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HPLC determination of polyphenols of the flowers of *Digitalis lamarckii, Xeranthemum annuum, Epilobium hirsutum* and *Silene compacta* from Bolu (Turkey)

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There have been a growing interest since decades in the medicinal plants in terms of preventive health care and treatment of various diseases. On the other hand, polyphenolic compounds present in medicinal plants are known to have antioxidant potency. In the current work, the quantitative HPLC analysis of the flowers of four plants grown in Bolu-Turkey, *Silene compacta, Digitalis lamarckii, Xeranthemum annuum* L., and *Epilobium hirsutum* L was carried out. Prior to HPLC quantification, they were detected by TLC with reference standard compounds, identified by LC-MS and compared for their profiles of phenolic acids and some flavonoids by using high-performance liquid chromatography (HPLC) with diode array detector. The highest contents of benzoic acids were detected in the flowers of *E. hirsutum* L. as 159 mg in 100 g dry flowers. As for cinnamic acids, the highest content value was found in *S. compacta* flowers as 292 mg in 100 g dry flowers. On the other hand, *E. hirsutum* L. flowers contained the highest total flavonoid value as much as 3890 mg. Total polyphenolic content was the highest (4104 mg/100 g dry plant) in *E. hirsutum* L., however, the lowest value was found as 492 mg/100 g dry flowers for *D. lamarcki*.

Key words: Polyphenols, HPLC, LC-MS, quantitative determination, *Silene compacta, Digitalis lamarckii, Xeranthemum annuum* L., *Epilobium hirsutum* L.

INTRODUCTION

The polyphenols comprise one of the largest and most diverse group of phytochemicals found in plants and they are secondary plant metabolites. Polyphenols can range from simple phenols, phenolic acids to coumarins, flavonoids and tannins (Ignat et al., 2011; Jaiswal et al., 2014; Kitada et al., 2003). These compounds usually occur in the form of glycosides or esters in plants. That is the reason for their tendency to be highly water-soluble

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(Harborne, 1998). Due to their importance in pharmacology, biochemistry and medicinal chemistry over the decades many reports have been published both in terms of synthesis and isolation from plant tissues (Handique and Baruah, 2002; Lu and Foo, 2002; Tomás-Barberán and Andrés-Lacueva, 2012; Ramassamy, 2006; Weinreb et al., 2004; Ferguson, 2001). Polyphenolic compounds are also usually responsible for the colorization in plants. They contain a part of antioxidants which are a large class of compounds which are effective to prevent cellular abrasion in the body of animals and human. Polyphenolics having strong antioxidant properties are also capable to repair the damage induced by oxidative reactions (Robards et al., 1999; Balasundram et al., 2006). Therefore, they can act against cardiovascular diseases, neurodegenerative diseases, diabetes and cancer as a preservative agent (Arts and Hollman, 2005; Pueyo and Calvo, 2009; Steinberg and Milardoviĉ, 2007). In addition, *Epilobium hirsutum* L. extracts were found to have anti-proliferative effect on a prostatic cancer cells (Vitalone et al., 2001).

To the best of our knowledge, there is no published report regarding the plant *Silene compacta* and its chemical components. On the other hand, as for the plant *Digitalis lamarckii*, there appears no study related to the quantitative analysis of polyphenolic compounds by means of HPLC and structure elucidation by mass spectrometric analysis. However, there is a limited number of research reports on the plants, *Xeranthemum annuum* L. and *E. hirsutum* L., for their chemical composition (Stanković et al., 2011; Karakaş et al., 2012; Stolarczyk et al., 2013; Cando et al., 2014; Battinelli et al., 2001).

Taking account of the aforementioned importance of the polyphenolics as natural products, we therefore focused on the quantitative determination of polyphenolic compounds of the four species by HPLC, namely, *D. lamarckii, X. annuum, E. hirsutum* and *S. compacta* grown in Bolu (Turkey).

In order to get insight into the diversity and to perform a comparison, a more detailed quantitative analysis of the chemical components of the plant flowers under investigation has been conducted in this study.

MATERIALS AND METHODS

Chemicals and reagents

All solvents and chemical reagents were purchased from Merck. Aqueous solutions were prepared using 0.055 μScm^{-1} deionized water produced by a Tka Smart 2 Pure water purification system.

Plants collection and extracts preparation

The plants used in this study are found widespread in Bolu province (Turkey) in the elevation of 900 to 2000 m. They were collected in July 2010 and 2011. *S. compacta* (**SC**) and *X. annuum* L. (**XA**) were collected from the roadsides between Bolu and Ankara provinces (Turkey). *D. lamarcki* (**DL**) was collected from near by Kıbrıscık (Bolu) and *E. hirsutum* L. (**EH**) from roadsides around Gölcük (Bolu). The dried flowers were stored in an airy place. The botanical identifications of the plants were made by the co-author Nursel İkinci. Voucher specimens are available at the herbarium of the Biology Department of Bolu Abant İzzet Baysal University with the numbers: *D. lamarckii* Ivanina (Plantaginaceae) N. İkinci 3794, *X. annuum* L. (Asteraceae) N. İkinci 3781, *S. compacta* Fisch. (Caryophyllaceae) N. İkinci 5030, and *E. hirsutum* L. (Onagraceae) N. İkinci 3797, respectively.

Figures 1 and 2 show some natural images of the collected plants.

General experimental procedures

LC-MC analysis

The conditions of LC-MS analysis of phenolic compounds: Waters Allians, Waters Micromass ZQ instrument was used. Negative electrospray ionization [ES(-)] method was applied. The HPLC column was XTerra® MS C-18 (4.6×250 mm, 5 µm) and mobile phase was water / acetonitrile / methanol (5:85:10, v/v). Flow rate was maintained at 0.75 mL/min. A photo-diode array (254 nm) detector was used. Analytical conditions were selected providing capillary voltage at 3.41 kV, cone voltage at 26 V, source temperature at 100°C and desolvation temperature at 350°C

HPLC analysis

Quantitative analysis of phenolic compounds was determined by high performance liquid chromatography (HPLC). The HPLC system used consisted of a Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with diode array detector. Phenolic acids were separated on a reversed phase column (C18, 25 cm × 4.6 mm I.D., 5 µm particle size, Advanced Chromatography Technologies Ltd., Scotland) with the mobile phase flow rate of 1 mL/min, operating at 30°C. Separation was achieved by elution gradient using the mobile phase (A) water/acetic acid (98:2, v/v) and (B) acetonitrile/water/acetic acid (80:19.6:0.4, v/v). The following linear gradient programme was used for the elution: from 0 to 10% B in 5 min, from 15 to 30% B in 20 min, from 30 to 50% B in 10 min, from 50 to 60% B in 5 min, and 60 to 90% B in 5 min followed by a return to the initial conditions in 5 min and reequilibration of the column (Bozan et al., 2008). Chromatograms were monitored at 280 nm (for benzoic acids), 320 (cinnamic acids), and 360 nm (flavonoids). Identification was based on comparing retention times and online spectral data in comparison with original standards. Reference phenolic acids and flavonoids were obtained from Sigma-Aldrich. Gallic, 3,4-di-OH-benzoic, protocatechuic, *p*-OH-benzoic, vanillic, isovanillic, and syringic acids were used as the reference compounds representing the benzoic acids. For cinnamic acids, five commercially available acids as caffeic, *p*-coumaric, ferulic, sinapinic, and *o*-coumaric acids were selected as reference compounds. In order to identify flavonoids, quercetin, luteolin, kaemferol and apigenin which were commercially available in their aglycone form, were used. Quantification was performed based on the the calibration curves of gallic acid for benzoic acids at 280 nm, chlorogenic acid for cinnamic acids at 320 nm, and quercetin for flavonoid aglycons at 360 nm. Two determinations were made on each extract.

Extraction

Qualitative studies

Dried flowers were extracted with methanol (3 g/300 mL) using a

Figure 1. *Silene compacta* from Bolu.
Source: Authors

Figure 2. *Digitalis lamarckii* from Bolu.
Source: Authors

Soxhlet apparatus for 2 days. Upon evaporation of methanol under reduced pressure, residual matter was taken up into water and extracted with petroleum ether (40-60°C), chloroform, diethyl ether and ethyl acetate, respectively. After prequalitative TLC assays, chemical components present in the ethereal, chloroform and ethyl acetate extracts were identified by LC-MS spectra (Figures 3 to 6).

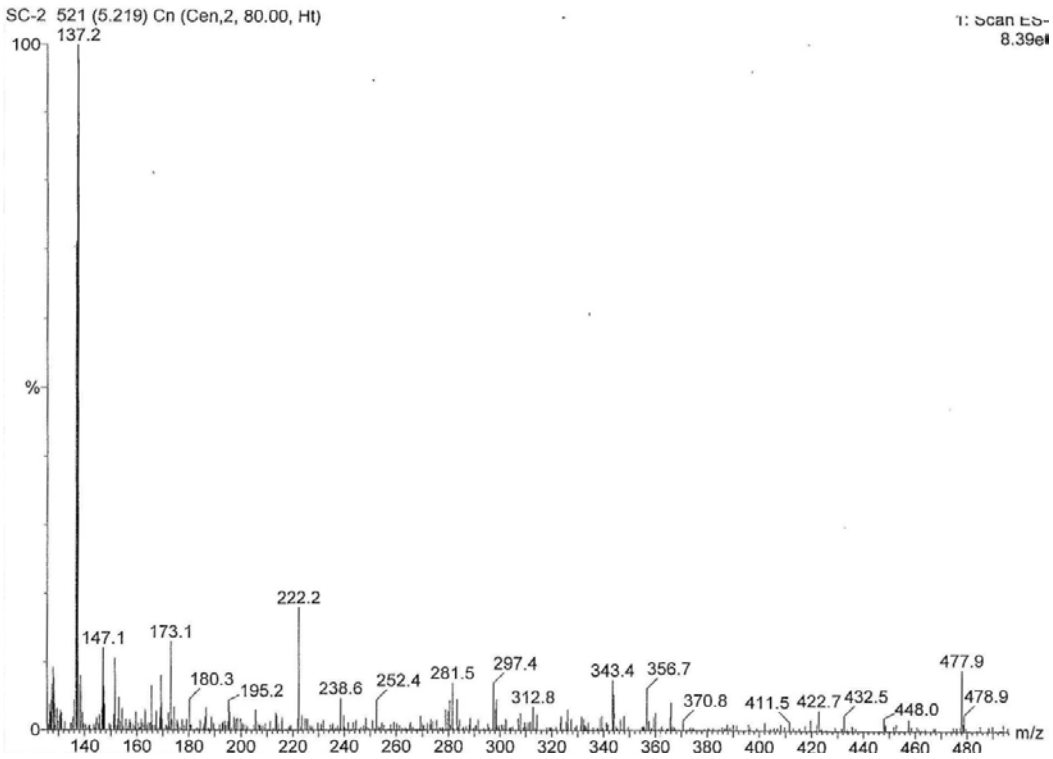

Figure 3. LC-MS spectrum of *p*-hydroxybenzoic acid detected in the Et_2O extract of *Silene compacta* flowers. Source: LC-MS instrument

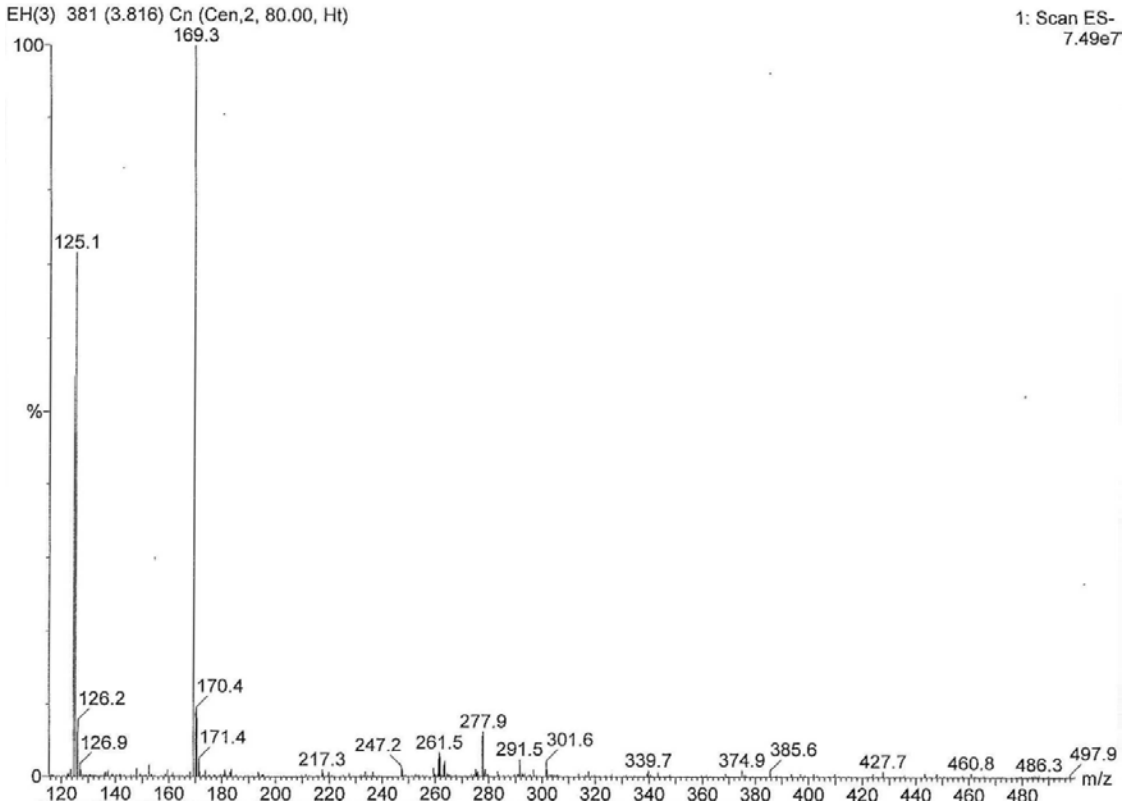

Figure 4. LC-MS spectrum of gallic acid detected in the EtOAc extract of *Epilobium hirsutum* flowers Source: LC-MS instrument

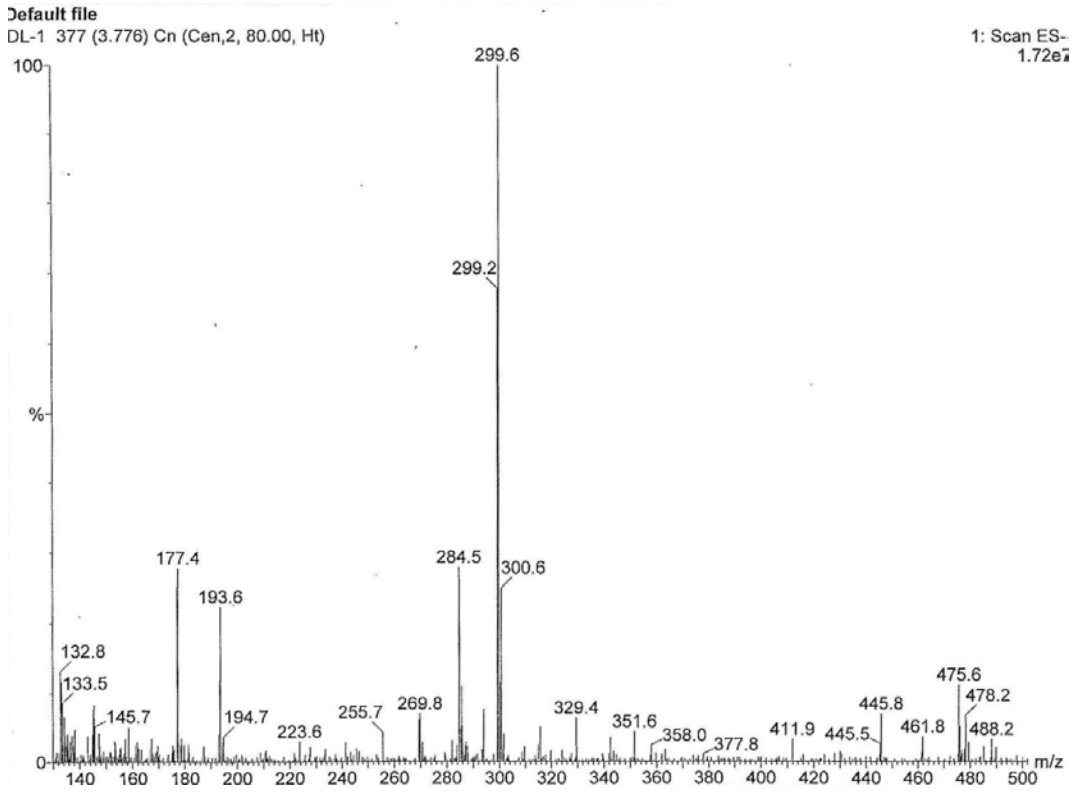

Figure 5. LC-MS spectrum of quercetin detected in the $CHCl_3$ extract of *Digitalis lamarcki* flowers.
Source: LC-MS instrument

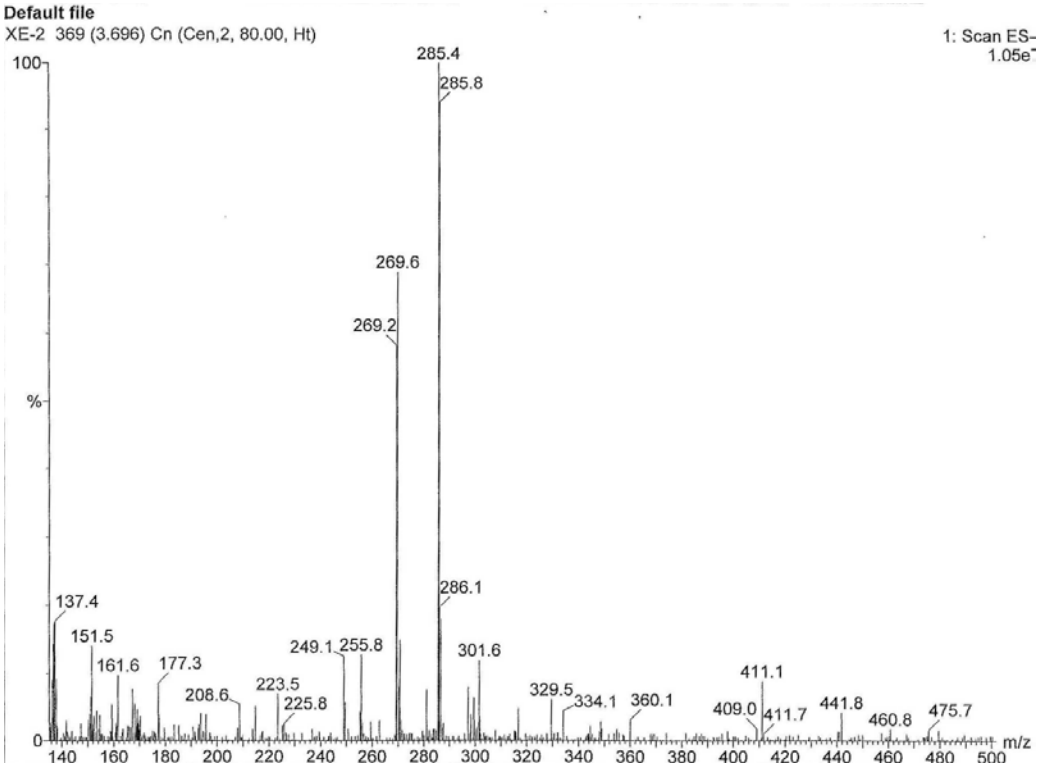

Figure 6. LC-MS spectrum of kaempferol detected in the Et_2O extract of *Xeranthemum annuum* flowers.
Source: LC-MS instrument

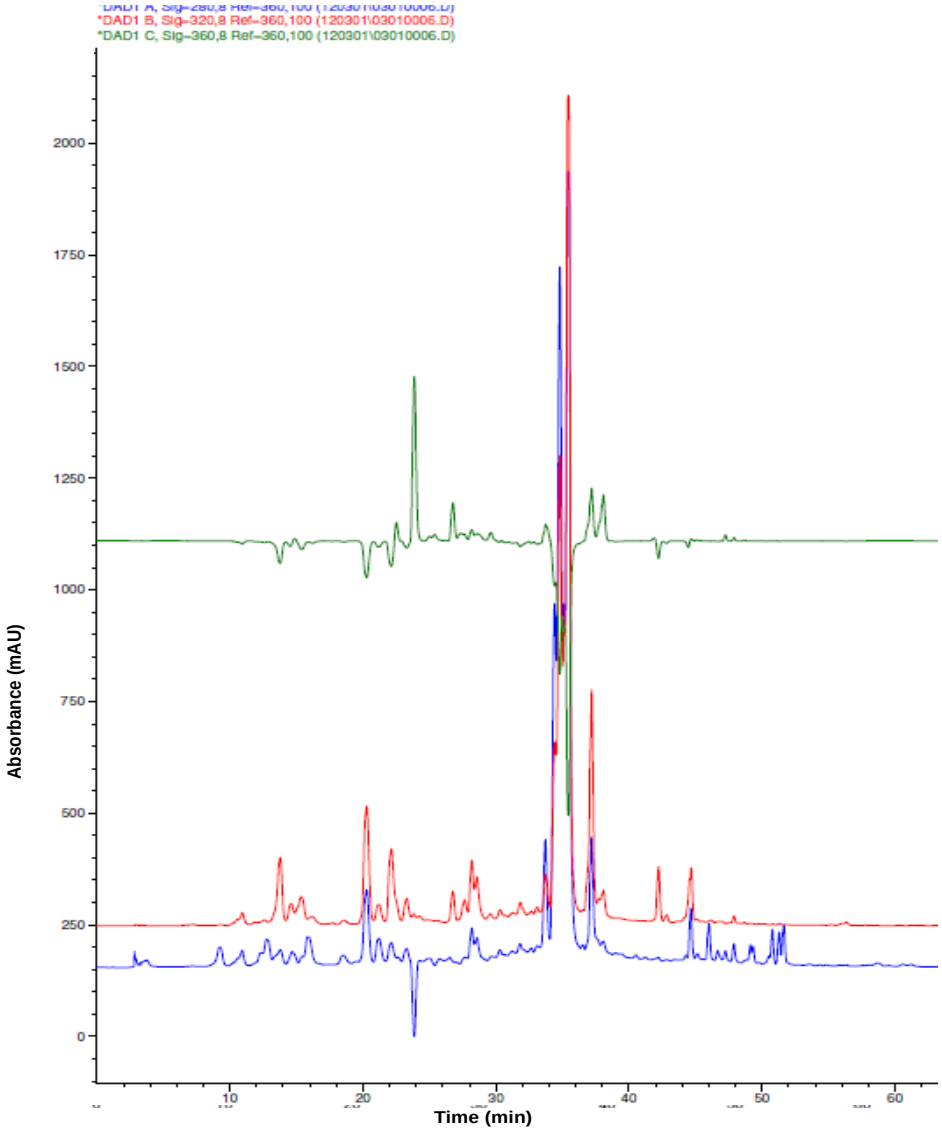

Figure 7. HPLC profile of the extract obtained from the flowers of *Digitalis lamarckii.*
Source: Authors

Quantitative studies

Two types of extracts for quantitative studies were prepared:

Extract 1: Dried flowers were extracted with petroleum ether (40-60°C) (3 g/300 mL) using a Soxhlet apparatus for 1 day. After drying, a certain amount of defatted plant material was exhaustively extracted with 70% aqueous MeOH stirring continuously and maintaining maximum at 40°C temperature. The obtained methanolic solution was evaporated at 40°C using a rotary evaporator until dryness. The dried extract was dissolved in H_2O and successively extracted with EtOAc. Combined EtOAc extracts were dried over anhydrous Na_2SO_4 overnight and solvent was removed and the dried residue was weighed. In this way, glycosides of small sized polyphenolics and some free aglycons were extracted.

Extract 2: A certain amount of dried and defatted flowers were heated in 1.2 M HCl in 50% aqueous MeOH on a water bath by stirring for 1 h. The extract obtained upon hydrolysis was filtered and evaporated. Then, the remaining aqueous part was successively extracted with ethyl acetate. Ethyl acetate extracts were combined, dried over Na_2SO_4 and evaporated under the reduced pressure and the residue was weighed. Upon this hydrolysis process which degrades sugars, only free aglycons are found in this extract.

RESULTS

HPLC chromatograms were monitored at UV wavelenghts of 280 nm (for benzoic acids), 320 nm (cinnamic acids), and 360 nm (flavonoids). They were overlayed as shown in Figures 7 to 14 where blue ones are run at 280, red ones at 320 and green ones at 360 nm, respectively.

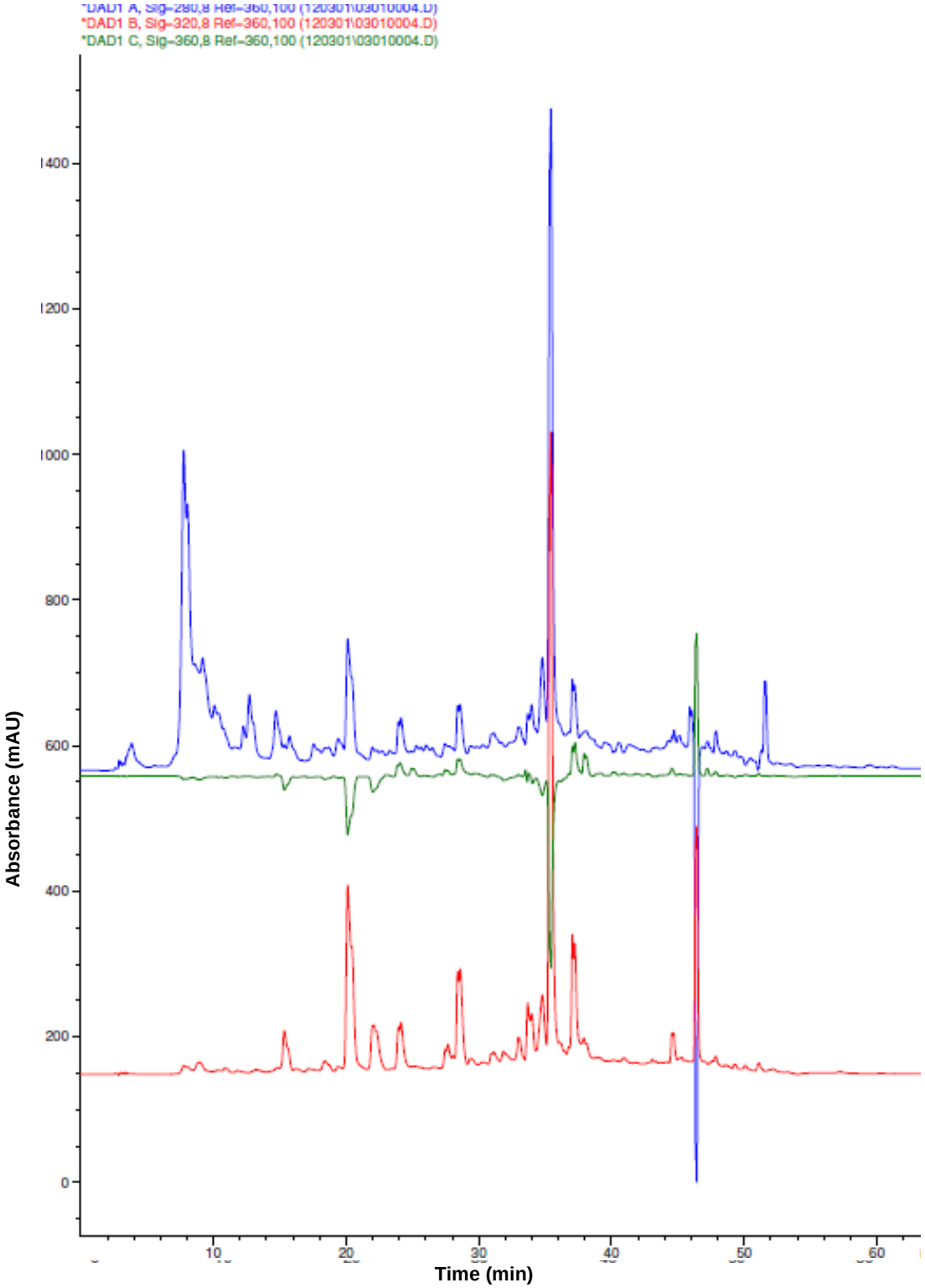

Figure 8. HPLC profile of the extract obtained upon hydrolysis from *Digitalis lamarckii.*
Source**:** Authors

Determination of the chemical components in plant tissues have frequently been utilized by means of HPLC comparatively with the standards (Fernández-Arroyo et al., 2011; Fecka and Turek, 2008; Nishitani and Sagesaka, 2004; Fecka and Turek, 2007; Boros et al., 2010; Peev et al., 2007). Prior to the HPLC determination

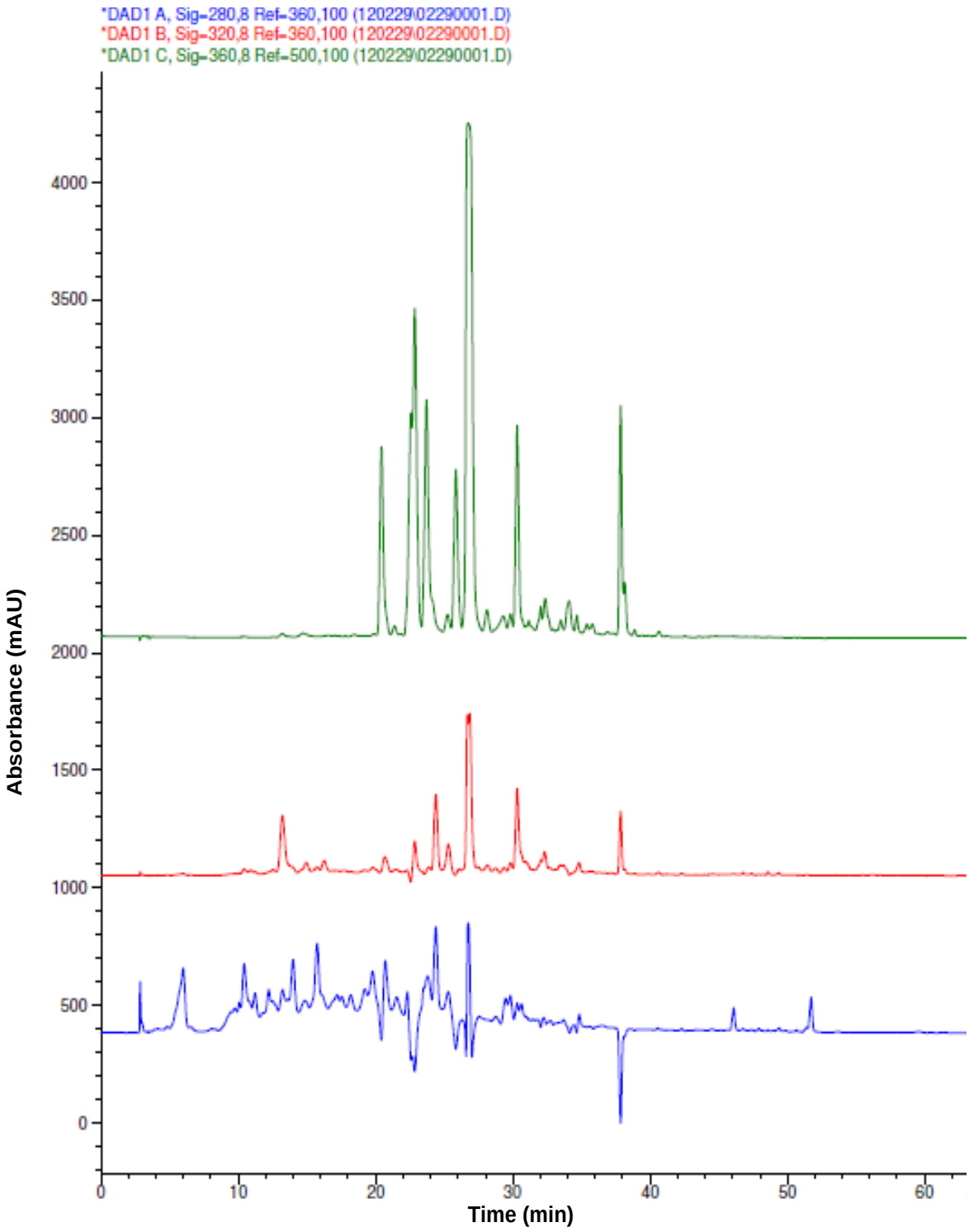

Figure 9. HPLC profile of the extract obtained from *Epilobium hirsutum.*
Source: Authors

in our work, polyphenolic contents of the extracts obtained from the dried flowers of the species were detected primarily by TLC, then, they were identified by LC-MS. As representative examples, LC-MS spectra of *p*-hydroxy benzoic acid from the ethereal extracts of the SC (*S. compacta*), gallic acid of EH (*E. hirsutum*), quercetin of DL (*D. lamarckii*) and kaempferol of XA (*X. annuum*) are as shown in Figures 3 to 6.

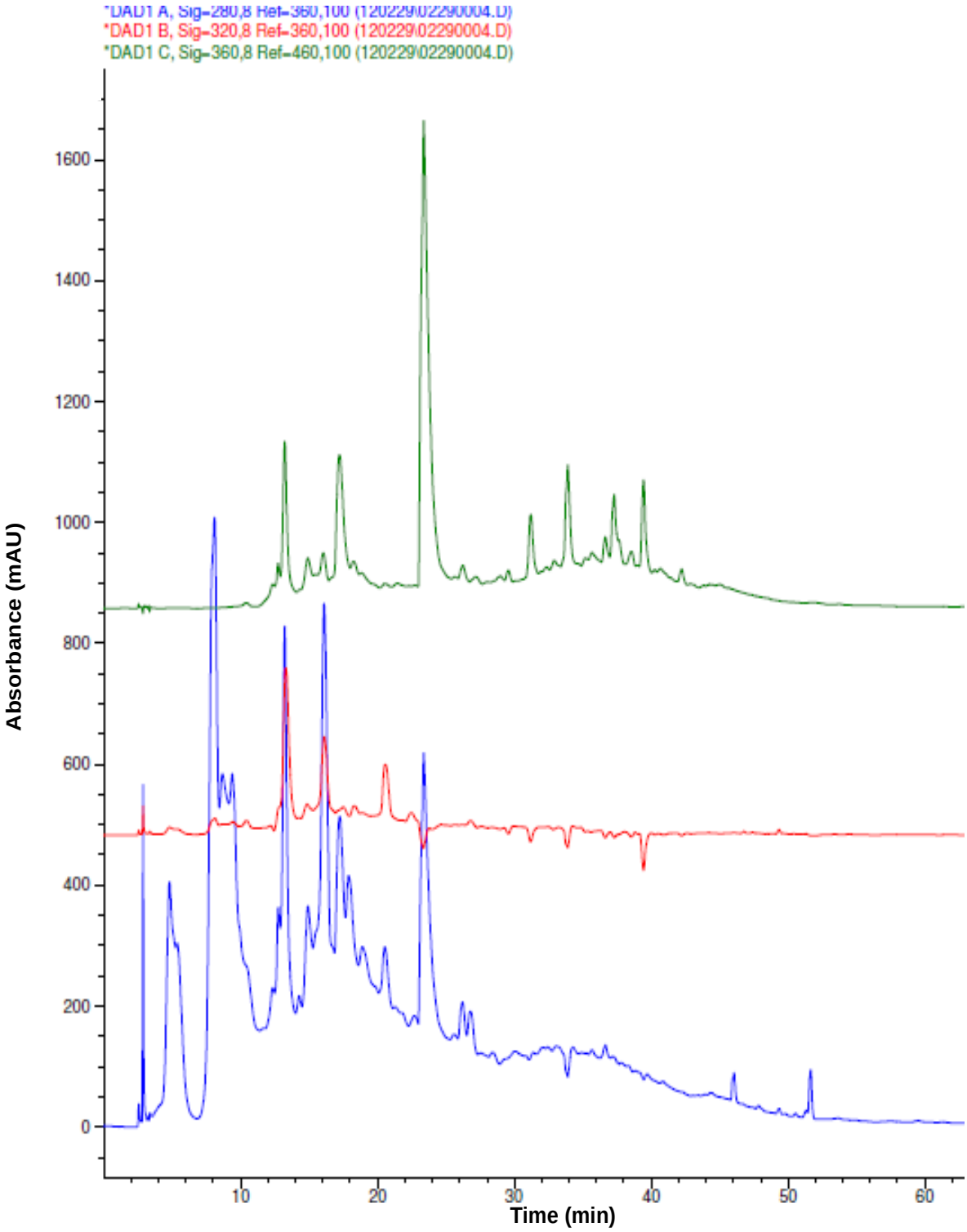

Figure 10. HPLC profile of the extract obtained upon hydrolysis from *Epilobium hirsutum.*
Source :Authors

DISCUSSION

Among the studied plants, *D. lamarckii* is used as medicinal plant in the Kıbrıscık county of Bolu where the plant was collected. In addition, it is also used for the treatment of jaundice, abdominal pain, constipation and swelling in the adjacent towns of Ankara (Simsek et al., 2004). Leaves and roots of *E. hirsutum* are used as pain

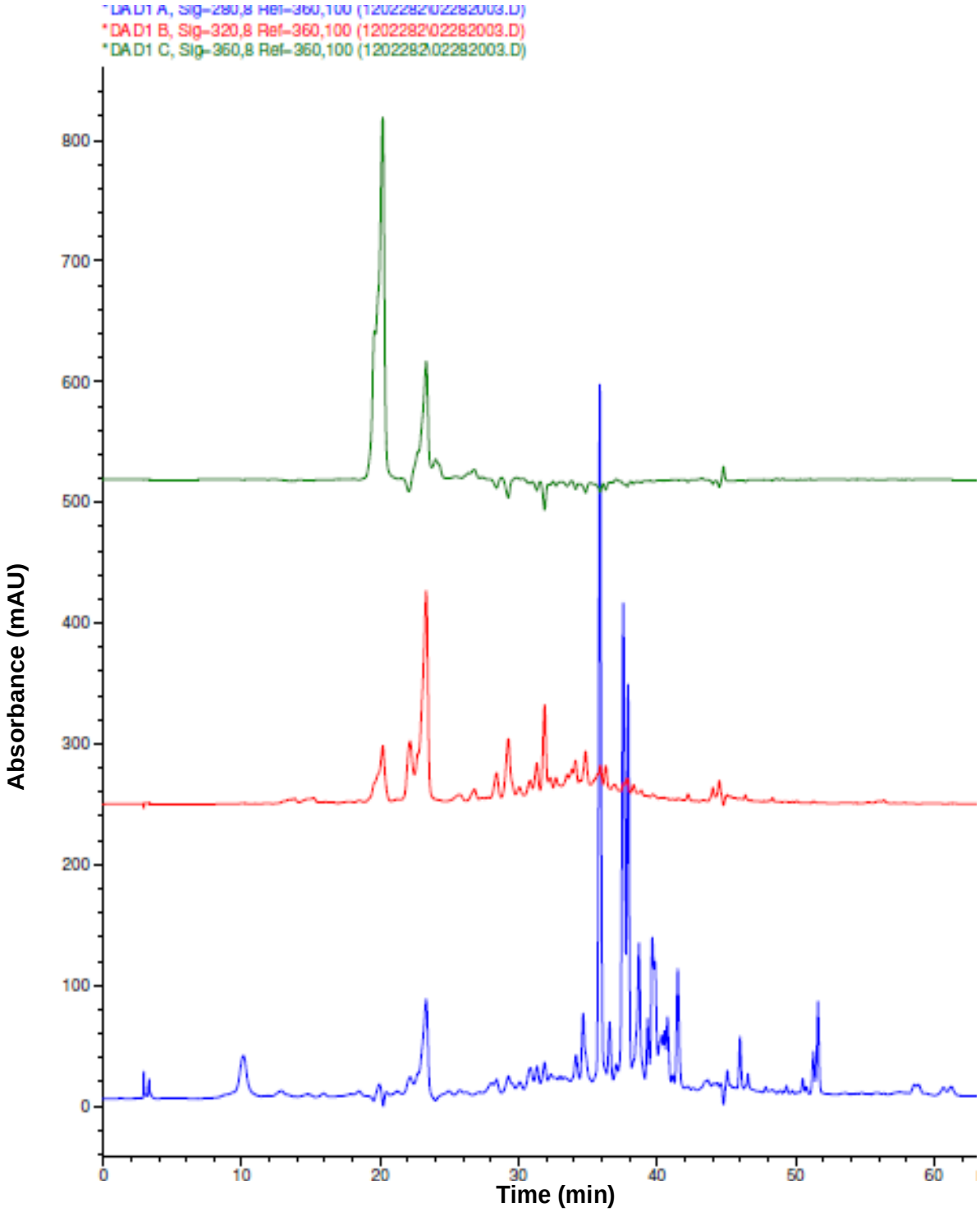

Figure 11. HPLC profile of the extract obtained from *Silene compacta*
Source: Authors

reliever and also for treatment of other diseases in Turkey (Celik et al., 2016). Orhan et al. (2016) reported that *Xeranthemum annuum* has a traditional usage in Turkey to treat burns and against toothache by mixing with tobacco. The genus *Silene* is also widely used as food and medicine throughout Turkey (Zengin et al., 2018).

The present study was aimed to determine polyphenolic

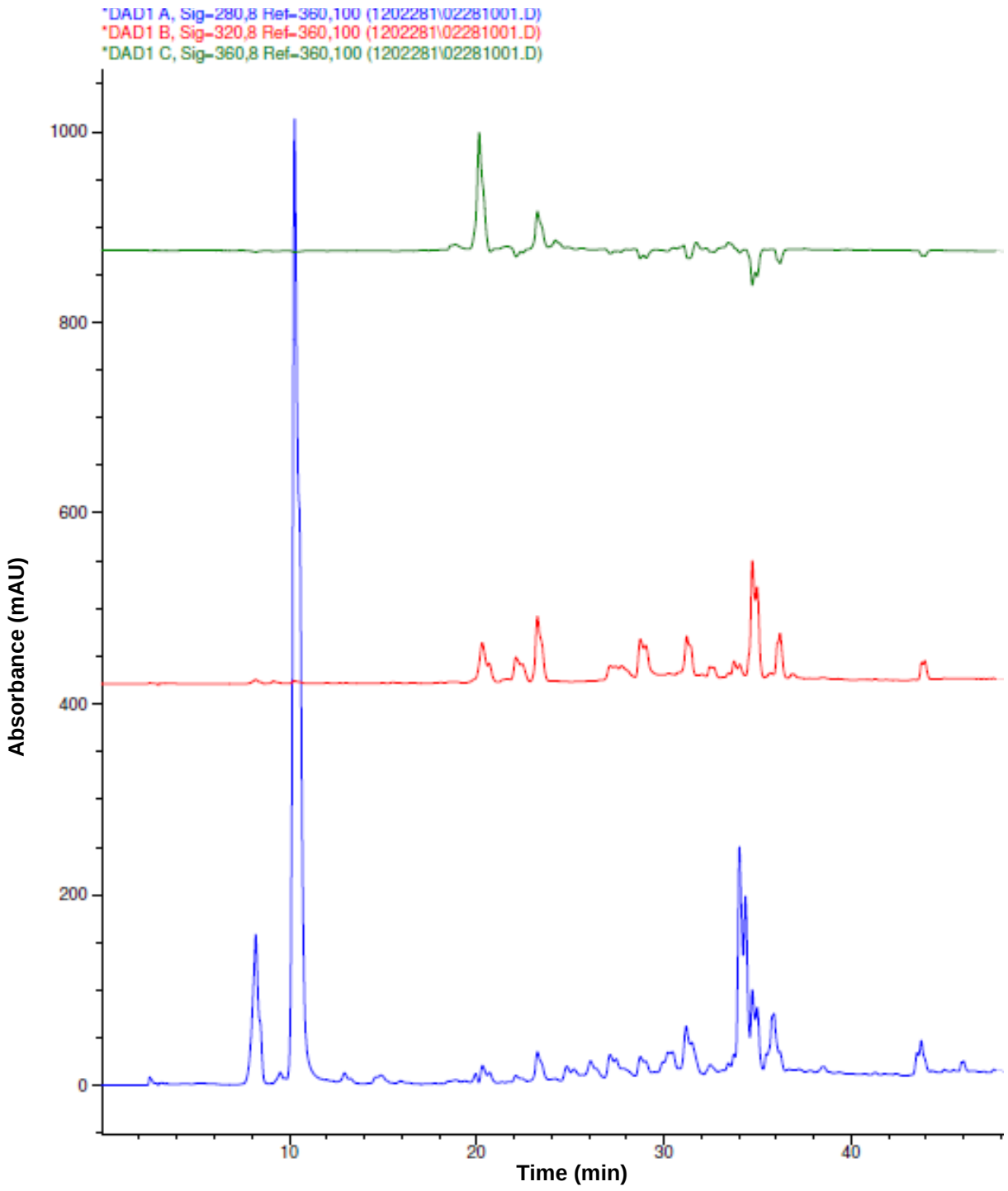

Figure 12. HPLC profile of the extract obtained upon hydrolysis from *Silene compacta*
Source: Authors

compounds in the flowers of four plants; *D. lamarckii, X. annuum, E. hirsutum* and *S. compacta* grown in Bolu province (Turkey).

In this regard, HPLC method is used and this work showed that the highest contents of benzoic acids were detected in the flowers of *E. hirsutum* L. as 159 mg in 100 g dry flowers (Table 2). But, as for cinnamic acids, the highest content value was found in *S. compacta* flowers as 292 mg in 100 g dry flowers. On the other hand, *E. hirsutum* L. flowers contained the highest total flavonoid

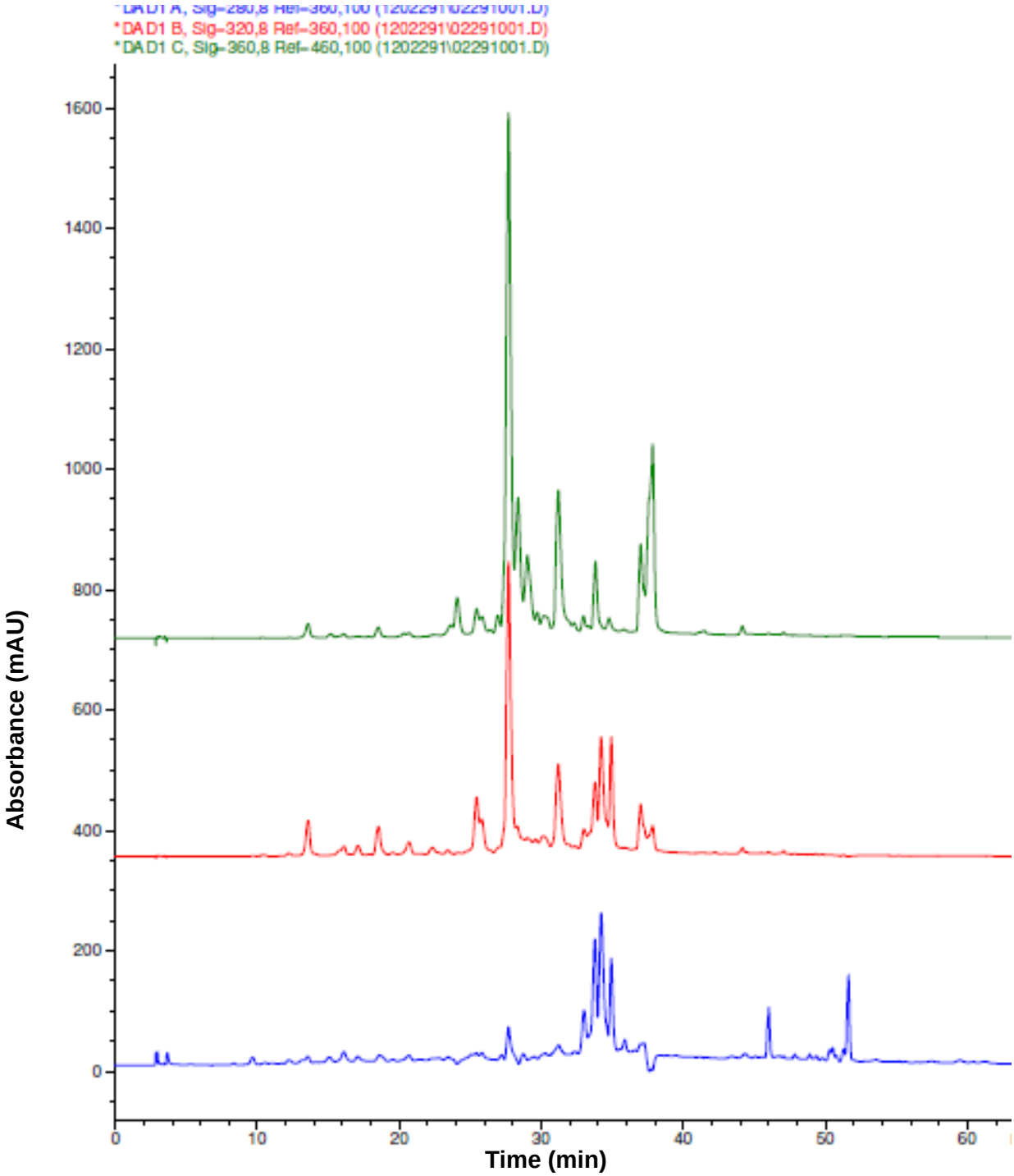

Figure 13. HPLC profile of the extract obtained from *Xeranthemum annuum.*
Source: Authors

value as much as 3890 mg. Although total polyphenolic content was the highest (4104 mg/100 g dry plant) in *E. hirsutum* L., however, the lowest value (492 mg/100 g dry flowers) has been found for *D. lamarcki*.

After LC-MS identification, from each of two different extractions (obtained by hydrolysis and without hydrolysis) applied to four different plants; benzoic acids, cinnamic acids in the extracts, the amounts of flavonoids have been determined respectively, in terms of gallic acid, chlorogenic acid and quercetin by HPLC and the results are shown in Table 1. In addition, total amounts in each extract and plants were summarized briefly in Table

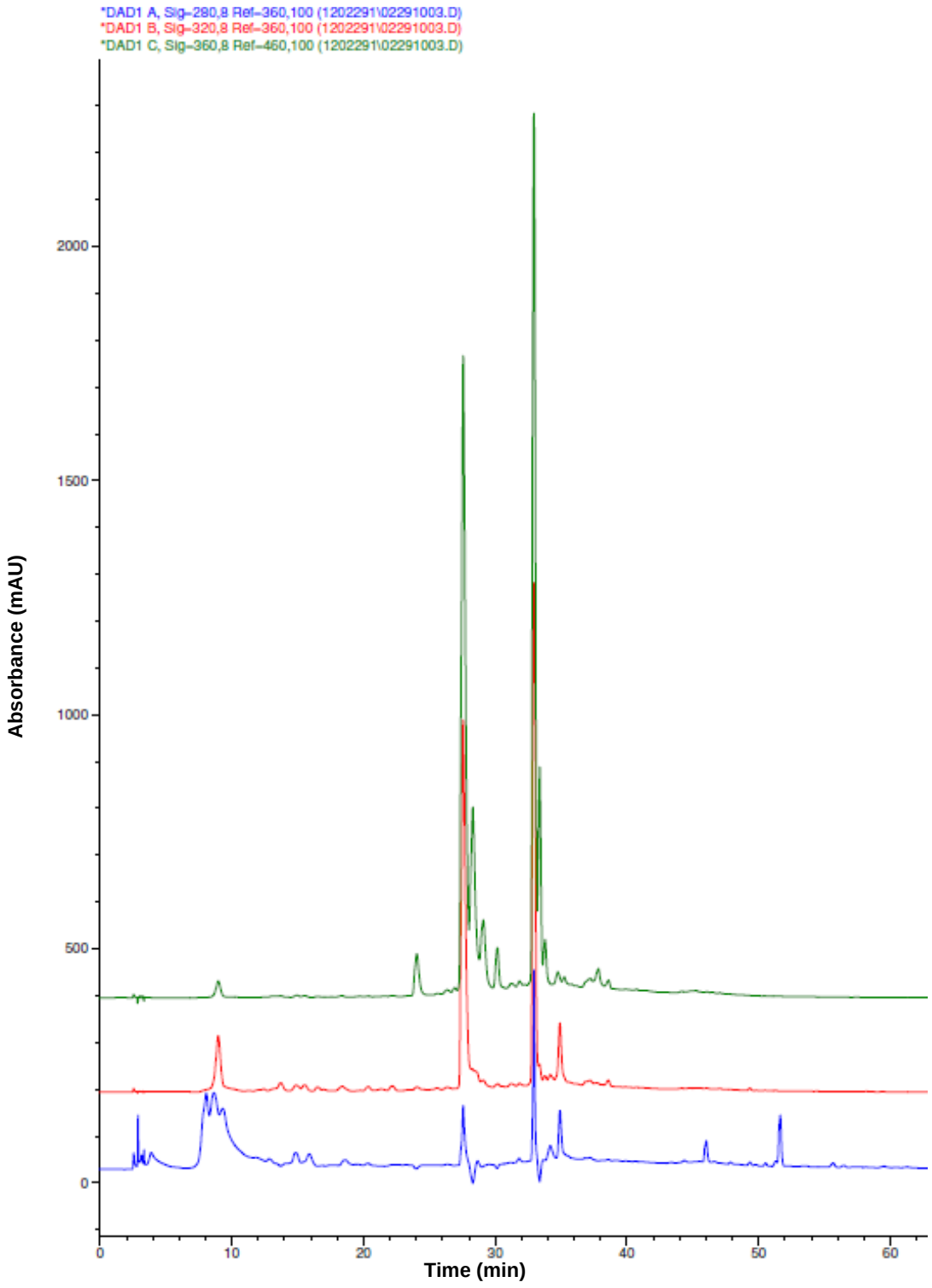

Figure 14. HPLC profile of the extract obtained upon hydrolysis from *Xeranthemum annuum*
Source: Authors

2.

In order to conduct quantitative HPLC determination, the calibration curves of gallic acid, chlorogenic acid and quercetin were constructed.

HPLC profiles of the extracts obtained from the flowers of each plant are indicated in Figures 7 to 14.

Table 1. The quantity of polyphenolic compounds of flowers of four plants (as mg/100 g dry flowers).

Polyphenolic compound	*Silene compacta* (SC)		*Digitalis lamarckii* (DL)		*Xeranthemum annuum* (XA)		*Epilobium hirsutum* (EH)	
	Extract 1	Extract 2	Extract 1	Extract 2	Extract 1	Extract 2	Extract 1	Extract2
Benzoic acids (as gallic acid equivalent)								
p-Hydroxy benzoic acid	1.88±0.69	2.01±0.19	4.53±1.12	-	-	-	-	-
Vanilic acid	1.08±0.01	3.48±0.16	1.86±0.20	4.81±0.10	1.58±0.08	2.49±0.08	2.94±0.09	-
Gallic acid	-	0.81±0.01	-	0.11±0.00	-	-	14.33±1.27	60.03±4.87
Protocatechuic acid	-	2.94±0.18	3.11±0.09	3.08±0.46	2.17±0.22	-	-	82.11±21.15
Total	2.96±0.69	9.24±0.31	9.50±1.14	8.00±0.47	3.75±0.23	2.49±0.08	17.27±1.27	142.14±21.70
Cinnamic acids (as chlorogenic acid equivalent)								
Ferulic acid	7.05±0.24	16.25±2.15	27.81±0.11	13.22±2.20	4.92 ± 0.67	1.77±0.02	-	-
trans-Cinnamic acid	-	21.04±4.44	-	-	-	-	-	-
Chlorogenic acid	-	-	25.89±0.10	-	18.42±0.21	-	31.59±1.76	-
Caffeic acid	-	-	13.36±2.47	8.37±0.54	5.76±1.93	2.55±0.00	8.14±0.00	-
Other								
Cinnamic acids	84.82±1.15	162.84±5.83	161.20±2.06	39.00±1.53	67.37±0.82	179.99±2.31	14.54±0.53	-
Total	91.87±1.17	200.13±7.64	228.26±3.22	60.59±2.73	96.47±2.21	184.31±2.31	54.27±1.84	0.00±0.00
Flavonoids (as quercetin equivalent)								
Flavan-3-ol(total)	17.00±0.10	508.76±40.34	-	4.15±0.08	14.94±13.90	56.42±6.69	36.55±1.74	14.37±0.65
Apigenin	-	-	40.66±0.57	9.50±0.29	-	-	-	-
Luteolin	-	-	-	-	-	35.48±3.10	-	-
Quercetin-3-O-rutinoside	632.29±96.97	318.82±15.07	-	-	-	-	231.41±0.33	-
Quercetin-3-O-glucoside	137.23±19.30	112.85±4.86	-	-	45.89±11.27	49.33±0.76	469.53±3.33	-
Apigenin-7-O-glucoside	-	-	20.12±0.33	-	857.88±6.93	-	-	-
Other								
Flavonoids	19.86±2.25	46.72±1.72	111.26±5.43	0.49±0.01	717.77±50.14	1933.74±22.05	1978.12±148.06	1160.14±24.29
Total	806.38±98.90	987.15±43.37	172.04±5.47	14.14±0.30	1636.48±53.69	2074.97±23.26	2715.61±148.11	1174.51±24.30

Extraction 1: with 70% aqueous MeOH; Extraction 2: with hydrolysis in 1.2 M HCl; Other indicates corresponding to different phenolics other than standards used in the study. Each value is the average of three measurements with standard deviations.
Source: Authors

Conclusion

In this study, polyphenolic constituents, flavonoids and benzoic acids of the flowers of the traditionally used plants grown in Bolu (Turkey) obtained via extraction by methanol and chloroform of the dry samples have been determined by HPLC method.

Results showed that *E. hirsutum* L., is the richest in polyphenolics and total flavonoids and benzoic

Table 2. Benzoic, cinnamic acid and flavonoid contents of the flowers investigated.

mg/100 g dry flowers	SC		DL		XA		EH	
	70% MeOH	Hydrolysis	70% MeOH	Hydrolysis	70% MeOH	Hydrolysis	70% MeOH	Hydrolysis
Benzoic acids	2.96	9.24	9.50	8.00	3.75	2.49	17.27	142.14
Total	12.20		17.50		6.24		159.41	
Cinnamic acids	91.87	200.13	228.26	60.59	96.47	184.31	54.27	-
Total	292		288.85		280.78		54.27	
Flavonoids	806.38	987.15	172.04	14.14	1636.48	2074.97	2715.61	1174.51
Total	1793.53		186.18		3711.45		3890.12	
Overall Total	2097.73		492.53		3998.47		4103.80	

Source: Authors

acids. *S. compacta* flowers are rich in terms of cinnamic acid. These results may be attributed to why indigeneous people living in the region where the flowers were collected, and the extracts of these plants are used to treat various health problems such as burns, jaundice, abdominal pain, constipation and swelling.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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