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Original Article

Simultaneous quantification of six flavonoids in four *Scutellaria* taxa by HPLC-DAD method

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ABSTRACT

The objective of this study was to develop and validate a new HPLC method to quantify several flavonoids in the methanol extract prepared from the aerial parts of four *Scutellaria* L. taxa from flora of Turkey. A simple, sensitive and precise reversed phase HPLC-DAD method was developed and validated for simultaneous determination of six main flavonoids; scutellarein 7-*O*-β-D-glucopyranoside, hispidulin 7-*O*-β-D-glucuronopyranoside, apigenin 7-*O*-β-D-glucopyranoside, hispidulin 7-*O*-β-D-glucopyranoside, luteolin and apigenin. All standard compounds showed a good linearity ($R^2 > 0.999$) in a relatively wide concentration range (1–120 μg/ml). The limit of detection of the compounds was in the range of 0.016–1.883 μg/ml and the limit of quantification was in the range of 0.232–3.368 μg/ml. The recoveries of the selected compounds were calculated in the range of 92.20–107.93%. The amounts of flavonoids showed variation in the extracts. The developed method was found to be accurate, precise, reproducible, and can be successfully applied to identify and quantify the flavonoid composition of *Scutellaria* species.

Introduction

Scutellaria L., Lamiaceae, species have long been the subject of many kinds of phytochemical, analytical and biological studies. It has a rich and diverse phytochemical profile which is mainly represented by flavonoids, phenylethanoid glycosides, diterpenes and iridoids (Shang et al., 2010).

The genus *Scutellaria* includes about 350 species commonly known as "skullcaps" (Shang et al., 2010). It is represented by 16 species in the flora of Turkey (Edmonson, 1972; Duman, 2000). Some *Scutellaria* species have been used in different folk medicines as antiviral, antiinflammatory, anticancer, hepatoprotective agents. In Turkey, *S. orientalis* is traditionally used for constipation, wound healing as well as due to its hemostatic and tonic properties (Baytop, 1999). In East Asia, particularly in Korea, China and Japan, some species of the genus *Scutellaria* (*S. baicalensis* and *S. barbata*) are used in traditional medicine due to their sedative, antiinflammatory, antiviral, antithrombotic and antioxidant activities (Shang et al., 2010). *Scutellaria barbata* is used as pain killer, to treat swelling throat, edema, and hemorrhoids in traditional Chinese medicine (Shang et al., 2010). The roots of *S. baicalensis* are used to eliminate stasis, activate blood circulation, induce diuresis and reduce edema as well as to clear away the heat evil and expel superficial evils in China (Shang et al., 2010). In China, Korea and India, *S. indica* is consumed for analgesia, detoxification and promoting blood circulation activities (Shang et al., 2010). Additionally, in China, there are many other applications of using skullcap in traditional medicine, in combination with various other plants. Some of them are curing menstrual diseases, thyphoid fever, diarrhea, dyspnea, headache, common cold, measles, toothache, abdominal pain, dysentery and eye diseases (Shang et al., 2010). Among the herbal medicine practitioners in the United Kingdom and Ireland, herba *S. lateriflora* is generally used against anxiety and stress; also it is used for spasms, digestive disorders, hypertension, irritable bowel syndrome, insomnia and allergies (Brock et al., 2012). The herb of the skullcap is primarily sold in the form of tea in health food stores as well as tonic or in combination with other medicinal plants such as valerian and passion flower in the form of tablets against sleeping disorders in Canada (Awad et al., 2003).

Due to the extensive use in different systems of traditional medicine, many pharmacological activity studies were conducted on extracts and the purified compounds of *Scutellaria* species reporting their antitumor, antiangiogenic, hepatoprotective, antioxidant, anticonvulsant and neuroprotective activities

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(Shang et al., 2010). Previous phytochemical studies on *Scutellaria* species led to the isolation of over 295 compounds (Shang et al., 2010). Since *Scutellaria* species are obviously important in the treatment of diseases, the need for quality assessment of these species procured. Many studies were performed for the evaluation of the marker and biomarker components (mainly flavonoids) up to now. Previously, Yu et al. (2007) developed a new capillary electrophoresis method for the quantification of three major flavonoids (baicalin, baicalein and wogonin) found in *S. baicalensis*. Dong et al. (2009) also developed a micellar electrokinetic chromatography (MEKC) method as well for the quantification of wogonin, oroxylin A, chrysin, 5,7,4′-trihydroxy-8-methoxyflavone and skullcaplavone II in *S. baicalensis* methanol (MeOH) extract within 12 min. Gao et al. (2008) also investigated the baicalin, baicalein and wogonin percentages in the tinctures of American (*S. lateriflora*) and Chinese skullcap (*S. baicalensis*) by high performance liquid chromatography (HPLC) with the assistance of ultraviolet (UV) detector. Moreover, Li et al. (2009) investigated the flavonoid profile (chrysin, baicalin, baicalein, scutellarin, wogonoside, oroxylin A, tenaxin I, skullcapflavon I, wogonin and derivatives) of the *S. baicalensis* roots by liquid chromatography–UV–Visible spectrophotometry/mass spectrometry (LC–UV/MS) Additionally, Qiao et al. (2011) developed a method in order to quantify several flavonoids (scutellarin, luteolin, apigenin, baicalein, wogonin, etc.) and phenolic acids in *S. barbata* simultaneously by LC–MS/MS.

In our very recent study, we have reported the identification and quantification of phenylethanoid glycosides in four *Scutellaria* taxa from the flora of Turkey by HPLC combined with diode array detector (DAD) (Bardakci et al., 2018). Since *Scutellaria* species may belong to different subgenera according to their secondary metabolite composition (Paton, 1990), and due to the broad pharmacological activity spectrum exerted by these species, the chemistry of the genus has gained importance, and the need for metabolomics studies emerged as well. In the continuation of our analytical studies on these species, on the premise of previous HPLC studies, monoglycosidic (7-*O*-glycosidic) flavone derivatives along with two flavone aglycones were quantified in four *Scutellaria* taxa: *Scutellaria hastifolia* L., *Scutellaria albida* subsp. *velenovskyi* (Rech.f.) Greuter & Burdet, *Scutellaria orientalis* L., and *Scutellaria albida* L. (*S. hastifolia*, *S. velenovskyi*, *S. orientalis* ssp. *pinnatifida* and *S. albida* ssp. *albida*, according to the Flora of Turkey and East Aegean Islands, respectively) by a new developed HPLC-DAD method.

Materials and methods

Chemicals and reagents

MeOH and acetonitrile (ACN) were HPLC gradient grade level and purchased from Sigma-Aldrich, St Louis USA. Acetic acid (AA) was obtained from Riedel de Haen. Ultrapure water was obtained from Millipore Simplicity system (Millipore, Bedford, MA, USA). The reference compounds, scutellarein 7-*O*-β-D-glucopyranoside (**1**), hispidulin 7-*O*-β-D-glucuronopyranoside (**2**), apigenin 7-*O*-β-D-glucopyranoside (**3**) and hispidulin 7-*O*-β-D-glucopyranoside (**4**) were previously isolated from *S. hastifolia* (Bardakci et al., 2015). Their purities were determined to be more than 95% by HPLC analysis. Luteolin (**5**) and apigenin (**6**) were purchased from Sigma. Membrane filters (Sartorius model 0.45 μm PTFE filter) were used for the filtration of the mobile phase, standards and samples prior to working with HPLC system.

Plant materials

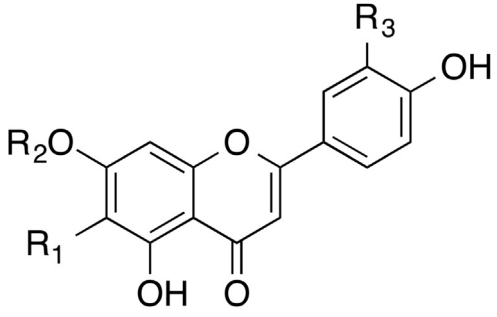
The localities where *Scutellaria* specimens were collected are as follows: *S. hastifolia* L. from Sakarya, Turkey in June 2011, *Scutellaria albida* subsp. *velenovskyi* (Rech.f.) Greuter & Burdet from Karabük, Turkey in July 2011, *S. albida* L. from Istanbul, Turkey in June 2013, *S. orientalis* L. from Eskişehir, Turkey in June 2013. The plant materials were dried at dark and fresh air ventilated rooms and stored at 25 °C in airtight containers till further use.

Plant materials were authenticated by one us (HK) before any process. Voucher herbarium specimens for *S. hastifolia* (YEF 11005), *Scutellaria albida* subsp. *velenovskyi* (YEF 11006), *S. albida* (YEF 13008) and *S. orientalis* (YEF 13009) were deposited at the herbarium of the Faculty of Pharmacy, Yeditepe University, Istanbul, Turkey.

Preparation of standard solutions

Stock solutions of the flavonoids were initially prepared as 1000 μg/ml in MeOH, and then diluted with MeOH (1–120 μg/ml). At least five concentration levels were selected for calibration curve of each compound.

Flavonoids utilized in the analysis were scutellarein 7-*O*-β-D-glucopyranoside (**1**), hispidulin 7-*O*-β-D-glucuronopyranoside (**2**), apigenin 7-*O*-β-D-glucopyranoside (**3**), hispidulin 7-*O*-β-D-glucopyranoside (**4**), luteolin (**5**) and apigenin (**6**). Working concentrations were 5, 15, 40, 75 and 120 μg/ml for scutellarein 7-*O*-β-D-glucopyranoside, 5, 15, 30, 40 and 75 μg/ml for hispidulin 7-*O*-β-D-glucuronopyranoside, 1, 5, 15, 30, 40, 75 and 120 μg/ml for apigenin 7-*O*-β-D-glucopyranoside, 1, 5, 15, 40 and 75 μg/ml for hispidulin 7-*O*-β-D-glucopyranoside and 1, 5, 15, 30, 40 and 75 μg/ml for the aglycones luteolin and apigenin. 10, 20 and 60 μg/ml concentrations of each standard flavonoid solution were selected as quality control (QC) concentrations at three different levels during validation of the method developed. Quercetin was selected as internal standard.



1 R_1=OH; R_2=Glc; R_3=H
2 R_1=OCH_3; R_2=GluA; R_3=H
3 R_1=H; R_2=Glc; R_3=H
4 R_1=OCH_3; R_2=Glc; R_3=H
5 R_1=R_2=H; R_3=OH
6 R_1=R_2=R_3=H

Preparation of sample solutions

All crude drug samples were powdered to a homogenous size by a mill. Each *Scutellaria* species were accurately weighed (1 g) and extracted twice with 20 ml of HPLC grade MeOH in ultrasonic bath for 30 min at 30 °C for each time. The pooled extracts were concentrated by rotary evaporator at 45 °C. The amounts of each extract and the yields of extractions (13.4–22.7%) were calculated. Each sample was dissolved in 5 ml of MeOH and filtrated through 45 μm Sartorius filters. Appropriate dilution processes were applied with

MeOH when necessary. The working solutions were kept protected from light at −20 °C until use.

Instrumentation and chromatographic conditions

The Agilent 1100 HPLC system (Germany) was used for quantification. The system included a JP13210680 degasser, a DE33224158 quaternary pump (Germany), a DE33223285 autosampler (Germany), a DE33235065 column compartment (Germany) and a DE33219705 DAD (Germany). The chromatographic data were obtained using Agilent Chem Station software.

The chromatographic separations were carried out on the Agilent Zorbax Eclipse-C18 (4.6 × 150 mm, 3.5 μm) column with the flow rate of 0.8 ml/min. The injection volume was set at 10 μl and at least three repetitive injections were performed for samples and standard solutions Chromatographic column temperature was maintained thermostated at 25 °C and the wavelength was selected as 340 nm. Due to the maximum absorption value in the spectra obtained by DAD detector of HPLC system. The mobile phase was filtered from 0.45 μm pore sized filter and ultrasonically degassed before use. For the simultaneous analysis of the analytes gradient elution program was applied. Phase A was H_2O, the mobile phase B was ACN both containing 0.02% AA. The gradient profile was set as follow: 0 min 15% B, 10 min 20% B, 13 min 20% B, 15 min 25% B, 20 min 30% B, 25 min 30% B, 30 min 50% B. Identification of the peaks were based on the comparison of retention times and the UV spectra presented in the chromatogram with those of standard compounds. In total, qualitative and quantitative analysis of six flavonoids in four *Scutellaria* extracts, was concluded in 29 min by using this method.

Statistics

All tests were performed in triplicates. Data are expressed as mean ± relative standard deviation. Slopes and intercepts of calibration graphs were calculated by linear regression. Correlation coefficient (r^2) values were obtained by using MS Office Excell programme.

Results

Optimization of the method

A variety of methods, solvents, times and column types were used in order to obtain the optimum separation of each standard in the extracts. MeOH:H_2O and ACN:H_2O combinations with different buffer agents (FA, AA), various flow rates (0.5–1.5 μl), column temperatures (10–30 °C) and column types (zorbax cyano column, zorbax C_{8-18} column, and zorbax phenyl column with diverse pore and column sizes) were employed in order to obtain best resolution and the shortest analysis time. AA was preferred as a buffer agent due to eliminate the peak tailing and for the high resolution of peaks.

During the study, samples were evaluated and controlled for each discussed experimental conditions. Because the experimental conditions can allow the analysis of standard compounds but they cannot be applicable for the samples. To verify the compounds

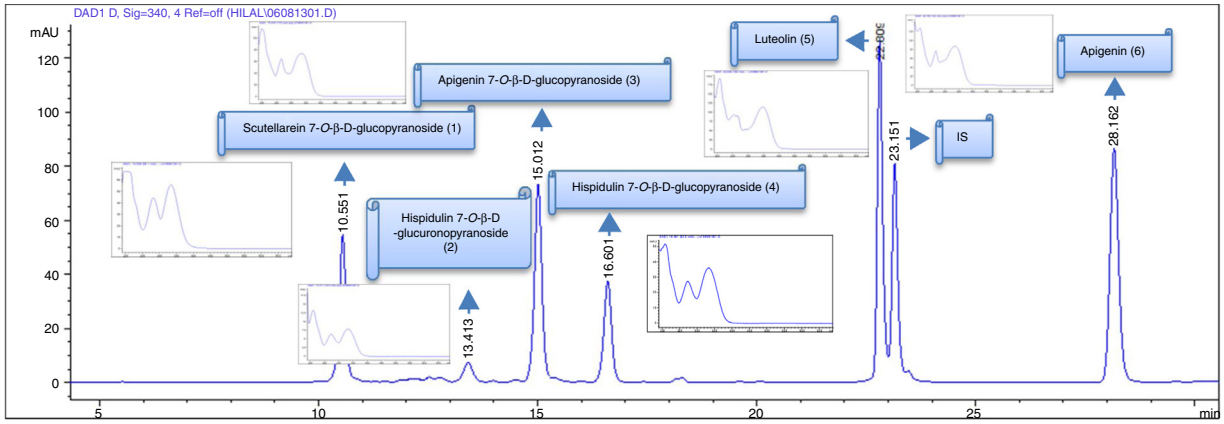


Fig. 1. HPLC chromatogram and UV spectra of the standard substances. Column: Zorbax XDB-C18 (4.6 × 150 mm, 3.5 μm), column thermostat: 25 °C, flow rate: 0.8 ml/min, wavelength: 340 nm, eluents: ACN (0.02% AA) and H_2O (0.02% AA) (15–50%).

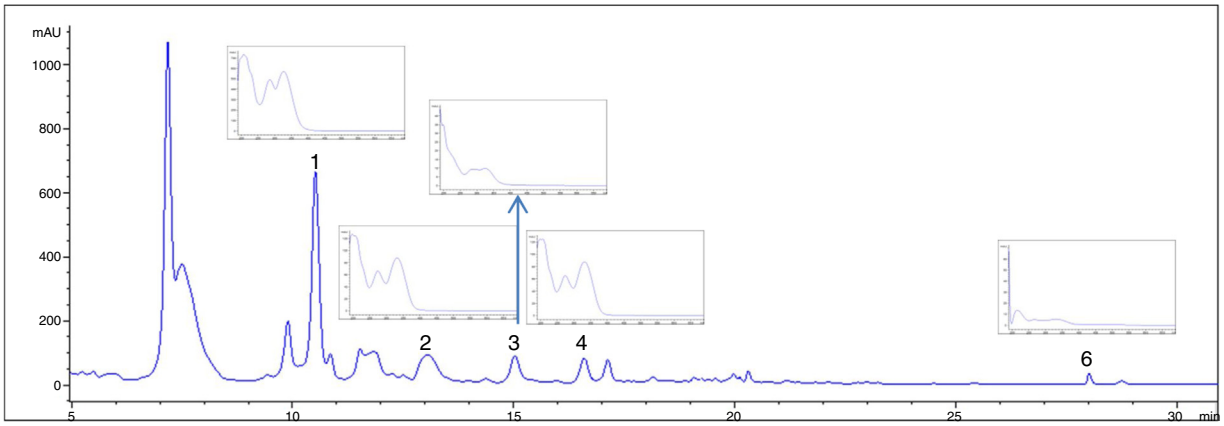


Fig. 2. HPLC fingerprint of the methanolic extract of *S. hastifolia*. Conditions: same as Fig. 1.

in the sample chromatogram, UV spectra of standard compounds were obtained and recorded for single injection of standard compound alone (Fig. 1). After obtaining the sample chromatogram, related compounds in the plant samples, were signed according to their retention time values (Fig. 2). Then, UV spectra of these signed peaks were investigated and compared with previously obtained spectra.

Under the proposed analytical conditions, good baseline resolution was obtained for all analytes. In total qualitative and quantitative analysis of six flavonoids in four *Scutellaria* species was lasted only for 29 min by this method. The correlation coefficient (r^2) of all the calibration curves were constantly greater than 0.999.

Method validation

The newly developed methods were validated in terms of linearity (calibration curve), sensitivity [limit of detection (LOD), limit of quantification (LOQ)], accuracy, precision, robustness and system suitability according to ICH and FDA guidelines (ICH, 2005; McPolin, 2009; Shabir, 2003).

System suitability

The system suitability is used to verify that HPLC system and method are capable of providing data with admissible quality. System suitability results are compared with the values given in the FDA guidelines (Shabir, 2003). The capacity factor ($k' > 2$), resolution ($R_s > 2$), tailing factor ($T \leq 2$) and theoretical plates ($N > 2000$) are evaluated in this respect and compared with the theoretical data. For this purpose standard mixture was analyzed six times and obtained values given in Table 1. According to these results, all parameters were in the appropriate range to show the system suitability.

Linearity, LOD and LOQ

The calibration curves were constructed from peak areas of the reference compounds versus their concentrations. The correlation coefficients (r^2) of all the calibration curves were consistently greater than 0.999 in the relatively wide concentration range (1–120 μg/ml). The limit of detection (LOD) was defined as the lowest concentration level that can be detected and limit of quantification (LOQ) was defined as the lowest concentration level that can be quantified. LOD and LOQ were calculated by using standard deviation of calibration curve intercept values (S_a) according to the ICH guidelines and following equations were used: $LOD = 3S_a/m$, $LOQ = 10S_a/m$. In these equations "m" was the slope of calibration curve. LOD values were calculated between 0.016 and 1.883 μg/ml and the LOQ values were found in the range of 0.232–3.368 μg/ml. All detailed analysis performance characteristics are given in Table 2.

Accuracy

Accuracy of the method was calculated for both intra- and inter-day variations using at least triplicate analysis. In order to check the accuracy of the developed method, recovery values were calculated by using quality control concentrations of standard substances. Calibration curve was used for this purpose. Triplicate experiments of three independent concentrations were performed for each substance. The intra- and the inter-day recoveries of the analytes were found between 92.2–107.83% and 92.42–107.93%, respectively. In addition, RSD values were found between 0.09–1.14% and 0.17–4.51%, respectively and given in Table 3. In recovery studies, recovery values should be between 90% and 110%. All results obtained are in this range. It shows that, this method is appropriate at determined concentration range.

Precision

Instrument precision was evaluated by analyzing the standard solutions in nine replicate injections. The retention times of the analytes were utilized to determine the intra- and inter-day variability of the method. Three different concentrations (10, 50 and 100 μg/ml) were prepared for each standard compound as QC samples. These levels were selected as low level (10 μg/ml), medium level (50 μg/ml) and high level (100 μg/ml) QC samples. The intra-day precision was calculated using nine replications prepared from the QC solutions within the day, while inter-day precision was performed over three consecutive days. The relative standard deviation (RSD) was taken as a measure of precision. The obtained data showed that intra-day RSD values were in the range of 0.04–0.30%

Table 1
System suitability parameters of standard compounds [scutellarein 7-*O*-β-D-glucopyranoside (**1**), hispidulin 7-*O*-β-D-glucuronopyranoside (**2**), apigenin 7-*O*-β-D-glucopyranoside (**3**), hispidulin 7-*O*-β-D-glucopyranoside (**4**), luteolin (**5**), apigenin (**6**)].

Parameter	1	2	3	4	5	6
k'	4.696	6.241	7.105	7.962	11.314	14.204
R_s previous peak	31.32	1.625	4.697	5.137	14.277	16.405
R_s next peak	4.954	4.684	5.153	4.57	1.546	15.797
T	0.922	1.208	1.005	0.981	1.283	1.114
N	20,875	16,339	33,206	38,463	115,251	855,528

Table 2
Calibration curve parameters for standard compounds [scutellarein 7-*O*-β-D-glucopyranoside (**1**), hispidulin 7-*O*-β-D-glucuronopyranoside (**2**), apigenin 7-*O*-β-D-glucopyranoside (**3**), hispidulin 7-*O*-β-D-glucopyranoside (**4**), luteolin (**5**), apigenin (**6**)] ($n = 3$).

Parameter	1	2	3	4	5	6
Dynamic range (μg/ml)	5–120	5–75	5–120	1–75	1–75	1–75
Slope	33.231	6.3613	47.756	23.153	54.017	56.681
Intercept	−83.832	−9.401	−148.11	−4.229	−12.128	−17.866
S_a[a]	0.829	1.328	3.891	0.807	1.332	1.786
S_b[b]	0.453	0.759	0.982	0.159	0.789	0.326
Limit of Detection (LOD, μg/ml)	1.709	1.883	1.500	0.016	0.058	0.234
Limit of Quantification (LOQ, μg/ml)	1.882	3.368	2.220	0.261	0.232	0.457
Correlation coefficient	0.999	0.999	0.998	0.999	0.999	0.999

[a] S_a, standard deviation of slope.
[b] S_b, standard deviation of intercept.

Table 3
Recovery and relative standard deviation values for scutellarein 7-*O*-β-D-glucopyranoside (**1**), hispidulin 7-*O*-β-D-glucuronopyranoside (**2**), apigenin 7-*O*-β-D-glucopyranoside (**3**), hispidulin 7-*O*-β-D-glucopyranoside (**4**), luteolin (**5**), apigenin (**6**)] ($n = 9$).

Analyte	Amount (μg/ml)	Intraday		Interday	
		Recovery %	RSD %	Recovery %	RSD %
1	10	99.71	0.09	98.06	0.89
	20	98.58	0.14	98.25	1.08
	60	99.83	0.50	98.08	01.08
2	10	92.57	1.14	92.42	0.27
	20	99.61	0.65	100.47	4.51
	60	92.2	0.32	93.12	0.89
3	10	100.11	0.16	100.33	2.53
	20	100.83	0.17	102.16	1.80
	60	99.74	0.31	98.26	1.42
4	10	99.36	0.25	98.19	0.17
	20	99.30	0.21	99.47	0.94
	60	100.99	0.29	100.48	0.86
5	10	107.26	0.10	107.93	0.65
	20	99.74	15	100.09	0.64
	60	101.05	0.30	101.82	0.93
6	10	107.83	0.19	102.12	2.28
	20	104.65	0.15	104.99	1.36
	60	103.42	0.29	100.78	0.92

RSD, relative standard deviation.

Table 4
Intraday and interday precision values of retention times of scutellarein 7-*O*-β-D-glucopyranoside (**1**), hispidulin 7-*O*-β-D-glucuronopyranoside (**2**), apigenin 7-*O*-β-D-glucopyranoside (**3**), hispidulin 7-*O*-β-D-glucopyranoside (**4**), luteolin (**5**), apigenin (**6**)] ($n = 9$).

	Intraday studies		Retention times ± RSD			
Amount (μg/ml)	**1**	**2**	**3**	**4**	**5**	**6**
10	10.55 ± 0.32	13.41 ± 0.30	15.01 ± 0.31	16.81 ± 0.18	22.80 ± 0.11	28.16 ± 0.05
20	10.55 ± 021	13.82 ± 0.24	15.06 ± 0.26	16.81 ± 0.18	22.80 ± 0.12	28.16 ± 0.09
60	10.55 ± 0.22	13.40 ± 0.21	15.06 ± 0.23	16.82 ± 0.17	22.79 ± 0.05	28.16 ± 0.04
	Interday studies		Retention times ± RSD			
10	10.56 ± 0.64	13.41 ± 0.27	15.01 ± 0.18	16.81 ± 0.39	22.80 ± 0.14	28.15 ± 0.17
20	10.55 ± 0.43	13.40 ± 0.29	15.01 ± 0.33	16.83 ± 0.23	22.79 ± 0.11	28.14 ± 0.12
60	10.55 ± 0.67	13.40 ± 0.57	15.08 ± 0.63	16.89 ± 0.54	22.78 ± 0.19	28.16 ± 0.19

RSD, relative standard deviation.

and the inter-day RSD values were found in the range of 0.11–0.67% as shown in Table 4. In precision studies, standard deviation of results should be less or equal than 2%. All values obtained provided this situation. It shows that, the developed method is a precise method.

Robustness

Robustness of the method was performed to document whether the method was susceptible to variations in method parameters or not. Robustness studies of the developed method were evaluated according to the ICH guidelines. Temperature (23–27 °C), acetic acid percentage (0.015–0.025%) and wavelength (330–350 nm) were changed deliberately and the effects of the changes were investigated via recovery values. Obtained values are presented in Table 5 which are in the range between 90% and 110%.

Quantification

Chromatographic investigation of the analytes was held by comparing the UV spectra of the corresponding peaks in the plant extracts and the standards (Figs. 1 and 2). The contents of the each *Scutellaria* sp. were determined by using the areas corresponding calibration curves and the calculated amounts are given in Table 6.

Hispidulin 7-*O*-β-D-glucuronopyranoside was found as major flavonoid (1.59 ± 0.04%) while apigenin was the minor one (0.005 ± 0.00%) in *S. hastifolia*. Scutellarein 7-*O*-β-D-glucopyranoside was found as major flavonoid (0.10 ± 0.012%) and apigenin 7-*O*-β-D-glucopyranoside was the minor one (0.028 ± 0.00%) in *S. velenovskyi*. Apigenin 7-*O*-β-D-glucopyranoside was found as major flavonoid (0.358 ± 0.00%) and hispidulin 7-*O*-β-D-glucuronopyranoside was found as the minor one (0.029 ± 0.05%) in *S. orientalis* ssp. *pinnatifida*.

Among the four plant samples, *S. hastifolia* showed the highest of hispidulin 7-*O*-β-D-glucuronopyranoside, scutellarein 7-*O*-β-D-glucopyranoside, hispidulin 7-*O*-β-D-glucopyranoside and apigenin 7-*O*-β-D-glucopyranoside content, *S. orientalis* showed the highest amount of luteolin content and finally *S. velenovskyi* showed the highest amount of apigenin content.

Discussion

Isolation, characterization and identification of a broad range of secondary metabolites from plant samples are mostly performed by HPLC analysis coupled with some other methods such as DAD, MS, etc. In this study, it was found that binary gradient solvent system could be used in separation of flavonoids in *Scutellaria* extracts.

Table 5
Results of robustness experiments ($n = 3$).

Analytes	Amount (μg/ml)	Temperature 23 °C	Temperature 27 °C	0.015% acetic acid	0.025% acetic acid	Wavelength 330 nm	Wavelength 350 nm
1	10	92.209 ± 0.213	92.636 ± 0.52	93.738 ± 0.14	92.375 ± 0.527	93.514 ± 0.934	94.073 ± 1.241
	20	99.11 ± 1.412	98.638 ± 0.579	97.742 ± 0.21	98.344 ± 0.097	98.734 ± 1.71	97.273 ± 0.288
	60	95.374 ± 0.639	97.135 ± 1.153	96.964 ± 1.028	97.3 ± 0.224	98.565 ± 1.804	98.644 ± 2.018
2	10	93.864 ± 0.271	92.301 ± 3.489	91.293 ± 0.241	95.77 ± 0.251	95.77 ± 0.215	94.296 ± 1.027
	20	100.896 ± 0.294	100.253 ± 1.658	98.59 ± 0.840	97.891 ± 0.669	97.891 ± 0.699	91.499 ± 1.571
	60	90.767 ± 1.074	92.39 ± 4.222	94.572 ± 1.232	94.861 ± 0.188	95.861 ± 0.188	93.811 ± 1.702
3	10	99.069 ± 0.477	101.056 ± 0.553	101.399 ± 0.299	99.946 ± 0.561	102.301 ± 0.575	102.984 ± 0.695
	20	101.442 ± 1.151	100.886 ± 0.644	102.084 ± 0.207	100.229 ± 0.144	101.879 ± 2.338	101.728 ± 2.817
	60	100.134 ± 0.606	101.68 ± 1.602	101.047 ± 0.823	101.996 ± 0.204	104.972 ± 1.506	105.095 ± 1.655
4	10	93.395 ± 0.085	93.059 ± 0.393	94.537 ± 0.213	94.535 ± 0.075	94.518 ± 0.529	94.426 ± 0.733
	20	99.229 ± 1.132	99.777 ± 0.688	98.165 ± 0.431	99.463 ± 0.269	99.411 ± 0.827	99.532 ± 0.483
	60	100.493 ± 0.622	100.745 ± 1.176	101.965 ± 1.356	101.078 ± 0.413	102.447 ± 1.427	102.971 ± 1.37
5	10	106.598 ± 0.133	107.126 ± 0.777	107.267 ± 0.108	106.842 ± 0.397	107.539 ± 0.837	107.369 ± 0.709
	20	100.331 ± 1.05	101.063 ± 0.736	100.152 ± 0.199	98.969 ± 0.094	99.193 ± 0.513	99.34 ± 0.44
	60	100.902 ± 0.66	101.622 ± 1.024	102.703 ± 0.698	102.142 ± 0.236	102.766 ± 1.58	102.805 ± 1.53
6	10	107.901 ± 0.086	108.74 ± 0.67	107.652 ± 0.146	106.736 ± 0.43	106.961 ± 0.597	107.062 ± 0.685
	20	106.587 ± 1.116	105.28 ± 0.673	105.881 ± 0.27	104.92 ± 0.129	104.718 ± 0.529	104.784 ± 0.546
	60	102.599 ± 0.652	102.253 ± 0.932	102.316 ± 0.468	101.861 ± 0.194	101.995 ± 1.624	102.056 ± 1.525

Table 6
Contents of standard compounds [scutellarein 7-*O*-β-D-glucopyranoside (**1**), hispidulin 7-*O*-β-D-glucuronopyranoside (**2**), apigenin 7-*O*-β-D-glucopyranoside (**3**), hispidulin 7-*O*-β-D-glucopyranoside (**4**), luteolin (**5**), apigenin (**6**)] in plant materials (*Scutellaria albida*; *S. hastifolia*; *S. orientalis*; *S. albida* subsp. *velenovskyi*) (w/w%). Results are given as the mean of triplicates ± S.D.

Plant samples	1	2	3	4	5	6
S. hastifolia	1.32 ± 0.021	1.59 ± 0.04	0.146 ± 0.014	0.262 ± 0.003	n.d.	0.005 ± 0.00
S. albida subsp. *velenovskyi*	0.10 ± 0.012	0.06 ± 0.08	0.028 ± 0.00	0.029 ± 0.021	0.041 ± 0.00	0.032 ± 0.00
S. orientalis	0.038 ± 0.00	0.029 ± 0.05	0.358 ± 0.00	n.d.	0.047 ± 0.00	0.20 ± 0.00
S. albida	n.d.	n.d.	0.036 ± 0.00	0.098 ± 0.021	n.d.	0.03 ± 0.00

S.D., standard deviation; n.d., not detected.

This solvent system is optimized after experiencing various solvent systems such as MeOH:H_2O mixtures with different buffer solvents such as acetic acid and formic acid. The details of chromatographic conditions were given in the Materials and Methods section. With the use of ACN:H_2O (0.02 AA%) as a mobile phase and optimum gradient system as well, separations were accomplished. This solvent system gives a better resolution, sharp and symmetrical peaks.

In some points, peaks did not completely separated due to the complex composition of plant samples although various different methods were applied. In this case, different techniques were performed. The amounts were calculated according to the heights of the peaks instead of areas. Similar results were seen with heights and areas. As it is seen, the peaks with lower abundance are attributed to the impurity in the sample or system. Since peak to valley ratio is high enough, it was able to apply valley-to valley integration method.

In hereby study, it was aimed to quantify major flavonoids in the MeOH extract of *S. hastifolia*, *S. velenovskyi*, *S. orientalis* and *S. albida*. Flavonoids used in this work were scutellarein 7-*O*-β-D-glucopyranoside, hispidulin 7-*O*-β-D-glucuronopyranoside, apigenin 7-*O*-β-D-glucopyranoside, hispidulin 7-*O*-β-D-glucopyranoside, luteolin and apigenin.

Previously, total flavonoid contents of selected species were measured. According to those results highest quercetin equivalent total flavonoid content was seen in *S. orientalis*, *S. hastifolia*, *S. velenovskyi* and *S. albida*, respectively. Except *S. hastifolia* due to most of the flavonoids, used in this study were isolated from *S. hastifolia*, the results were consistent with the previous study (Bardakci et al., 2015). The highest flavonoid content of this study was seen in *S. orientalis*, *S. velenovskyi* and *S. albida*, respectively, showing similar results with the previous one.

In *S. hastifolia*, the quantities of the isolated flavonoids, namely, scutellarein 7-*O*-β-D-glucopyranoside, hispidulin 7-*O*-β-D-glucuronopyranoside, apigenin 7-*O*-β-D-glucopyranoside, hispidulin 7-*O*-β-D-glucopyranoside were calculated, plus apigenin aglycone was detected and the amount was calculated. It was predictable because its glucose derivative was isolated in this plant. In *S. velenovskyi*, all the tested flavonoids were detected and quantified. As mentioned above, in a study concerning the chemical composition of the MeOH extract of *S. velenovskyi*, Skaltsa et al. isolated glucuronic acid derivatives of scutellarein and hispidulin verifying findings of this study (Matsa et al., 2013). In *S. orientalis*, among the tested flavonoids, except hispidulin 7-*O*-β-glucopyranoside, all of them were detected. Eventually, in *S. albida*, apigenin 7-*O*-β-glucopyranoside, hispidulin 7-*O*-β-glucopyranoside and apigenin were quantified.

Although there are plenty of HPLC-DAD studies for few of the selected flavonoids in the literature (especially apigenin, luteolin and apigenin glucoside) there is no study concerning all the flavonoids utilized in this study. Moreover, studies concerning the *Scutellaria* species mostly cover their chemical composition especially their terpenic and volatile oil composition not related their phenolic composition analysis. To the best of our knowledge, there is no other study related with the neither HPLC analysis of selected species nor selected flavonoids. Due to this information, there is no other study for us to compare the results.

The quantification of the mentioned flavonoids in *Scutellaria hastifolia*, *S. velenovskyi*, *S. albida* ssp. *albida*, *S. orientalis* ssp. *pinnatifida* by HPLC is being reported for the first time which might be used in the future for the chemotaxonomic and quality control studies of the extracts prepared from these species.

Authors contributions

HK contributed in collecting and identifying plant materials, herbarium sample preparation, study design, evaluation of results and critical reading of the manuscript. HBA prepared the working samples and standard solutions, performed the chromatographic analysis, method validation, evaluated the results and wrote the manuscript. ETA performed the chromatographic analysis and performed method validation studies. All authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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