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Phenolics of Aerial Parts of *Gentiana lutea* L. and Their Biological Activity

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Abstract: *Gentiana lutea* L. is a medicinal plant the roots of which are primarily used in treatments of various human digestive disorders, but also the production of alcoholic liquors. The roots (radix) of *G. lutea* are described in the European Pharmacopeia, but knowledge about the chemical composition and biological activities of its aerial parts is still limited. Thus, until today aerial parts of this species have not been used in medical treatments or consumed. Therefore, flowers, leaves, and stems extracts of *G. lutea* obtained by using four different extraction solvents (petrol ether, chloroform, ethanol, and water) were examined for their chemical composition and biological activities. High concentrations of salicylic acid, apigenin, and naringenin were recorded for ethanol stem extracts, while significant amounts of kaempferol were detected in leaves and flowers in chloroform and water extracts, respectively. The highest antioxidant potential was recorded for flower and stem petrol ether extracts with the lowest IC₅₀ values, ranging from 94.46 ± 9.45 to 105.38 ± 10.54 µg/mL. Ethanol extracts of flowers and stems showed moderate antioxidant activity (IC₅₀ 143.15 ± 14.32 and 146.90 ± 14.69 µg/mL) as well as strong antimicrobial activity against *Candida albicans* (21.00 ± 1.00 and 27.50 ± 1.78 mm inhibition zones, respectively). In addition, ethanol extracts had higher antimycotic activity compared to naturally occurring phenolic compounds that are used as positive controls. Moreover, statistical analysis of the activities of plant extracts and single compounds showed that levels of chlorogenic and caffeic acids strongly correlate with the biological activities of the extracts, i.e., they are the main carriers of these biological activities. The presented results indicate the possible use of aerial parts of *G. lutea* as a natural preservative, as well as a antimicrobial agent, which significantly amplifies the benefits of this medicinal crop and greatly affects the sustainability of cultivated *Gentiana* plantation.

Keywords: aerial parts; antimicrobial activity; antioxidant activity; chemical composition; *Gentiana lutea* L.

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1. Introduction

Members of the family Gentianaceae, which comprises more than 400 species, can be found from central and southern Europe, America, Australia, and New Zealand up to 2500 m of altitude. They are widely cultivated in China, continental and northern Europe, as well as in the Balkans [1]. Yellow gentian (*Gentiana lutea*) is an herbaceous perennial plant that grows in mountainous regions, on meadows and open slopes of the Eastern part of the Alps, and in the Balkan Peninsula [2,3].

The root of yellow gentian (Gentian root; *Gentianae radix*) is an official drug of European pharmacopeia [4], containing bitter active principles, and is primarily used in treatments of various human digestive disorders. Large amounts of the plant are consumed by the industry of alcoholic drinks for liquors and bitters [5]. It is noted that gentian extracts can stimulate neuritogenesis, without cytotoxic activity [6]. Petrol ether and alcohol extracts have been tested on rats as an oral medication for carrageenan-induced rat paw oedema, xylol-induced mouse ear oedema, and cotton pellet-induced chronic inflammatory models as inflammatory remedies [7]. The choleric activity was recorded for ethanol root extracts, with significant hepatoprotective activity [8]. Active components of gentian root extracts are mainly secoiridoid bitter compounds. Root extracts exhibit remarkable antioxidant activity estimated by DPPH scavenging assay and Trolox equivalent antioxidant capacity [9]. Also, an inhibitory effect on the enzyme myeloperoxidase was recorded [10]. The antimicrobial activity of leaves and flowers was also recorded against Gram-positive and Gram-negative bacteria and yeast *Candida albicans* [11]. Extensive research of bioactive properties of *Gentiana lutea* extracts shows several additional effects such as anti-tubercular activity [12], radio-protective activity [13], CNS stimulation [14], anti-atherosclerotic effects [15], gastroprotective effects [16], and as ingredient in traditional remedies for atherosclerosis [15].

With the evidence for bioactive properties of gentian roots and some indications that above-ground parts can be used as a replacement for the radix in other *Gentiana* species [17], such as *G. punctata* and *G. hederacea* [18], this study aimed to evaluate the effect of the different solvents of extraction efficiency, adequate plant organ for extraction, and bioactive properties of obtained extracts.

2. Materials and Methods

2.1. Collection of Samples and Extract Preparation

Inflorescence samples of *Gentiana lutea* were collected at Mt. Bjelašnica in Bosnia and Herzegovina (Coordinates: 43.709376° latitude; 18.578821° longitude), during the flowering stage in August 2019. Specimens for Voucher were deposited under no. 374. Flowers, leaves, and stems were separated and air-dried for 7 days at room temperature (23 °C) in a shaded, well-ventilated room. Dried samples were powdered in the mill and stored at +4 °C until use.

For each sample, 500 mg of powdered plant material was soaked in 12.5 mL of solvent (petrol ether, chloroform, ethanol, and water) and sonicated for 30 min at 23 °C. The supernatant was removed after centrifugation (5000 rpm, 15 min) and sediment was again soaked into the same solvent. Supernatants were combined for further analysis. Due to the high evaporation rate of petrol ether and chloroform, these extracts were evaporated to dryness and resuspended in dimethyl sulfoxide (DMSO).

2.2. UHPLC-MS/MS Analysis

UHPLC-MS/MS analysis of the extracts was performed on Nexera X2 UHPLC (Shimadzu Handels GmbH, Kyoto, Japan) coupled with an MS-8050 (Shimadzu Handels GmbH, Kyoto, Japan). Chromatographic separation was performed on an UHPLC Acquity BEH C18 (150 × 3.0 mm; 1.7 µm particle size) column (Waters Corp., Milford, MA, USA) with the temperature set at 40 °C. The mobile phase contained 10 mmol/L formic acid in water (A) and acetonitrile (B). All 32 target compounds were separated using a binary gradient starting at 5% B for 3 min, increasing to 25% B for 4 min, then increasing to 30% B for 6 min, then increasing to 35% B for 4 min, then increasing to 60% B for 6 min, then increasing to 100% B for 4 min, and isocratic run for 1.5 min, and then back to 5% B for 0.1 min, and equilibration for 3.4 min. The flow rate was 0.4 mL/min and the injection volume 10 µL.

All analytes were detected in negative ionization mode ESI. Multiple reaction monitoring (MRM) mode was used for their quantification. The spray voltage was 3 kV, and the vaporizer the ion transfer tube temperatures were 320 °C.

Standard solutions of compounds (apigenin, 2,3-dihydroxybenzoic acid, caffeic acid, catechin, chlorogenic acid, chrysin, ferulic acid, galangin, gallic acid, hesperidin, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 5-hydroxyferulic acid, kaempferol, methyl *p*-coumarate, morin, myricetin, naringenin, naringin, *p*-coumaric acid, pinocembrin, quercetin, quercitrin, rosmarinic acid, rutin, salicylic acid, salicylic acid 2-O- β -D-glucoside, sinapic acid, syringic acid, *trans*-cinnamic acid, and vanillic acid), purchased from Sigma Aldrich Company, Germany, were firstly prepared in methanol at 1 mM concentrations, and solutions were gradually diluted in the mobile phase to the working concentrations that ranged from 0.01 to 50 μ M. Quantification was performed by the isotope diluting method using *p*-coumaric acid- d_6 and salicylic acid- d_4 .

2.3. GC/MS Analysis

Volatile compounds were analyzed by GC/MS using a Hewlett-Packard GC/MS system (GC 7890 A; MSD 5975C series II, Palo Alto, CA, USA). For the GC conditions we used a fused silica HP-5MS UI column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m), carrier gas He (1.1 mL/min), with temperature program 3 $^{\circ}$ C/min from 60 $^{\circ}$ C to 240 $^{\circ}$ C; the temperature of the injection port was 250 $^{\circ}$ C and detector 280 $^{\circ}$ C. Ionization was performed in the EI mode (70 eV). The linear retention indices (RI) were determined by injection of the sample of C₈–C₂₆ n-alkanes. Compound identification was accomplished by comparing retention indices and mass spectra with literature data [19], by a computer library search (HP Chemstation computer library NBS75K.L, NIST/EPA/NIH Mass Spectral Library 2.0 and Mass Finder 4 Computer Software and Terpenoids Library), and in the laboratory's own database.

2.4. DPPH (2,2-Diphenyl-1-picrylhydrazyl Radical) Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl radical) antioxidant capacity was evaluated for all four extracts and standards according to Meda et al. [20]. Antioxidant potential was evaluated according to the absorbance change of the free DPPH radical that is reduced by the potent antioxidant. Each extract (compound) was evaluated at 4 different concentration levels, and its antioxidant potential is presented as IC₅₀ value (concentration of extract that scavenges 50% of DPPH radicals). Single phenolic compounds, i.e., morin, rosmarinic acid, chlorogenic acid, ferulic acid, coumaric acid, salicylic acid, 4-hydroxybenzoic acid, caffeic acid, quercetin, naringenin, and caffeic acid were used as positive controls.

2.5. Antimicrobial Assay

The agar well diffusion method was used to evaluate the antimicrobial activity of plant extracts and standards according to the National Committee for Clinical Laboratory Standards [21]. Each well contained 100 μ L of extract (0.1 mg/mL). Bacterial strains used in the analysis included Gram-positive: *Enterococcus faecalis* ATCC[®] 19433TM, *Staphylococcus aureus* subsp. *aureus* ATCC[®] 6538TM and Gram-negative bacteria: *Salmonella abony* NCTC[®] 6017TM, *Escherichia coli* ATCC[®] 8739TM, and the yeast *Candida albicans* ATCC[®] 10231TM. Bacterial strains were used as standardized inoculum of 5×10^5 CFU/mL using McFarland standard.

Müller-Hinton and Sabouard media were used for the cultivation of bacterial strains and yeast, respectively. Ampicillin was used as positive standards for bacterial strains and nystatin for *Candida albicans*. Ethanol and DMSO were used as negative controls. Antimicrobial activity of morin, rosmarinic acid, chlorogenic acid, ferulic acid, coumaric acid, salicylic acid, 4-hydroxybenzoic acid, caffeic acid, quercetin, naringenin, and caffeic acid in the concentration of 0.1 mg/mL was also evaluated. Antimicrobial effect was expressed as a diameter of inhibition zone in mm reduced by the inhibition zone of negative controls (solvents) if appropriate.

2.6. Statistical Analysis

All data were analyzed using the STATISTICA 10.0 software (Statsoft Inc., Tulsa, OK, USA). Experimental results were presented in tables and graphs as the mean $\pm$ standard deviation of three independent replications. Data obtained were subjected to variance analysis (ANOVA) and the Newman–Keuls post hoc test was carried out to identify significant differences between extract types. Mean values with $p < 0.01$ were considered statistically significant. Pearson correlations were performed to observe the possible correlation between the phenolic profile, antioxidant capacity, and detected antimicrobial activity at the level of significance $p < 0.01$.

3. Results

The phenolic composition of different extracts of flowers, leaves, and stems are reported in Table 1. A high concentration of phenolic acids was recorded in water extracts for flowers, with caffeic acid as the most abundant ($84.74 \pm 7.86 \mu\text{mol/g}$).

Significant amounts of this acid were also found in less polar extracts of flowers. Chlorogenic and 4-hydroxybenzoic acids were also found in significant amounts in all examined extracts. In general, flowers contain the highest amounts of these acids, while leaves contain the least.

Comparison of identified flavonoid components in different plant parts and different solvents showed a significant content of hesperidin in flower ($190.05 \pm 0.136 \mu\text{mol/g}$), and naringenin in stem water extracts ($164.91 \pm 1.36 \mu\text{mol/g}$), (Table 1). Considerable amounts of naringenin were recorded for stems and leaves ethanolic extracts (102.61 ± 0.72 and $91.00 \pm 6.99 \mu\text{mol/g}$, respectively), while high levels of apigenin were recorded for flowers ($166.5 \pm 1.19 \mu\text{mol/g}$) and stems ($158.13 \pm 20.79 \mu\text{mol/g}$) ethanol extract and in stems in chloroform extract ($114.90 \pm 3.61 \mu\text{mol/g}$).

GC/MS analysis of terpenoids indicated the presence of phytol in a higher percentage in leaves (28.60%) and higher alkanes, *n*-heneicosane (0.92–1.29%), *n*-docosane (2.78–3.84%) *n*-tricosane (10.57–16.75%), and in flowers and stems, respectively. Volatile terpenoids were not recorded in any of investigated plant extracts, which is in the agreement with the literature data [6,22,23]. However, these extracts were obtained from the roots of *G. lutea*, while the presented results apply for aerial parts.

The results of the antioxidant capacity of *G. lutea* extracts and single compounds found in the extracts are summarized in Tables 2 and 3. Total antioxidant capacity is expressed as IC_{50} value ($\mu\text{g/mL}$ concentration of the extract that inhibits 50% of DPPH radicals). Petrol ether flower and stem extracts showed the lowest IC_{50} values ($105.38 \pm 10.54 \mu\text{g/mL}$ and $94.46 \pm 9.45 \mu\text{g/mL}$, respectively) compared to other solvents used. The highest IC_{50} value was recorded for water leaves extract ($727.71 \pm 72.77 \mu\text{g/mL}$), which is in agreement with previously published data [24], where authors investigated the root extracts of yellow gentian.

Antioxidant activity of petrol ether extracts is strongly correlated to the presence of caffeic acid ($r = 0.999$), and hesperidin ($r = 0.999$), while for chloroform extracts correlation of antioxidant activity and presence of chlorogenic acid was recorded ($r = 0.998$), (Table 4).

The content of 4-hydroxybenzoic acid in ethanol extracts was highly correlated ($r = 0.999$) to the antioxidant capacity of these extracts. For water extracts antioxidant activity correlated to the content of chlorogenic ($r = 0.996$) and caffeic acid ($r = 0.995$). The antioxidant capacity of chlorogenic and caffeic acid, as well as hesperidin, was also evident (Table 4), which is in agreement with previously published results [24–26].

Table 1. Phenolic composition ($\mu\text{mol/g}$) of extracts of aerial parts of *Gentiana lutea*.

Compound	Petrol Ether			Chloroform			Ethanol			Water		
	F	L	S	F	L	S	F	L	S	F	L	S
GA	nd	nd	nd	0.16 $\pm$ 0.00 *	nd	nd	nd	nd	nd	nd	nd	nd
CGA	1.26 $\pm$ 0.02	9.32 $\pm$ 1.61	nd	16.24 ^c $\pm$ 1.79	12.32 ^d $\pm$ 0.04	0.63 ^f $\pm$ 0.01	21.62 ^b $\pm$ 0.32	18.99 ^{bc} $\pm$ 1.32	15.64 ^c $\pm$ 1.48	35.57 ^a $\pm$ 0.24	7.00 ^e $\pm$ 0.91	11.32 ^d $\pm$ 0.11
4HBA	9.70 ^c $\pm$ 0.69	18.94 ^b $\pm$ 1.57	nd	11.25 ^c $\pm$ 1.03	nd	12.87 ^c $\pm$ 1.46	22.25 ^b $\pm$ 0.41	nd	nd	22.90 ^b $\pm$ 0.53	54.55 ^a $\pm$ 7.71	50.11 ^a $\pm$ 2.36
CA	3.67 ^d $\pm$ 0.02	nd	nd	10.69 ^d $\pm$ 0.22	6.18 ^d $\pm$ 0.63	18.81 ^c $\pm$ 2.48	21.03 ^{bc} $\pm$ 1.25	8.95 ^d $\pm$ 0.16	26.98 ^b $\pm$ 3.83	84.74 ^a $\pm$ 7.86	14.89 ^c $\pm$ 0.42	26.77 ^b $\pm$ 1.41
3HBA	4.35 ^a $\pm$ 0.42	nd	nd	2.81 ^b $\pm$ 0.07	nd	nd	2.94 ^b $\pm$ 0.17	nd	nd	nd	nd	nd
pCA	13.30 ^c $\pm$ 0.77	36.58 ^a $\pm$ 0.06	nd	4.81 ^e $\pm$ 0.57	11.39 ^{cd} $\pm$ 1.19	5.73 ^e $\pm$ 0.98	8.04 ^d $\pm$ 0.07	9.42 ^d $\pm$ 0.40	20.78 ^b $\pm$ 0.13	14.35 ^c $\pm$ 0.78	nd	12.91 ^c $\pm$ 0.27
FA	18.48 ^c $\pm$ 1.23	nd	nd	23.83 ^{bc} $\pm$ 1.53	5.75 ^e $\pm$ 0.26	nd	27.05 ^{ab} $\pm$ 4.27	5.56 ^e $\pm$ 0.58	nd	32.73 ^a $\pm$ 4.23	4.60 ^e $\pm$ 0.33	13.95 ^d $\pm$ 0.91
RA	nd	nd	nd	nd	nd	nd	nd	nd	nd	55.27 ^a $\pm$ 2.98	35.34 ^b $\pm$ 0.92	36.28 ^b $\pm$ 0.29
SA	11.33 ^{fg} $\pm$ 0.72	14.95 ^{fg} $\pm$ 1.25	nd	23.94 ^{de} $\pm$ 2.69	27.36 ^d $\pm$ 0.31	10.77 ^g $\pm$ 1.75	73.14 ^a $\pm$ 0.7	62.30 ^b $\pm$ 6.41	54.18 ^c $\pm$ 5.30	21.01 ^{cd} $\pm$ 0.18	19.16 ^{ef} $\pm$ 0.16	14.49 ^{fg} $\pm$ 0.61
HSP	2.77 ^c $\pm$ 0.08	nd	nd	nd	nd	1.41 ^c $\pm$ 0.04	nd	18.79 ^b $\pm$ 0.96	nd	190.05 ^a $\pm$ 1.36	nd	nd
RUT	2.50 ^a $\pm$ 0.06	nd	nd	nd	nd	1.88 ^b $\pm$ 0.15	nd	nd	nd	nd	nd	nd
MOR	nd	nd	nd	nd	nd	85.17 $\pm$ 1.04	nd	nd	nd	nd	nd	nd
APG	nd	nd	nd	114.90 ^b $\pm$ 3.61	nd	nd	166.50 ^a $\pm$ 1.19	nd	158.13 ^a $\pm$ 20.79	0.76 ^d $\pm$ 0.04	nd	83.61 ^c $\pm$ 7.09
NRG	nd	nd	nd	13.84 ^e $\pm$ 0.58	nd	nd	31.64 ^d $\pm$ 1.07	91.00 ^c $\pm$ 6.99	102.61 ^b $\pm$ 0.72	1.80 ^f $\pm$ 0.06	164.91 ^a $\pm$ 1.03	nd
KMP	nd	nd	nd	nd	156.56 ^a $\pm$ 3.13	nd	nd	nd	nd	123.49 ^b $\pm$ 5.80	nd	nd

* Data represent means of three replicates ($\pm$ standard deviation). Values within one row followed by the same letter do not differ significantly after factorial analysis of variance (ANOVA) post hoc Newman–Keuls analysis at a significance level of $p < 0.01$; F—flowers; L—leaves; S—stems; GA—gallic acid; CGA—chlorogenic acid; 4HBA—4-hydroxybenzoic acid; CA—caffeic acid; 3HBA—3-hydroxybenzoic acid; pCA—p-coumaric acid; FA—ferulic acid; RA—rosmarinic acid; SA—salicylic acid; HSP—hesperidin; RUT—rutin; MOR—morin; APG—apigenin; NRG—naringenin; KPH—kaempferol; nd—not detected.

Table 2. Antioxidant and antimicrobial activities of extracts of aerial parts of *Gentiana lutea*.

Solvent	Plant Organ	Antioxidant Activity (IC ₅₀ µg/mL)	Antimicrobial Activity (Inhibition Zone mm)				
			<i>S. abony</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>S. aureus</i>	<i>C. albicans</i>
PE	F	105.38 ± 10.54 *	12.00 ^c ± 0.00	13.67 ^{bc} ± 1.15	14.00 ^d ± 0.00	12.33 ^e ± 0.58	16.00 ^g ± 0.00
	L	458.32 ± 45.83	nd	nd	nd	12.33 ^e ± 0.58	17.67 ^f ± 1.53
	S	94.46 ± 9.45	nd	13.67 ^{bc} ± 1.15	13.00 ^{ef} ± 0.00	12.67 ^e ± 0.58	17.33 ^f ± 0.58
CH	F	454.56 ± 45.46	13.00 ^{bc} ± 1.00	13.00 ^{bc} ± 1.00	15.67 ^d ± 1.53	16.33 ^{cd} ± 0.58	21.67 ^{cd} ± 2.89
	L	506.14 ± 50.61	13.33 ^{bc} ± 0.58	12.33 ^c ± 0.58	13.33 ^e ± 1.53	12.67 ^e ± 0.58	19.33 ^d ± 1.15
	S	254.26 ± 25.43	12.00 ^c ± 0.58	13.67 ^{bc} ± 0.00	14.33 ^e ± 0.58	13.00 ^e ± 1.29	20.00 ^d ± 1.78
ET	F	146.90 ± 14.69	14.00 ^b ± 1.00	15.67 ^a ± 1.15	16.00 ^d ± 1.00	17.00 ^c ± 2.65	21.00 ^{cd} ± 1.00
	L	455.40 ± 45.54	13.33 ^{bc} ± 0.58	14.00 ^{ab} ± 1.73	15.67 ^d ± 0.58	15.00 ^d ± 0.00	29.33 ^a ± 1.15
	S	143.15 ± 14.32	13.67 ^{bc} ± 0.58	nd	15.33 ^d ± 0.58	17.00 ^c ± 1.29	27.50 ^b ± 1.78
W	F	223.29 ± 22.33	nd	nd	nd	nd	nd
	L	727.71 ± 72.77	nd	nd	nd	nd	nd
	S	257.08 ± 25.71	nd	nd	nd	nd	nd

* Data represent means of three replicates (±standard deviation). Values within one row followed by the same letter do not differ significantly after factorial ANOVA post hoc Newman–Keuls analysis at a significance level of $p < 0.01$; PE—petrol ether; CH—chloroform; ET—ethanol; W—water; F—flowers; L—leaves; S—stems, nd—not detected.

Table 3. Antioxidant and antimicrobial activities of the phenolic constituents of *G. lutea*.

Compound	Antioxidant Activity (IC ₅₀ µg/mL)	Antimicrobial Activity (Inhibition Zone mm)				
		<i>S. abony</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>S. aureus</i>	<i>C. albicans</i>
4-Hydroxybenzoic acid	45.60 ± 0.41 *	12.33 ^{bc} ± 1.53	12.00 ^c ± 0.00	12.67 ^f ± 1.15	nd	18.67 ^{ef} ± 0.58
Salicylic acid	2.71 ± 0.04	13.50 ^{bc} ± 0.71	13.00 ^{bc} ± 1.73	13.00 ^{ef} ± 1.73	nd	25.33 ^b ± 0.58
<i>p</i> -Coumaric acid	17.76 ± 1.78	13.67 ^{bc} ± 0.58	11.33 ^c ± 0.58	13.00 ^{ef} ± 0.00	nd	17.00 ^f ± 0.00
Ferulic acid	7.36 ± 0.74	nd	14.67 ^{ab} ± 0.58	nd	nd	nd
Rosmarinic acid	5.47 ± 0.55	13.00 ^{bc} ± 0.00	13.33 ^{bc} ± 1.15	12.67 ^f ± 1.15	12.00 ^e ± 0.00	17.33 ^f ± 1.15
Chlorogenic acid	5.62 ± 0.56	13.00 ^{bc} ± 0.00	nd	nd	nd	14.33 ^h ± 0.58
Caffeic acid	28.55 ± 1.04	14.33 ^b ± 0.58	11.33 ^c ± 0.58	18.0 ^c ± 1.73	12.00 ^e ± 1.00	19.67 ^d ± 1.52
Morin	5.03 ± 0.50	nd	nd	15.67 ^d ± 1.53	13.67 ^e ± 0.58	18.00 ^{ed} ± 1.73
Naringenin	7.04 ± 0.18	nd	12.33 ^c ± 0.58	23.00 ^a ± 1.00	27.33 ^b ± 2.31	22.33 ^c ± 1.52
Ampicillin	-	17.00 ^a ± 1.41	14.00 ^{ab} ± 1.83	19.50 ^b ± 0.71	34.00 ^a ± 2.83	-
Nystatin	-	-	-	-	-	20.00 ^{cd} ± 1.41

* Data represent means of three replicates (±standard deviation). Values within one row followed by the same letter do not differ significantly after factorial ANOVA post hoc Newman–Keuls analysis at a significance level of $p < 0.01$.

Table 4. Correlations of the most abundant phenolic compounds and antibacterial and antioxidant activities of extracts of *G. lutea*.

Compound	Extract	Antibacterial Activity					Antioxidant Activity
		<i>S. abony</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>S. aureus</i>	<i>C. albicans</i>	DPPH
CGA	PE	0.992 *	0.388	0.446	−0.604	− 0.950	−0.363
	CH	0.999	− 0.961	−0.161	0.195	−0.001	0.998
	ET	−0.439	0.851	0.558	−0.829	0.141	0.835
	W	N/A	N/A	N/A	N/A	N/A	0.996
	PE	0.858	−0.014	0.049	−0.873	−0.746	0.040
4HBA	CH	−0.386	0.115	−0.848	− 0.980	− 0.922	−0.429
	ET	−0.866	0.413	0.000	− 1.000	0.670	0.999
	W	N/A	N/A	N/A	N/A	N/A	− 0.997
	PE	−0.500	− 1.000	−0.997	−0.500	0.654	0.999
	CH	0.151	0.128	0.951	0.998	0.988	0.198
CA	ET	−0.657	−0.813	− 0.981	−0.192	0.857	0.182
	W	N/A	N/A	N/A	N/A	N/A	0.995
	PE	0.687	−0.285	−0.223	−0.972	−0.537	0.310
	CH	0.905	−0.752	0.274	0.595	0.424	0.924
	ET	−0.569	0.763	0.427	− 0.904	0.288	0.908
SA	W	N/A	N/A	N/A	N/A	N/A	0.676
	PE	−0.500	−1.000	− 0.997	−0.500	0.654	0.999
	CH	− 0.970	0.866	−0.082	−0.427	−0.240	− 0.981
	ET	0.866	0.581	0.866	0.500	− 0.977	−0.490
	W	N/A	N/A	N/A	N/A	N/A	0.998

* Bolded correlations are significant at $p < 0.05000$, $N = 3$ (Casewise deletion of missing data); CGA—chlorogenic acid; 4HBA—4-hydroxybenzoic acid; CA—caffeic acid; SA—salicylic acid; HSP—hesperidin; PE—petrol ether extract; CH—chloroform extract; ET—ethanol extract; W—aqueous extract, N/A—not analyzed.

The results of the antimicrobial activity evaluation for *G. lutea* extracts are presented in Table 2. Leaves', flowers' and stems' petrol ether, chloroform and ethanol extracts inhibited the growth of all tested microorganisms depending on extract type. Most efficient were chloroform extracts exhibiting antimicrobial activity against all tested organisms; flower extracts having more prominent effects ranging from 13.00 ± 0.00 mm inhibition zones against *Salmonella abony* to 21.67 ± 2.89 mm for *Candida albicans*. All ethanol extracts showed a similar inhibitory effect against all tested microorganisms with higher efficiency against *Candida albicans* especially for leaves and stem extracts (29.33 ± 1.15 and 27.50 ± 1.78 mm inhibitory zone, respectively) compared to antimycotic nystatin (20.00 mm inhibitory zone). By contrast, water extracts showed no activity. The efficiency of the extract against bacteria is usually dependent upon the ability of the extract to act on the bacterial membrane by disrupting or increasing membrane permeability. It has been recorded that extracts containing acetone, ethanol, and methanol cause disruption and increase membrane permeability [27], while water alone cannot cause such damage to the bacterial membrane.

Positive correlations were recorded between the antimicrobial effect of chloroform extracts against *Staphylococcus aureus* and *Salmonella abony* and chlorogenic acid ($r = 0.999$), (Table 4).

4. Discussion

Gentian roots are widely used in traditional medicine creating great demand and in parallel raising concerns about species' extinction. In Europe, different levels of concern are raised in the context of local use and exploitation levels [28]. For most European soils, data suggest an unfavorable or inadequate level of conservation and further steps need to be taken. For this study, collection of the samples was undertaken following guidelines on how to handle plants with unfavorable/endangered conservation status [29], therefore, population size was not impacted.

A high concentration of active compounds in flowers was previously recorded for methanol extracts [30], where the concentration of isogenistin was 10 times higher in flowers than in leaves. The high content of naringenin and hesperidin has been previously reported for *Gentiana* bitter liquors and by-products [31], where the concentration of naringenin and hesperidin were higher in by-products compared to the liquor. The biological activity of naringenin and hesperidin has been previously recorded as antioxidants and chemopreventive agents.

Within biological systems different primary reactive oxygen species (superoxide radical) and secondary ROS (hydroxyl radical, hydrogen peroxide, and singlet oxygen) are generated through enzyme or metal-catalyzed processes. For this reason, assessment of extract capacity for scavenging superoxide radicals is important. Differences between reducing the power of extracts can be attributed to solvent nature, quantitative and qualitative phytochemical profiles of different plant parts, plant age, method, and system used through the experiment [10,24,32]. The most effective were flower and stem extracts for petrol ether, ethanol, and water extracts with the same efficiency in radical scavenging, while for chloroform extracts stem extracts were most efficient with the lowest IC_{50} value. Higher efficiency of leaves' extracts in *G. lutea* compared to root extracts was previously reported [32], where IC_{50} value of 7.2 mg/mL for leaves and 19.0 mg/mL for root extracts were recorded compared to the present study. Lowest IC_{50} value was recorded for petrol ether flower extracts (0.10 mg/mL). Pure compounds had significantly lower values of IC_{50} , compared to extracts, which is in agreement with the fact that synergism and concentration ratios of active compounds in plant extracts plays important roles in their biological activities [33].

The antimicrobial effect of this acid is probably the result of irreversible permeability changes in the cell membrane and due to the binding of the acid to DNA which results in inhibition of cellular functions. *Salmonella abony* is a Gram-negative bacteria, and the outer membrane of these bacteria consists of lipopolysaccharides (LPs) and proteins. Anionic substances can remove cations from the binding sites of LPs disrupting the membrane activ-

ity. It was suggested that chlorogenic acid, due to its negative surface charge, binds to the outer membrane disrupting the membrane and leading to the loss of barrier function [30], which is related to data presented in this study. However, plant extracts may contain hundreds or even thousands of components, and identifying the compounds responsible for a given biological effect represents a significant challenge. Some studies have shown that the overall activity of plant extracts can result from mixtures of compounds with synergistic, additive, or antagonistic activity, and it is very common that when extracts are fractionated activity is lost [33,34].

Nevertheless, among individual naturally occurring components analyzed, the compound with the widest spectrum of activity was found to be rosmarinic acid, while the most potent compound was salicylic acid against *C. albicans* (25.33 ± 0.58 mm inhibitory zone). The efficiency of ethanol extracts against *Candida albicans* could be attributed to the presence of higher amounts of salicylic acid, apigenin, and naringenin as suggested by the results obtained for naturally occurring compounds and the presence of these compounds in extracts with high antimicrobial properties (Table 2). This fact is confirmed by correlating their levels and antimicrobial activities (Table 3). The antimicrobial effect of salicylic acid against bacteria and yeast is related to the enhancement of the effect of other extract compounds as well through salicylic acid's ability to prevent the adherence of bacteria and yeast to surfaces [35]. A combination of higher concentrations of salicylic acid and apigenin could be responsible for the high efficiency of the ethanol extracts against *C. albicans*, since apigenin can induce cell shrinkage in *C. albicans* [36].

5. Conclusions

Evaluation of the bioactive potential of aerial parts of *Gentiana lutea* suggested that ethanol extracts are the most diverse in chemical compounds with strong biological activity. Flowers' and stems' ethanol extracts showed moderate antioxidant activity with strong antimicrobial activity against *Candida albicans*. Ethanol extracts had higher antimycotic activity compared to isolated compounds. The highest antioxidant potential was recorded for flower and stem petrol ether extracts with the lowest IC₅₀ values, while single compounds showed much lower IC₅₀ values compared to all extracts. To conclude, in addition to Gentian radix, aerial parts of this medicinal plant could be used in the pharmaceutical and food industries, as they are rich sources of natural preservatives and antimicrobial agents. Our results indicate the possible use of aerial parts of *G. lutea* as a replacement for the roots, which in return would have a great effect on the sustainability of cultivated *Gentiana* plantation.

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