są głównie do aromatyzowania alkoholi. W związku z dużym zapotrzebowaniem na ten surowiec oraz zanikaniem populacji turówki leśnej na stanowiskach naturalnych wskazane jest wprowadzenie jej do uprawy. W niniejszej pracy wytypowano do badań 12 populacji turówki leśnej i oceniono je pod kątem przydatności do uprawy. Określono skład gatunkowy roślin towarzyszących turówce oraz intensywność nasłonecznienia na zajmowanych stanowiskach. Gatunek ten zidentyfikowano w trzech typach zbiorowisk leśnych. Powietrznie sucha masa liści w przeliczeniu na roślinę wahała się u badanych populacji od 1,73 do 11,07 g. Były one wyraźnie zróżnicowane pod względem ogólnej zawartości zidentyfikowanych grup związków czynnych, tj. kumaryn (0,36–0,96%), flawonoidów (0,09–0,26%) i kwasów polifenolowych (0,13–0,29%). Zróżnicowanie dotyczące zawartości kumaryny, będącej wskaźnikiem jakości surowca, było jeszcze wyższe. Zawartość tego związku wahała się od 52,03 do 275,74 mg/100 g s.m. Spośród badanych populacji najbardziej interesujące pod względem cech jakościowych wydają się być populacja nr 12 (Jedwabno) i populacja nr 10 (Ruciane), charakteryzujące się relatywnie wysoką masą liści jak i najwyższą zawartością zidentyfikowanych związków czynnych, w tym kumaryny.

Słowa kulczowe: *turówka leśna, populacje, światło, kumaryny, flawonoidy, kwasy polifenolowe*