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Chemical composition, antioxidant and anticancer activity of licorice from Fruska Gora locality

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ABSTRACT

The present study was undertaken in order to evaluate the potential application of fresh and dry roots and leaves ethylacetate extracts of licorice from locality of Fruska Gora (Serbia) as a new source of valuable bioactive compounds with health benefits. Forty bioactive compounds have been quantified using LC–MS–MS and remarkable differences have been found among the extracts. The most abundant compounds were observed in the extract of fresh root. Therefore, this extract showed the strongest antioxidant potential and was the most effective that induced noticeable necrosis on breast MDA-MB-361 adenocarcinoma cell line and exhibited considerable proapoptotic property on SiHa ovarian cancer cell line. All presented results indicate fresh root of tested plant could have pharmacological potential, especially as a valuable source of natural antioxidants and/or phytoestrogens, which could indicate its long term use in prevention and/or therapy of oxidative stress-related diseases including some types of cancer.

1. Introduction

Licorice (*Glycyrrhiza glabra* L.) is spread on a wide part of Northwest Europe, North Africa, Siberia and the Caucasus, as well as in Asia. In Serbia, it is rarely found. Fruska Gora mountain, Cortanovci forest (45° 9′ 16” North, 20° 1′ 14” East) was locality where plant material was harvested for present study. Licorice is one of the medicinal plants known and used for centuries. Ancient people used licorice as a healing agent, but also for flavoring drinks. Roots and leaves of *G. glabra* contain a wide spectrum of bioactive constituents such as triterpenes (glycyrrhetic acid, glycyrrhizin), phenols (including liquiritigenin, liquiritin, isoliquiritigenin, isoliquiritin) and many others, detected by different chromatography techniques (Biondi et al., 2005; Jiang et al., 2013; Liao et al., 2012a,b; Siracusa et al., 2011; Wang et al., 2015; Wang and Yang, 2007; Zadeh and Kor, 2013), which are most likely responsible for therapeutic properties mentioned below.

Many reports presented antioxidant (Dong et al., 2014; Gupta et al., 2008; Martins et al., 2015; Parvaiz et al., 2014; Visavadiya and Narasimhacharya, 2006), antimicrobial (Gupta et al., 2008; Saraf et al., 2013; Sultana et al., 2010) activity – mainly reflected through anti-Helicobacter pylori (Asha et al., 2013; Fukai et al., 2003), antiviral (Cheel et al., 2010; Wang et al., 2015), antiinflamatory (Biondi et al., 2005; Farag et al., 2015; Zheng et al., 2014). as well as antiproliferative activity (Basar et al., 2015; Chin et al., 2007; Dunlap et al., 2015; Farag et al., 2015; Huang et al., 2014; Yan et al., 2014) related to the content of bioactive compounds from *G. glabra*. Standardized licorice extract is used in sweets, tobacco industry and cosmetics, as well as an additive to different beverages (liqueur, brandy, etc.) because of its sweet taste (Chin et al., 2007). Many phenols present in licorice extracts such as liquiritigenin, liquiritin, isoliquiritigenin, isoliquiritin, glabridin, formononetin are responsible for phytoestrogen activity of this plant extracts (Tang et al., 2015). They can selectively bind to peroxisome

Abbreviations: AAE, ascorbic acid equivalents; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DPPH, 2,2 diphenyl-1-picrylhydrazyl; dw, dry weight; EDTA, ethylenediamine tetraacetic acid; ESI, electrospray ionization; FC reagent, Folin-Ciocalteu reagent; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; HO, Hoechst 33258; LP, lipid peroxidation; MDA, malondialdehyde; MRM, multiple reaction monitoring; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADH, nicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium; TFC, total flavonoid content; TPC, total phenolic content; PBS, phosphate-buffered saline; PI, propidium iodide; PMS, phenazine methosulfate; QE, quercetin equivalents; SRB, sulforhodamine B; TBA, 2-thiobarbituric acid; TPTZ, 2,4,6-tripyridil-*s*-triazine

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proliferator-activated receptor gamma or estrogen receptor, causing lipid reduction and having some good effects on hormone imbalances or menopausal symptoms in women. Hence, licorice is also used in hormone replacement therapy (Boonmuen et al., 2016; Simmler et al., 2013). Although the research attention is focused mostly on the healing properties of roots, the leaves should not be neglected, because they also contain bioactive substances such as flavonoids (naringein, astragalin, isoquercetrin, vicenin, etc.) (Biondi et al., 2005; Siracusa et al., 2011).

Until now, there were no reports on study of phytochemical properties of the licorice extracts and connection with their biological activity, from any localities. We performed such a study of licorice from Fruska Gora mountain. Thus, this could be a valuable study and contribution to the use in human nutrition and also in the prevention and treatment of many diseases of this rarely – found plant. The present study was undertaken to characterize and quantify selected bioactive compounds in extracts of *G. glabra* L. leaves and root and evaluate their antioxidant and anticancer potential.

2. Material and methods

The plant material (root and leaves) was collected during spring 2014 on Fruska Gora (Cortanovaci forest), Vojvodina, Serbia. The plant was determinate by Dr. Sanja Vasiljevic and a voucher specimen of the plant was confirmed and deposited at Herbarium of Department of Applied Botany, at Faculty of Agriculture; University of Belgrade. The samples are abbreviated as **L1**–fresh root, **L2**- dry root; **L3**- fresh leaves; **L4**-dry leaves.

The extracts were prepared by Microwave-assisted (MW) extraction. The method was used before for isolation of phenolic compounds. Using microwaves instead of steam, the interaction of electromagnetic fields with the liquid present in the cell vacuoles leads to their cracking and rapid exit of the contents (Flamini et al., 2007). This method has a few advantages over conventional extraction: the extraction time is shorter and the process is less expensive.

Previously chopped dried and fresh plant material (5 g) was placed in the flask (100 mL) and coated with 50 mL of 70% ethyl acetate. The extraction lasted 10 min. After the filtration the solvent evaporated *in vacuo* at 45 °C. Dried extracts were dissolved in 80% ethanol (v/v) to obtain 100 mg/mL (stock solution). Extraction was performed using modified, previously described MW assistant extraction (Vlaisavljevic et al., 2016).

2.1. Quantitative LC–MS/MS analysis of the selected bioactive compounds

New method for quantification of 17 bioactive compounds was hereby developed. The optimized compound-specific parameters for quantification of 17 compounds in dynamic MRM (MRM (multiple reactions monitoring) mode – retention time, precursor ion, product ion, voltage of fragmentor, collision voltage) are given in Table 1.

Further, the method for quantification of 45 plant phenolics (Orčić et al., 2014) was used for quantitative determination of phenolic compounds in the *G. glabra* extracts. Prior to the analysis, all extracts were diluted in a mixture of water and methanol premixed in 1:1 ratio, to obtain a final concentration of 2 mg/mL. For both mixes of 45 and 17 compounds, fifteen working standards, ranging from 1.53 ng/mL to 25.0×10^3 ng/mL, were prepared by serial 1:1 dilutions of standard water-methanol mixture (1:1). All samples and standards were analyzed using Agilent Technologies (AT) 1200 Series high-performance liquid chromatography coupled with AT 6410A Triple Quad tandem mass spectrometer with electrospray ion source, and controlled by AT MassHunter Workstation software – Data Acquisition (ver. B.03.01). Into the system 5 µL of the samples/standards were injected, and compounds were separated on Zorbax Eclipse XDB-C18 (50 mm × 4.6 mm, 1.8 µm) rapid resolution column held at 50 °C. Mobile phase consisted of A: 0.05% aqueous formic acid and B: methanol, was delivered at flow rate of 1 mL/min in gradient mode (0 min 30% B, 6 min 70% B, 9 min 100% B, 12 min 100% B, re-equilibration time 3 min). Eluted compounds were detected by ESI–MS, using the ion source parameters as follows: nebulization gas (N_2) pressure 50 psi, drying gas (N_2) flow 10 L/min and temperature 350 °C, capillary voltage 4 kV, in negative polarity (negative ionization mode, NI). Data were acquired in dynamic MRM mode, using the optimized compound-specific parameters (Orčić et al., 2014). For all the compounds, peak areas were determined using Agilent MassHunter Workstation software – Qualitative Analysis (ver. B.03.01.). Calibration curves were plotted and samples' concentrations calculated using the OriginLabs Origin Pro (ver. 9.0) software.

Table 1
Optimized dynamic MRM parameters for 17 compounds.

Compound	Precursor *m/z*	Product *m/z*	$V_{fragmentor}$ (V)	$V_{collision}$ (V)	t_R (min)
Equol	241	119	110	25	3.72
Daidzein	253	208	145	30	3.43
Liquiritigenin	255	119	100	20	3.33
Isoliquiritigenin	255	119	100	20	4.89
Formononetin	267	252	100	15	5.49
Genistein	269	133	145	30	1.72
Glycitein	283	268	140	15	3.55
Calycosin	283	268	140	15	3.74
Biochanin A	283	268	140	20	5.98
Enterolactone	297	253	160	20	3.8
Enterodiol	301	253	140	20	3.5
Pinoresinol	357	151	140	15	3.33
Daidzin	415	253	200	15	1.3
Genistin	431	269	180	15	1.72
Glycitin	445	282	200	25	1.41
Glycyrrhetinic acid	469	425	280	40	8.93
Glycyrrhizin	821	351	220	40	7.41

2.2. Determination of the total bioactive components

The total Phenol Content (TFC) was performed using the method described previously customized for 96-well microplates (Fukumoto and Mazza, 2000). The aluminum chloride colorimetric method (Jia et al., 1999), adapted for 96-well plates, was used to determine the total content flavonoids content (TFC).

The total phenol content was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (dw) of extract, calculated according to the standard calibration curve.The mean values of flavonoid content were expressed as milligrams of quercetin equivalents (QE) per gram of dry weight (dw) of extract, calculated according to the standard calibration curve.

2.3. DPPH assay

Plant extracts were tested for the scavenging effect on the DPPH radical (Sánchez-Moreno et al., 1999). The percentage of inhibition I (%) for each radical species was calculated using the following equation: $I\ (\%) = 100 \times (A_{blank} - A_{sample})/A_{blank}$, where A_{blank} was the absorbance of the control reaction and A_{sample} was the absorbance of the examined samples, corrected for the value of the blank probe. From the obtained I (%) values, the IC_{50} values (which represented the concentrations of the examined extracts that caused 50% neutralization) were determined by linear regression analysis, using Origin software, version 9.0.

2.4. Hydroxyl-radical (HO·) scavenger capacity

Scavenging capacity for HO radical of the *G. glabra* leaves and root extracts was determined by monitoring the chemical degradation of 2-deoxy-D-ribose (Cheesman et al., 1998). The percentage of inhibition I (%) for each radical species was calculated using the following

equation: I (%) = 100 × (A_{blank} − A_{sample})/A_{blank}, where A_{blank} was the absorbance of the control reaction and A_{sample} was the absorbance of the examined samples, corrected for the value of the blank probe. From the obtained I (%) values, the IC_{50} values (which represented the concentrations of the examined extracts that caused 50% neutralization) were determined by linear regression analysis, using Origin software, version 9.0.

2.5. Superoxide anion ($O_2\cdot^-$) scavenger capacity

The capability of extracts to neutralize superoxide anion formed by the reduction of nitroblue tetrazolium (NBT) with NADH mediated by phenazine methosulfate (PMS) under aerobic conditions was conducted according to Cos et al. (1998). The percentage of inhibition I (%) for each radical species was calculated using the following equation: I (%) = 100 × (A_{blank} − A_{sample})/A_{blank}, where A_{blank} was the absorbance of the control reaction and A_{sample} was the absorbance of the examined samples, corrected for the value of the blank probe. From the obtained I (%) values, the IC_{50} values (which represented the concentrations of the examined extracts that caused 50% neutralization) were determined by linear regression analysis, using Origin software, version 9.0.

2.6. NO scavenger capacity

Test of nitric oxide radical (NO·) scavenging capacity was based on method of Green et al. (1982), adapted for 96-well plates. The percentage of inhibition I (%) for each radical species was calculated using the following equation: I (%) = 100 × (A_{blank} − A_{sample})/A_{blank}, where A_{blank} was the absorbance of the control reaction and A_{sample} was the absorbance of the examined samples, corrected for the value of the blank probe. From the obtained I (%) values, the IC_{50} values (which represented the concentrations of the examined extracts that caused 50% neutralization) were determined by linear regression analysis, using Origin software, version 9.0.

2.7. Reducing power – FRAP assay

To evaluate the reducing power of extracts, the ferric ion reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996), modified for 96-well plates, was undertaken. Mean values of reducing power were expressed as milligrams of ascorbic acid equivalents (AAE) per gram of dry weight of extract calculated according to the standard calibration curve.

2.8. Statistical analysis

All of the results were expressed as mean ± SD of three different trials. A comparison of the group means and the significance between the groups were verified by one-way ANOVA. Statistical significance was set at $p < 0.05$.

2.9. Cell culturing

Human breast cancer cell lines (MCF7, T47D, MDA-MB-231 and MDA-MB-361), a cervical cancer cell line (HeLa) and an ovarian cancer cell line (A2780) were purchased from ECACC (European Collection of Cell Cultures, Salisbury, UK), while SiHa cells (cervical cancer) were obtained from ATCC (American Tissue Culture Collection, Manassas, Virginia, USA). Cells were cultivated in minimal essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids and an antibiotic-antimycotic mixture. All media and supplements were obtained from Lonza Group Ltd. (Basel, Switzerland). The cells were maintained at 37 °C in humidified atmosphere containing 5% CO_2.

2.10. Antiproliferative activity measured by MTT assay

The growth-inhibitory activity of the extracts was determined by MTT method against a panel of human cancer cell lines of gynecological origin (Mosmann, 1983). Briefly, all types of used cells were seeded into 96-well plates at a density of 5000 cells/well, with exception for MDA-MB-361 cells, which were seeded at 10000/well. After an overnight preincubation cells were incubated with the tested extracts at 10 and 30 μg/mL. After incubation for 72 h, 5 mg/mL MTT solution was added and the samples were incubated for another 4 h. The precipitated formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was measured at 545 nm with a microplate reader. Stock solutions of the extracts were prepared with DMSO (10 mg/mL) and the highest concentration of the solvent (0.3%) has no substantial action on the cell viability. Two independent experiments with 5 wells in each condition were performed.

2.11. Hoechst 33258–propidium iodide double staining

On the basis of the results of MTT, SiHa and MDA-MB-361 cell lines were selected for treatment with licorice fresh root extract **L1**. Near-confluent SiHa or MDA-MB-361 cells were seeded into a 96-well plate (5000 cells/well). After incubation for 24 h with the tested extract, Hoechst 33258 (HO) and propidium iodide (PI) were added to the culture medium to give final concentrations of 5 and 3 μg/mL, respectively. The cells were incubated with the staining mixture for 1 h at 37 °C and were then photographed by means of a Nikon Eclipse microscope equipped with an epifluorescence attachment containing the appropriate optical blocks and a QCapture CCD camera. The staining allowed the identification of live, early-apoptotic, late-apoptotic/necrotic cells. Hoechst 33258 permeates all the cells and makes the nuclei appear blue. Apoptosis was revealed by nuclear changes such as chromatin condensation and nuclear fragmentation. The necrotic and the late-apoptotic cells were identified as cells with propidium iodide uptake, which indicates loss of membrane integrity, leading the cell nuclei being stained red (Ribble et al., 2005).

2.12. Analysis of cell cycle by flow cytometry

On the basis of the results of MTT and the double staining assays, the cell cycle phase distribution of SiHa cells treated with extract **L1** was determined by flow cytometry. Cells were seeded into 6-well plates at a density of 300000 cells/well. After 24 h of treatment the cells were washed twice with cold phosphate-buffered saline (PBS), harvested by trypsinization and centrifuged at 1500 rpm for 10 min. After washing in PBS, the cells were fixed in 1 mL cold 70% ethanol for 30 min. Samples were stained with 1.0 mL dye solution containing 0.02 mg/mL RNAse A, 0.1 mg/mL PI, 0.003 ml/ml Triton-X and 1.0 mg/ mL sodium citrate in distilled water, and the mixture was incubated in the dark for 60 min at room temperature. Cells were analyzed by a Partec CyFlow instrument (Partec GmbH, Münster, Germany). In each analysis, 20000 events were recorded, and the percentages of the cells in the different cell cycle phases (subG1, G1, S and G2/M) were determined using ModFit Software. The subG1 fraction was regarded as the apoptotic cell population (Vermes et al., 2000).

Statistical analyses were performed by GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) using ANOVA followed by Newman-Keuls Comparison Test.

3. Results and discussion

3.1. LC–MS–MS analysis: quantitative determination of bioactive compounds in G. glabra extracts

Earlier reports on the bioactive compounds content in extracts of *G. glabra* from different localities are based on narrow spectrum of phenols

Table 2
LC–MS–MS quantification of bioactive compounds presented in *G. glabra* extracts (μg/g dw).

	Compounds	L1	L2	L3	L4
Phenolic acids	p-Hydroxybenzoic acid	52.89 ± 0.01[d]	17.93 ± 0.01[c]	8.51 ± 0.025[b]	6.02 ± 0.06[a§]
	Protocatechuic acid	2.81 ± 0.07[d]	2.06 ± 0.07[c]	1.13 ± 0.03[b]	0.38 ± 0.10[a]
	2.5-Dihydroxybenzoic acid	1.61 ± 0.11[b]	0.35 ± 0.02[a]	3.20 ± 0.01[c]	1.98 ± 0.32[b]
	p-Coumaric acid	73.82 ± 1.31 [d]	20.56 ± 1.02 [c]	19.53 ± 0.02 [b]	9.79 ± 0.01 [a]
	Vanillic acid	9.40 ± 0.05[c]	5.08 ± 0.04[b]	4.30 ± 0.1[a]	n.d.
	Gallic acid	5.41 ± 0.19[a]	4.97 ± 0.06[b]	4.55 ± 0.05[b]	4.15 ± 0.08[b]
	Caffeic acid	1.20 ± 0.06 [c]	1.18 ± 0.07[c]	0.77 ± 0.03[b]	0.55 ± 0.03[a]
	Quinic acid	730.89 ± 0.33 [d]	9.22 ± 0.01 [c]	4.33 ± 0.01[b]	2.21 ± 0.10 [a]
	Ferulic acid	22.95 ± 0.04 [d]	9.46 ± 0.04 [c]	1.13 ± 0.13 [a]	1.92 ± 0.08[b]
	Syringic acid	8.53 ± 0.04 [b]	3.02 ± 0.1 [a]	n.d.	n.d.
	Chlorogenic acid	3.37 ± 0.05 [c]	1.44 ± 0.03 [b]	n.d.	0.55 ± 0.01 [a]
	Cinnamic acid	n.d.	n.d.	n.d.	n.d.
	o-Coumaric acid	n.d.	n.d.	n.d.	n.d.
	3.4-Dimethoxycinnamic acid	n.d.	n.d.	n.d.	n.d.
	Sinapic acid	n.d.	n.d.	n.d.	n.d.
Flavonoids	Apigenin	3.44 ± 0.07 [d]	2.68 ± 0.05 [c]	0.71 ± 0.06 [a]	1.15 ± 0.02[b]
	Naringenin	186.35 ± 0.08 [d]	130.67 ± 0.01 [c]	9.36 ± 0.03 [b]	4.82 ± 0.06 [a]
	Luteolin	2.58 ± 0.07 [c]	2.34 ± 0.03[c]	1.88 ± 0.05 [b]	0.28 ± 0.2 [a]
	Kaempferol	2.46 ± 0.04 [b]	2.70 ± 0.54[b]	0.99 ± 0.05[a]	1.41 ± 0.06 [a]
	Epicatechin	11.10 ± 0.13[a]	n.d.	n.d.	n.d.
	Chrysoeriol	0.36 ± 0.10 [b]	0.34 ± 0.02[b]	n.d.	0.71 ± 0.07[a]
	Isorhamnetin	8.19 ± 0.09[a]	8.00 ± 0.12[a]	8.05 ± 0.05 [a]	7.98 ± 0.01 [a]
	Vitexin	22.88 ± 0.06[c]	2.15 ± 0.01[b]	0.22 ± 0.01 [a]	0.33 ± 0.03[a]
	Apigenin-7-*O*-β-glucoside	11.67 ± 0.14[d]	1.51 ± 0.09[c]	0.49 ± 0.07[b]	0.34 ± 0.11[a]
	Luteolin-7-*O*-β-glucoside	48.00 ± 0.00[b]	3.17 ± 0.12[a]	n.d.	n.d.
	Kaempherol-3-*O*-glucoside	9.41 ± 0.07[c]	1.62 ± 0.24[b]	1.16 ± 0.03[b]	0.97 ± 0.10[a]
	Hyperoside	10.17 ± 0.90[b]	1.32 ± 1.28[a]	n.d.	n.d.
	Quercetin-3-*O*-glucoside	29.00 ± 1.35[b]	3.38 ± 2.43[a]	n.d.	n.d.
	Amentoflavone	1.02 ± 0.02[b]	0.28 ± 1.44[a]	0.13 ± 0.09[a]	n.d.
	Apigenin	n.d.	n.d.	n.d.	0.37 ± 1.31[a]
	Rutin	284.63 ± 1.07[b]	28.30 ± 0.08[C]	n.d.	n.d.
	Baicalein	n.d.	n.d.	n.d.	n.d.
	Catechin	n.d.	n.d.	n.d.	n.d.
	Quercetin	n.d.	n.d.	n.d.	n.d.
	Myricetin	n.d.	n.d.	n.d.	n.d.
	Baicalin	n.d.	n.d.	n.d.	n.d.
	Quercitrin	n.d.	n.d.	n.d.	n.d.
	Liquiritigenin	1766.33 ± 1.17 [c]	124.08 ± 2.13 [b]	13.10 ± 0.07[a]	12.40 ± 0.09[a]
	Isoliquiritigenin	1013.02 ± 1.93 [c]	219.14 ± 0.07 [b]	16.70 ± 0.10 [a]	16.60 ± 0.08[a]
Isoflavonoids	Daidzein	86.10 ± 1.02[b]	7.75 ± 0.2[a]	n.d.	n.d.
	Genistein	577.01 ± 5.2 [d]	407.12 ± 4.04 [c]	364.34 ± 7.30 [b]	324.05 ± 8.0 8 [a]
	Formononetin	1081.11 ± 4.58 [c]	112.24 ± 5.03 [b]	14.20 ± 0.05 [a]	13.80 ± 0.05[a]
	Equol	n.d.	n.d.	n.d.	n.d.
	Glicitein	114.09 ± 1.53[c]	13.20 ± 0.05[b]	0.52 ± 0.02 [a]	0.21 ± 0.06[a]
	Calycosin	337.54 ± 2.34 [c]	115.03 ± 1.56 [b]	11.30 ± 1.01 [a]	11.02 ± 0.09[a]
	Biochanin A	1013.02 ± 2.51 [c]	219.45 ± 0.08 [b]	16.70 ± 0.07 [a]	16.60 ± 0.10 [a]
	Daidzin	324.25 ± 3.00 [c]	200.10 ± 1.00 [b]	17.20 ± 1.05 [a]	15.60 ± 0.75[a]
	Glicitin	n.d.	n.d.	n.d	n.d.
Lignans	Enterolacton	n.d.	n.d.	n.d.	n.d.
	Pinoresinol	120.15 ± 0.05 [b]	117.12 ± 0.03 [b]	53.01 ± 0.02 [a]	n.d.
Triterpenoids	18- β Glycyrrhetic acid	2049.05 ± 1.17 [a]	n.d.	n.d	n.d.
	Glycyrrhizin	n.d.	n.d.	n.d.	n.d.
Coumarine	Aesculetin	1.230.08 [b]	0.58 ± 0.01 [a]	n.d.	n.d.
	Scopoletin	0.83 ± 0.09[c]	0.38 ± 0.01 [b]	n.d.	0.12 ± 0.0 7 [a]
	Umbelliferon	n.d.	n.d.	n.d.	n.d.

[§] Values are means ± SD of three measurements; a,b,c,d,e means in the same row not sharing the same superscript are significantly different ($p < 0.01$); dw-dry weight.

Table 3
TPC and TFC.

Extracts	L1	L2	L3	L4
Total phenolics (mg GAE/g of dw)	37.27 ± 0.55 [a]	31 ± 0.27[b]	16.5 ± 0.78[c]	13.23 ± 0.36[d]
Total flavonoids (mg QE/g of dw)	5.90 ± 0.05 [a]	3.83 ± 0.17 [b]	3.68 ± 0.23[c]	2.69 ± 0.11[d]

Data are means ± SD of three measurements; a,b,c,d,e means in the same row not sharing the same superscript are significantly different ($p < 0.05$); dw-dry weight; GAE-gallic acid equivalent; QE- quercetin equivalents

and other bioactive compounds. Considering that, the number of identified and quantified compounds in fresh and dry root and leaf extracts of *G. glabra* from Fruska Gora, Serbia (**L1**-**L4**, respectively) has been expanded in the present work (Table 2).

The results indicate that the major bioactive compound in *G. glabra* extracts was 18-β glycyrrhetic acid, detected only in the fresh licorice root extract, sample **L1** (2049.05 μg/g). The presence of this compound was noticed before in most licorice extracts; it showed anticancer

Table 4
Antioxidant potential of *G. glabra* extracts.

	Extracts				Standards	
	L1	L2	L3	L4	BHA	BHT
DPPH·	11.5 ± 0.34 [c]	46.17 ± 0.53 [d]	58.93 ± 0.7 [e]	206.52 ± 0.60 [f]	9.64 ± 0.04[b]	8.23 ± 0.28[a]
HO·	315.27 ± 0.17 [c]	415.79 ± 0.04 [d]	616.14 ± 0.0 [e]	715.77 ± 0.23 [f]	117.17 ± 0.03 [a]	120.49 ± 1.82[b]
$O_2^{\cdot -}$	37.23 ± 0.10 [c]	44.51 ± 0.18 [d]	48.87 ± 0.5 [e]	84.33 ± 0.16 [f]	35.23 ± 0.09 [b]	23.55 ± 0.18[a]
NO·	36.37 ± 0.25 [c]	57.46 ± 0.3[d]	502.72 ± 0.0 [e]	775.2 ± 0.65 [f]	23.52 ± 0.21 [a]	28.12 ± 0.1[b]
Reducing power (mg of ascorbic acid equivalents (AAE)/g of dw)						
FRAP	55 ± 1.20	38,48 ± 0.42	5.17 ± 0.9	2.66 ± 0.77	107.18 ± 0.30	145.23 ± 0.30

Data are means ± SD of three measurements. a,b,c,d,e means in the same row not sharing the same superscript are significantly different($p < 0.05$); dw-dry weight

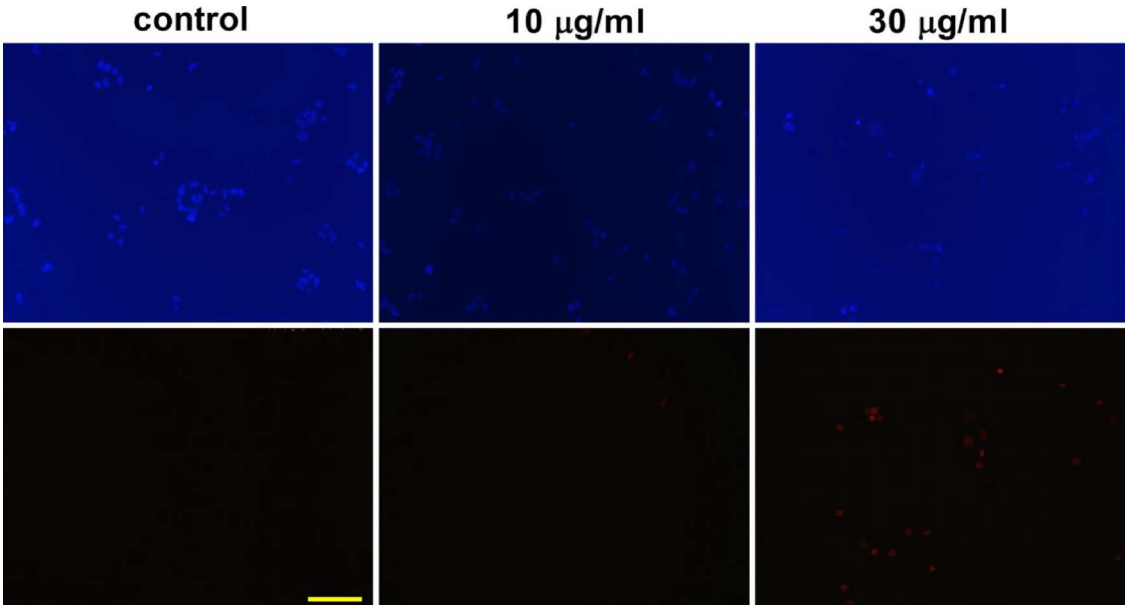

Fig. 1. Fluorescent microscopic images of MDA-MB-361 cells treated with **L1** extract for 24 h. Two separate pictures from the same field were taken for the two fluorescent markers. Blue fluorescence (upper panels) relates to HO while red coloration (lower panels) reflects PI accumulation. The bar in the PI control panel indicates 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

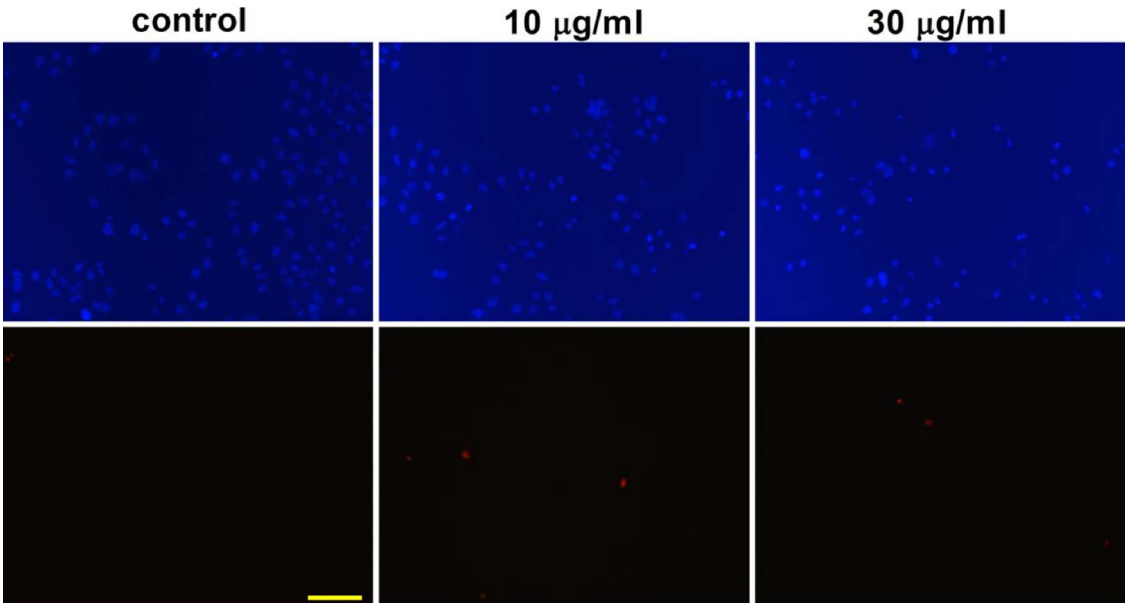

Fig. 2. Fluorescent microscopic images of SiHa cells treated with **L1** extract for 24 h. The photos were taken in the same way as in the case of Fig. 1. Blue fluorescence (upper panels) relates to HO while red coloration (lower panels) reflects PI accumulation. The bar in the PI control panel indicates 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

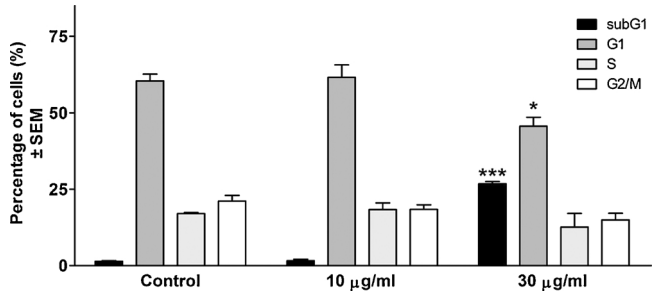

Fig. 3. The effect of **L1** extract on the cell cycle distribution of SiHa cells after 24 h of incubation. * and *** indicate $p < 0.05$ and $p < 0.001$ as compared with the control cells, respectively.

activity in several *in vitro* and *in vivo* cancer chemopreventive models (Wang and Yang, 2007; Yang et al., 2015). Other biologically active compounds that were detected and quantified in high amount belong to the class of phenolic compounds and acting as phytoestrogens. Flavonon liquiritigenin and chalcone-type flavonoid isoliquiritigenin are the most abundant flavonoids in licorice extracts. Liquiritigenin was found as highly selective agonist of β-estrogen receptor, while isoliquiritigenin has a potential as cancer chemopreventive agent (Cheel et al., 2010). The greatest level of these bioactive compounds was noticed in sample **L1**, namely extract of fresh licorice root (1766.33 μg/g and 1013.02 μg/g, respectively). Both isoliquiritigenin and liquiritigenin were identified in earlier studies of licorice originating from other regions, but the content of these compounds was lower than that in the present study (Liao et al., 2012a,b; Wang and Yang, 2007; Zhang and Ye, 2009; Zheng et al., 2014). The presence of the best-known health benefit flavonoids such as rutin, quercetin, lutein, naringenin, kaempherol-7- *O*-glucoside in licorice was detected in earlier reports, but was not quantified (Siracusa et al., 2011; Zhang and Ye, 2009). In this research these compounds were quantified in the root extract samples **L1** and **L2**, in significantly higher amount than in leaf extract samples **L3** and **L4**.

Further, isoflavonoids as a widespread and the most abundant group of phytoestrogens, play an important role in human nutrition due to their valuable health benefits. Presence of formononetin in licorice root was reported in earlier studies (Chin et al., 2007). The main isoflavonoids presented in the samples studied here were formononetin and biochanin A, followed by genistein. These compounds were noticed as dominant in fresh root of *G. glabra*, **L1** (1081.11, 1013.54, 577.01 μg/g dw, respectively). O-methylated isoflavonoid calycosin and glucoside of daidzein, daidzin, were found in similar concentration and were also most abundant in the sample **L1**. These isoflavonoids were reported to possess primarily phytoestrogenic activity, but also antioxidant, anti-inflammatory and anticancer activity (Miadoková, 2009). They exert their anti-estrogen activity through binding to estrogen receptors α and β (ER α and ER β) (Choi and Kim, 2014; Dixon, 2004; Dixon and Ferreira, 2002; Pilsková et al., 2010; Tang et al., 2010).

Phenolic acids were also observed in high level, where the antioxidant, quinic acid, found in the highest amount in the sample **L1** (730.89 μg/g) followed by *p*-coumaric acid (73.82 μg/g) and *p*-hydroxybenzoic acid (52.89 μg/g). The rest phenolic acids were found in quite lower amount ranged from 0.35–22.95 μg/g dw.

Unlike the other detected compounds, the lignan pinoresinol, was observed in the extracts of fresh and dry root (53.01–120.15 μg/g) and fresh leaves in very similar concentration, which was not found in previous reports. Pinoresinol is recognized as strong anti-inflammatory agent in colon cell lines (During et al., 2012). It also affects the estrogen receptor in MCF-7 cells, that contribute to its estrogen activity (Hu et al., 2009).

Coumarines scopoletin and umbelliferon were detected in very low amount in all licorice extract samples. These results were in accordance with previous report (Cruz-Vega et al., 2009).

Based on the results presented in Table 2, it can be concluded that the most bioactive compounds, in the highest amounts, are found in the extract of *G. glabra* fresh root, **L1**. It could be explained by the fact that drying process can change bioactive compounds content and therefore biological activities of the extracts. Thus, drying of the plant material may lead to enzymatic degradation of secondary metabolites and this could result in the decrease of their content. The identified compounds could contribute prevention and treatment of many pathophysiological conditions. Previous studies indicated that only root of licorice contains a great content of bioactive compounds (Basar et al., 2015; Boonmuen et al., 2016; Cruz-Vega et al., 2009; Dai and Mumper, 2010; Khoddami et al., 2013; Martins et al., 2015; Zheng et al., 2014). In our study these compounds were identified and quantified also in fresh and dry leaves, though in much lower content. This part of plant could also be considered as a potential phytoremedy.

3.2. TPC, TFC and antioxidant activity

Phenolic compounds express antioxidant and/or radical scavenging ability, thanks to electron-donating phenol groups, stabilizing radical form. In the licorice extracts, we evaluated content of total phenolic compounds (TPC) and content of total flavonoid compounds (TFC) (Table 3). The greatest content of TPC in *G. glabra* extracts from Fruska Gora is noticed in the extract of the fresh root **L1** (37.27 ± 0.55 mg GA

Table 5
The antiproliferative action of the tested extracts.

Cell line	Conc. (μg/mL)	Growth inhibition (%) ± SEM			
		L1	**L2**	**L3**	**L4**
HeLa	10	–*	–	–	–
	30	24.13 ± 0.84	–	–	–
SiHa	10	–	22.96 ± 2.67	–	–
	30	74.87 ± 1.26	39.54 ± 1.82	–	19.94 ± 2.56
MCF7	10	–	–	–	–
	30	56.67 ± 1.29	–	–	–
T47D	10	22.81 ± 1.84	13.61 ± 2.04	–	13.07 ± 2.90
	30	54.35 ± 1.03	36.13 ± 0.88	19.18 ± 2.97	28.87 ± 1.17
MDA-MB-231	10	–	–	–	12.61 ± 2.08
	30	64.26 ± 1.77	13.65 ± 1.87	–	13.80 ± 2.74
MDA-MB-361	10	15.42 ± 1.66	–	–	–
	30	85.36 ± 0.63	14.17 ± 2.78	16.91 ± 2.39	14.01 ± 2.80
A2780	10	–	–	–	–
	30	63.42 ± 1.78	–	–	–

*: Extracts exhibiting growth inhibition lower that 10% are considered ineffective and their data are not given numerically for clarity.

eq/g of dw). The same extract was the richest in flavonoids as well (5.90 ± 0.05 mg QE eq/g of dw).

Some studies reported moderate to high capacity for neutralization of DPPH, NO, OH and $O_2\cdot^-$ radicals of dry root extracts of *G. glabra* from different localities, compared to synthetic antioxidants (Saraf et al., 2013; Siracusa et al., 2011; Sultana et al., 2010; Visavadiya and Narasimhacharya, 2006). In order to determine antioxidant potential and/or scavenging capacity of *G. glabra* roots and leaves extracts under study, several tests were performed: DPPH, NO, OH and $O_2\cdot^-$ and FRAP assay. The obtained results, are shown in Table 4. It can be noticed that the extracts of *G. glabra* roots (fresh and dry) exhibited markedly higher antioxidant potential than that of *G. glabra* leaves extracts. Antioxidant activity of tested samples was comparable with synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Extract of fresh *G. glabra* root (**L1**) showed the strongest scavenging capacity towards all radicals, while the most significant was the neutralization of DPPH radical (IC_{50} 11.50 μg/mL) and $O_2\cdot^-$ (IC_{50} 37.23 ± 0.10 μg/mL) radicals, followed by, NO (IC_{50} 36.37 ± 0.25 μg/mL). The obtained results indicate that the root of *G. glabra* from Fruska Gora possesses strong antioxidative potential, thanks to its high content of phenolic compounds, and could be used as a great source of various antioxidants in reducing oxidative damage in human body.

3.3. Antiproliferative activities of the tested extracts

The antiproliferative action of the prepared extracts were determined by means of MTT assay on a panel of human adherent cell lines of gynecological origin, containing four breast (T47D, MCF7, MDA-MB-231 and MDA-MB-361), two cervical (HeLa and SiHa) and one ovarian cancer cell line (A2780). It was found that **L1** fresh root licorice extract exhibited a substantial action (> 50% growth inhibition) at 30 μg/mL against all of the treated cell lines, with exception of HeLa cells, while other extracts exerted a modest action or were ineffective (Table 4). Based on these data, **L1** extract was selected for additional investigations in order to characterize its action on the treated cancer cells.

The pronounced cell growth inhibitory property of **L1** extract against MDA-MB-361 and SiHa cells justified further *in vitro* investigations including morphological studies. Therefore, fluorescent staining with Hoechst 33258 (HO) and propidium iodide (PI) dyes was performed after 24 h of treatment in order to obtain data concerning the possible mode of the antiproliferative action (Figs. 1 and 2).

The nuclei of control MDA-MB-361 or SiHa cells were homogenously stained with HO dye, with no PI uptake, indicating no changes in chromatin as well as intact membrane function. On the other hand, after 24 h treatment of estrogen-receptor positive (ER+) human breast cancer MDA-MB-361 cell line with 10 or 30 μg/mL of the extract **L1**, a small number of the nuclei were more intensively stained by HO dye, indicating low rate of chromatin condensation and, accordingly, apoptosis in a low rate. Further, intensive PI uptake, evidenced by reddish fluorescence, especially with higher dose of the extract, is indicating the deterioration of the membrane function in treated MDA-MB-361 cells, which is characteristic feature of necrosis or secondary necrosis. This finding can be explained by a high necrotic potential of the extract against this cell line (Fig. 1).

After the same treatment of SiHa cervical cancer cells with 10 or 30 μg/mL of the extract **L1**, a portion of the nuclei was more intensively stained, indicating the condensation of chromatin, especially in case of higher dose treatment (Fig. 2). Accordingly, conclusion is that the extract induced apoptosis of the treated cells. PI uptake was detected at very low level.

Since the presence of apoptosis is a crucial feature of almost all antiproliferative or cancer preventive substances, the proapoptotic property of the most effective extract (**L1**) has been confirmed by means of cell cycle analysis in SiHa cells after labeling the cellular DNA with PI (Fig. 3). The licorice fresh root extract **L1** after 24 h treatment of SiHa cervical cancer cells exerted no substantial effect at 10 μg/mL. The treatment with a higher concentration (30 μg/mL) of **L1** resulted in a substantial and significant increase of hypodiploid (subG1) population on the expense of cell in G1 phase. The accumulation of the subG1 population is generally considered as a consequence of apoptotic self-decomposition and therefore a marker of the programmed cell death. Accordingly, these findings are in agreement and are confirming results from double staining test.

Based on presented results concerning cell lines tests, it can be summarized that fresh licorice root extract **L1** has a pronounced antiproliferative action against a broad range of cancer cell lines of gynecological origin. Induction of apoptosis or necrosis was evidenced in different cell lines as a component of this growth inhibitory action, indicating that the plant extract could be considered as a potential source of novel anticancer lead molecules and/or agent for phytomedical treatment of malignances (Table 5).

4. Conclusion

Biological potential of this plant material, licorice from Fruska Gora mountain, is connected to and based on its phytochemical properties, especially concerning the most active licorice part – fresh root, rich in phenolic compounds liquiritigenin, isoliquiritigenin, biochanin A, formononetin, quinic acid and others, and triterpenoid 18-β glycyrrhetic acid, all confirmed as biologically and pharmacologically active compounds. The obtained results indicate that fresh root of the plant could be considered as potentialphytoremedy, specially very useful in treatment of problems and diseases of female reproductive tissues.

Conflict of interest

The authors declare that they have no conflict of interest.

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