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Determination and Comparison of Bioactive Compounds in Different Parts of *Glycyrrhiza* Species

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ABSTRACT
Glycyrrhiza spp., one of the most widely used herbal medicine for centuries in the world, contain a large number of bioactive such as triterpene saponins and flavonoids which are the main constituents and show broad biological activity. The present study aimed to evaluate the phytochemical profile of extracts from different parts (roots, stems and leaves) of all wild *Glycyrrhiza* spp. grown in Turkey and to reveal that other parts besides the roots are a rich source of bioactive compounds with potential use in the pharmaceutical and food industries. For this purpose, extracted bioactive compounds from different parts of five *Glycyrrhiza* spp. collected in different provinces of Turkey were determined and compared. The microwave-assisted extraction method, which is mentioned as a green technique and requires low solvent and extraction time, was applied. Determination of bioactives was performed using liquid chromatography- electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS). Among the collected *Glycyrrhiza* spp.; in leaf parts, the highest glycyrrhizin (GL) (2.05±0.07 mg g^{-1}) and glycyrrhetinic acid (GA) (0.107±0.005 mg g^{-1}) contents were found in *Glycyrrhiza flavescens* ssp. *flavescens*; the highest carbenoxolone (CBX) (0.133±0.006 mg g^{-1}) and liquiritin (LQ) (1.644±0.014mg g^{-1}) contents were in *Glycyrrhiza glabra* grown in Polatlı. In stem parts, the highest GL (2.735±0.04 mg g^{-1}), CBX (0.069±0.004 mg g^{-1}) and LQ (0.602±0.010 mg g^{-1}) contents were determined in *G. glabra* plant growing in Ankara. In root parts, the highest GL (14.68±0.09 mg g^{-1}) and LQ (9.735±0.046 mg g^{-1}) contents were detected in *G. glabra* plant growing in Gaziantep while the highest GA (0.136±0.005 mg g^{-1}) and CBX (0.188±0.067 mg g^{-1}) contents in *Glycyrrhiza flavescens* ssp. *antalyensis*. Thus, it was determined which location in Turkey and which parts of *Glycyrrhiza* spp. that grow wild in Turkey can be used as a priority for the food and pharmaceutical industry with this study.

Keywords: *Glycyrrhiza, microwave-assisted extraction, bioactive compounds, LC-ESI-MS/MS*

1. Introduction

The *Glycyrrhiza* genus consists of about 30 species, which are distributed worldwide and are mainly native to the Mediterranean countries, central to southern Russia, and certain regions of Asia (Messier et al. 2012), is a species of perennial plant belonging to the *Leguminosae* family (Russo et al. 2014). Licorice has been used commercially as a depigmentation agent in cosmetics and as a flavoring and sweetening agent in food products which has been classified as "generally recognized as safe" by the Food and Drug Administration (Jiang et al. 2016).

Licorice is one of the oldest and most popular herbal medicines in the world. More than 20 triterpenoids and 300 flavonoids, which possess several pharmacological properties including anti-inflammatory (Cheel et al. 2010), antispasmodic (Cho et al., 2010), expectorant (Kim et al. 2006), antiallergic (Liao et al. 2012), antidepressive (Zhang & Ye 2009), antiviral, antioxidative, antimicrobial, antidiabetic, antiasthma, and anticancer activities as well as immunomodulatory, gastroprotective, hepatoprotective, neuroprotective, and cardioprotective effects (Hosseinzadeh & Nassiri-Asl 2015), have been isolated from *Glycyrrhiza* spp. (Yang et al. 2015). In the flora of Turkey, the genus *Glycyrrhiza* is represented by six species (*Glycyrrhiza glabra*, *G. echinata*, *G. aspera*, *G. iconica*, *G. flavescens*, *G. asymmetrica*) three of which are endemic (*G. iconica*, *G. flavescens* ssp. *antalyensis*, *G. asymmetrica)* (Duran et al. 2012). But, *G. aspera* is no longer available as the area where the plant grows is permanently under water. The distribution of these species in Turkey is presented in Figure 1.

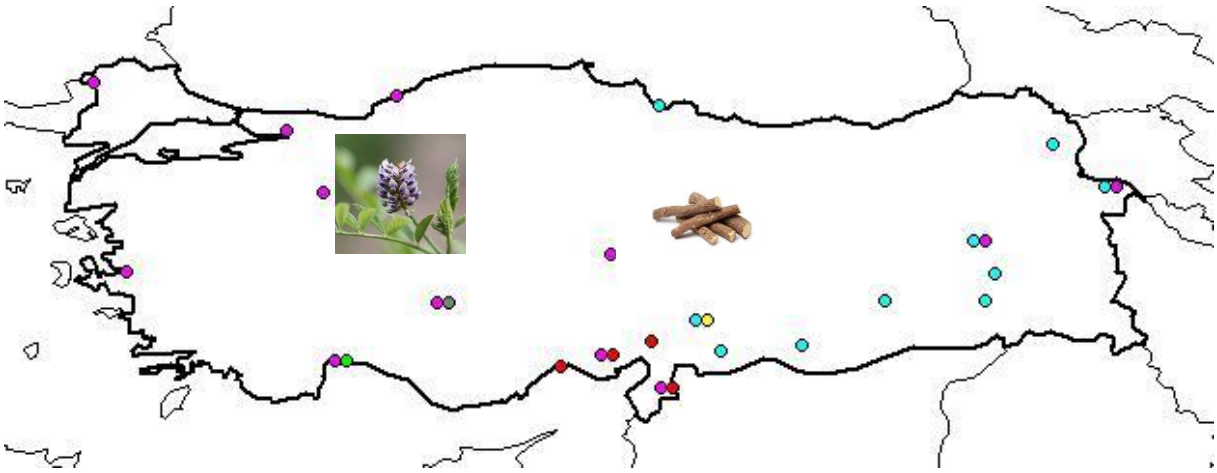

Figure 1- The distribution of *Glycyrrhiza* species in Turkey

Glycyrrhizin (GL), the most important constituent of licorice, is the saponin of the pentacyclic triterpene derivative of the oleanane type (Maatooq et al. 2010). It exhibits potent hydrocortisone-like anti-inflammatory, antiulcer, antiviral and antihepatotoxic activities (Cinatl et al. 2003; Sasaki et al. 2002). Glycyrrhetinic acid (GA), aglycone metabolite of GL, is a potent antibiotic against ulcer-causing *Helicobacter pylori* (Krausse et al. 2004). Also, GA and its derivatives have cytotoxic, anti-inflammatory, immuno-modulating, antitumor and apoptosis-inducing effects (Maatooq et al. 2010; Csuk et al. 2010). In addition, liquiritin (LQ), which is among the most important phenolic compounds, is mostly responsible for antioxidant and antitumor activities of licorice (Wang & Nixon 2001). Furthermore, carbenoxolone (CBX), a semi-synthetic compound derived from GL, has a wide scope of pharmacological activities. Although it possesses anti-inflammatory, antimicrobial and antiulcer properties at low doses, it induces adverse effects including cytotoxicity (Bharathala et al. 2021).

The present study aimed at the evaluation of the phytochemical profile of bioactive compounds in extracts from different parts (roots, stems and leaves) of five *Glycyrrhiza* spp. Thus, it was determined which locations and parts of all *Glycyrrhiza* spp. that grow wild in Turkey can be used as a priority for the food and pharmaceutical industry with this study.

To date, several methods have been developed for the quantification of bioactive in licorice, including high-performance thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) coupled with other techniques, capillary electrophoresis (CE) and gas chromatography (GC) in tandem with other methods. But it is the first time, the detection of the GL, GA, CBX and LQ contents in different parts of all species and varieties of *Glycyrrhiza* growing wild in Turkey via liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) was carried out at the same time.

2. Material and Methods

2.1. Plant materials and chemicals

Glycyrrhiza spp. were collected from different provinces of Turkey. Only *G. aspera* could not be collected because the area where the plant grows was permanently under water during the field studies. Identification and collection of the plant material were performed by botanist Dr. Ozlem CETIN. Voucher specimens were deposited at Gazi University Herbarium (GAZI), Ankara. The species, codes, collection dates, collected parts, locations and coordinates of licorice plants grown in Turkey were shown in Table 1. *Glycyrrhiza* spp. were air-dried before being analysed.

GL, GA, CBX and LQ were obtained from Sigma Aldrich (St. Louis, MO, United States). Methanol (for HPLC, ≥ 99.9%) was purchased from Merck (Darmstadt, Germany).

Table 1- The species, codes, collection dates, locations, coordinates and collected parts of licorice plants grown in Turkey

Plant	*Codes*	*Collection date*	*Location*	*Coordinates*	*Part*
Glycyrrhiza x iconica (endemic)	GXI	01/06/2010	B4 Konya: Sarayonu, between Sarayonu-Gozlu Farm, 992 m, field edge	38º 26' 51' 'N 32º 27' 58' 'E	Leaf, Stem, Root
G. echinata ssp. *echinata*	GEE	10/06/2011	C3 Konya: Seydisehir, Golyuzu village, 1100 m	37º 19' 57' 'N 31º 56' 19' 'E	Leaf, Stem, Root
G. echinata ssp. *macedonica*	GEM	01/06/2010	C2 Mugla: Dalyan, Iztuzu beach road	36º 49' 27.82' 'N 28º 38' 48.43' 'E	Leaf, Stem
G. flavescens ssp. *antalyensis* (endemic)	GFA	07/05/2011	C3 Antalya: Kemer, Between Tekirova-Cirali, Ancient road, 4 m	36º 28' 07' 'N 30º 30' 17' 'E	Leaf, Stem, Root
G. flavescens ssp. *flavescens*	GFF	16/04/2011	C6 Osmaniye: Osmaniye-Yarpuz road, 10th km, 835 m	37º 016' 20' 'N 36º 27' 433' 'E	Leaf, Stem, Root
G. asymmetrica (endemic)	GA	23/05/2010	C3 Antalya: Kemer	36º 37' 47.35' 'N 30º 33' 14.32' 'E	Root
	GAE	22/05/2011	C3 Antalya-Aksu road, 1 km before Aksu, roadside	36º 56' 45.34' 'N 30º 50' 30.98' 'E	Leaf, Root
G. glabra	GGC6	15/06/2010	C6 Gaziantep: Nizip, 7 km before Nizip	37º 02' 21.69' 'N 37º 54' 21.66' 'E	Root
	GGC2	24/06/2010	C2 Denizli: Pamukkale-Denizli road, roadside	37º 53' 52.09' 'N 29º 07' 32.69' 'E	Root
	GG1036	22/05/2011	C6 Kahramanmaras: Between Kahramanmaras-Turkoglu, Before reaching the Aksu bridge, 448 m, roadside	37º 29' 38' 'N 36º 53' 42' 'E	Root
	GG1047	22/05/2011	C6 Kahramanmaras: Between Kahramanmaras-Turkoglu, After the Aksu bridge	37º 29' 38' 'N 36º 53' 42' 'E	Root
	GGC7	01/02/2012	C7 Adıyaman: Isikli village, field edge	37º 26' 13.87' 'N 38º 13' 45.21' 'E	Root
	GG1055	01/06/2012	C7 Sanliurfa: Between Sanliurfa-Suruc, 12 km before Suruc	37º 06' 603' 'N 38º 49' 934' 'E	Leaf, Stem
	GGB4	23/06/2012	B4 Ankara: Polatlı-Eskişehir road, Sakarya riverside, 680 m	39º 35' 00' 'N 31º 56' 58' 'E	Leaf, Stem

2.2. Preparation of licorice extracts

According to the data obtained as a result of the study conducted by Özcan et al. (2020), the extraction process was performed by microwave-assisted extraction method, which is the extraction method with the highest yield compared to other extraction methods. Finely ground 1 g of *Glycyrrhiza* samples was added to 10 mL of 50% MeOH (MeOH: H_2O; 1: 1, v/v). The samples were extracted at room temperature (25±1 ºC) in a microwave oven (Milestone Start D Microwave Digestion System, USA) for 7 minutes with 100-watt energy. The extracts obtained were diluted 10000 times and passed through a 0.2 µm membrane filter. The same process was repeated without diluting the extracts to determine bioactive compounds which exist at low concentration. Filtered extracts were taken into vials and given to LC-ESI-MS/MS.

2.3. Preparation of calibration standard solutions

Stock solutions (mg mL^{-1}) of the four bioactive standards were prepared by dissolving in methanol. Stock solutions were mixed and then diluted with methanol to give solutions containing 1, 25, 100, 500 and 1000 ng mL^{-1} of mixed standard solution. Calibration curves were constructed by injecting each standard solution at each concentration level in triplicate and plotting peak areas versus concentrations. A representative chromatogram is given in Figure 2.

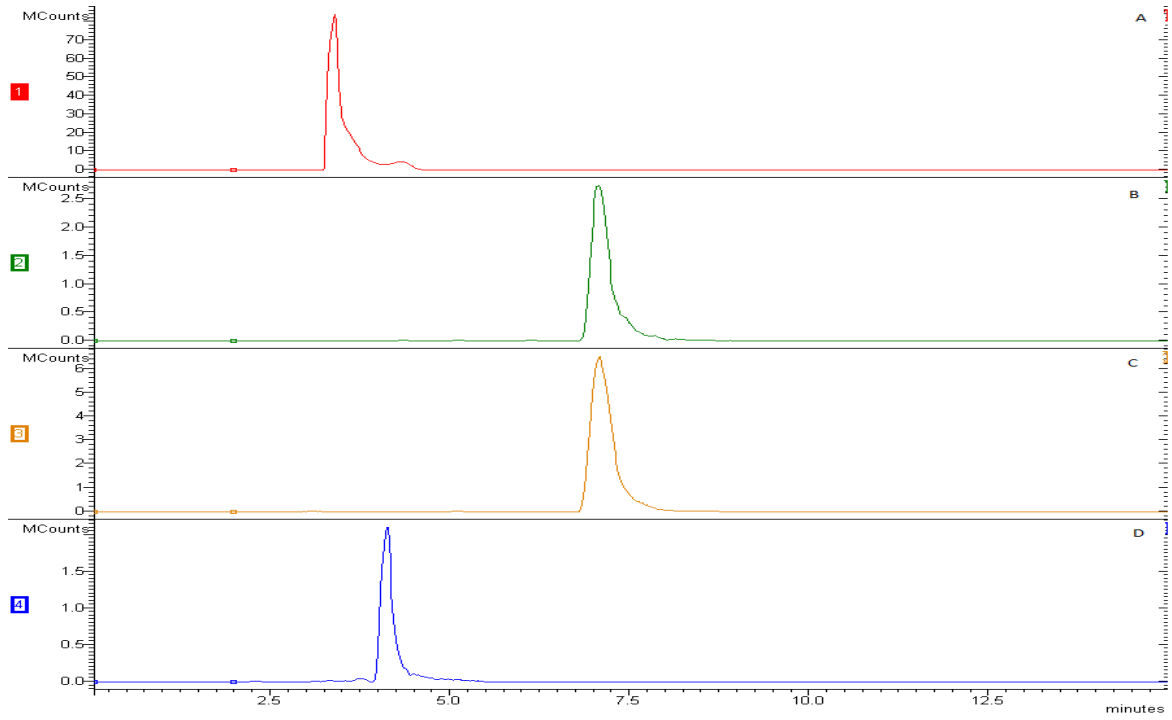

Figure 2- Representative chromatogram of 25 µg/L standard solutions. A: Liquiritin, B:Glycyrrhetinic acid, C: Carbenoxolone, D: Glycyrrhizin

2.4. Chromatographic conditions and mass spectrometric determination

Quantitative analyses of four bioactive compounds were performed on Zivak Tandem Gold (Istanbul, Turkey) system equipped with a Waters Symmetry C18 column (2.1 mm × 150 mm I.D., particle size 5 µm; Waters, Milford, MA, USA). The mobile phase consisted of deionised water (A) and methanol (B) and a gradient elution program was programmed as the following steps for chromatographic separation; 0:00 – 2:30 min, 10% B; 2:30 – 4:00 min, 50% B; 4:00 –10:00 min, 90% B; 10:00 – 12:00 min, 90% B; 12:00 – 15:00 min, 50% B; 15:00 – 20:00 min, 10% B with a constant flow 300 µL min^{-1}.

The instrument was operated in negative ion mode with an ion spray voltage of 5000 V, nebuliser gas (nitrogen) of 35 psi, source temperature of 50 ºC, drying gas temperature of 350 ºC. The column effluent was injected directly into the ESI source and spotted with multiple reaction monitoring (MRM). Mass parameters were optimised by using standard solutions of GL, GA, CBX and LQ in Özcan et al. (2020). All the transition ions and parameters, which were determined after optimisation by using standard solutions of each compound, were listed in Table 2.

Table 2- Precursor-product ions and parameters for MRM of compounds used in the present study

Analytes	*Precursor ion (m / z)*	*Screening ion (m / z)*	*Confirmation ion (m / z)*	*Capillary voltage (V)*	*Collision energy (eV)*
Glycyrrhizin	822.1	351	193	80	50
Glycyrrhetinic acid	470	425	355	60	40
Liquiritin	417.5	254.9	135.1	60	20
Carbenoxolone	570.1	469.7	100.2	80	30

2.5. Statistical analysis

Each sample was analyzed in three replicates and all results were presented as mean values ± standard deviations and analyzed by SPSS Statistics software 23 (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) was performed using the Tukey test for any significant differences between the means. Differences between the means at 1% ($P<0.01$) level were evaluated significantly.

3. Results and Discussion

The content of GL, GA, CBX and LQ in different plant parts of *Glycyrrhiza* spp. was calculated and listed in Table 3 which showed significant variations among the contents ($P< 0.01$).

Table 3- Quantitative LC-MS/MS analysis of bioactive compounds from different parts of *Glycyrrhiza* spp. grown in Turkey

Codes	*Glycyrrhizin (GL)*			*Glycyrrhetinic acid (GA)*			*Carbenoxolone (CBX)*			*Liquiritin (LQ)*		
	Leaf	***Stem***	***Root***	***Leaf***	***Stem***	***Root***	***Leaf***	***Stem***	***Root***	***Leaf***	***Stem***	***Root***
GXI	0.67±0.07 ^{C}	0.75±0.04 ^{C}	2.60± 0.09 ^{G}	0.074±0.01 ^{A,B}	0.063±0.002 ^{A}	0.062± 0.009 ^{C}	0.070±0.005 ^{C}	0.019±0.030 ^{B}	0.013± 0.003 ^{D}	0.32±0.001 ^{D}	0.323±0.005 ^{B}	0.266± 0.006 ^{G}
GEE	0.33±0.03 ^{D}	0.65±0.06 ^{C}	7.65±0.05 ^{E}	0.067± 0.022 ^{B}	0.064±0.02 ^{A}	0.088±0.010 ^{B,C}	0.023± 0.006 ^{D}	0.015±0.010 ^{B}	0.026±0.013 ^{D}	0.292± 0.012 ^{E}	0.230±0.009 ^{D}	0.260±0.004 ^{G}
GEM	0.91±0.05 ^{B}	1.01±0.09 ^{B}		0.066±0.008 ^{B}	0.070±0.004 ^{A}		0.010±0.003 ^{D}	0.012±0.003 $^{B. C}$		0.392±0.003 ^{B}	0.262±0.008 ^{C}	
GFA	< LOD ^{E}	< LOD ^{E}	1.59±0.02 ^{H}	0.070± 0.003 ^{B}	0.065± 0.004 ^{A}	**0.136±0.005** ^{A}	0.020± 0.004 ^{D}	0.015± 0.008 ^{B}	**0.188±0.067** ^{A}	< LOD ^{F}	< LOD ^{E}	0.378±0.003 ^{F}
GFF	**2.05±0.07** ^{A}	0.441±0.02 ^{D}	1.22±0.07 ^{I}	**0.107±0.005** ^{A}	0.065±0.011 ^{A}	0.068±0.003 ^{B,C}	0.098±0.004 ^{B}	0.013±0.006 $^{B. C}$	0.086±0.065 ^{C}	0.397±0.008 ^{B}	0.266±0.004 ^{C}	0.261±0.005 ^{G}
GA			0.87±0.03 ^{J}			0.070±0.003 ^{B,C}			0.014±0.005 ^{D}			0.259±0.002 ^{G}
GAE	< LOD ^{E}		1.08±0.07 ^{I,J}	0.070±0.01 ^{B}		0.065±0.012 ^{B,C}	0.014±0.006 ^{D}		0.128±0.126 ^{B}	< LOD ^{F}		0.264±0.002 ^{G}
GGC6			**14.68±0.09** ^{A}			0.081±0.008 ^{B,C}			0.012±0.017 ^{D}			**9.735±0.046** ^{A}
GGC2			11.10±0.07 ^{D}			0.090±0.009 ^{B}			0.012±0.005 ^{D}			3.380±0.011 ^{D}
GG1036			12.02±0.06 ^{C}			**0.133±0.006** ^{A}			0.012±0.001 ^{D}			4.917±0.014 ^{C}
GG1047			7.28±0.09 ^{F}			0.076±0.007 ^{B,C}			0.015±0.001 ^{D}			3.212±0.018 ^{E}
GGC7			12.34±0.12 ^{B}			0.072±0.007 ^{B,C}			0.012±0.001 ^{D}			8.424±0.053 ^{B}
GG1055	< LOD ^{E}	< LOD ^{E}		0.060±0.009 ^{B}	0.058±0.007 ^{A}		0.015±0.003 ^{D}	< LOD ^{C}		0.352±0.005 ^{C}	0.248±0.005 $^{C, D}$	
GGB4	0.718±0.1 ^{C}	**2.735±0.04** ^{A}		0.061±0.004 ^{B}	0.075±0.01 ^{A}		**0.133±0.006** ^{A}	**0.069±0.004** ^{A}		**1.644±0.014** ^{A}	**0.602±0.010** ^{A}	

mg g^{-1}, LOD<1, *Values are mean ± SE. Different letters mean the statistical difference between groups

The quantitative analysis results indicated GGC6 (14.68±0.09 mg g^{-1}) had the highest GL content in roots of *Glycyrrhiza* spp., followed by GGC7 (12.34±0.12 mg g^{-1}) and GG1036 (12.02±0.06 mg g^{-1}). As shown in Table 3, GL was quantified maximum in stems of GGB4 (2.735±0.04 mg g^{-1}) and then GEM (1.01±0.09 mg g^{-1}), respectively. Likewise, GL was detected highest in the leaves of GFF (2.05±0.07 mg g^{-1}). The GL was identified as the most prominent compound among quantified bioactive compounds of *Glycyrrhiza* spp. In the comparison among different parts of each *Glycyrrhiza* spp., GL was found maximum in roots of GGC6 (14.68±0.09 mg g^{-1}).

As stated in Table 3, GA was determined maximum in roots of GFA (0.136±0.005 mg g^{-1}) and GG1036 (0.133±0.006 mg g^{-1}). On the other hand, according to the comparative analysis results on stem parts, no significant difference was observed in GA contents among *Glycyrrhiza* spp. ($P<0.01$). In addition, the highest GA content in leaf parts was obtained in GFF (0.107±0.005 mg g^{-1}) and GXI (0.074±0.01 mg g^{-1}).

Among the roots of all *Glycyrrhiza* spp., the highest CBX content was found in GFA (0.188±0.067 mg g^{-1}), followed by GAE (0.128±0.126 mg g^{-1}) and GFF (0.086±0.065 mg g^{-1}), respectively. Also, CBX was quantified maximum in stems of GGB4 (0.069±0.004 mg g^{-1}). No significant difference was observed in CBX contents among other *Glycyrrhiza* spp. ($P<0.01$). Comparing the leaf parts of all species, the highest CBX content was found in GGB4 (0.133±0.006 mg g^{-1}), followed by GFF (0.098±0.004 mg g^{-1}) and then GXI (0.070±0.005 mg g^{-1}).

LQ content was detected maximum in roots of GGC6 (9.735±0.046 mg g^{-1}), followed by GGC7 (8.424±0.053 mg g^{-1}) and GG1036 (4.917±0.014 mg g^{-1}). In the comparison of the leaf and stem parts of all species, it was observed that the highest LQ contents were in GGB4 (1.644±0.014 mg g^{-1} and 0.602±0.010 mg g^{-1}, respectively). For the stem parts, GXI (0.323±0.005 mg g^{-1}) was the second species with the maximum LQ after this species. On the other hand, for leaf parts, GFF (0.397±0.008 mg g^{-1}) was the second with the highest LQ content, following GGB4.

Esmaeili et al. (2020) collected 28 licorice (*Glycyrrhiza glabra* L.) populations from various wild areas across of Iran and quantified the bioactive compounds by high-performance liquid chromatography–photodiode array detection (HPLC–PDA). According to the findings of their study, minimum (11.7 mg g^{-1}) and maximum (74.0 mg g^{-1}) amount of GL were determined in Haji Abad and Eghlid populations, while minimum (0.14 mg g^{-1}) and maximum (2.01 mg g^{-1}) amount of LQ were quantified in Saqez and Eghlid populations. Furthermore; Souri et al. (2016) reported that average content of GL varied between 1.38% and 3.40% in their study with different populations from Iran. In another study, Hayashi et al. (2003) the quantity of GL in their study was higher than reported in Italy (1.6–3%), Spain (0.7–4.4%) and Uzbekistan (4.76%–6.13%).

On the other hand, Alsaadi et al. (2020), extracted *G. glabra* collected from Hatay province of Turkey by sonication with 50% ethanol and then quantified three bioactive compounds (GL, LQ and glabridin) via HPLC. According to their results, GL content varied from 0.54% to 2.40%, while LQ content varied from 0.18% to 1.85%.

In addition, Quintana et al. (2020) also extracted phytochemicals from *G. glabra* L. by supercritical antisolvent (SAS) technique. The LQ content ranged from 0.71 mg g^{-1} to 9.61 mg g^{-1} while GL content ranged from 0.18 mg g^{-1} to 0.56 mg g^{-1} in their work.

Li et al. (2016) determined the bioactive substances by UHPLC-MS/MS in six different *G. glabra*, five different *G. uralensis* and *G. inflata*. In their study, the GL content of the *G. glabra* samples ranged from 16.28 mg g^{-1} to 31.40 mg g^{-1} and LQ content varied from 1.27 to 8.14 mg g^{-1}. On the other hand, GA content obtained a maximum of 0.10 mg g^{-1}, the other five samples were detected below the LOD value (Li et al., 2016).

Montoro et al. (2011) determined the bioactive compounds in *G. glabra* collected from Turkey by LC- ESI-MS/MS and found GL content as 32.52 mg g^{-1} and LQ content as 2.38 mg g^{-1}.

In the light of all these results, large variations in these bioactive compounds have been mainly thought to be related to differences in used extraction methods and parameters as well as genetics (Esmaeili et al., 2020), harvesting time and also environmental and/or soil factors (Hou et al. 2018; Hosseini et al. 2014). In addition to all this, Jt et al. (2011) noted that the physicochemical characterization of soil is the foremost factor concerning the accumulation of bioactive ingredients in this plant.

4. Conclusions

In this study, for the first time leaf, stem and root parts of different species of *Glycyrrhiza* grown wild in Turkey were compared with each other in terms of the highest GL, GA, CBX and LQ contents. As a result of the comparison of the leaf parts of the plant, it was found that *Glycyrrhiza flavescens* ssp. *flavescens* contained the highest GL and GA, while *Glycyrrhiza glabra* included the maximum CBX and LQ. Among the *Glycyrrhiza glabra* spp. collected from different provinces of Turkey, the species grown especially in Ankara was determined as the species with the highest bioactive substance content. In a

comparison of stem parts of the plant; *Glycyrrhiza glabra*, grown in Ankara, had the highest content of bioactive substances and there was no statistically significant difference between species in terms of GA content.

Comparing the root parts of the plant, *Glycyrrhiza glabra* was the species with the highest bioactive substances except for CBX. *G. glabra*, which had the highest GL and LQ contents in the root parts of the plant, were collected from Nizip district of Gaziantep, and *G. glabra* with the highest GA was collected from Kahramanmaras. On the other hand, the species with the highest CBX in the root part was *Glycyrrhiza flavescens* ssp. *antalyensis*.

Glycyrrhiza glabra spp., grown in Gaziantep and Kahramanmaras provinces, was ahead of other species due to the fact that the leaf parts were less than the other parts of the plant and it had the highest efficiency in terms of these four bioactive substances content.

This is the first study to evaluate and compare the extracts obtained from the leaves, roots and stems of all different wild *Glycyrrhiza* spp. grown in Turkey in terms of bioactive substances.

Furthermore, we have demonstrated that different parts of *Glycyrrhiza* spp., which are a rich source of bioactive compounds with potential usage in pharmaceuticals and the food industry. Briefly, in this work, the populations with high-amount of each bioactive ingredient in root, stem and leave parts of *Glycyrrhiza* spp. were identified, which can be exploited depending on the purpose of usage.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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