Article

Chemical Characterization, Antioxidant Activity, and Cytotoxicity of Wild-Growing and In Vitro Cultivated *Rindera umbellata* (Waldst. & Kit.) Bunge

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Abstract: The aim was to comparatively analyze chemical composition and biological activity of wild- and in vitro grown *Rindera umbellata*. Explants were cultivated on 0.003-0.3 M sucrose, fructose or glucose. HPLC-DAD for quantifying rosmarinic (RA) and lithospermic B (LAB) acids and GC-MS/FID for qualitative pyrrolizidine alkaloids (PAs) detection were used. Antioxidant activity (DPPH and ABTS assays) and cytotoxicity (MTT test) were monitored. Identified PAs were 7-angeloyl heliotridane, lindelofine, 7-angeloyl heliotridine, 7-angeloyl-9-(+)-trachelanthylheliotridine, punctanecine and heliosupine, with the higher variability in wild-growing samples. Total phenolic contents (TPCs) were comparable in wild-growing and in vitro, but total flavonoid (TFC) and RA levels were multifold higher in in vitro samples. Notably high concentration of LAB was detected in wild-growing roots. 0.3 M and 0.1 M sucrose were optimal for TFC and RA production, while maximal antioxidant activity was monitored in plants grown on 0.3 M sucrose. MTT test indicated colorectal HT-29 as more sensitive than A549 lung adenocarcinoma and normal MRC-5 cells, showing selective sensitivity to wild-growing and 0.3 M sucrose samples. In conclusion, PAs in vitro, as well as TPC, TFC, RA and LAB in both growing conditions were detected for the first time in *R. umbellata*.

Keywords: ex situ conservation; rosmarinic acid; lithospermic acid B; pyrrolizidine alkaloids; biological activity

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Lastname

Received: date
Revised: date
Accepted: date
Published: date



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1. Introduction

Rindera umbellata (Waldst. & Kit.) Bunge, also known as *Mattia umbellata* (Waldst. & Kit.) Schult. belongs to the Boraginaceae family and its distribution is limited to sandy habitats of south-east Europe, from Central Balkan Peninsula to south-west Ukraine. In Serbia, *R. umbellata* is rare and critically endangered species, found only in Deliblato and Kladovo Sands. Due to its rarity and extinction risk, *R. umbellata* is under National State Protection Legislation [1]. Propagation of *R. umbellata* in nature is limited and laborious due to fungal infections and insufficient seed maturity [2]. To preserve existing gene fond and provide plant material for further molecular and phytochemical analysis, establishment of the adequate ways of conservation is necessary. One of the ex situ conservation strategies of rare and endangered plant species is cultivation of plants in tissue culture. Method of in vitro cultivation provides production of healthy plant material suitable for

environmental recultivation and further phytochemical research. Moreover, in vitro cultivated plants can be considered as a valuable source of biologically active compounds that can be applied in different fields, i.e. as pharmaceuticals, flavors and fragrances, food additives, agrochemicals, and biopesticides [3,4]. However, in vitro cultivation requires some specificity, such as necessity to add carbohydrates in a culture media. This can be addressed to limited photosynthetic activity, due to low light intensity and restricted CO_2 amount in a closed system used for propagation [5]. Moreover, concentration of carbohydrates in culture media does not affect yield of plant material only, but also the level of secondary metabolites production [6]. Carbohydrate types and concentrations in culture media induce metabolic changes and consequently, the adequate conditions stimulating production of secondary metabolites can be achieved [7]. This is especially important since the abundant secondary metabolites are embedded with numerous biological activities and could be used as a raw material for different pharmaceutical products [3].

Plant phenolic compounds are important group of secondary metabolites which production strongly depends on carbohydrate content [8]. Phenolics are chemically heterogeneous group of compounds broadly distributed in the plant kingdom, showing a great diversity of structure and roles for the plants themselves [9]. Numerous literature data indicate that phenolic compounds possess strong antioxidant potential and could remove free radicals and promote chelation of redox active metal ions, consequently preventing lipid peroxidation and Fenton reaction [10]. Another important group of plant secondary metabolites are alkaloids, chemical entities classified into several subgroups, with a primary function in protecting against herbivores and microbial infections. [11]. Both, phenolic compounds and alkaloids, could affect human health due to diverse biological activities [12,13].

Among phenolic compounds, rosmarinic acid (RA), an ester of caffeic and 3,4-dihydroxyphenyllactic acid, and a dimer of rosmarinic acid denoted as lithospermic acid B (LAB, also known as salvianolic acid B), are well known for variable biological activities. Literature review showed different effects of RA, such as analgesic, anticancer, antiallergic, neuroreparative, cardioprotective, antidiabetic; many of these activities can be attributed, at least partially, to its potent antioxidative and anti-inflammatory properties [14]. Similarly, LAB is well known to induce antioxidant, anti-inflammatory, anticancer, antihypertension and cardioprotective effects [15]. Both substances are featured with notable anti-thrombosis and antiviral (anti-HIV) properties, and could be used as aldose reductase inhibitors, being active as eye protectors in diabetic patients [16].

In a previous research conducted by Perić et al. [17], *R. umbellata* has been introduced in in vitro culture and growth conditions for its propagation were optimized. Furthermore, its acclimatization to greenhouse and field was successfully established. Some of our previous research detected pyrrolizidine alkaloids (PAs) and fatty acids of wild-growing *R. umbellata* [18,19]. However, literature review revealed no publications dedicated to phytochemical composition of in vitro growing *R. umbellata*. Considering the abovementioned facts, the subject of this study was to comparatively~~e~~ analyze the chemical composition of methanol extracts of wild-harvested~~growing~~ and in vitro cultivated *R. umbellata*, as well as to investigate the effect of selected carbohydrates, namely sucrose, glucose and fructose, on the production of phenolic compounds. Furthermore, selected extracts were examined for the antioxidant capacity and cytotoxic effect against lung and colorectal adenocarcinoma cells (A549 and HT-29 lines, respectively). Our investigation has been conducted to optimize culture conditions for providing a source of valuable biologically active compounds. In addition, *R. umbellata* in vitro culturing could diminish its uncontrolled harvesting from nature, contributing to protection and ex situ conservation of this rare and endangered species.

2. Materials and Methods

2.1. Wild-Growing Plant Material

R. umbellata plants were collected from a population distributed in Deliblato Sands, Serbia, at Latitude N 44°57'58'' and Longitude E 21°1'48'', in accordance with permission for collecting of strictly protected and protected plant species for scientific research purposes No. 353-01-184/2011-03. Collected plant material was~~and~~ dried at room temperature. Different parts of air-dried plants (flowers, stems, leaves and roots) were used for the extracts preparation (Figure 1 A, B).

2.2. In vitro Culture Establishment

In vitro culture was established as described earlier [17], via embryos excised from immature seeds. Stems, 1cm long, were used as primary explants. They were provided from adventitious buds propagated on basal medium (BM) containing 3% sucrose, 0.7% agar, MS salts and vitamins [20], with addition of 1 mg L^{-1} 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} indole-3-acetic acid (IAA). Explants were cultivated on media without growth regulators containing different carbohydrate sources (i.e. sucrose, fructose, or glucose, ranging from 0.003 to 0.3 M). After six weeks dry aerial parts (rosette leaves) were used for the extraction (Figure 1 C, D).



Figure 1. *Rindera umbellata* from the natural habitat (**A**, **B**) and grown in vitro (**C**, **D**).

2.3. Preparation of Plant Extracts

Dry plant samples (0.1 g each) were powdered in liquid nitrogen and extracted at room temperature using 80% methanol (1:10, w:v). After centrifugation for 20 min at 15000 g supernatants were collected and filtered through 0.45 μm PVDF filters (Econofilter, Agilent Technologies, Santa Clara, USA). For cytotoxicity evaluation they were additionally evaporated at room temperature and stored at +4 °C until use. To analyze presence of PAs

in the extracts of wild and in vitro growing plant material, the samples have been prepared in accordance with Mandić et al. [21].

2.4. Total Phenolic and Flavonoid Content Determination

Total phenolic content (TPC) and total flavonoid content (TFC) of plant methanol extracts were determined as reported earlier [22]. Briefly, for TPC estimation the extracts were mixed with 5% sodium carbonate and incubated for 5 min at room temperature. The extracts were then oxidized with 50% Folin-Ciocalteu reagent (Fermentas International Inc) and subsequently incubated for one hour at room temperature, in dark. The absorbance of the resulting mixture was measured at 724 nm using HP Agilent 8453 Spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). Quantification was based on the standard curve of gallic acid. Results were expressed as milligram of gallic acid equivalent per g of dry weight (mg GAE g^{-1} DW).

Aluminum chloride complex forming assay (aluminum chloride colorimetric method) was used to determine TFC. Plant extract were mixed with 5% KNO_2 and incubated at room temperature. After addition of 10% $AlCl_3$, a yellow complex solution was formed, which then turned immediately to red after addition of 1 M NaOH. The absorbance of mixture was measured at 510 nm. TFC content in the extract was calculated from the standard curve based on rutin, and the results were expressed as milligram of rutin equivalent per g of dry weight (mg RE g^{-1} DW).

2.5. HPLC-DAD analysis of Phenolic Compounds

Chromatographic analysis of methanol extracts was performed on an Agilent HPLC system, model 1100, with DAD, using Hypersil column BDS-C18 (5 μm), 125 mm x 2 mm I.D (Phenomenex, USA). The samples were filtered through a 0.2 μm cellulose filters (Agilent technologies, Santa Clara, CA, USA) prior to analysis. The mobile phase contained 0.1% phosphoric acid and acetonitrile (Acros Organics, Geel, Belgium). Phosphoric acid (A) and acetonitrile (B) were applied in elution gradient as follows: 7.5% B (0.00 min), 20% B (20.00 min), 25% B (25.00 min), 7.5% B (30.00 min), with the flow rate 0.500 ml min-1. Chromatograms were monitored at different wavelengths (210, 266, 310 and 326 nm). Quantification was performed using a standard curve prepared with five different concentrations of standard mixtures, containing coniferyl alcohol, rutin hydrate, gallic, chlorogenic, caffeic, *p*-coumaric, ferulic and rosmarinic acid (Sigma-Aldrich, Oakville, ON, Canada). Chromatogram analysis was conducted by HP Chemstation chromatographic software (Palo Alto, CA, USA).

2.6. GC-MS/FID Analysis of Pyrrolizidine Alkaloids

Qualitative analysis GC-MS analyses were performed on an Agilent 7890A GC system equipped with a 5975C inert XL EI/CI MSD and a FID detector connected by capillary flow technology through a 2-way splitter with make-up gas. An HP-5 MS capillary column (Agilent Technologies, Santa Clara, CA, USA, 25 mm i.d., 30 m length, 0.25 μm film thickness) was used. Samples were injected in splitless mode. The injection volume was 1 μL and 3 μL, for the extracts obtained from wild and in vitro grown plants, respectively, while the injector temperature was 250 °C. The carrier gas (He) flow rate was 1.1 mL min^{-1}, whereas the column temperature was programmed linearly in a range of 100–325 °C at a rate of 10 °C min^{-1}. The transfer line temperature was 320 °C. The FID detector temperature was 300 °C. EI mass spectra (70 eV) were acquired in *m*/*z* range of 45–450, and the ion source temperature was 230 °C.

2.7. Determination of Antioxidant Activity

Free radical scavenging activity of the selected extracts was measured using DPPH and ABTS antioxidant capacity assays, as previously described by Šiler at al. [22]. Results are expressed using the EC_{50} value, defined as the concentration of antioxidant that causes a 50% decrease in the DPPH or ABTS radical absorbance. RA was used as the reference compound. Results are expressed as mM of RAE per g of DW (mM RAE g^{-1} DW). All the analyses were run in triplicate and mean values were calculated.

2.8. Cytotoxicity Assay

The human cell lines used in cytotoxicity assay were human lung adenocarcinoma epithelial cells A549 (ATCC CCL-185), human colorectal adenocarcinoma cells HT-29 (ATCC HTB-38) and normal human fetal lung fibroblasts MRC-5 (ECACC 84101801). Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin mixture (all purchased from Sigma-Aldrich, Steinheim, Germany). Cell lines were maintained in an incubator at 37 °C with 5.0% CO_2 and 100% humidity. The cells were sub-cultured at 90% confluence, twice each week, using 0.1% trypsin. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were used as an indicator of metabolic activity/cell viability.

The cytotoxic effect of the selected extracts was measured by MTT assay~~, as described by Nikolić et al. (2015)~~ [23]. Briefly, cells were inoculated into 96-well plates at a density 2 x 10^4 cells per well. After 24 h of pre-incubation at 37 °C in 5% CO_2, used to provide a cell monolayer, cells were washed with phosphate buffer saline (PBS). After that, fresh medium with tested concentrations of the extracts (within the range 0.125-4 mg mL^{-1}) was added and the treatment lasted for following 24 h at 37 °C in 5% CO_2. After treatment, MTT solution (0.5 mg mL^{-1}) was added and additional 3h incubation was provided in order to form formazan crystals. They were then dissolved in DMSO and absorbance was measured at 570 nm, indicating cell viability.~~Extracts were tested in the concentration range 0.125-4 mg mL^{-1}~~. For each test substance, three independent experiments with six replicates were performed. Furthermore, cytotoxicity data obtained on different cell lines were used to calculate selectivity index (SI), as previously described by Badisa et al ~~(2009)~~ [24]. It was calculated according to equation SI=IC_{50} for normal cells/IC_{50} for cancer cells. SI>1 indicates higher toxicity against cancer cells.

2.9. Statistical analysis

GraphPad Prism 6.01 software (Software, Inc.) was used and one-way ANOVA with Dunnet's and Tukey's post hoc tests was applied to test statistical significance at $p<0.05$ level.

3. Results

3.1. Phenolic Compounds Content

Content of phenolic compounds was analyzed by determining the quantity of TPC and TFC, as well as the contribution of RA and LAB, as the dominant constituents (Figure 2).

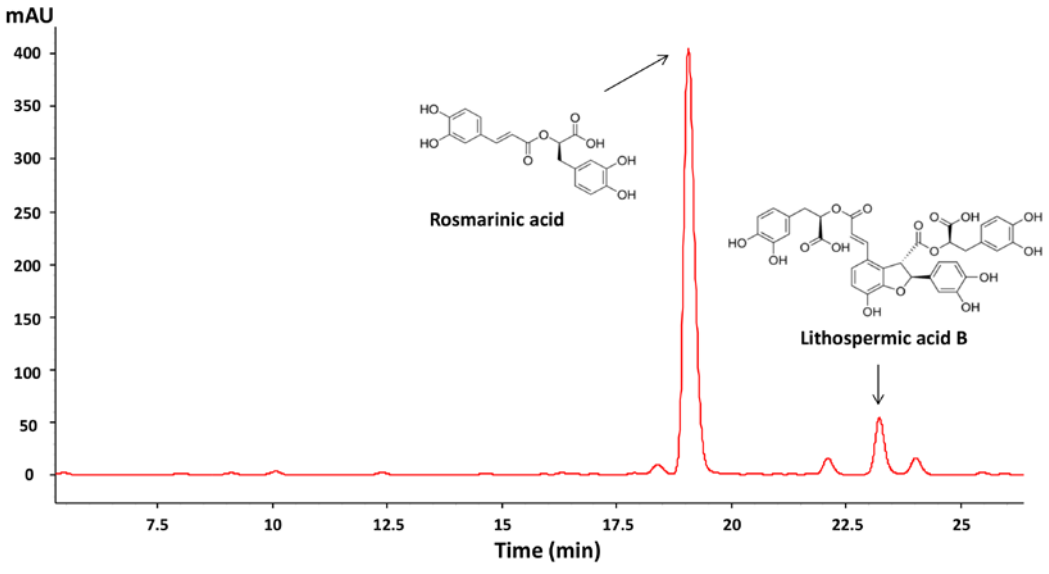


Figure 2. HPLC–DAD chromatogram of the methanol extract of *Rindera umbellata* from the natural habitat. Detection was performed at 326 nm. Major peaks corresponding to rosmarinic acid (Rt=19.05 min) and lithospermic acid B (Rt=23.25 min) are labeled.

Results revealed that quantities of all measured compounds varied between growth conditions and tested plant parts. While for wild-growing plants the content was dependent on plant part used for extract preparation (Table 1), for in vitro cultivated plants it was dependent on the type and concentration of carbohydrates used in cultivation media (Table 2). Concerning the wild-growing plants, the highest TPC (32.37 mg GAE g^{-1} DW) was detected in the leaves, with 0.86% consisting of RA (0.28 mg g^{-1} DW) and 0.12% consisting of LAB (0.04 mg RAE g^{-1} DW). In their roots total phenols were detected in the amount of 26.93 mg GAE g^{-1} DW, with the highest proportion of LAB (4.15%, i.e. 1.12 mg RAE g^{-1} DW). Moreover, roots contained higher amount of LAB compared to leaves, while it was not detected in other tested plant parts (flowers and stem). In addition, there was a significant participation of flavonoids in TPC, with the highest content detected in leaves (22.87 mg RE g^{-1} DW).

Table 1. Phenolic content in different parts of wild growing *R. umbellata* plants. Results represent the mean values ± SE of the three repeated measurements. Different letters designate statistical difference at $p \leq 0.05$ level of significance. TPC-total phenolic content; TFC-total flavonoid content; RA-rosmarinic acid; LAB- lythospermic acid B.

Plant parts	TPC (mg GAE g^{-1} DW)	TFC (mg RE g^{-1} DW)	RA (mg g^{-1} DW)	LAB (mg RAE g^{-1} DW)
Leaves	32.37 ± 0.15 *c*	22.87 ±2.12 *c*	0.28 ± 0.04 *b*	0.04 ± 0.02 *a*
Flowers	25.31 ± 1.70 *b*	13.41 ± 2,31 *b*	0.24 ± 0.04 *b*	Nd
Roots	26.93 ± 0.22 *b*	19.10 ± 3.15 *bc*	0.06 ± 0.01 *a*	1.12 ± 0.21 *b*
Stems	7.26 ± 1.17 *a*	0.81 ±0.44 *a*	0.02 ± 0.01 *a*	Nd

Nd - not determined

On the other hand, TPC of in vitro grown plants was measured only in rosette leaves, considering that root development of this species in culture is a slow process resulting in low biomass production (data not shown). The effect of different carbohydrates concentrations in the growth media was monitored for three commonly used carbohydrates, namely sucrose, glucose and fructose. The highest TPC was detected in leaves of *R. umbellata* grown on media containing 0.03 M sucrose and fructose, as well as 0.3 M glucose (29.58 mg GAE g^{-1} DW, 24.50 mg GAE g^{-1} DW and 26.74 mg GAE g^{-1} DW, respectively, Table 2).

Table 2. Phenolic content in leaves of *R. umbellata* plants grown in vitro using different carbohydrate sources. Results represent the mean values ± SE of the three repeated experiments. For each sugar and phenolic compound the values with the different letters are significantly different at the $p \leq 0.05$ level. TPC-total phenolic content; TFC-total flavonoid content; RA-rosmarinic acid; LAB-lythospermic acid B.

	Carbohydrate concentration (M)	TPC (mg GAE g^{-1} DW)	TFC (mg RE g^{-1} DW)	RA (mg g^{-1} DW)	LAB (mg RAE g^{-1} DW)
Sucrose	0.003	18.63 ± 1.78 *a*	15.37 ± 2.86 *b*	0.43 ± 0.04 *ab*	0.05 ± 0.02 *a*
	0.01	29.37 ± 0.96 *b*	8.37 ± 1.48 *a*	0.22 ± 0.03 *a*	0.01 ± 0.01 *a*
	0.03	29.58 ± 1.11 *b*	16.65 ± 0.38 *bc*	0.42 ± 0.01 *ab*	0.01 ± 0.01 *a*
	0.06	26.08 ± 1.86 *b*	17.76 ± 2.29 *bc*	0.46 ± 0.04 *ab*	0.07 ± 0.01 *a*

	0.1	27.87 ± 1.33 *b*	22.15 ± 1.33 *c*	0.71 ± 0.11 *b*	0.10 ± 0.02 *a*
	0.3	27.95 ± 1.40 *b*	61.97 ± 1.49 *d*	2.52 ± 0.17 *c*	0.11 ± 0.11 *a*
Glucose	0.003	13.92 ± 0.01 *a*	10.83 ± 1.56 *abc*	0.23 ± 0.06 *a*	0.04 ± 0.02 *a*
	0.01	14.43 ± 0.21 *a*	8.23 ± 0.98 *ab*	0.18 ± 0.01 *a*	0.01 ± 0.01 *a*
	0.03	18.02 ± 0.46 *b*	13.55 ± 2.61 *bc*	0.30 ± 0.06 *ab*	0.02 ± 0.02 *a*
	0.06	21.45 ± 0.20 *c*	14.40 ± 2.56 *c*	0.40 ± 0.06 *b*	0.02 ± 0.01 *a*
	0.1	20.26 ± 0.01 *c*	12.56 ± 0.66 *bc*	0.28 ± 0.05 *ab*	0.00 ± 0.00 *a*
	0.3	26.74 ± 0.97 *d*	6.60 ± 1.69 *a*	0.23 ± 0.06 *a*	0.00 ± 0.00 *a*
Fructose	0.003	17.95 ± 0.17 *b*	14.10 ± 0.32 *ab*	0.23 ± 0.01 *a*	0.04 ± 0.15 *a*
	0.01	19.25 ± 0.86 *b*	11.26 ± 1.46 *a*	0.26 ± 0.04 *a*	0.06 ± 0.02 *ab*
	0.03	24.50 ± 0.97 *c*	10.68 ± 0.32 *a*	0.24 ± 0.04 *a*	0.05 ± 0.01 *a*
	0.06	20.63 ± 1.53 *bc*	14.94 ± 3.08 *ab*	0.34 ± 0.08 *a*	0.07 ± 0.02 *b*
	0.1	13.75 ± 1.42 *a*	18.43 ± 1.99 *b*	0.46 ± 0.10 *a*	0.08 ± 0.05 *b*
	0.3	11.24 ± 1.18 *a*	31.21 ± 2.55 *c*	1.51 ± 0.22 *b*	0.05 ± 0.05 *a*

For TFC and RA, an increased amount was observed only at 0.3 M sucrose and 0.3 M fructose (61.97 and 31.21 mg RE g^{-1} DW of TFC, and 2.52 and 1.51 mg g^{-1} DW of RA, respectively), while LAB was detected only in trace amounts, with the slightly higher content detected on media containing 0.1 M and 0.3 M sucrose (0.10 mg RAE g^{-1} DW and 0.11 mg RAE g^{-1} DW).

3.2. Pyrrolizidine Alkaloids Presence in Wild and In Vitro Growing Plants

Identification of pyrrolizidine alkaloids (PAs) in wild and in vitro growing *R. umbellata* plants was performed by GC-MS analyses of obtained methanol extracts. In wild-growing plants six PAs: namely 7-angeloyl heliotridane, 7-angeloyl heliotridine, lindelofine, 7-angeloyl-9-(+)-trachelanthylheliotridine, punctanecine and heliosupine were identified (Figure 3).

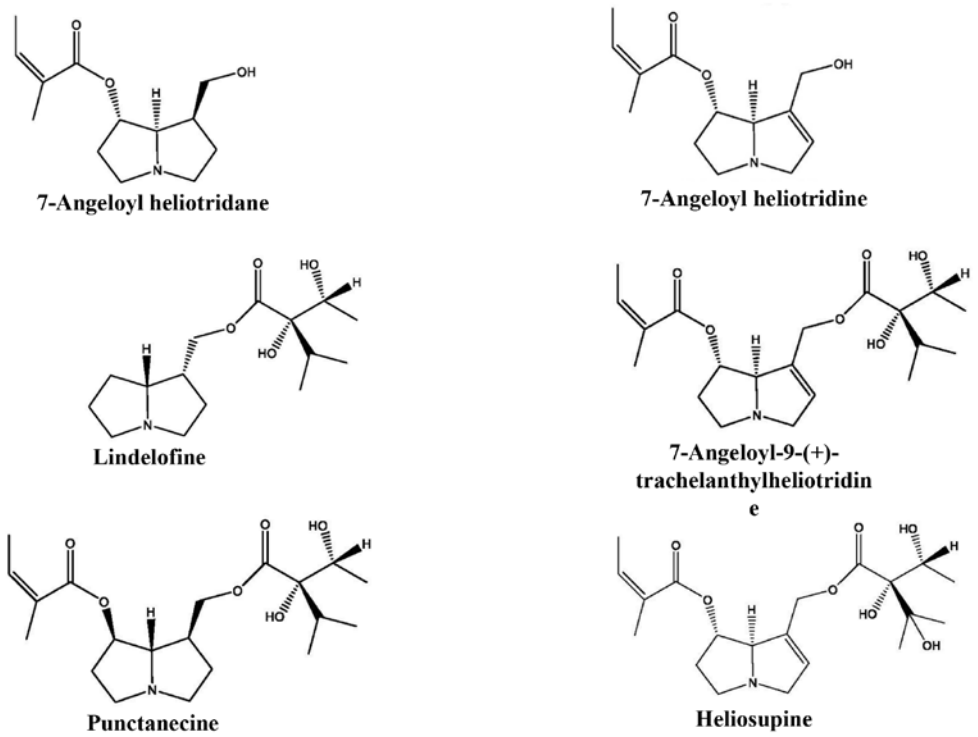


Figure 3. Structures of pyrrolizidine alkaloids detected in wild-growing *R. umbellata*.

They were mostly detected in leaves and flowers, with the exception of heliosupine found only in leaves, and lindelofine detected in all tested plant parts (Table 3). Apart from the identified compounds, 9 saturated and 4 unsaturated PAs of unknown structure were detected in leaves of wild-growing plants (data not shown). On the other hand, PAs presence and variability in in vitro plants grown under common conditions (0.1 M sucrose) were notably lower. Namely, only 7-angeloyl heliotridane and lindelofine were detected in leaves and roots, respectively.

Table 3. Pyrrolizidine alkaloids detected in samples of wild and in vitro growing *R. umbellata* plants. Flw-flowers; lv-leaves; rt-roots.

Pyrrolizidine alkaloids	Retention time(min)	Wild-growing plants	In vitro plants
7-angeloyl heliotridane	12.349	flw, lv	lv
7-angeloyl heliotridine	13.171	flw, lv	
lindelofine	15.176	flw, lv, rt	rt
7-angeloyl-9-(+)-trachelanthylheliotridine	19.571	flw, lv	
punctanecine	19.746	flw, lv	
heliosupine	20.288	lv	

3.3. Antioxidant Activity

~~Taking into account the high content of phenolic compounds in the extracts, some antioxidant potential was anticipated and it was~~In further work, antioxidant properties were evaluated for both wild-growing and in vitro cultivated *R. umbellata* plants. ~~For that reason~~Actually, DPPH and ABTS assays were used and IC_{50} values were determined (Table 4).

Table 4. Antioxidant activity of methanol extracts of *R. umbellata* plants from the natural habitat and grown in vitro. Results represent the mean values ± SE of the three repeated measurements. Different letters designate statistical difference at $p \leq 0.05$ level of significance.

	IC_{50} (mM RAE g^{-1} DW)	
Wild growing plants	***DPPH•***	***ABTS•+***
Leaves	2.31 ± 0.23 *a*	0.77 ± 0.01 *a*
Flowers	4.14 ± 0.77 *a*	1.15 ± 0.28 *a*
Roots	1.71 ± 0.54 *a*	1.03 ± 0.17 *a*
Stems	52.56 ± 3.57 *b*	10.06 ± 3.35 *b*
Plants grown in vitro		
0.3 M sucrose	0.52 ± 0.04 *a*	0.27 ± 0.01 *a*
0.1 M sucrose	2.07 ± 0.33 *b*	1.06 ± 0.16 *b*

Results obtained from extracts derived from wild-growing plants showed that all plant parts, except stems, had high antioxidant activity; the lowest IC_{50} were determined for leaves in ABTS, and roots in DPPH test (0.77 and 1.71 mM RAE g^{-1} DW, respectively). Antioxidant capacity of the leaves was enhanced in the in vitro conditions, but depended on the applied sucrose concentrations (Table 4). IC_{50} values pointed out that 0.3 M sucrose could be considered as the most appropriate one (0.52 and 0.27 mM RAE g^{-1} DW in DPPH and ABTS assay, respectively). Comparison of the antioxidant activity with TPC, TFC, RA, and LAB values indicated that only TPC were significantly correlated to the antioxidant capacities of plant extracts, with $R^2 = 0.9351$ and $R^2 = 0.9384$, as determined by the DPPH and ABTS assay, respectively. ~~Comparison of the antioxidant activity and TPC, TFC and RA content indicated that clear correlation cannot be observed, but it seems that TFC and RA contributed to the antioxidant capacity more strongly than TPC.~~

3.4. Cytotoxic activity

Results obtained in MTT assay showed that the most susceptible cell line was HT-29, where IC_{50} values were determined for all tested extracts (leaves harvested from wild-growing plants and the ones grown in vitro on 0.3 M and 0.1 M sucrose, Table 5).

Table 5. Cytotoxic effect of *R. umbellata* leaves extracts towards selected cell lines. IC_{50} values are expressed in mg mL^{-1}. SI was calculated as the ratio of IC_{50} values determined for normal MRC-5 cells and for each cancer cell line. The value >1 indicates higher toxicity against cancer cells, while the value ≥ 2 shows high selectivity to cancer cells.

	HT-29		**A549**		**MRC-5**
	IC_{50} (mg mL^{-1})	**SI**	**IC_{50} (mg mL^{-1})**	**SI**	**IC_{50} (mg mL^{-1})**
Wild-growing leaves	1.65 ± 0.18	Na	Nd	Na	Nd
In vitro grown leaves					
0.3 M sucrose	1.25 ± 0.14	2.56	4.00 ± 0.17	0.80	3.20 ± 0.15
0.1 M sucrose	3.19 ± 0.11	0.90	3.50 ± 0.22	0.80	2.90 ± 0.20

Nd - not determined in applied concentration range (up to 4 mg mL^{-1})

Na - not applicable, due to the fact that IC_{50} value was not determined in tested concentration range, at least for one cell line

Actually, the extract produced from wild-growing leaves was effective only against HT-29 cells. On the other hand, extracts of in vitro samples were cytotoxic against all three

tested cell lines. The lowest IC_{50} value was determined in the case of HT-29 cells treated with 0.3 M sucrose extract. Furthermore, activity of the extracts obtained from leaves harvested from 0.3 M sucrose and from nature was comparable (IC_{50} values 1.25 mg mL^{-1} and 1.60 mg mL^{-1}, respectively), whilst 0.1 M sucrose showed notably lower effect. In addition, the calculated selectivity indices (SIs), being used to compare activity on cancer and normal cells, indicated that the selectivity for cancer cells was observed in the case of 0.3 M sucrose extract and HT-29 cell line. Although SI was not calculable for the wild-growing plant extract, it can be noted that the selectivity against HT-29 cell line was high, due to the lack of cytotoxicity on normal MRC-5 cells.

4. Discussion

Production of plant secondary metabolites using in vitro technology has a lot of advantages. It does not depend on the seasonal variations and/or the influence of the environmental factors, and additionally, enables the production of plant material without disturbing the environment. Growing in strictly controlled, optimized conditions could increase yield of some pharmaceutically important secondary metabolites, or even production of certain compounds that are usually not detected in plants from the natural habitats [~~23~~25,~~24~~26] *R. umbellata*, that is considered as rare and endangered in Serbia, has already been successfully introduced in in vitro culture; moreover, protocol for optimized growth and biomass production has been established in our previous work [17]. However, until now, the comparative analysis of chemical composition and biological activities of the extracts from in vitro grown plants or plants from nature was lacking.

Concerning the PAs presence, it is worth noting that Ganos et al. [~~25~~27] detected echinatine, echinatine N-oxide and rinderine N-oxide in *R. graeca* samples collected from nature. In addition, previous investigations of our group on wild-growing *R. umbellata* showed that the extracts contained versatile PAs, including 7-angeloyl-9-(+)-trachelanthylheliotridine, lindelofine, punctanecine, 7-angeloyl heliotridane, 7-angeloyl heliotridine, heliosupine, lindelofine-N-oxide, heliosupine-N-oxide and 9-(+)-trachelanthyllaburnine, echinatine and 7-angeloyl rinderine, but their presence was variable and depended on harvesting season, plant part and extraction procedure [18,19]. Indeed, it can be assumed that relating to harvesting season; detected PAs content depend on different abiotic factors, such as temperature, light and air humidity, as well as stages of plant development [~~26~~28]. This is in line with the results obtained in this work for the methanol extracts of leaves, flowers and roots of wild-growing *R. umbellata*. Among detected PAs, six were of identified structure: 7-angeloyl heliotridane, 7-angeloyl heliotridine, lindelofine, 7-angeloyl-9-(+)-trachelanthylheliotridine, punctanecine and heliosupine. Taking into account that plant material and season of collection were not the same in previous studies [18,19] and this work, observed differences are to be expected. Since plant material in this work was collected separately for different organs, one could note that the highest variability of PAs is present in the leaves and flowers.

On the other hand, *R. umbellata* grown in vitro in common conditions (0.1 M sucrose) produced small amounts of non-versatile PAs, namely 7-angeloyl heliotridane and lindelofine. Scarce detection of PAs is in line with the literature data indicating that certain compounds, such as nicotine, some PAs and tropane alkaloids were produced only in small amounts, or not being produced at all, in in vitro culture [2~~9~~7]. In contrast, some literature data indicated that in vitro growing plants could also produce PAs in certain conditions; for example, Graikou et al. [~~28~~30] detected echinatine, echinatine N-oxide and rinderine N-oxide in seedlings and hairy roots of *R. graeca*. However, considering that some alkaloids are strongly toxic for mammals, inducing neurotoxic, hepatotoxic, mutagenic and carcinogenic effects [~~26~~28,~~29~~31], in vitro plant culturing which could prevent or decrease alkaloids production is of great importance, especially in the case of medicinal

plants. Accordingly, this method could be considered suitable for the production of some nontoxic, pharmaceutically important secondary metabolites of *R. umbellata*.

Phenolic profile analysis of wild-growing *R. umbellata* plants was performed for the first time in this study. Results showed that the distribution of TPC is the same in roots and flowers, while the highest content was measured in leaves samples. In addition, the TFC of different parts was determined in the following descending order: leaves > roots > flowers, while in stem it was determined only in traces. Furthermore, RA was detected in leaves and flowers, while LAB was determined only in roots. Similar results, with high values of TPC, TFC and RA, but lack of LAB, were determined for aerial parts of *R. graeca* collected from nature [~~25~~27].

Concerning determination of phenolic compounds in in vitro grown plants, it is worth noting that the previous research pointed out that the optimization of culturing conditions could increase their production [~~30~~32,~~31~~33]. Indeed, level of secondary metabolites depends on numerous factors and among them are the availability of carbohydrates in the media, used as a source of carbon and energy [~~30~~32]. High concentrations of carbohydrates can affect cell metabolism and change osmotic potential, consequently inducing an increase in secondary metabolites production [~~30~~32,~~32~~34,~~33~~35]. Considering this, we have cultivated plants in the presence of variable amounts of different carbohydrates. Namely, sucrose, glucose and fructose were added, all in the concentration range 0.003-0.3 M. Their effect was variable, but comparison of all tested phenolics in wild growing and in vitro cultivated plants pointed out that TFC and RA content were notably increased in leaves of plants cultivated on media containing 0.3 M sucrose. If this fact is taken into consideration together with the result showing that TPC in plants grown at 0.3 M sucrose is still high and comparable with the values determined in leaves collected from nature, it can be denoted as the most appropriate medium for the production of the phenolic compounds. However, according to our previous results [17], 0.3M sucrose considerably reduced *R. umbellata* in vitro growth and biomass production in comparison to optimal sucrose concentration (0.1 M). This is in line with previously published data, also indicating inverse proportionality of RA content and biomass production. This was confirmed for *Perilla frutescens* [~~34~~36] and for *Salvia officinalis* [~~35~~37]. Possible explanation for this phenomenon could be found in the fact that high concentrations of carbohydrates act as stressors and induce changes in cell metabolism and consequently inhibit plant growth, but at the same time stimulate the production of secondary metabolites [5]. Consequently, compromise between secondary metabolites content and biomass production should be made when the optimal conditions for in vitro production of bioactive substances is established. In accordance with that, both 0.1 M and 0.3 M sucrose were selected for further investigation of biological activities. The first one was selected due to the highest biomass yield which was previously confirmed [17], but also due to relatively high amount of all determined phenolic compounds. The second concentration was chosen as the one which provided the highest secondary metabolites production. Results of DPPH and ABTS assays performed with the extracts obtained from the wild growing plants showed that moderate antioxidant activity was detected for the ones prepared from roots, leaves and flowers, while antioxidant effect of stem extract was weak. Concerning plants grown in vitro, it is observable that antioxidant properties were the best for 0.3 M sucrose. If the values determined for TPC, TFC, RA and LAB are taken into consideration, it seems that notable increase of ~~RA and TFC~~TPC contributed to elevated antioxidant activity. Actually, numerous literature data indicate that TPC, including flavonoids~~,~~ and ~~as well as~~ RA, possess strong antioxidative potential and could protect cells from oxidative stress and its possible consequences [~~36~~38,~~37~~39]. In accordance with the fact that oxidative stress is involved in numerous disorders, such as cardiovascular, neurological, respiratory and kidney diseases, as well as diabetes mellitus, rheumatoid arthritis, cancer, aging and aging-related diseases [40], potential applications of *R. umbellata* extracts could be numerous. Further on, to determine the cytotoxic potential of *R. umbellata*, comparative analysis of both wild-growing and in vitro cultivated plants was carried out. As for the antioxidant

properties, the extracts of leaves obtained at 0.1 M and 0.3 M sucrose were used, but the extract of wild-growing plants' leaves was also involved. Efficacy was screened against two cancer cell lines, namely lung (A549) and colorectal adenocarcinoma (HT-29). However, considering that the potential chemotherapeutics should possess stronger cytotoxicity against cancer than normal cells, normal lung fibroblasts were involved as well. Although the overall activity was weak, some observations could be made. If comparison of the cell line susceptibility is made, one could note that HT-29 cells were highly sensitive, at least to leaves extract of wild-growing and 0.3 M sucrose plants. Simultaneously, the lack of activity and notably lower activity on normal MRC-5 cell line, of the wild-growing and 0.3 M extracts, respectively, together with the literature data pointing out that SI ≥ 2 is an indication of high selectivity [~~38~~24], showed that these extracts could be considered as effective against colorectal cancers. Certainly, high values of determined IC_{50} against HT-29 indicated that crude methanol extracts could be denoted as a solid base for searching of active cytotoxic principle/principles being potentially applicable at least against colorectal adenocarcinoma. On the other hand, lung carcinoma A549 cells were highly resistant to all tested extracts and moreover, their sensitivity was even lower compared to normal MRC-5 cells. High resistance of this cell line could be attributed to the elevated level of catalase activity and glutathione, responsible for high intrinsic level of antioxidant protection, which is multifold higher in this cell line than in the normal lung fibroblasts [~~39~~41].

Since the bioactive substances are responsible for the observed cytotoxicity, extract obtained from nature should be separately discussed from the ones prepared from in vitro grown leaves. When it comes to the leaves of wild-growing plants, both phenolics and PAs probably contributed to the observed activity, while in the case of the in vitro leaves extracts, the latter one should be omitted. Actually, phenolic compounds, although well known as potent antioxidants, in certain circumstances (high concentrations and/or the presence of metal ions), could induce pro-oxidative effect [~~40~~42]. Pro-oxidant agents could cause strong oxidative stress which consequently could lead to cell death [~~41~~43]. Furthermore, literature data shows that RA and LAB also possess cytotoxic potential [14,15], indicating that they probably influenced the observed activity, especially in the case of the extracts of in vitro grown plants, since their levels were notably higher within them than within the extracts obtained from leaves from nature. In addition, PAs of the leaves collected from nature could also contribute to overall cytotoxic properties. This is supported by the fact that alkaloids, including PAs, are characterized by anticancer activity [~~42~~44]. Mechanisms of their anticancer activity could be diverse, including the effect on tubulin polymerization, as it was shown in our previous work for lindelofine-N-oxide, isolated from *R. umbellata* [18].

General view on the presented findings could be helpful for all the researchers being interested in ex situ conservation using in vitro technique, and in utilization of biological activity of rare and endangered plant species. Considering possible use of *R. umbellata*, it is worth noting that its growing in in vitro conditions opens new possibilities for the production of biological active compounds. This would significantly reduce the pressure on the wild growing *R. umbellata* populations and prevent its further uncontrolled harvesting from nature. In addition, in vitro technique could enable *R. umbellata* reintroduction to natural habitats.

5. Conclusions

Chemical characterization revealed for the first time contents of total phenolics, flavonoids, rosmarinic and lythospermic B acids in both wild and in vitro grown *R. umbellata*, as well as PAs presence in in vitro cultivated plants. Further on, this research pointed out that 0.1 M and 0.3 M sucrose in the growth media provided the highest yield of bioactive natural products with antioxidant properties. Although cytotoxic effect was mild and its selectivity was limited to colorectal HT-29 cell line, obtained result encourage further

study that should be directed to determination of active principals being responsible for this activity, as well as to screening on numerous cancer cell lines.

Author Contributions: S.T. has made research conceptualization, collected data and statistically analyzed them, written and prepared the final version of the manuscript; M.P. has performed the in vitro experiments, measured total phenolic and flavonoid content and determined antioxidant activity; B.N. has given the concept and supervised research of the cytotoxicity, written and prepared the final version of the manuscript; B.M. has accomplished GC-MS/FID analysis of PAs; S.C. has performed the cytotoxicity experiments and the literature research; M.B. prepared extracts and tested biological activity; S.Ž. has performed HPLC-DAD analysis of phenolic compounds. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (grants: 451-03-47/2023-01/ 200007; 451-03-47/2023-01/ 200178)

Data Availability Statement: Raw data are available by request via email to slatod@ibiss.bg.ac.rs.

Conflicts of Interest: The authors declare no conflict of interest.

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