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Anatomical characteristics and antioxidant properties of *Euphorbia nicaeensis* ssp. *glareosa*

Research Article

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Abstract: Anatomical analyses found that leaves of *Euphorbia nicaeensis* ssp. *glareosa* are isolateral, amphistomatous, with two layers of palisade cells on the adaxial and one on the abaxial side. Laticifers are present by vascular bundles, in palisade and spongy tissue. Stem laticifers are located in the pericyclic ring, adjacent to the phloem, in cylinder parenchyma and medullar rays. The structure of pleiochasium and dichasium peduncle is similar to the stem structure. Plants from typical steppe habitat show more xeromorphic features. Phytochemical screening of extracts showed presence of catecholes, flavonoids, tannins, saponins, free quinone derivatives and absence of anthocyanins, leucoanthocyanins, alkaloids, steroid compounds and essential oils. Our results showed that the examined taxon was partially susceptible to the action of reactive oxygen species, such as $O_2^{\cdot-}$ and $^{\cdot}OH$. The higher quantities of ROS thus provoked an antioxidative response from the plant, both in an enzymatic and non-enzymatic manner. Stable anatomical structure, presence and distribution of laticifers and effective antioxidant properties when exposed to ROS, make *Euphorbia nicaeensis* subsp. *glareosa* potentially interesting for further pharmaceutical and phytochemical examinations.

Keywords: *Anatomy • Antioxidant activity • Euphorbia • E. nicaeensis*

1. Introduction

Euphorbia nicaeensis All. is a herbaceous, perennial plant species. Two subspecies are differentiated: *E. nicaeensis* subsp. *nicaeensis*, distributed in the south Europe, and subsp. *glareosa* (Pallas et Bieb.) A. R. Sm., a Pontic-balkanian subspecies, distributed in eastern Europe and eastern parts of Central Europe [1,2]. The latter also grows in Serbia, where it is dispersed throughout the territory, occuring more frequently on dry steppe habitats in the Pannonian region [3]. This subspecies is a member of *Salvio-Festucetum sulcatae* Zólyomi and *Astragalo-Festucetum sulcatae* Soó communities [4]. According to the ecological indicator values given by Borhidi [5], these plants are xero-tolerant, adapted to xeroterm habitat conditions on steppes, with low water supply and high temperature and insolation. Such habitat conditions induce formation of helio-xeromorphic anatomical structure of plants, including higher number of stomata, thicker cuticle, denser indumentum, highly developed photosynthetic, mechanical and vascular tissue, and smaller size of cells [6].

Euphorbia L. is a large and widely distributed genus, with species containing white, milky latex in vacuoles of specialized secretory cells called laticifers [1,7]. The latices of *E. antiquorum* L., *E. nerifolia* L. and *E. tirucalli* L. are highly valued in Indian medicine, mainly as purgatives, in addition to their other therapeutic applications [8]. Some metabolites of *E. antiquorum* showed inhibitory effects on Epstein-Barr virus. Components of *E. myrsinites* L. latex showed moderate levels of HIV-1 reverse transcriptase inhibition [9]. In Turkish folk medicine, latices of many *Euphorbia* species have been used as purgatives, but since they

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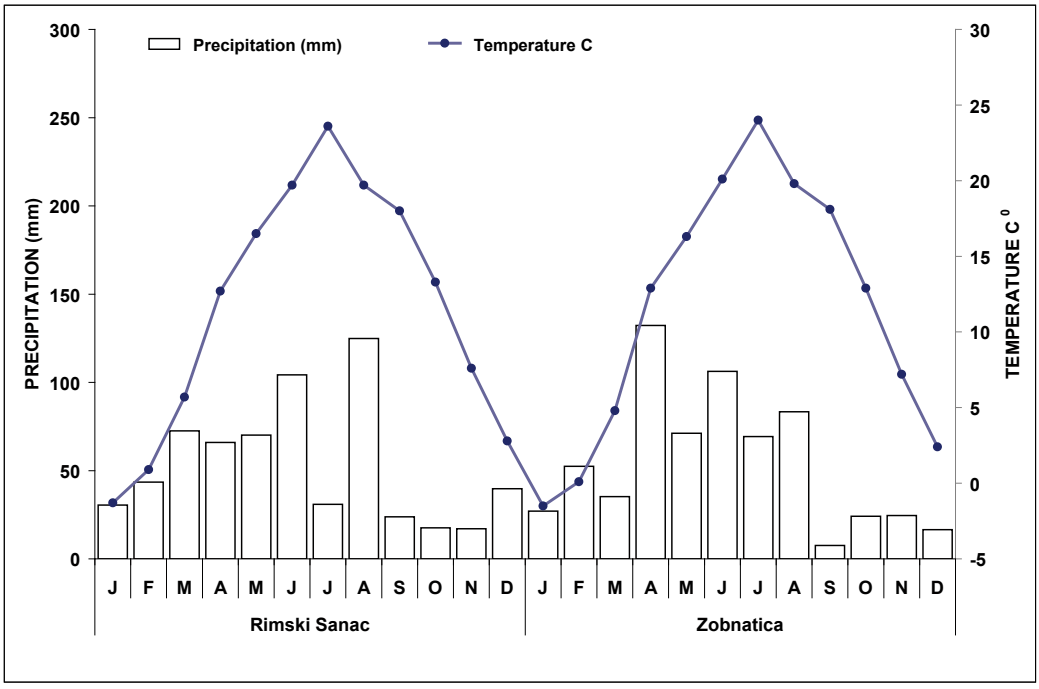

Figure 1. Mean monthly air temperature and total monthly rainfall on collecting sites during the year 2006.

are highly irritant they are currently used only to treat warts [10]. *E. kansui* L. has been used in Chinese folk medicine for treatment of cancer, and its root extracts demonstrated antileukemic activity [11]. The extracts of roots of *E. nicaeensis* subsp. *glareosa* showed significant cytotoxic activity, whilst extracts of the aerial parts showed only moderate activity. Pharmacological studies of extracts of *E. nicaeensis* s. l. showed some *in vitro* cytostatic and anti-inflammatory activity, including inhibition of ear edema in mice, especially due to the activity of glyceroglycolipids [12,13].

Several authors previously investigated the anatomy of some *Euphorbia* taxa [14-21]. Laticiferous cells are present in the pith and primary cortex of the axis, as well as in the leaf veins and mesophyll of mature plants [15]. The same authors reported the presence of segmented and unsegmented laticiferous vessels in the phloem of both stem and leaf, at the margin of the pith, in the primary cortex, and sometimes in the leaf mesophyll. Taxa of the genus *Euphorbia* have nonarticulated branched laticifers [16]. Analyses of stem, leaf, and root anatomy of *E. cyparissias* L. and *E. helioscopia* L. [17] and of *E. thymifolia* L. [18] showed non-articulated unbranched and branched laticifers in leaves, near the vascular tissue and in stems, in cortex parenchyma. Laticifers occur throughout *E. pulcherrima* Willd., being well represented in certain parenchymatous tissues and phloem [19]. The mature laticifers have a vital protoplast, which encloses a large central vacuole containing the milky latex fluid. In *E. supina* Raf. laticifers of the mature stem are found only in the cortex, the widest one being in contact with the phloem but not xylem [20]. Within the lamina, the laticifers follow the vascular system and branch from one vein to another. Some *Euphorbia* species could be clearly separated on the basis of latex microscopic features by their characteristic starch grain patterns [8,21]. Samman *et al.* [22] found that *E. nicaeensis* displayed three types of variation in the architecture of the inflorescence axis, as well asdifferences in flowering times and the quantity of seeds, induced by the local environmental heterogeneity.

However, despite the fact that *Euphorbia* species are interesting from a medicinal and pharmacological point of view, the majority of wild-growing species of this genus are still biologically, phytochemically and pharmacologically uninvestigated. Therefore, the aim of this study was to investigate structural characteristics of *E. nicaeensis* subsp. *glareosa* organs, distribution and percentage of secretory tissues, antioxidant properties and medicinal value as a potential source of natural antioxidants.

2. Experimental Procedures

Plant material was collected during the flowering season from native populations in North Serbia. Plants were identified and voucher specimens deposited in the

Herbarium of the Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad (BUNS). In order to validate the structural analyses, due to the fact that this taxon inhabits uniform type of steppe habitats of the same geological origin in Pannonian part of Serbia, duplicate samples were obtained from two populations. One population originated from Rimski Sanac (collection date: 15. VII 2006., leg et det: Boza, P.) and the other from Zobnatica steppe habitat (collecting date: 12. VII 2006., leg et det: Boza, P.). Both localities had the same soil type (chernozem) and were directly insolated. The meteorological data for temperature and rainfall for both localities were obtained from the Rimski Sanac station in Novi Sad, and the Zobnatica station in Palic (Figure 1).

Thirty plants from each population were used for anatomical studies. Leaves from the middle part of the stem, stem segments from the middle part of the plants and median peduncle segments were isolated and fixed in 50% ethanol. Leaf epidermal characteristics were observed under light and scanning electron microscope (SEM). For light microscopy observations, leaf epidermal prints were made following the procedure described by Wolf [23]. For SEM analysis, small pieces of dry leaves of ten plants per population were sputter coated with gold for 180 seconds, 30 mA (BAL-TEC SCD 005) and viewed with JEOL JSM-6460LV electron microscope at an acceleration voltage of 20 kV.

Cross sections were made using Leica CM 1850 cryostat, at temperature -18°C-20°C, at cutting intervals of 25 µm. Light microscopy observations and measurements were performed on thirty cross sections of each organ and thirty leaf epidermal prints using Image Analyzing System Motic 2000. Stomata were counted on five randomly selected areas of the adaxial and abaxial surfaces, excluding main veins, and calculated per mm^2 of the leaf surface. Relative proportions were calculated for each tissue, and expressed as a ratio of the whole cross section area of each organ, to leaf thickness or to stem and peduncle diameter.

Quantities of superoxide ($O_2^{\cdot-}$) and hydroxyl ($^{\cdot}OH$) radicals, malonyl-dialdehyde (MDA), activity of antioxidant enzyme (SOD) and content of total flavonoids were determined in the leaves and cyatia of the investigated plant. The potential antioxidant activity of the test sample was assessed based on scavenging activity of the stable DPPH free radicals. In addition, a phytochemical screening for the presence of secondary biomolecules was performed [24]. Superoxide radicals were studied by the inhibition of adrenaline autooxidation [25]. Hydroxyl radicals were measured by the inhibition of deoxyribose degradation [26]. The lipid peroxidation (LP) was measured as MDA production at 532 nm with thiobarbituric acid (TBA) as described by Placer *et al.* [27]. Total flavonoids were estimated according to Marckam [28]. SOD activity was measured by monitoring the inhibition of nitroblue tetrazolioum (NBT) reduction at 560 nm [29]. Total potential antioxidant activity of the investigated aqueous acetone extracts was assessed based on their scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, using a modified DPPH assay [30].

Data were statistically processed by analysis of variance and means, standard errors and coefficients of variation were calculated using STATISTICA for Windows version 7.0. The significance difference of measured parameters between the populations was determined using t-test ($P \leq 0.05$ and $P \leq 0.01$). The general structure of sample variability was established by Principal Component Analysis (PCA), based on correlation matrix.

3. Results

3.1 Anatomical characteristics of leaf, stem, pleiochasium and dichasium peduncle

The leaves have an isolateral structure. The single-layer epidermis is covered with a wax-like substance, which is thicker on the abaxial side (Figure 2). The epidermal cells are papillose, with the papillae more prominent and more densely distributed on the abaxial side. Leaves are amphistomatous, with nearly equal number and size of stomata on both surfaces (Table 1). The stomata are of anomocytic type, surrounded by limited number of cells that are indistinguishable in size and shape from other epidermal cells. Significant differences between the two populations were recorded only in the number of stomata, which were more numerous on samples from Rimski Sanac, but not in their size.

The palisade tissue is composed of elongated, cylindrical cells. They are slightly larger and arranged in two rows on the adaxial side, whilst in one row on the abaxial side. Spongy tissue cells are irregular in shape, arranged in 3-4 rows (Figure 3a). Differences in parameters of photosynthetic tissue and cells are not significant between the populations. Vascular bundles are categorized in three groups, according to their size. Bundles smaller than 5000 μm^2, which were the most numerous, were assigned as small, between 5000 and 10 000 μm^2 as medium, while larger than 10 000 μm^2 as large. The main vein, which is not very prominent, contains one large vascular bundle, surrounded with laticifers, especially along the phloem. Laticifers are also present along small and medium vascular bundles, but

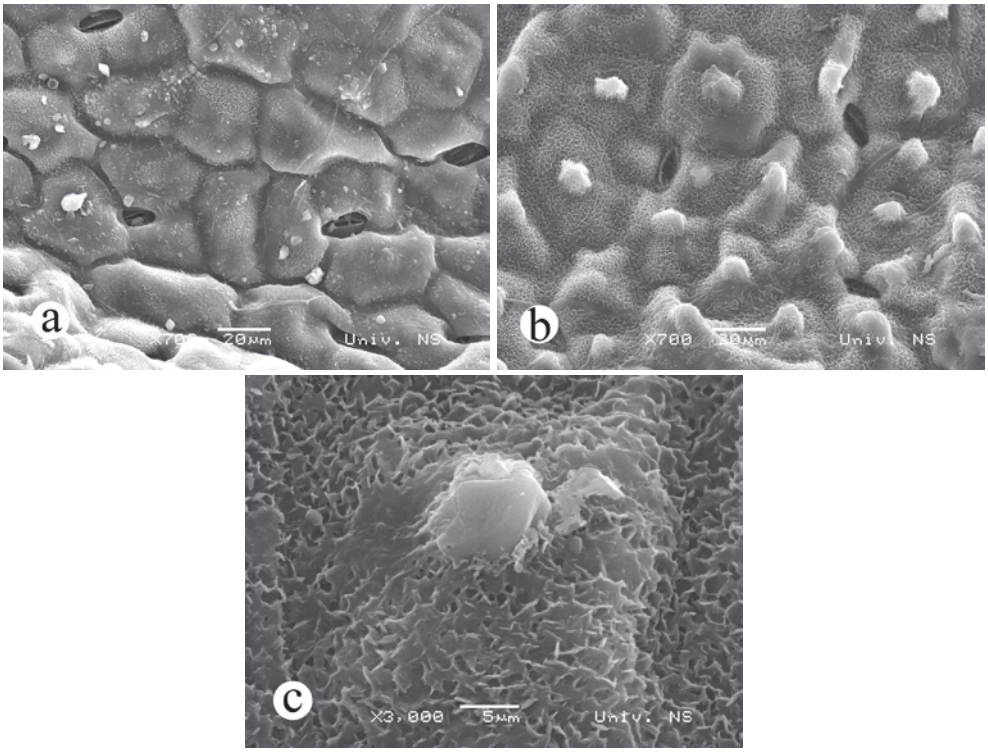

Figure 2. Scanning electron micrographs of leaf epidermis. a) adaxial epidermis; b) abaxial epidermis; c) papilla and wax deposits on abaxial epidermis.

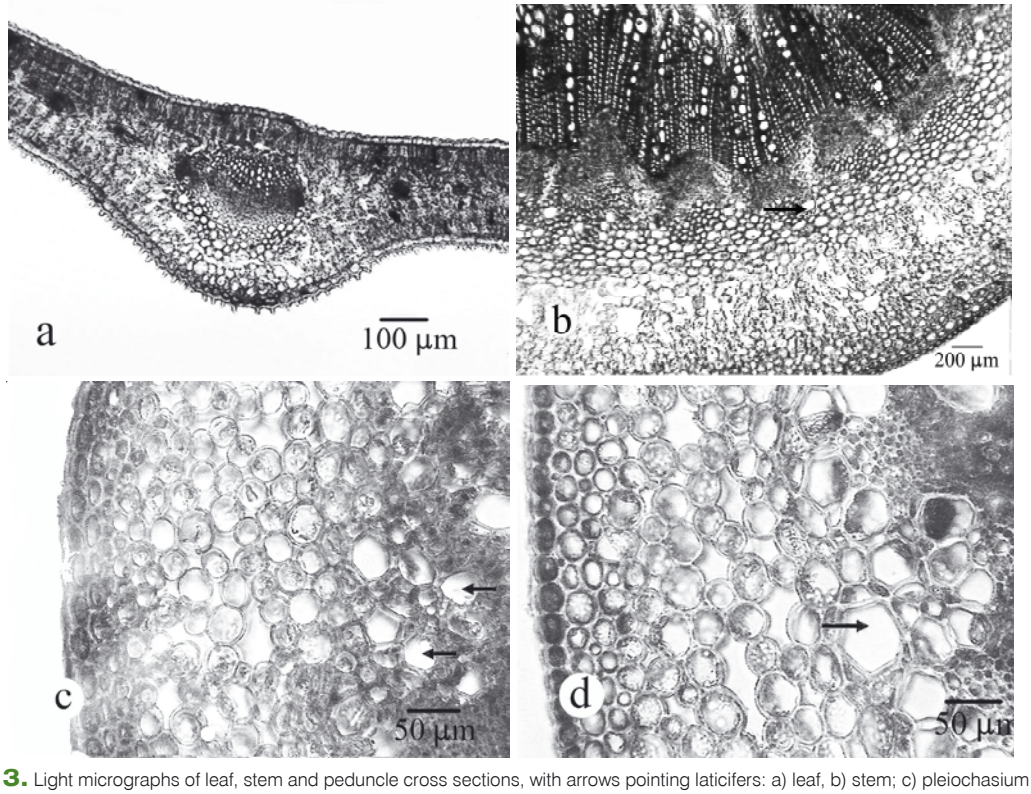

Figure 3. Light micrographs of leaf, stem and peduncle cross sections, with arrows pointing laticifers: a) leaf, b) stem; c) pleiochasium peduncle; d) dichasium peduncle.

		Sample size / population	Rimski Sanac	Zobnatica	t-test
Leaf	cross section area (mm^2)	30	2.94 ± 0.1 (13.6)	3.47 ± 0.4 (34.6)	ns
	cross section thickness (μm)	30	269 ± 11.0 (12.9)	268 ± 4.4 (5.2)	ns
Adaxial epidermis	number of stomata/mm^2	150	169 ± 7.4 (13.7)	125 ± 11.5 (29.1)	**
	stomata length (μm)	150	14.0 ± 0.4 (9.3)	14.3 ± 0.6 (12.6)	ns
	stomata width (μm)	150	5.9 ± 0.2 (10.2)	6.2 ± 0.4 (21.0)	ns
	% epidermal thickness	30	7.4 ± 0.4 (17.6)	8.6 ± 0.5 (19.8)	ns
Abaxial epidermis	number of stomata/mm^2	150	168 ± 12.9 (24.3)	117 ± 10.9 (29.3)	**
	stomata length (μm)	150	14.0 ± 0.8 (18.6)	13.1 ± 0.9 (22.1)	ns
	stomata width (μm)	150	6.3 ± 0.4 (17.5)	7.0 ± 0.4 (15.7)	ns
	% epidermal thickness	30	8.2 ± 0.5 (18.3)	10.0 ± 0.4 (13.0)	ns
Mesophyll	% adaxial palisade tissue thickness	30	28.3 ± 1.1 (12.0)	28.6 ± 1.0 (10.8)	ns
	% abaxial palisade tissue thickness	30	15.6 ± 1.1 (21.8)	15.9 ± 0.6 (12.6)	ns
	% of spongy tissue thickness	30	40.0 ± 2.7 (21.3)	36.3 ± 1.0 (9.1)	ns
	number of vascular bundles: small	30	39.0 ± 3.0 (24.1)	42.0 ± 5.4 (40.7)	ns
	middle	30	4.0 ± 0.5 (42.5)	5.0 ± 0.9 (56.0)	ns
	large	30	2.0 ± 0.2 (35.0)	3.0 ± 0.4 (40.0)	ns
	% vascular bundles + sclerenchyma	30	4.9 ± 0.5 (32.7)	6.5 ± 0.4 (21.5)	ns
	% laticifers	30	0.5 ± 0.04 (20.0)	0.2 ± 0.04 (28.0)	**
Cell cross-section area (μm^2)	palisade tissue, adaxial	150	383 ± 27.0 (22.2)	411 ± 16.2 (12.5)	ns
	palisade tissue, abaxial	150	374 ± 23.6 (20.0)	365 ± 9.4 (8.1)	ns
	spongy tissue	150	338 ± 14.0 (13.1)	352 ± 15.0 (13.4)	ns

Table 1. Leaf anatomical characteristics (means, standard errors and coefficients of variation %).

***, ns - differences between the populations significant at P≤0.01, or not significant*

		Rimski Sanac	Zobnatica	t-test
Stem	cross-section area (mm^2)	10.7 ± 1.2 (36.4)	12.7 ± 0.4 (11.0)	ns
	% of epidermal thickness	1.2 ± 0.1 (16.7)	0.9 ± 0.03 (11.1)	**
	% of collenchyma thickness	6.8 ± 0.4 (17.6)	4.7 ± 0.1 (8.5)	**
	% of cortex parenchyma	29.9 ± 4.0 (42.5)	38.0 ± 2.4 (20.3)	ns
	% of sclerenchyma	7.7 ± 0.7 (27.3)	5.2 ± 0.4 (23.1)	**
	% of vascular tissue	30.7 ± 1.7 (17.3)	31.3 ± 1.8 (18.8)	ns
	% of cylinder parenchyma	24.1 ± 1.9 (24.9)	21.8 ± 1.7 (25.2)	ns
Pleiochasium	cross-section area (mm^2)	1.0 ± 0.04 (10.0)	1.0 ± 0.03 (10.0)	ns
peduncle	% of epidermal thickness	2.5 ± 0.2 (20.0)	2.4 ± 0.1 (16.7)	ns
	% of collenchyma thickness	8.5 ± 0.4 (14.1)	8.4 ± 0.4 (15.5)	ns
	% of cortex parenchyma	53.1 ± 1.1 (6.8)	48.5 ± 1.4 (8.9)	ns
	% of sclerenchyma	5.8 ± 0.7 (37.9)	6.0 ± 0.5 (25.0)	ns
	% of vascular tissue	21.2 ± 1.0 (15.1)	25.7 ± 1.1 (13.6)	**
	% of cylinder parenchyma	4.6 ± 0.3 (19.6)	5.2 ± 0.4 (25.0)	ns
Dichasium	cross-section area (mm^2)	0.7 ± 0.06 (28.6)	0.7 ± 0.06 (28.6)	ns
peduncle	% of epidermal thickness	2.6 ± 0.1 (15.4)	2.8 ± 0.1 (14.3)	ns
	% of collenchyma thickness	9.1 ± 0.7 (24.2)	9.2 ± 0.3 (8.7)	ns
	% of cortex parenchyma	55.5 ± 2.4 (13.7)	54.1 ± 2.3 (13.3)	ns
	% of sclerenchyma	8.1 ± 1.0 (37.0)	6.0 ± 0.4 (23.3)	ns
	% of vascular tissue	19.2 ± 1.0 (16.1)	20.5 ± 0.9 (13.2)	ns
	% of cylinder parenchyma	4.6 ± 0.6 (39.1)	4.1 ± 0.5 (34.1)	ns

Table 2. Stem and peduncle anatomical characteristics (means, standard errors and coefficients of variation %, 30 specimens per population).

***, ns - differences between the populations significant at P≤0.01, or not significant*

Characters		Factor 1	Factor 2	Factor 3
Leaf	cross section area (mm^2)	-0.1018	0.5243	-0.4407
	cross section thickness (μm)	0.4800	0.3177	-0.3901
	adaxial epidermis			
	no of stomata/mm^2	0.3797	-0.5401	-0.1885
	stomata length (μm)	-0.0764	0.2804	-0.3140
	stomata width (μm)	-0.0256	0.4814	-0.4487
	% epidermal thickness	-0.6033	-0.1194	-0.1698
	abaxial epidermis			
	no of stomata/mm^2	0.4153	-0.3681	-0.3192
	stomata length (μm)	0.4556	0.3524	-0.1197
	stomata width (μm)	0.2411	0.6403	-0.1313
	% epidermal thickness	**-0.7563***	-0.1833	0.2777
	mesophyll			
	% adaxial palisade tissue thickness	-0.3403	0.0631	0.1131
	% abaxial palisade tissue thickness	-0.0421	0.3408	-0.0022
	% of spongy tissue thickness	0.6206	0.1838	-0.3761
	no. of small vascular bundles	-0.1026	0.3324	-0.4265
	no. of middle vascular bundles	-0.1962	0.4189	-0.6702
	no. of large vascular bundles	-0.4048	0.2491	-0.3327
	% vascular bundles + sclerenchyma	-0.6080	-0.2195	0.0030
	% laticifers	0.4892	-0.5392	-0.3846
	cell cross-sec.area pal. tiss.adax	0.3043	**0.7405***	-0.0592
	cell cross-sec.area pal. tiss.abax	0.2032	0.3874	-0.0209
	cell cross- sec. area spongy tiss.	-0.0422	0.4997	-0.2940
Stem	cross-section area (mm^2)	-0.6935	0.1322	-0.2400
	% of epidermal thickness	**0.8359***	-0.1534	0.0209
	% of collenchyma thickness	**0.8419***	-0.1699	0.0559
	% of cortex parenchyma	**-0.7743***	-0.12167	-0.4558
	% of sclerenchyma	**0.8596***	-0.1822	0.0758
	% of vascular tissue	0.2522	0.5723	0.2757
	% of cylinder parenchyma	0.6789	0.2175	0.2860
Pleiochasium peduncle	cross-section area (mm^2)	-0.1382	0.0819	0.0900
	% of epidermal thickness	0.2334	0.4651	0.1164
	% of collenchyma thickness	0.1248	0.4522	-0.2798
	% of cortex parenchyma	0.1482	-0.6963	-0.3610
	% of sclerenchyma	-0.4185	0.0635	0.2419
	% of vascular tissue	-0.3086	0.6229	0.3721
	% of cylinder parenchyma	-0.0471	0.4233	0.5405
Dichasium peduncle	cross-section area (mm^2)	0.0326	0.3099	0.1613
	% of epidermal thickness	-0.3196	0.1533	0.2522
	% of collenchyma thickness	-0.1085	-0.1471	-0.3767
	% of cortex parenchyma	0.3690	0.1597	0.4398
	% of sclerenchyma	-0.0164	-0.4806	0.0026
	% of vascular tissue	0.1151	0.4789	-0.3016
	% of cylinder parenchyma	0.0546	-0.4597	-0.4567
Percentage of total variance explained		0.1816	0.1485	0.094
Cumulative percentage of total variance		0.1816	0.3301	0.4241

Table 3. Principal components analysis (PCA) of measured parameters. Factor coordinates of the variables, based on correlations and cumulative percentages of the vectors (marked loadings are >0.700).

	Leaf		t-test	Inflorescence		t-test
	Rimski Sanac	Zobnatica		Rimski Sanac	Zobnatica	
Superoxide-radical ($O_2^{\cdot-}$) nmol $O_2^{\cdot-}$ g^{-1} fr. w.	17.6 ± 1.0 (10.4)	19.6 ± 1.4 (12.6)	ns	29.9 ± 0.5 (3.1)	27.2 ± 3.3 (21.0)	ns
Hydroxyl-radical (OH) nmol OH g^{-1} fr. w.	246.2 ± 14.4 (10.1)	259.7 ± 20.4 (13.6)	ns	273.3 ± 15.4 (9.7)	229.4 ± 10.6 (8.0)	ns
Superoxide-dismutase (SOD) U g^{-1} fr. w.	1396 ± 144 (17.9)	1573 ± 106 (11.6)	ns	1592 ± 46.4 (5.0)	1346 ± 27.5 (3.5)	*
Lipid peroxidation (LP) nmol MDA g^{-1} fr. w.	154.6 ± 5.7 (6.4)	179.2 ± 142 (13.6)	ns	114.7 ± 5.5 (8.2)	91.5 ± 0.9 (1.6)	*
Flavonoids g rutine 100 g^{-1} d.w.	0.57 ± 0.01 (3.5)	0.41 ± 0.0 (0.0)	**	0.84 ± 0.03 (5.9)	0.67 ± 0.02 (5.9)	**
(1.1-diphenyl-2-picryl hydrazil) DPPH % neutralised radicals	39.7 ± 14.8 (64.7)	56.5 ± 7.7 (23.5)	ns	54.9 ± 7.8 (24.5)	57.8 ± 8.6 (25.6)	ns

Table 4. Antioxidant parameters of leaf and inflorescence (means ± standard errors and coefficients of variation %).

**, **, ns - differences between the populations significant at P≤0.05, P≤0.01, or not significant*

never between the cells of vascular tissue. Laticifers are recorded in all other parts of the mesophyll, in palisade tissue, between palisade tissue and phloem and in spongy tissue. They have significantly larger percentage of cross-section area in plants from Rimski Sanac (Table 1).

In the cross section, the primary stem is rounded to oval in shape, without ribs. It has a single layer of epidermis, covered with a cuticle. The epidermal cells have thickened walls, and possess conical papillae. Collenchyma tissue is present subepidermally. Cortex parenchyma is composed of several layers of thin-walled cells, with numerous intercellular spaces. Vascular bundles in the central cylinder are close to each other, forming a vascular ring (Figure 3b). Only narrow medullar rays are sometimes visible between them. Pericyclic groups of sclerenchyma tissue occur above the phloem region of vascular bundles. Central cylinder parenchyma cells are thin-walled, circular and with numerous intercellulars, with no central cavity present.

Numerous laticifers are recorded in the pericyclic ring, especially along the phloem, in central cylinder parenchyma, and even in medullar rays. Differences in tissue proportions between the two populations were statistically significant only for proportions of epidermis, collenchyma and sclerenchyma (Table 2). Those values were significantly higher in plants from Rimski Sanac.

The structure of pleiochasium and dichasium peduncle was generally very similar to the stem structure (Figure 3c-d). Differences in measured parameters were not significant between the populations, except for percentage of vascular tissue in pleiochasium peduncle, which was significantly higher in plants from Zobnatica.

The results of Principal Component Analysis (PCA) showed that examined characters had generally low variability, since the first three axes explained only 42.4% of total variation (18.16%, 14.85% and 9.40%, respectively). The most variable were stem parameters (percentages of epidermis, mechanical tissue and cortex parenchyma), which defined the first principal component (Table 3).

3.2 Phytochemical and antioxidant parameters analysis

Phytochemical screening of *E. nicaeensis* ssp. *glareosa* extracts showed the presence of catecholes, flavonoids, tannins, saponins, free quinone derivatives, and the absence of anthocyanins, leucoanthocyanins, alkaloids, steroid compounds and essential oils.

Our results showed that the examined taxon was partially susceptible to the action of reactive oxygen species (ROS), such as $O_2^{\cdot-}$ and $\cdot OH$. The results obtained have shown high oxygen radicals quantities, but there were no significant differences among examined populations. The $O_2^{\cdot-}$ quantity in examined organs varied from 17.6 to 29.9 μmol $O_2^{\cdot-}$ g^{-1} fr.w., and it was higher in cyatia for both localites. The quantity of $\cdot OH$ radicals ranged from 229 to 273 nmol g^{-1} fr. w. Higher values were recorded for cyatia from Rimski Sanac and leaves from Zobnatica (Table 4).

In addition to normal metabolic activity, ROS can result from cellular exposure to various environmental stimuli. Such factors are known to induce free radical formation in most aerobic organisms. ROS were produced in larger quantities probably due to high outside temperatures in mid-July when the plant material

was collected. These observations can be supported by the fact that MDA quantity in the plant material ranged from 91.5 to 179.2 nmol MDA g^{-1} fr. w. The activities of antioxidant enzyme SOD in leaves and inflorescences were also high, and they ranged from 1346 to 1592 U g^{-1} fr. w., depending on the locality. The content of total flavonoids was determined and for both examined organs it was higher in plants from Rimski Sanac. Flavonoids may act as strong $O_2^{\cdot-}$ scavengers as well as $^1O_2$ quenchers, and together with other phenols, such as phenolic acids, they behave as active antioxidants.

4. Discussion

Low variability was observed between the two populations of *E. nicaeensis* subsp. *glareosa*, with differences that were not statistically significant.

Korsmo [17] provided anatomy data for two other *Euphorbia* species, *E. cyparissias* and *E. helioscopia*. Comparison of these data with data obtained from our analysis showed that these species had dorsiventral, while *E. nicaeensis* subsp. *glareosa* had isolateral leaves. Although they differed in leaf anatomy, their primary stem anatomy was very similar.

According to Korsmo [17] and Paliwal and Kakkar [18], three *Euphorbia* species analyzed had laticifers in leaves near the vascular tissue, and in stems in the cortex parenchyma. Rosowski [20] reported that *E. supina* stems had laticifers only in cortex. Our results confirmed these findings and revealed the presence of laticifers in leaves near the vascular tissue and in stem cortex parenchyma. Moreover, we recorded that *E. nicaeensis* subsp. *glareosa* also had laticifers in the leaf palisade tissue, between palisade tissue and phloem, in the spongy tissue, in stem central cylinder parenchyma, and in medullar rays.

This study found that plants from Rimski Sanac had a significantly higher percentage of leaf laticifers, higher number of stomata on both leaf epidermal sides, higher stem epidermal thickness, and higher percentage of stem mechanical tissue. According to the descriptions of xeromorphic characteristics given by Fahn and Cutler [6], higher stomata density, smaller stomata size, thicker epidermis, and well-developed sclerenchyma tissue in the stem are adaptations to dry and more insolated habitats. Therefore, the structure of plants from Rimski Sanac could be classified as more xeromorphic than the structure of plants from Zobnatica. The observed characteristics could be adaptations to the somewhat drier habitat conditions on the steppe of Rimski Sanac, with lower precipitation during the growing season.

Previous experiments provided evidence of an association between LP and different types of oxidative stresses in plants [31-33]. Consequently, ROS may react with unsaturated fatty acids of cell membranes both in seeds and leaves, resulting in different degrees of peroxidation. MDA, being the main end-product of LP, can cause the cross-linking and polymerisation of membrane components. Owing to its diffusibility it can also react with free amino groups in protein and DNA bases. Our results suggest that MDA quantity in *E. nicaeensis* subsp. *glareosa* was higher compared to MDA quantities in some other wild-growing species, e.g. in *Ocimum basilicum* L. (15.16 nmol g^{-1} f.m.), *Urtica dioica* L. (26.64 nmol g^{-1} f.m.), *Nuphar luteum* L. (46.80 nmol g^{-1} f.m.) or *Lillium martagon* L. (75.50 nmol g^{-1} f.m.) [34], but significantly lower compared to other species such as wild growing *Allium* spp. [35]. The higher quantities of ROS triggers an antioxidative response from the plant, both in an enzymatic and non-enzymatic manner. Compared to the SOD activities in some well-known medicinal and aromatical plants such as *Allium sativum* L. (803.37 U g^{-1}), *Achillea millefolium* L. (399.00 U g^{-1}) or *Ocimum basilicum* L. (235.42 U g^{-1}) [36], the SOD activity in *E. nicaeensis* subsp. *glareosa* was significantly higher (1346-1592 U g^{-1}).

DPPH-radical scavenging activity is a measure of non-enzymatic antioxidant activity. Higher levels of DPPH activity have been correlated with tolerance to different stress conditions [36]. In this assay system, antioxidants can react with the stable free DPPH radical and produce 1,1-diphenyl-2-picrylhydrazine. The change of absorbance produced in this reaction is assesed to evaluate the antioxidant potential of test samples. According to Lee *et al.* [37], the preliminary distribution pattern of antioxidant activity in plant extracts may be categorized as: active (>80% inhibition), moderately active (50-80% inhibition), and inactive (<50% inhibition). Our samples showed more than 50% inhibition which categorize them as moderately active, except for leaves from Rimski Sanac, which were inactive. Results indicate that plant phenolics and other non-enzymatic antioxidants were active and sufficient to prevent increased oxygen radicals in leaves from Rimski Sanac.

Overall results, based on stable structure, percentage and distribution of laticifers and effective antioxidant property when exposed to negative influence of ROS, suggest *Euphorbia nicaeensis* subsp. *glareosa* is a potentially interesting candidate for further pharmaceutical and phytochemical examinations.

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