Journal of Medicinal Plants Research Vol. 6(20), pp. 3664-3671, 30 May, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR12.298
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Qualitative and quantitative determination of polyphenol content of *Scilla bifolia*

Maria-Luiza Bălăşoiu[1], Daniela Călina[2,4]*, Laurian Vlase[3] and Maria-Viorica Bubulică[2]

[1]Hermes Pharmacy, Buzău, Romania.
[2]Faculty of Pharmacy, University of Medicine and Pharmacy, Craiova, Romania.
[3]Faculty of Pharmacy, Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania.
[4]2-4 Petru Rares Street, 200638 Craiova, Romania.

Accepted 7 March, 2012

High-performance liquid chromatography with mass spectrometry and UV detection was applied for qualitative of eighteen polyphenols and quantitative determination of fourteen of them in *Scilla bifolia* bulbs and aerial parts. Caffeic acid, p-coumaric acid, ferulic acid, luteolin and apigenin were found in all hydrolysed and unhydrolysed sample extracts. Sinapic acid was detected and quantified only in hydrolyzed extract of bulbs and aerial parts indicating that it is present only as ester.

Key words: *Scilla bifolia*, polyphenol, high-performance liquid chromatography-diode-array detection-mass spectrometry (HPLC-DAD-MS).

INTRODUCTION

Lately, there have been published a series of articles involving phytocomponents isolated from natural sources for the synthesis of new bio and nanomaterials with biological activities, which are of fundamental interest to modern science due to their vast applications in controlled drug release (Mihaiescu et al., 2011), drug targeting (Grumezescu et al., 2011), inhibition of microbial biofilm growth (Saviuc et al., 2011a), biosensors (Chirea et al., 2011), antimicrobial therapy (Saviuc et al., 2011b) or medical diagnostics. It is necessary that all these components be quantified in order to obtain exactly the desired properties.

Scylla genus includes about 125 species dispersed in temperate zone of Europe, Asia and North Africa. Among the species of *Scilla* genus, the most known and used is *Scilla maritima* that contains numerous active compounds: Cardiac glycosides, flavonoids, stigmasterol, anticyanosides and mucilages (Norn and Kruse, 2004).

Scilla bifolia (chives, two-leaf-squill) species is characterized by a relatively small size (10 to 30 cm), the aerial parts are developed from a globular bulb (Popovici et al., 1993). It grows through deciduous forests. Stem has two basal leaves. The blue flowers (1 to 7) are grouped in raceme.

Two-leaf-squill is a melliferous, ornamental and medicinal plant. Fresh flowers are used for painting in blue of natural fibers.

Liquid chromatography is a separation technique suitable for analysis of low or nonvolatile molecules, thermally fragile compounds, with multiple applications in pharmaceutical science domain (Fodorea et al., 2005; Ding et al., 2007).

When a liquid-chromatograph is coupled with a mass spectrometer, analysis can be made with high sensitivity and specificity comparative with other LC detectors. Liquid chromatography–mass spectrometry (LC-MS) can also identify and quantify compounds without chromophore group. Recently, high-performance liquid chromatography (HPLC) with diode-array detection (DAD) and electrospray ionization mass spectrometry techniques were described for determination of various compound classes (Termentzi et al., 2008; Oszmiański et al., 2011).

The method presented in this study is based on a method already published (Ivanescu et al., 2010; Parvu et al, 2010).

As far as we know, there are no previous studies regarding chemical composition of *S. bifolia* with the exception of traditional medicine. However, based on

*Corresponding author. E-mail calinadaniela@gmail.com, Tel/Fax: 0040251533713.

chemotaxonomy's principles and its affinity with some other species of *Scilla,* chives offer the perspective of discovering some active principles. The aim oh this study was to determine the plant's pharmacological value base on its polyphenol content.

MATERIALS AND METHODS

Plant material

Aerial parts and bulbs of *S. bifolia* were collected at the flowering stage from the spontaneous flora around Craiova City, Dolj County, Romania between March and April 2010. The plant was identified and two voucher specimens (SBB2010 and SBH2010) were deposited in our institution herbarium.

Samples were washed with doubly distilled water in order to remove dust and damaged portions. The leaves, flowers, stems and bulbs were air-dried (25 to 27°C) in a dark room. After drying, the collected samples were ground to fine powder and passed through a sieve (24 mesh) then the ground samples were dried again in a convection oven at 45°C until there was no change in weight (Mohd et al., 2002).

Extract preparation

Unhydrolyzed ethanolic extract

About 10 g of powdered aerial parts or bulbs were refluxed separately with 100 ml volumes of ethanol 70%, 3 times successively at room temperature for 60 min. Combined extracts were brought to 10 ml by rotary evaporation under reduced pressure.

Hydrolyzed ethanolic extract

Hydrolysis was carried out according to the following protocol: Half of the ethanolic extract is diluted with an equal amount of 2 N HCl solution and maintained on a water bath at 80°C for 60 min. The volume of liquid evaporated was filled during hydrolysis by adding an appropriate amount of purified water. The hydrolyzed extract obtained was injected into the chromatographic system (Ivanescu et al., 2010).

Chemicals and reagents

All chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich (USA), Merck (Germany), Dalton (USA) or Roth (Germany).

Standard references includes caftaric acid, gentisic acid (2,5-dihydroxybenzoic acid), caffeic acid, chlorogenic acid, p-coumaric acid, feluric acid, sinapic acid, hyperoside, isoquercitrin, rutin, myricetol, fistein, quercitrin, quercetol, patuletin, luteolin, kaempferol and apigenin.

Chromatographic conditions

Equipment

Equipment used includes HPLC coupled with diode array detector and mass spectrometry: HP 1100 Series system equipped with binary pump, autosampler (HP 1100 Series) and thermostat (HP 1100 Series).

Detectors

Detectors used include:

1. UV detector (HP 1100 Series);
2. Mass spectrometer: Agilent Ion Trap 1100 V.

HPLC working conditions

For the separations, a Zorbax SB-C18 reversed-phase (100 mm × 3.0 mm i.d., 3.5 µm) column was used, with a Zorbax SB-C18 precolumn. The mobile phase was prepared from methanol:acetic acid 0.1% (v/v), gradient elution (beginning with 5% methanol, end ending with 42% methanol for 38 min, return to initial condition). The flow rate was: 1 ml/min at 48°C; Injection volume was 5 µl.

Detection: UV was done at 330 nm for the first 17 min and at 370 nm for the next 21 min; while the MS working conditions includes:

1. Ion source: Electrospray ionization interface (ESI); ionization mode: Negative;
2. Nebuliser: Nitrogen; pressure: 70 psi;
3. Drying gas: Nitrogen; flow: 12 L/min; temperature: 360°C;
4. Capillary potential: +3000 V;
5. Analysis mode: Monitoring specific ions (polyphenolcarboxylic acids) or AUTO MS (flavonoids and their aglycones) (Zehl et al., 2011).

Method validation

Calibration curves (five points) with good linearity (R>0.999) were used for each compound. Standard deviation ranged from 0.04 to 0.19 min for retention times. The detection limit (LOD) for each standard was calculated on the basis of signal to noise ratio greater than 3. The quantification limit (LOQ) was calculated as the amount of analyte giving a signal ten times higher than the noise level. Inter-day variations were less than 4% for all analytes indicating that the method has good reproducibility. Chemstation and data analysis software was used for data processing.

RESULTS AND DISCUSSION

Determination of polyphenols was performed both on hydrolyzed ethanolic extracts and on unhydrolyzed extracts.

Generally, some flavone aglycones or some poly-phenolcarboxylic acids are found bound in the form of glycosides, esters, etc. Achievement of acid hydrolysis of these compounds released them and provided more information about the chemical composition (polyphenols) of the product studied.

The method is applicable for qualitative analysis (18 compounds) and quantitative analysis (14 compounds).

Analysis of polyphenols by UV detection

Compounds from each class were detected at a wavelength corresponding to the maximum absorption of

Table 1. Chromatographic characteristics of analyzed polyphenols.

S/N	Compound	Retention time (min)	MS mode	Specific ions for identification $[M-H]^-$ > Ions from the spectrum
1	Caftaric acid	2.10	MRM*	311>148.6, 178.6
2	Gentisic acid	2.15	MRM	153>108.7
3	Caffeic acid	5.6	MRM	179.4>134.7
4	Chlorocgenic acid	5.6	MRM	353.5>178.7, 190.7
5	P-Coumaric acid	8.7	MRM	163> 118.7
6	Feluric acid	12.2	MRM	193.2> 133.7, 148.7, 177.6
7	Sinapic acid	14.3	MRM	223.4>148.6, 163.6, 178.7, 207.7
8	Hyperoside	18.6	SIM**	463.1
9	Isoquercitin	19.6	SIM	463.1
10	Routine	20.2	SIM	609.1
11	Myricetol	20.7	SIM	317.1
12	Fistein	22.6	SIM	285.1
13	Quercitin	23.0	SIM	447.1
14	Quercetol	26.8	SIM	301.1
15	Patuletin	28.7	SIM	331.1
16	Luteolin	29.1	SIM	285.1
17	Kaempferol	31.6	SIM	285.1
18	Apigenin	33.1	SIM	269.2

* **MRM** = "multiple reaction monitoring", monitoring specific ion of the substance monitorizare, through a double MS process (isolation + fragmentation); ** **SIM** = "single ion monitoring" involves a single MS stage, isolation of the interest compound and then scanning without fragmentation stage.

the UV spectrum. Thus, polyphenol carboxylic acids (caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid and sinapic acid) were detected at the length of 330 nm while the flavonoids (hyperoside, isoquercitrin, rutoside) and their aglycones (myricetol, fistein, quercitrin, quercetol, patuletin, luteolin, kaempferol and apinegin) were detected at 370 nm.

For quantitative evaluation, calibration curve (six points) was performed for each compound in the concentration range 0.5 to 50 mg/ml. Good linearity was obtained for all calibration curves with regression coefficients > 0.999. For example, the calibration curve for ferulic acid was y = - 1.016 + 39.55 x, where y is peak area (mAu × s) and x is concentration (μg ml^{-1}). Since in the chromatographic conditions used, there are two pairs of substances incompletely separated (caftaric acid - gentisic acid and caffeic acid - chlorocgenic acid); for these compounds, only qualitative determination based on MS information was performed. In Table 1 are presented retention times for the analyzed compounds.

Analysis of polyphenols by MS detection

All analyzed compounds can lose a proton (converting itself into negative ions $[M-H]^-$ and can therefore be analyzed by negative ionization. In the case of plant extracts, phenolic compounds can be identified by analyzing MS spectra. Initially was checked the molecular weight minus one, $[M-H]^-$, and if it corresponded, the mass spectra of the unknown compound was compared with the one of the standard. If the ions from the mass spectrum were the same, the compound was considered identified.

MS analysis mode and specific ions based on which sample identification was done are presented in Table 1. For example, in Figure 1 is presented the mass spectra of ferulic acid and the extracted ions chromatogram (Figure 2). As shown in Figure 1, the ion corresponding to the deprotonated molecule (m / z = 191) splits into three major ions with m / z: 133.6, 148.6, 177.6. At the retention time of 12.5 min another signal is observed, probably corresponding to cis-ferulic acid, formed over time by ferulic acid isomerization.

For polyphenolcarboxylic acids, which were analyzed in MS - MRM mode, were extracted main ion chromatograms and mass spectra. For the other compounds, analyzed in AUTO - MS mode, the identification was done only on spectral base.

In Figures 3 to 6 are presented chromatograms of hydrolysed and unhydrolysed samples.

The results for the contents of the polyphenols in hydrolyzed and unhydrolyzed extracts are summarized in Tables 2 to 3.

HPLC-MS analysis revealed in *S. bifolia* several polyphenols - caffeic acid, chlorogenic acid, p-coumaric

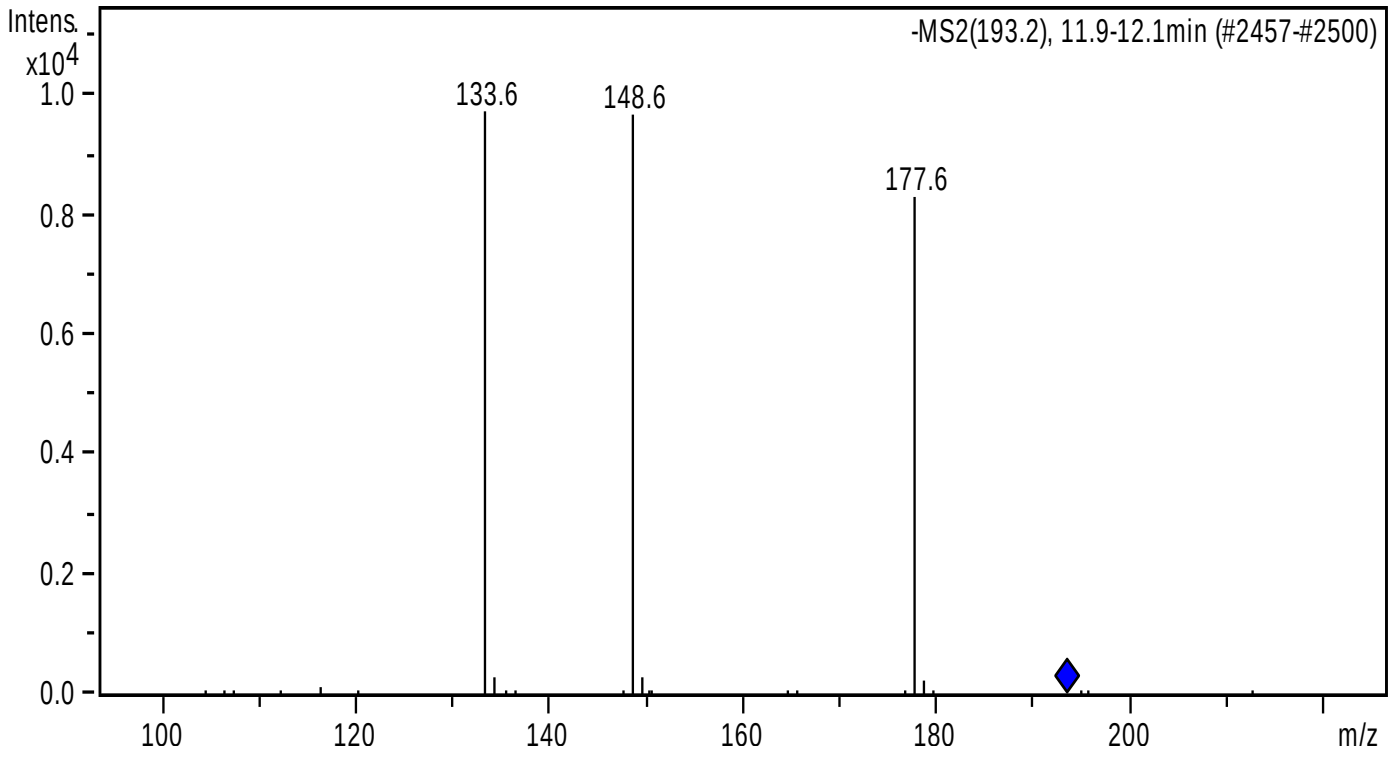

Figure 1. Mass spectra of ferulic acid.

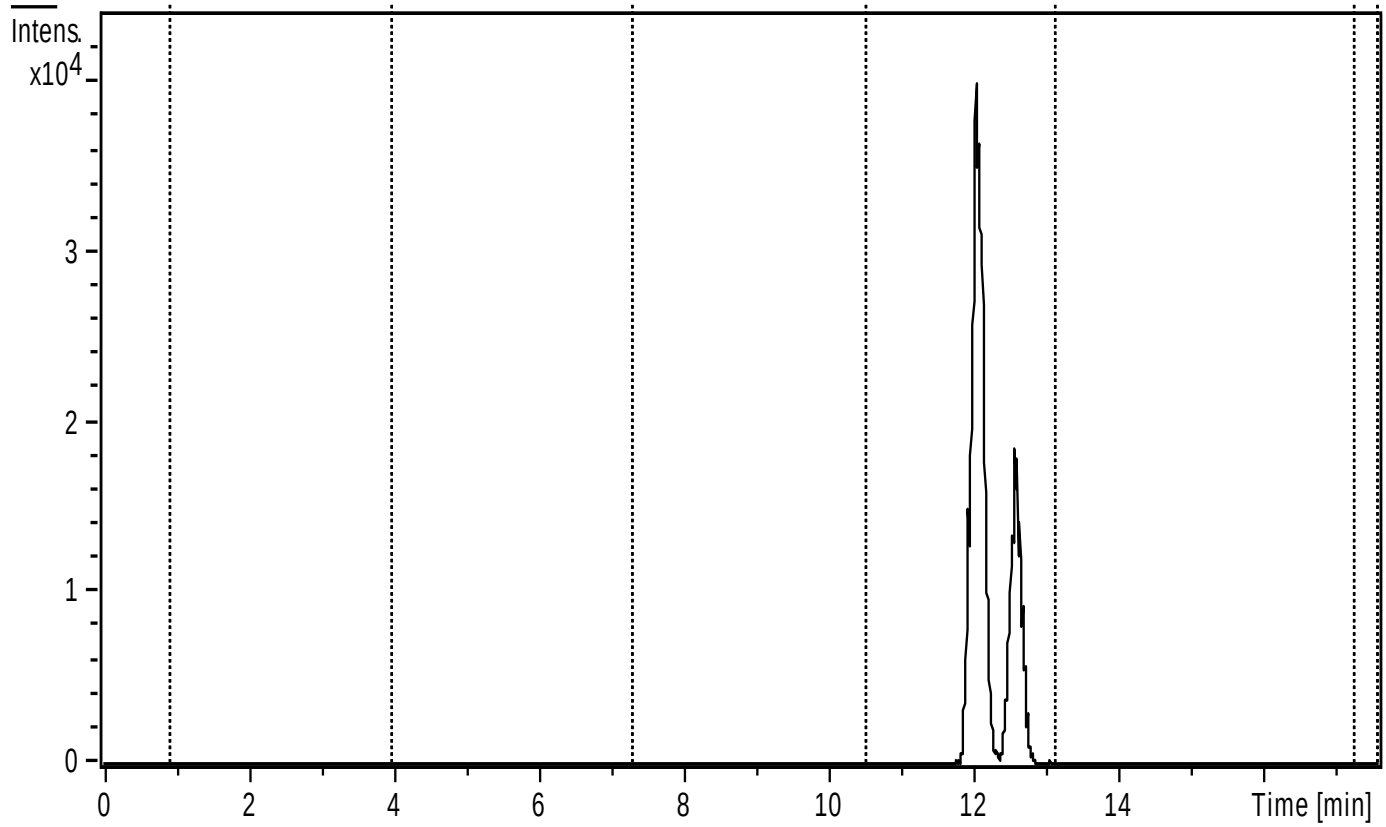

Figure 2. Extracted chromatogram of ferulic acid.

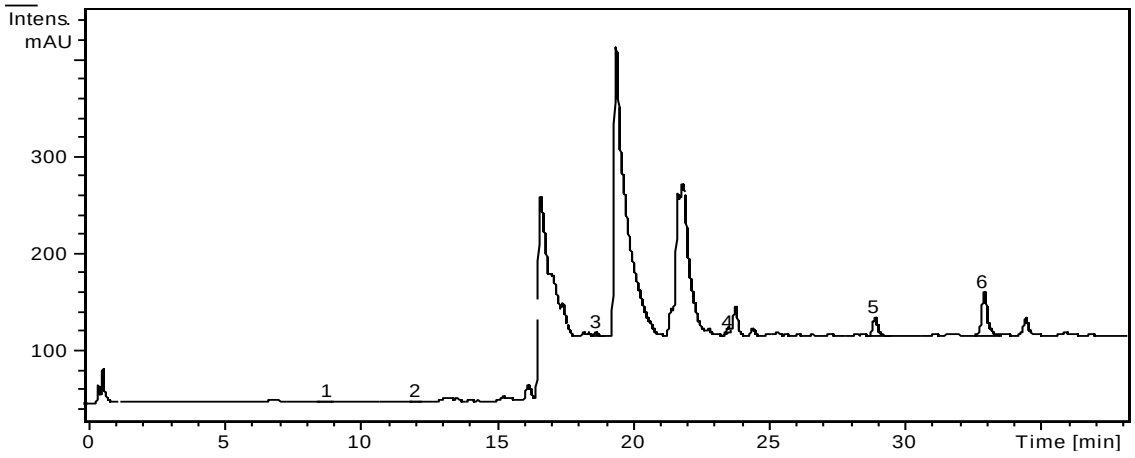

Figure 3. Chromatogram of *S. bifolia* unhydrolysed extract from aerial parts.

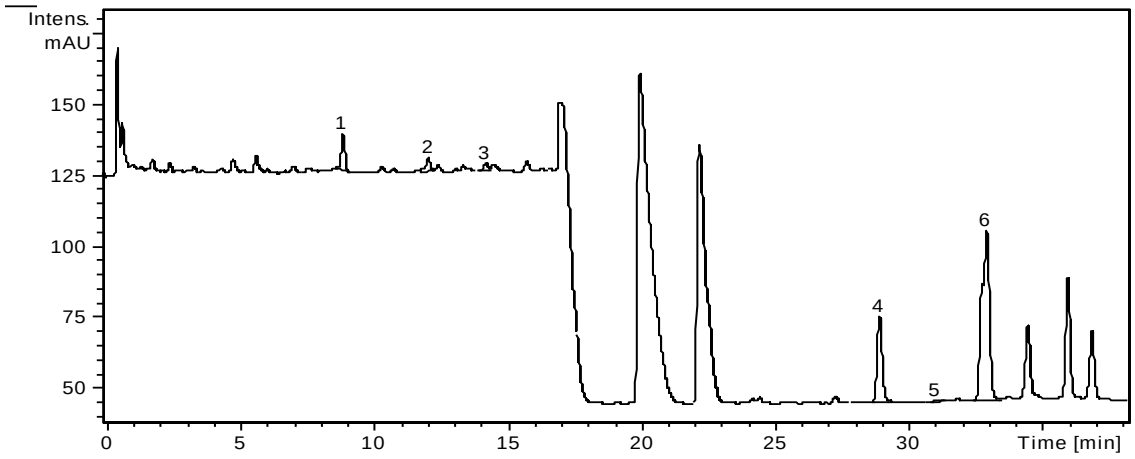

Figure 4. Chromatogram of *S. bifolia* hydrolysed extract from aerial parts.

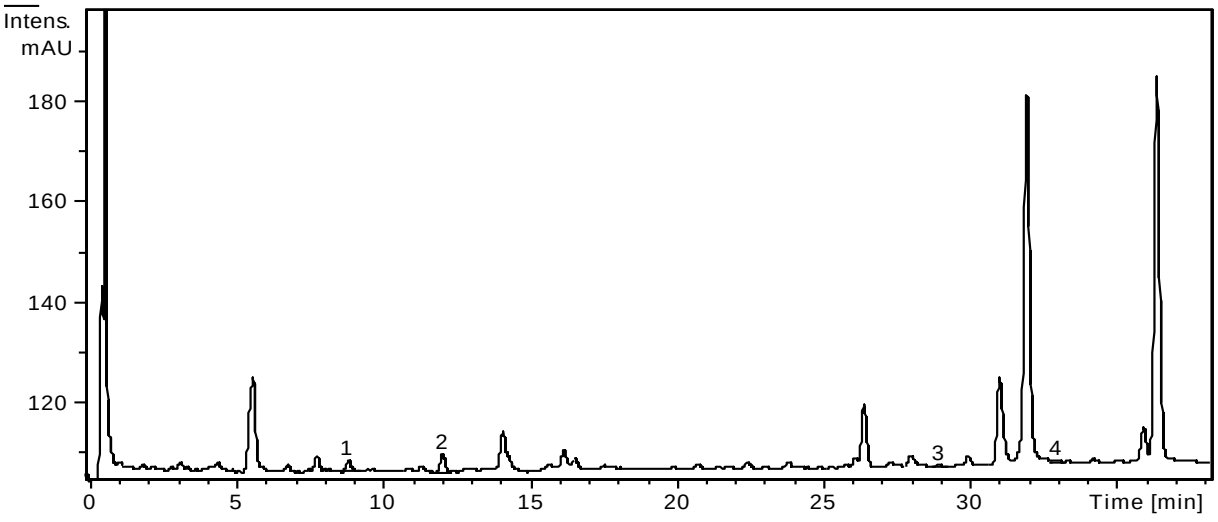

Figure 5. Chromatogram of *S. bifolia* unhydrolysed extract from bulbs.

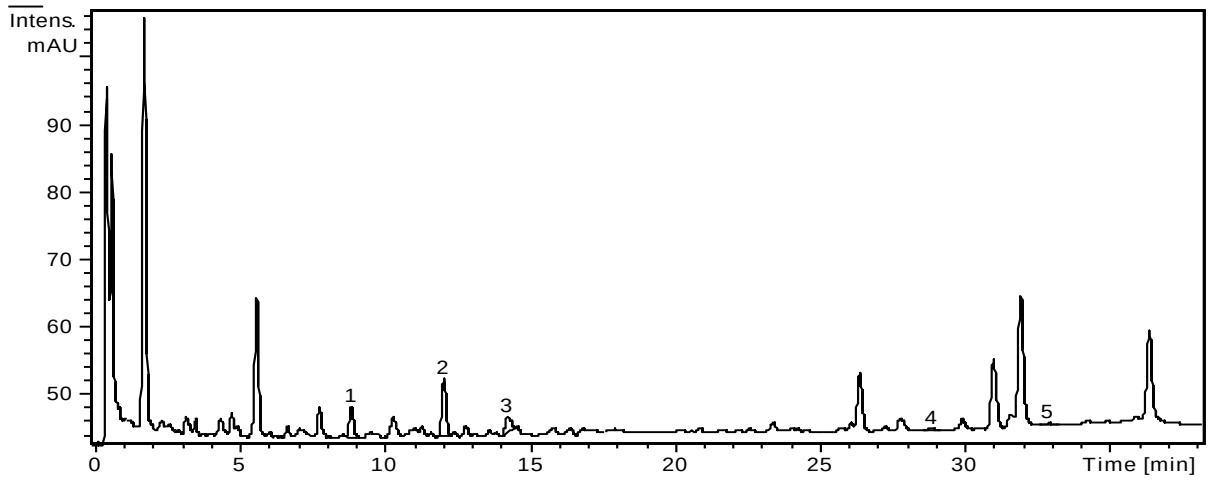

Figure 6. Chromatogram of *S. bifolia* hydrolysed extract from bulbs.

Tabel 2. Identified/quantified polyphenols from *S. bifolia* aerial parts.

Number on chromatogram	Compound	UV identified	MS identified	Concentration (µg/ml)
Unhydrolysed extract				
-	Gentisic acid	No	Yes	Qualitative
-	Caffeic acid	No	Yes	Qualitative
-	Chlorogenic acid	No	Yes	Qualitative
1	P-Coumaric acid	Yes	Yes	0.822
2	Ferulic acid	Yes	Yes	0.455
3	Hyperoside	Yes	Yes	1.808
4	Quercitrin	Yes	Yes	5.417
5	Luteolin	Yes	Yes	13.197
6	Kaempferol	Yes	Yes	0.142
7	Apigenin	Yes	Yes	56.360
Hydrolysed extract				
-	Gentisic acid	No	Yes	Qualitative
-	Caffeic acid	No	Yes	Qualitative
-	Chlorogenic acid	No	Yes	Qualitative
1	P-Coumaric acid	Yes	Yes	3.531
2	Ferulic acid	Yes	Yes	1.441
3	Sinapic acid	Yes	Yes	0.680
4	Luteolin	Yes	Yes	9.568
5	Apigenin	Yes	Yes	36.657

acid and ferulic acid acid in herba. Their concentrations are usually below 1 µg / ml.

Sinapic acid was detected and quantified only in hydrolyzed extract of bulbs and aerial parts indicating that it is present only as ester.

In herba also appear hyperoside, quercitin, luteolin, apigenin and kampferolul. After hydrolysis increased concentration of luteolin and apigenin and the appearance of quercetol due to flavonoid hydrolysis and highlighting their aglicons was observed. Luteolin and apinegin are the most abundant constituents in the *S. bifolia* aerial parts in both hydrolyzed and unhydrolized extracts and are found in high concentrations range of tens of micrograms per milliliter (in esteric forms are in much smaller quantities). Patuletin, fistein, routine, isoquercitin, myricetol and caftaric acid were not detected in these samples. In bulbs were found especially caffeic acid, p-coumaric and ferulic acid and small amounts of

Table 3. Identified/quantified polyphenols from *S. bifolia* bulbs.

Number on chromatogram	Compound	UV Identified	MS Identified	Concentration (µg/ml)
Unhydrolysed extract				
-	Caffeic acid	No	Yes	Qualitative
1	P-Coumaric acid	Yes	Yes	0.732
2	Ferulic acid	Yes	Yes	1.087
3	Luteolin	Yes	Yes	0.165
4	Apigenin	Yes	Yes	0.241
Hydrolysed extract				
-	Caffeic acid	No	Yes	Qualitative
1	P-Coumaric acid	Yes	Yes	1.334
2	Ferulic acid	Yes	Yes	2.250
3	Sinapic acid	Yes	Yes	0.842
4	Luteolin	Yes	Yes	0.268
5	Apigenin	Yes	Yes	0.290

luteolin and apigenin. Bulbs contained caffeic acid in both esteric and acid form while aerial parts contained caffeic acid and chlorogenic acid.

Polyphenols, present in *S. bifolia*, are believed to act as antioxidant (Seddik et al., 2010), antibacterial (Kasim et al., 2011) and antineoplazic agents (Custódio et al., 2011) even at the small quantities that are achievable through the diet.

The values obtained for other medicinal plants and reported in literature were comparable to the values obtained in our study. For example, ferulic acid is in *S. bifolia* in similar amounts to those determined by RP-HPLC in *Phyllostachys pubescen* (Jin et al., 2011).

Conclusion

Eighteen polyphenols were identified and fourteen of them were quantified for the first time in *S. bifolia* bulbs and aerial parts. The phenolic compounds found in plant samples have strong antiradical properties suggesting that the plant may be beneficial to human health although further studies are required.

REFERENCES

Chirea M, Pereira EM, Pereira CM, Silva F (2011). Synthesis of poly-lysine/gold nanoparticle films and their electrocatalytic properties. Biointerface Res. Appl. Chem., 1: 119-126.

Custódio L, Escapa AL, Fernandes E, Fajardo A, Aligué R, Alberício F, Neng N, Nogueira JMF, Romano A (2011). *In vitro* cytotoxic effects and apoptosis induction by a methanol leaf extract of carob tree (Ceratonia siliqua L.). J. Med. Plants Res., 5(10): 1987-1996.

Ding B, Zhou T, Fan G, Hong Z, Wu Y (2007). Qualitative and quantitative determination of ten alkaloids in traditional Chinese medicine *Corydalis yanhusuo* W.T. Wang by LC-MS/MS and LC-DAD. J. Pharm. Biomed. Anal., 45: 219-226.

Fodorea CS, Vlase L, Leucuta SE, Tamas M (2005). Phytochemical study on some polyphenols of *Geranium pyrenaicum*. Chem. Nat. Compd., 41(4): 400-402.

Gray A, Waigh R (2011). Antimicrobial agents from the leaf of Struchium sparganophora (Linn) Ktze, Asteraceae. J.M.A., 3(1): 13-17.

Grumezescu AM, Saviuc C, Holban A, Hristu R, Coritoru C, Stanciu G, Chifiriuc C, Mihaiescu D, Balaure P, Lazar V (2011). Magnetic chitosan for drug targeting and *in vitro* drug delivery response. Biointerface Res. Appl. Chem., 1: 160-165.

Ivanescu B, Vlase L, Corciova A, Lazar MI (2010). HPLC-DAD-MS study of polyphenols from *Artemisia absinthium, A. annua, and A.vulgaris.* Chem. Nat. Compd., 46(3): 468-470.

Jin Y, Liu H, Yuan K (2011). Simultaneous determination of seven effective constituents in the leaves of bamboo by reversed phase high performance liquid chromatography (RP-HPLC). J. Med. Plants Res., 5(23): 5630-5635.

Kasim LS, Ferro VA, Odukoya OA, Drummond A, Ukpo GE, Seidel V, Gray A, Waigh R (2011). Antimicrobial agents from the leaf of Struchium sparganophora (Linn) Ktze, Asteraceae. JMA. 3(1): 13-17.

Mihaiescu DE, Grumezescu AM, Mogosanu DE, Traistaru V, Balaure PC, Buteica A (2011). Hybrid inorganic/organic nanomaterial for controlled cephalosporins release. Biointerface Res. Appl. Chem., 1: 41-47.

Mohd-Zin Z, Abdul-Hamid A, Osman A (2002). Antioxidative activity of extracts from mengkudu (*Morinda citrifolia* L.) root, fruit and leaf. Food Chem., 6: 227-231.

Norn S, Kruse, PR (2004). Cardiac glycosides: From ancient history through Withering's foxglove to endogeneous cardiac glycosides. Dan. Medicinhist Arbog., 1: 119-132.

Oszmiański J, Wojdyło A, Gorzelany J, Kapusta I (2011). Identification and Characterization of Low Molecular Weight Polyphenols in Berry Leaf Extracts by HPLC-DAD and LC-ESI/MS. J. Agric. Food Chem., 59(24): 12830-12835.

Parvu M, Toiu A, Vlase L, Parvu EA (2010). Determination of some polyphenolic compounds from Allium species by HPLC-UV-MS. Nat. Prod. Res., 24(14): 1318-1324.

Popovici L, Moruzi C, Toma I (1993). Botanical Atlas. Bucharest: Didactical and Pedagogical, P. 161.

Saviuc C, Grumezescu AM, Chifiriuc MC, Bleotu C, Stanciu G, Hristu R, Mihaiescu D, Lazăr V (2011a). *In vitro* methods for the study of microbial biofilms. Biointerface Res. Appl. Chem., 1: 31-40.

Saviuc C, Grumezescu AM, Holban A, Chifiriuc C, Mihaiescu D, Lazar V (2011b). Hybrid nanostructurated material for biomedical applications. Biointerface Res. Appl. Chem., 1(2): 064-071.

Seddik K, Nadjet I, Abderrahmane A, Daoud H, Lekhmici A (2010). Antioxidant and antibacterial activities of extracts from Artemisia herba alba Asso. leaves and some phenolic compounds. J. Med.

Plants Res., 4(13): 1273-1280.
Termentzi A, Kefalas P, Kokkalou E (2008). LC-DAD-MS(ESI+) analysis of the phenolic content of *Sorbus domestica* fruits in relation to their maturity stage. Food Chem., 106: 1234-1245.
Zehl M, Braunberger C, Conrad J, Crnogorac M, Krasteva S, Volger B, Beifuss U, Krenn L (2011). Identification and quantification of flavonoids and ellagic acid derivates in therapeutically important Drosera species by LC-DAD, LC-NMR, NMR, and LC-MS. Anal. Bioanal. Chem., 400: 2565-2576.