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Full Length Research Paper

Determination of cardiac glycosides in *Scilla bifolia* (*Liliaceae*) by two different analytical techniques: Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography - Mass Spectrometry (HPLC-MS)

LUIZA BĂLĂŞOIU[1], DANIELA CĂLINA[2]*, ANCA DOCEA[2], EMILIA PATRU [2], LAURIAN VLASE[1], MARIA VIORICA BUBULICA[2], HONORIUS POPESCU[1]

[1]University of Medicine and Pharmacy „Iuliu Haţieganu", Faculty of Pharmacy, 8 Victor Babeş Str, 400012, Cluj-Napoca, România.

[2]University of Medicine and Pharmacy, 2-4 Petru Rares Str. 200349, Craiova, Romania.

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In this study was performed chemical analysis on alcoholic extracts of the *Scilla bifolia* (Liliaceae family) species harvested from spontaneous flora, with the purpose of determining bioactive cardiac glycosides, particularly proscillaridin A. The used analysis techniques were thin layer chromatography (TLC) and high performance liquid chromatography coupled with mass spectrometry (HPLC-MS). After performing TLC analysis, the presence of the scillarosides was confirmed, but only in traces, in higher amounts in herba than in bulbs. The presence of proscillaridin A has not been determined in any analysed samples, either before or after hydrolysis by HPLC-MS. Thus, we can conclude that, the bulbs and aerial parts of *Scilla bifolia* do not contain proscillardin A.

Key words: HPLC-MS analysis, alcoholic extract, proscillaridin A, *Scilla bifolia,* TLC analysis.

INTRODUCTION

Scilla genus includes about 125 species widespread in Europe, North Africa and Asia Minor. The most widely used is *Scilla maritime* (Violet).

In Romania, *Scilla bifolia* (blue snowdrops, alpine squill) is spontaneous or cultivated as ornamental plant (Parvu, 2005). Bulbs of this plant are used for medicinal purposes, *bulbus Scillae*, knowing that they contain cardiac glycosides such as scillirosides (Verbiscar et al., 1986), mucilage and flavonoids (Majinda et al., 1997), thus justifying their tonic action and diuretic actions (Iizuca et al., 2001).

The pharmaceutical industry uses these plants from which are extracted by special techniques active principles acting similar to secondary metabolites from *Urginea maritima* that has therapeutic indications in heart failure in the elderly (Kropp et al., 1996).

Bioactive glycosides are widespread in the plant kingdom. They are found both in the aerial parts: leaves, flowers, fruits, seeds, wood and in roots or bulbs (Khatun et al., 2011; Ajayi et al., 2011). Glycosides are solid, crystalline, colorless or colored substances (depending on the aglycon type), with a bitter taste in general. Some have a specific flavor. Glycosides have optical activity.

Most are soluble in water, alcohol and acetone and insoluble in ether. They are easily hydrolysed with acid or base or under the action of enzymes (Ciulei et al., 1993).

*Corresponding author. E-mail: calina.daniela@gmail.com

Many glycosides have physiological action on living beings (curative or harmful) and are used since antiquity as medicines or poisons (Toplis et al., 2003).

However the cardiac glycosides of the *S. bifolia* species have not yet been determined. Thus, the aim of this study is to determine the cardiac glycosides of the *S. bifolia* species, particularly proscillaridine A by two different techniques of analysis: thin layer chromatography (TLC) and high performance liquid chromatography coupled with mass spectrometry (HPLC-MS).

MATERIALS AND METHODS

Herbal product

The aerial parts and bulbs of *S. bifolia* species were collected at full bloom between March and April, in two successive years (2008 to 2009) from the spontaneous flora of the Podari Bucovăţ forest, Dolj County, Romania. Herbal product was selected by hand and dried naturally at constant temperature and humidity. The plant was identified by the botanist Prof. Stefania Trasca and four voucher specimens (SBB2008, SBH2008, SBB2009, SBH2009) were deposited in our institution herbarium.

Processing samples

Dried and grounded plant material was extracted under stirring at room temperature using methanol/ethanol 70°C. Extraction ratio was 1:5 (plant material:solvent). After extraction, the tincture was filtered.

Preliminary screening of possible cardiac glycosides

Phytochemical screening of sterols or triterpenoids (alkaloids, saponosides, cardiac glycosides) (Evans, 1996) was carried out using Bourchard reagent.

Thin layer chromatography (TLC)

Stationary phase: Silica gel with fluorescent indicator at 254 nm UV type SilG F254 (Merck). Samples: *S. bifoliae* Herba, methanolic extract, 2008 (SBH2008M), *S. bifoliae* Herba, methanolic extract, 2009 (SBH2009M), *S. bifoliae* Bulbus, methanolic extract, 2008 (SBB2008M), *Scillae bifoliae* Bulbus, methanolic extract, 2009 (SBB2009M); *S. bifoliae* Herba, ethanolic extract, 2008 (SBH2008E), *S. bifoliae* Herba, ethanolic extract, 2009 (SBH2009E), *S. bifoliae* Bulbus, ethanolic extract, 2008 (SBB2008E), *S. bifoliae* Bulbus, ethanolic extract, 2009 (SBB2009E). We used 30 µl from each sample. Standards: 10 µl Proscillaridin A (Figure 1), (5 mg Proscillaridin A purchased from Roth Germany were dissolved in 1 mL of equal volumes of ethyl acetate and methanol).

Eluent: purified water - methanol (Merck) - ethyl acetate (Merck) (8:11:81 v/v). Distance migration: 15 cm. Anisaldehide was sprayed with reagent and was heated to 105-110°C for 5 to 10 min. Chromatogram images was taken under visible 254 nm UV modes.

High performance liquid chromatography – mass spectrometry analysis (HPLC-MS)

Equipment: HPLC coupled with mass spectrometer; HP 1100 Series binary pump; HP 1100 Series autosampler; HP 1100 Series thermostat; Ion Trap mass spectrometer Agilent 1100 SL.

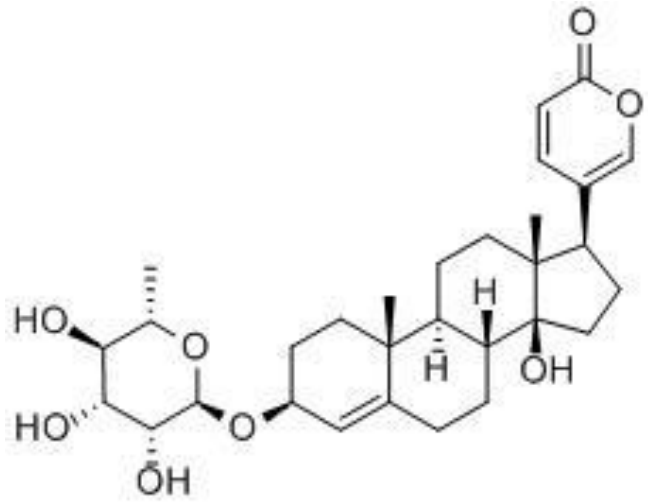

Figure 1. Proscillaridin A structure.

HPLC working conditions: Analytical column, Gemini NX 50 mm × 2.0 mm id, 3 mm (Phenomenex, USA); On-line 0.2 micron filter (Agilent); Mobile phase, mixture 1 mM ammonium formate solution and acetonitrile 64/36 (v/v), isocratic elution; Flow rate, 0.5 ml/min, temperature, 35°C; Detection - MS/MS - isolation and fragmentation of the ion with *m/z* 531.6 corresponding to protonated molecule of the proscillaridine A and then monitoring the ions with *m/z* 349.3 and 367.3 of the MS/MS spectrum of the analyte; Injection volume: 4 µl.

MS working conditions: Ion Source, ESI (electrospray ionisation); ionization mode, positive; nebulizer nitrogen, pressure 55 psi; drying gas nitrogen, flow 12 L/min, drying temperature 350°C; Capillary potential, -5000 V; analysis mode, monitoring transitions m/z 531.6 > (m/z 349.3 + m/z 367.3).

RESULTS AND DISCUSSION

Proscillaridin A was first obtained from *S. maritima* and it was assumed that this cardiac glycoside is contained by the bulbs of other plants belonging to the *Scilla* genus (Kedra and Kedrowa, 1968; Vahdettin and Vahit, 2010).

As known from literature, glycosides are easily extractable in alcohol (Oluwaniyi and Ibiyemi, 2007) and for this reason ethanol and methanol extracts were made. TLC and HPLC-MS for quantitative determination of proscillaridin A in alpine squill.

Preliminary screening

Bourchard reaction revealed the presence of alkaloids, saponosides and cardiac glycosides in all extracts, with the appearance of a brown ring being observed after achieving specific reaction, in concordance with the literature (Sollner and Fresnesius, 1963).

Table 1. R_f values for proscillaridin A and for the analyzed samples.

Sample	R_f values[a]
Proscillaridin A	0.41
SBB2008M	0.048; 0.2; 0.82
SBB2008E	0.08; 0.22; 0.82
SBB2009M	0.18; 0.23; 0.34; 0.41; 0.48; 0.54; 0.66; 0.77
SBB2009E	0.16; 0.69
SBH2008M	0.08; 0.22; 0.45; 0.50; 0.67; 0.77
SBH2008E	0.05; 0.21; 0.35; 0.45; 0.68; 0.77
SBH2009M	0.05; 0.09; 0.18; 0.23; 0.34; 0.38; 0.41; 0.57; 0.63; 0.69; 0.77
SBH2009E	0.05; 0.09; 0.18; 0.23; 0.31; 0.41; 0.57; 0.63; 0.69; 0.77

[a] at 254 nm UV.

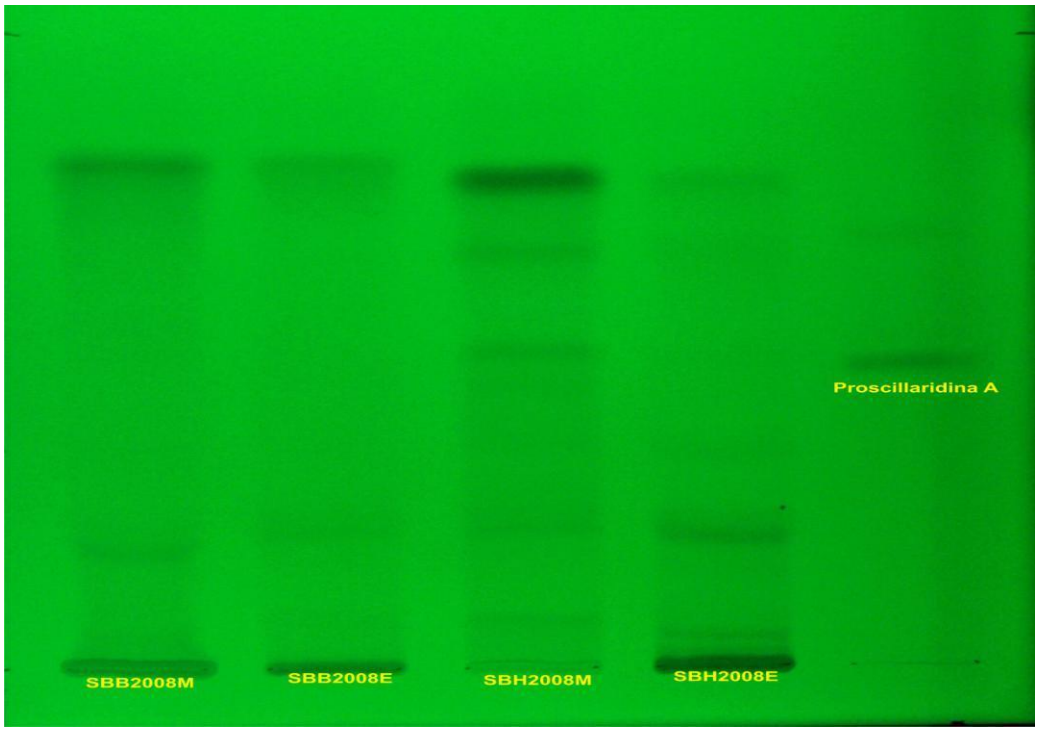

Figure 2. The chromatogram of the extracts of *Scilla bifolia* harvested in 2008 at UV 254 nm.

Thin layer chromatography (TLC)

Table 1 presents the Rf values for proscillaridin A and in Figures 2 to 5 are presented chromatograms obtained at 254 nm in UV, fluorescence and visible light. By this method were detected traces of cardiac glycosides in greater amounts in Herba than in bulbs. The results are similar to those obtained for *Scilla maritima* species (*Urginea maritime*), where the concentration of cardiac glycosides with bufadienolidic nucleus was 0.4 to 0.6% (Krenn et al., 1989).

High performance liquid chromatography – mass spectrometry analysis (HPLC-MS)

For more conclusive results, a HPLC-MS analysis was performed. The obtained extracts were hydrolyzed with hot HCl 2 N (1:1). By hydrolysis, proscillaridin A (bound as glycoside) was released from the bound form. All analyzed samples were injected after centrifugation for 3 min at 12,000 rpm. Mass spectrum (full scan) of a standard solution of proscillaridin A, as described is shown in Figure 6. Because in the ionization conditions

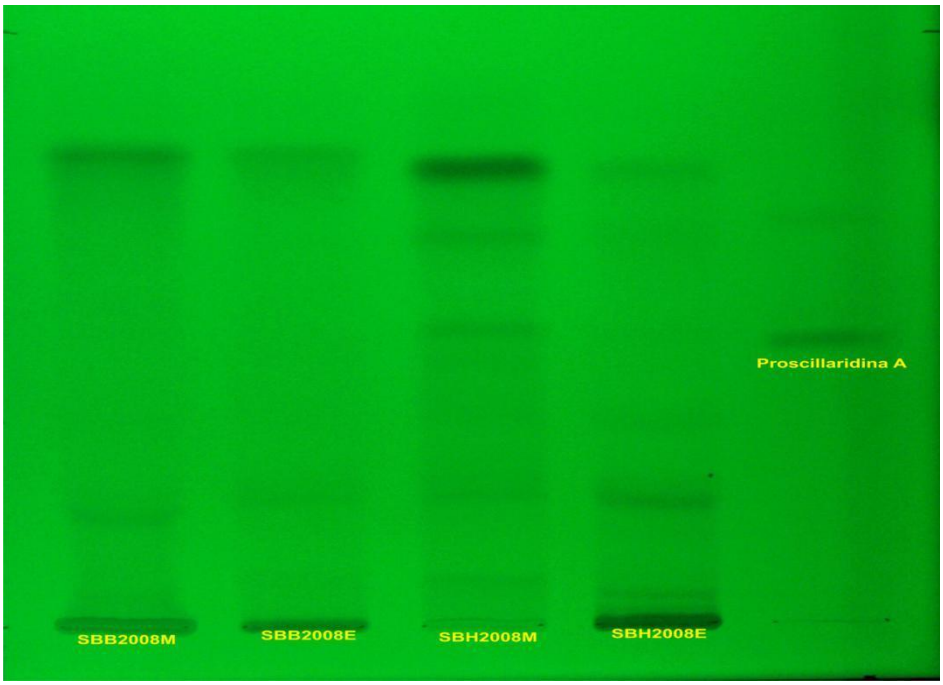

Figure 3. The chromatogram of the extracts of *Scilla bifolia* harvested in 2009 at UV 254 nm.

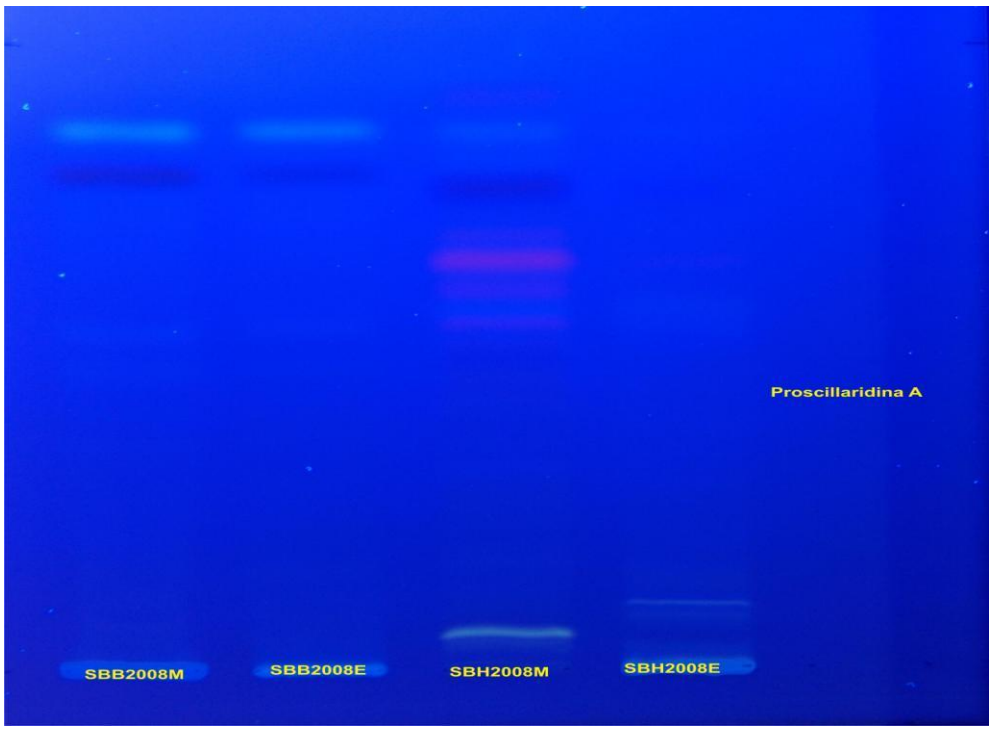

Figure 4. The chromatogram of the extracts of *Scilla bifolia* harvested in 2008, in fluorescence.

proscillaridin A molecule is protonated, the ions detected by the spectrophotometer are in the form $[M+H]^+$. The ion expected, according to analyte molecular mass (M = 530.6), is the ion with m/z 531.6, corresponding to the

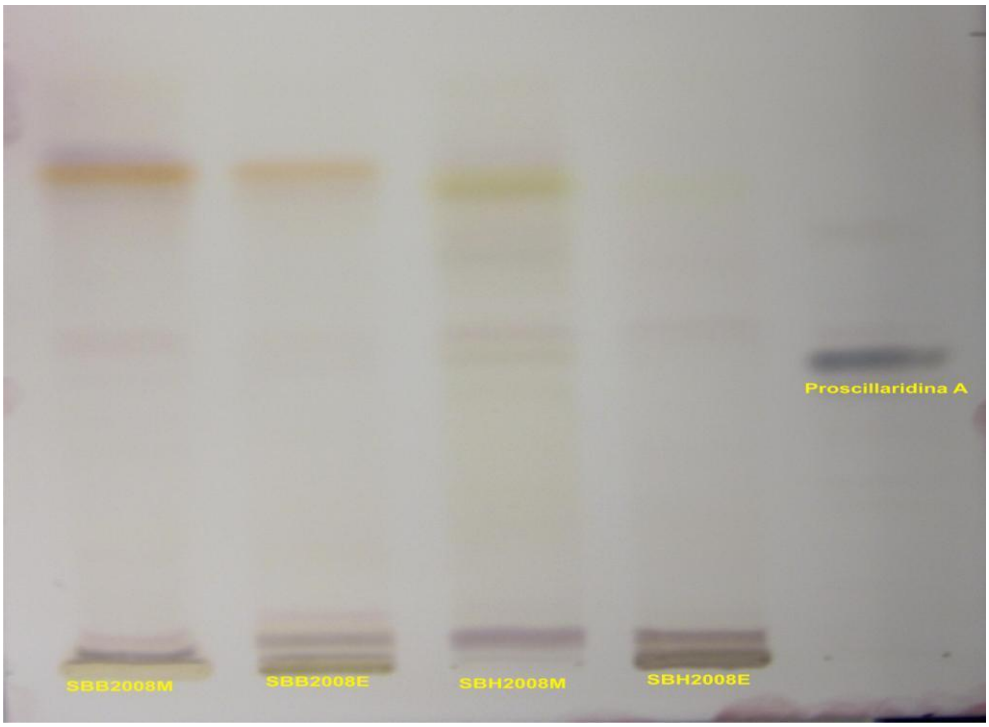

Figure 5. The chromatogram of the extracts of *Scilla bifolia* harvested in 2008, in visible light.

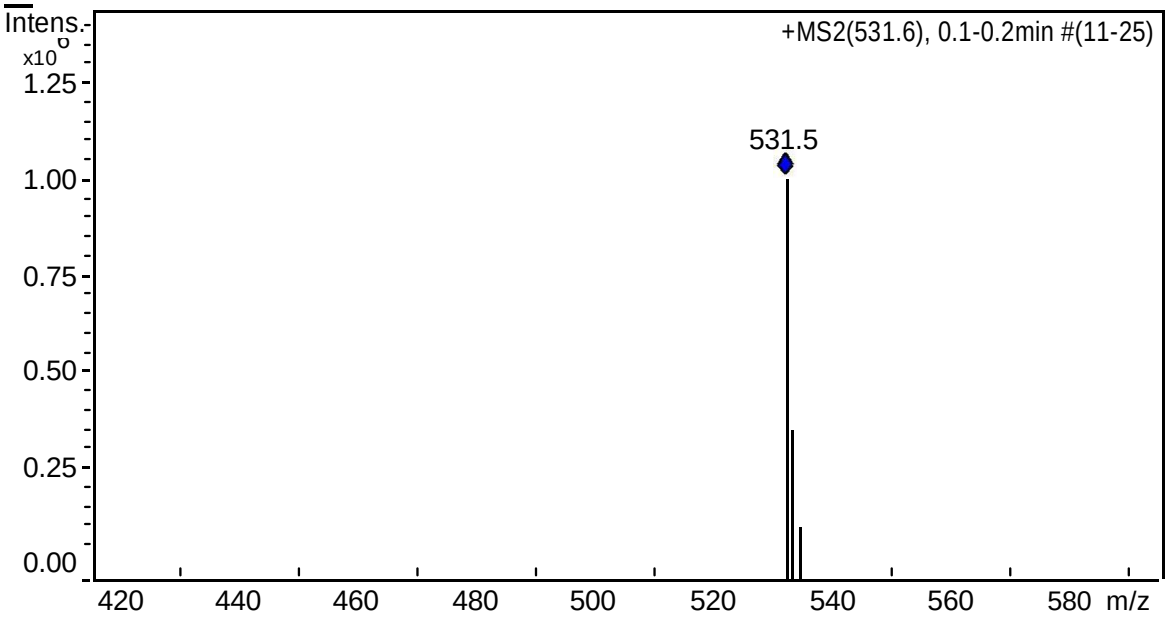

Figure 6. Full-scan spectrum of type Proscillaridin A in the mobile phase.

protonated molecule. To increase the selectivity of the analytical method, the specific ion has been fragmented (Figure 7), and based on the fragments from the MS spectrum the extracted chromatogram was drawn (Figure 8).

From MS/MS spectra of proscillaridin A, the main ions with *m/z* 349.3 and 367.3 were used in quantification. The proscilaridine A calibration curve was performed in the concentration range 12 to 768 ng/ml. All solutions were prepared in distilled water. The quadratic obtained

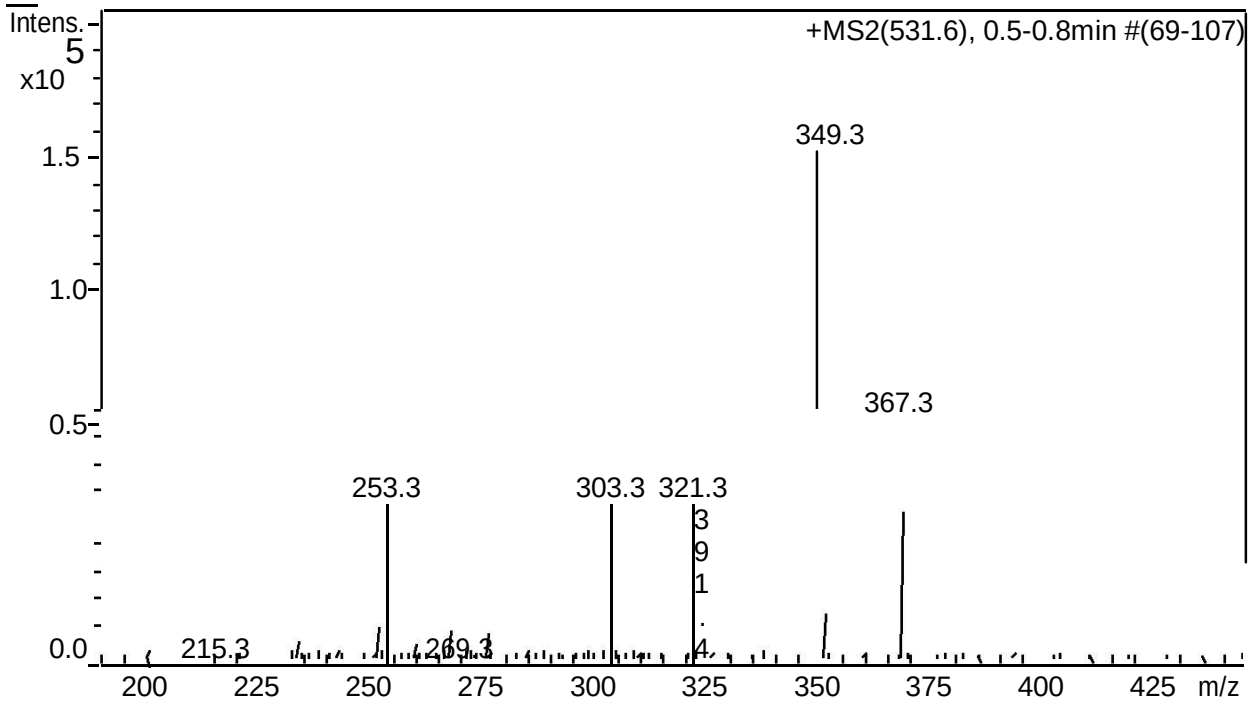

Figure 7. The MS/MS spectrum of proscillaridin A in the mobile phase.

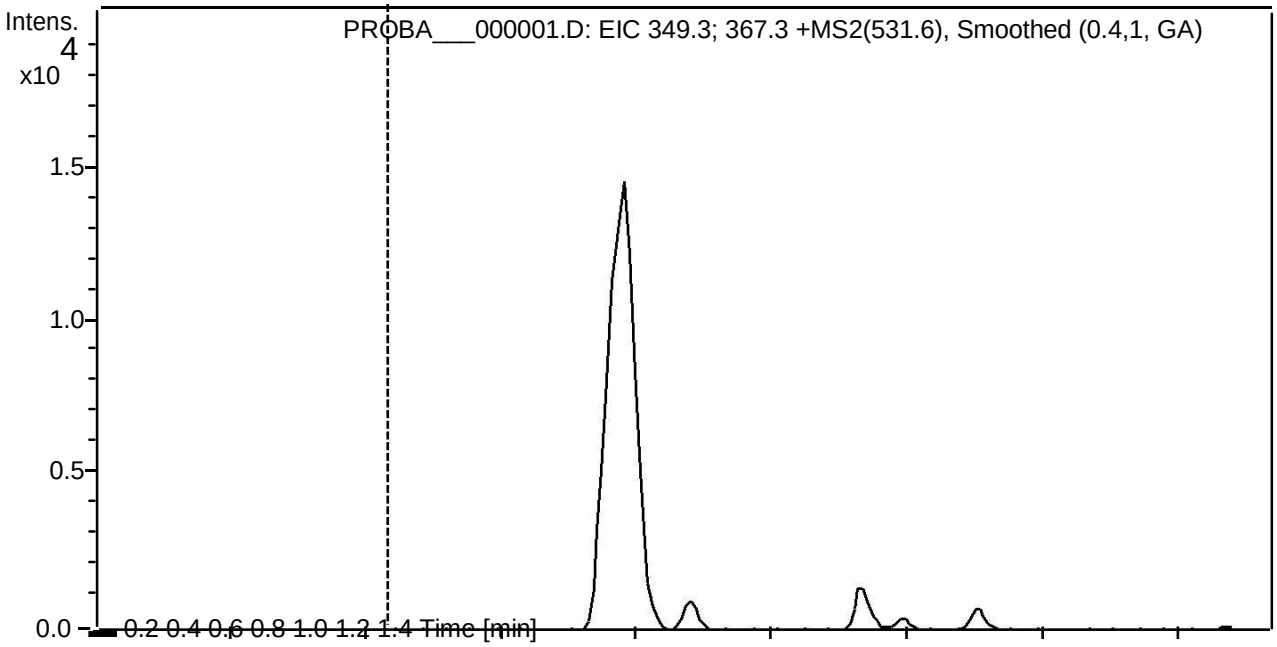

Figure 8. Extracted chromatogram of proscillaridin A, analyte retention time: 0.8 minutes.

were positive for all prepared extracts but this result might be due to the presence of alkaloids or saponosides. After determination of proscillaridin A, a natural compound with cardiotonic action. Initially the reactions with Bourchard reagent used to identify sterol or triterpenoid structure compounds (alkaloids, saponosides, cardiac glycosides)

performing TLC analysis, the presence of the scillarosides was confirmed, but only in traces, higher in Herba than in bulbs. For more conclusive results, the extracts were analysed using HPLC-MS but this analysis did not reveal the presence of proscillaridin A in any sample either before or after hydrolysis. The obtained

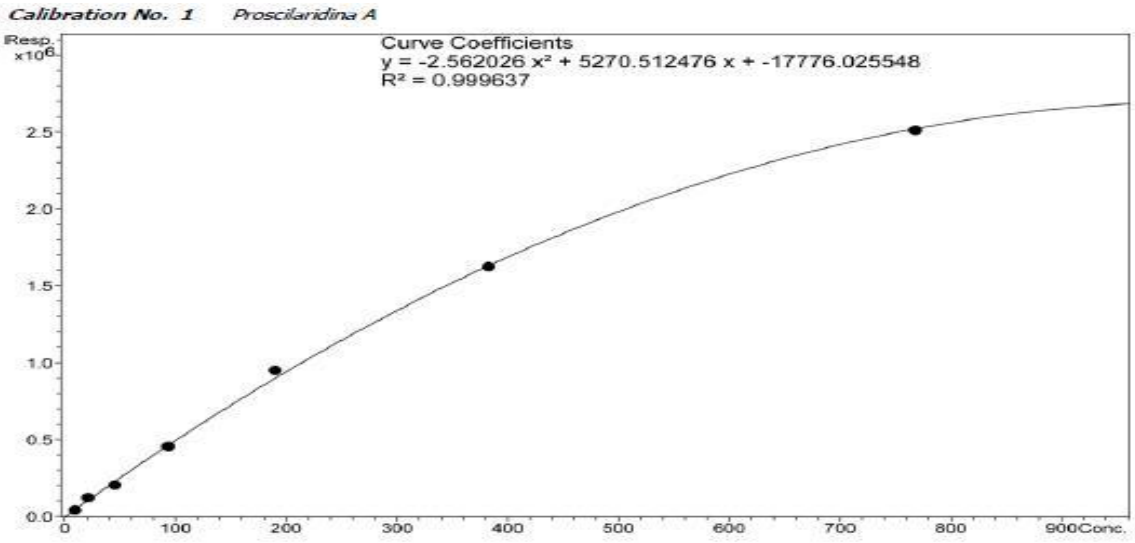

Figure 9. Proscillaridin A calibration curve, detection MS/MS.

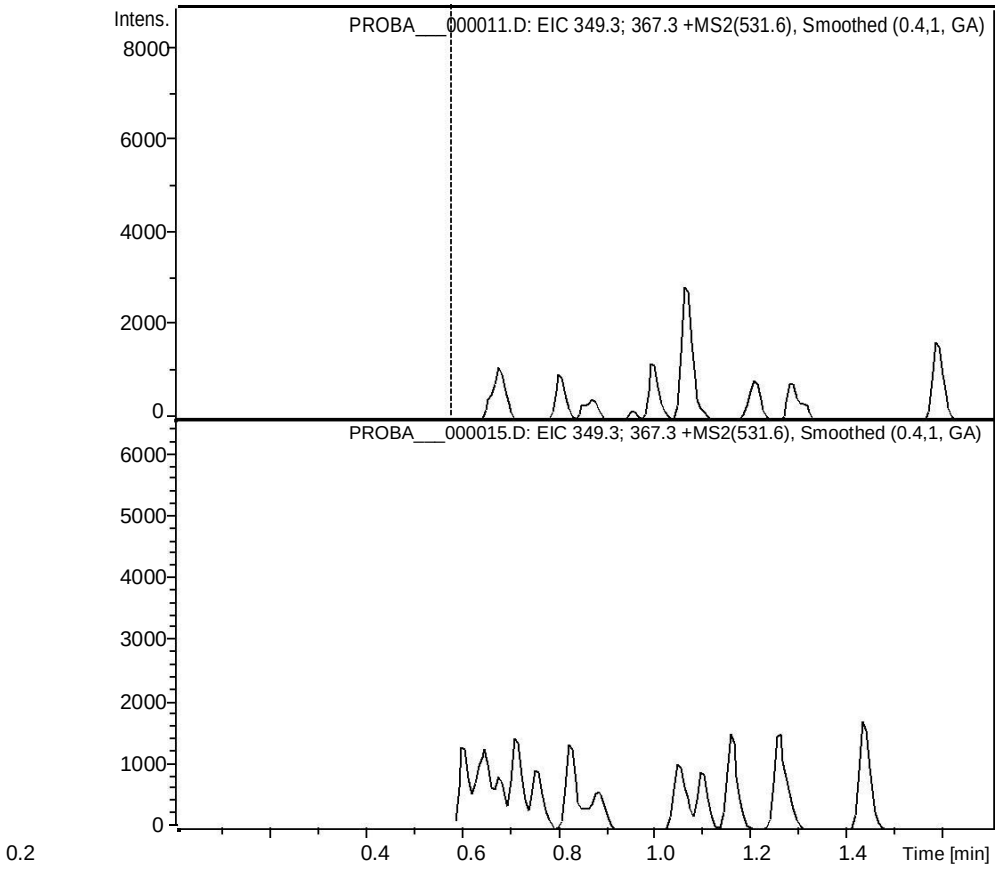

Figure 10. The proscillaridin A chromatograms of unhydrolyzed SBB2009E sample (top) and hydrolyzed sample (bottom).

calibration curve ($y = ax^2 + bx + c$) is shown in Figure 9. A good linearity and good accuracy were obtained. The accuracy of analytical method calculated separately for each concentration (n = 7) is shown in Table 2. In Figure 10, typical chromatograms of the SBB2009E sample are shown. There was no evidence of the proscillaridin A

Table 2. Accuracy of analytical method for quantitative determination of proscillaridin A.

	Calibration Curve	
Theoretical concentration (ng/ml)	**Concentration found (ng/ml)**	**Acuracy**
12.00	11.78	98.1
24.00	27.36	114.0
48.00	43.45	90.5
96.00	94.68	98.6
192.00	204.27	106.4
384.00	382.50	99.6
768.00	761.02	99.1
Coefficient X^2	-2.562	-
Coefficient X	5270.5	-
Intercept	-17776.0	-
Correlation coefficient r^2	0.99963	-

presence in any analyzed sample, either before or after hydrolysis. Analyzed extracts do not contain measurable concentrations of proscillaridin A (do not contain higher concentrations of 12 ng/ml extract).

CONCLUSION

Methanolic extracts and ethanolic extracts obtained from *Scilla bifolia* species were analyzed by two different techniques: TLC and HPLC-MS for quantitativeresults led us to the issuance of a final conclusion that the bulbs and aerial parts of *S. bifolia* do not contain proscillardin A.

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