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Chromatographic Methods for the Determination of Primula Acid 1 Content in *Primulae extractum fluidum*

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Abstract: *Primula veris* L. (*Primulaceae*) is healing plant, whose root is officially used to treat cough associated with cold. Other reported indications are respiratory, thoracic and mediastinal disorders. These effects are result of high contents of triterpenoid saponins and phenolic glycosides. Primula acid 1 (PA 1, also primulasaponin 1) is main active component in *Primula elatior* L. This paper presents an optimized high pressure liquid chromatography (HPLC) method for the determination of primula acid 1 content in *Primulae extractum fluidum.* TLC was performed to check for the presence of the substance of interest. The determination was performed by reversed phase chromatography using C18 as a stationary phase. The mobile phase used for separation consisted of 0.2% H_3PO_4 and acetonitrile. This method was validated through different parameters. The detection limit for primula acid 1 was LD=10.41 μg/ml, and the quantification limit was LQ=34.69 μg/ml. In order to determine the content of primula acid 1, a calibration curve was constructed, and the content of primula acid 1 was calculated by the equation of the calibration curve and was 0.2793 mg per gram of extract. The results and simple preparation of sample show that HPLC is the method of choice for this type of analysis.

INTRODUCTION

According to European Pharmacopoeia, herbal drug, primula root (Primulae radix) is descibed as dried root and rhizome of *Primula veris* L. (*Primula officinalis*) and/or *Primula elatior* L. Hill*., Primulaceae* (Coran and Mulas, 2012; Eur. Ph. 7.0, 2012). *Primula veris L.* is a well-known healing plant which grows on the lawns all over Europe and Western Asia and is officially used to treat cough associated with cold. Other reported indications for primula root are respiratory, thoracic and mediastinal disorders. These pharmacological effects are the result of a high contents of triterpenoid saponins (usually between 3-10%) and phenolic glycosides, located mostly in the roots. Important saponins in *Primula veris* L. are primacrosaponin and priverosaponin B, while in *Primula elatior* L. the main active component is Primula acid 1 (PA 1, also primulasaponin 1) This acid has a triterpenoid structure which is the skeleton of oleanolic acid. The sugar component is bound to this triterpenoid aglycon (protoprimulagenine A- Fig. 1) and together form primulasaponin heteroside (Coran and Mulas, 2012; Okršlar, Plaper, Kovač, *et al.*, 2007; Başbülbül, Ozmen, Biyik, *et al.*, 2008). The sugar chain consists of: D-glucose, D-galactose, D-glucuronic acid and L-rhamnose, and binds to the hydroxyl group of protoprimulagenin A at the C-3 atome. (Fig. 1.) (Coran and Mulas, 2012; Okršlar, Plaper, Kovač, *et al.*, 2007; Başbülbül, Ozmen, Biyik, *et al.,* 2008). Saponins help expectoration due to their capability to dissolve bronchial mucus and can therefore be used in syrups to treat respiratory deseases. Primula extract (*Primulae extractum*) is widely used in the production of medicinal pharmaceutical formulations and herbal products. It can be used alone or in combination with thyme extract or thyme tinctura (*Thymi extractum* ili *Thymi tinctura*, *Thymi herba*) (Gruenwald, Graubaum and Busch, 2005).

Indications for the use of primula extract and its combination with thyme tincture are mostly respiratory diseases, especially when it comes to chronic cough associated with chronic bronchitis and mucus arrest, pertussis and asthma. It has been found to have the highest secretolityc properties as primula acid 1 and primula acid 2 (Coran and Mulas, 2012; Okršlar, Plaper, Kovač, *et al.*, 2007; Başbülbül, Ozmen, Biyik, *et al.*, 2008; EMEA, 2008).

The mechanism of action of primulasaponin is not yet completely clear. In literature, there is a general agreement that saponins irritate gastric mucosa locally causing a reflex to increase bronchial secretion which dilutes mucus and reduces the viscosity. Irritation of the mucous membranes in throat and respiratory tract could also cause increased bronchial secretion (EMEA, 2012).

High pressure liquid chromatography (HPLC) analysis on normal and reversed phases are most commonly used to identify and purify saponins. To achieve the best separation of saponins, HPLC on reversed phases is usually used. This technique is fast, selective and very sensitive. The separation of saponins by HPLC can be affected by different stationary and mobile phases (Negi, Singh, Pant, *et al.*, 2011; Oleszek, 2002; Zhao, Zhao, Yuan, 2012).

The aim of this paper was to develop and optimize the HPLC method for the identification and quantification of primula acid 1 and to determine the content of primula acid 1 in Primulae extractum fluidum.

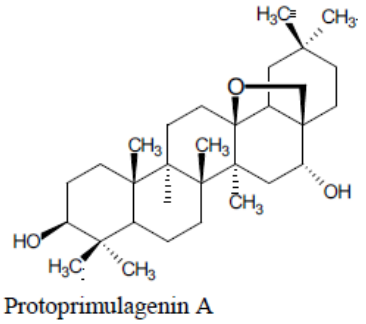

Figure 1. Aglycone part of saponin of *Primula veris* L. (protoprimulagenin A) (Wikimedia, 2019)

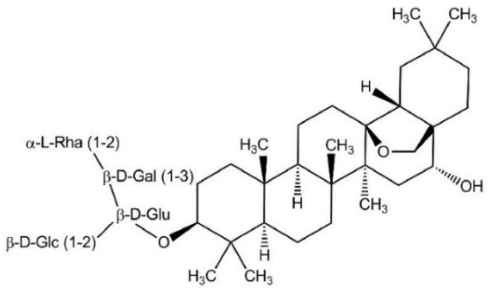

Figure 2. Structural formula of primula acid 1 (Wikimedia, 2019)

EXPERIMENTAL

Apparatus

The analysis was performed on HPLC apparatus with UV/VIS detector (HPLC system Prominence, type: 3-079, Shimadzu). The stationary phase was C18, dimensions of 250 x 4.6 mm, 5 µm Microsorb-Varian. Class-VP 7.4 software was used for signal analysis and statistical processing. Disposable filters CROMAFIL ® O-45/25- MachereyNagel, Germany; HPTLC plates 20x10 Kieselgel 60 F_{254}-Merck, USA; Microcapillaries 10 µl Camag, Swiss; UV/VIS lamp DesagaHeidelberg, Germany.

Chemicals

Primula acid 1 (≥99,9% purity)- Phytolab, Germany; Silica gel 60 GF_{254}- Merck, USA; Vanillin- Roth, Germany; Acetonitrile (HPLC grade)- Sigma Aldrich, USA; Methanol (HPLC grade)- Sigma Aldrich, USA; Sulphuric acid 96%- Lachema, Czech Republic; Ethanol absolute- Merck, USA; Purified water for HPLC.

Thin-Layer Chromatography (TLC)

Initially, screening of herbal extract was done to check for the presence of the substance of interest. Thin layer chromatography analysis was performed. For their preparation, HPTLC plates for analysis were kept overnight immersed in methanol, after which they were dried. Five samples were applied on the plate. The plate was placed in a chromatographic bath, where a mobile phase was previously prepared. When separation was completed, the plate was observed under a UV lamp at 254 nm. The plate was then sprayed with vanillin/ sulfuric acid reagent and again observed under UV lamp.

TLC conditions

HPTLC plates: 20x10 Kieselgel 60 F_{254}- Merck
Mobile phase: ethyl-acetate:formic acid:purified water (ratio 50:10:10 v/v)
Detection: UV/Vis light 254, 366 nm
Detection reagent: vanillin/sulfuric acid reagent

Sample preparation for TLC

1 ml from 5 g of liquid extract of primrose was dissolved in methanol in volumetric flask of 10 ml. 10 µl of this extracts was placed on HPTLC plate and then was separated.

Preparation of vanillin/sulfuric acid reagent

0.5 g of vanillin was dissolved in 80 ml of sulfuric acid and 20 ml of ethanol, cooled and used to visualize the TLC plates.

Chromatographic conditions

Stationary phase: column C18 (4.6 x 250 mm, 5 µm) Microsorb-Varian,
Mobile phase: 0.2% H_3PO_4 and acetonitrile (in 60:40 ratio v/v – isocratic),
Flow rate: 1.25 ml/min,
Injection volume: 20 µl,
Column temperature: 25˚C,
Detection: 195 nm.

Preparation of standard solution of primula acid 1

The stock solution of primula acid standard has been prepared at a concentration 1.0 mg/ml in methanol. For complete dissolution, solutions were placed in an ultrasonic bath for 15 minutes and then cooled. For the calibration curve, concentrations were prepared in the range of 50 to 150 µg/ml.

Preparation of *Primulae extractum fluidum* (Hagiwara, 2000).
Primulae radix 4.00 kg (v/v=0.1990)
Ethanolum (96 per centum) 8.80 kg (v/v=0.4378)
Aqua purificata 7.27 kg (v/v=0.3616)
Ammoniae solutio concentrata (10 per centum) 33.00 g (v/v=0.0016)

Total: 20.10 kg (v/v=1)

The prescribed ammounts of 96% ethanol and purified water were weighed and mixed. The ammonia solution was combined with alcohol and water. Th weighed ammount of drug (*Primulae radix*) which was previosly sieved and rinsed with cold water was poured over with 2 kg of combined solution and left to swell for 2 hours.
The drug was transferred to the percolate, the extraction solution was then poured and left to macerate for 12 hours. After maceration, percolation and filtration were performed. 5 g of liquid extract was obtained, so the drug extract ratio (DER) was 800:1. Used solvents were: ethanolum, aqua purificata and ammoniae solutio concentrata.
This prescription was used in the industrial preparation of intermediate product, from which 5 g was taken for the each analysis (HPLC and TLC), due to the concentration of primula acid in the final product (syrup).

Sample preparation of *Primulae extractum fluidum*
5 g of liquid extract of primula root (Primulae extractum fluidum, *Primula veris* L., *Primulaceae*) was dissolved in methanol in volumetric flask of 50 ml.

Contet calculation of Primula acid 1 in *Primulae extractum fluidum*
Content of Primula acid 1 in Primulae extractum fluidum can be calculated by the following formula:

$$\text{Content of Primula acid 1 } \left[{}^{\text{mg}}/_{\text{g}}\right] = \frac{X * V * 10^{-3}}{m}$$

$m_p = x * v$
m_p – mass of primula acid 1 in the sample in μg
X- concentration of primula acid 1 in the sample in μg/ml, calculated by the equation of calibration curve
V- sample volume in mililiters
m- mass of sample in grams
10^{-3} – used in formula to convert μg in mg

RESULTS AND DISCUSSION

The results of TLC analysis are shown in Figure 3. and Figure 4., where it is clearly seen the presence of primula acid 1. Rf value was 0.3.
Identification and quantification of primula acid 1 in the Primulae extractum fluidum can be proceeded by chromatographic method. Mueller et al. (2006) described the first liquid chromatographic method suitable for the characterization of bioactive compounds, i.e., saponins and phenolic glycosides, present in *Primula veris* and *Primula elatior*. On the other hand, Apel et al. (2017) developed a high performance liquid chromatography-diode array detection/mass spectrometry (HPLC/MS) method to analyze and compare the secondary metabolite profile of *Primula*.

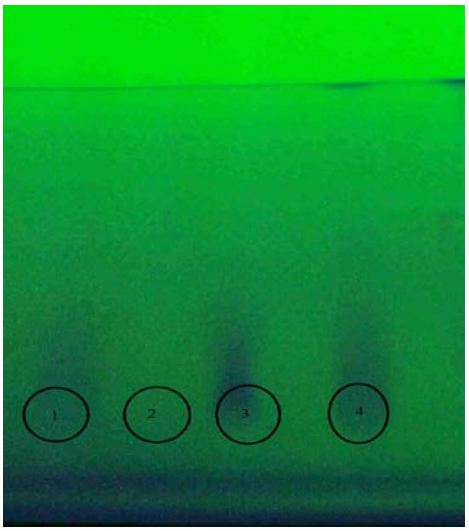

Figure 3. Chromatogram of standard of primula acid 1 (1), placebo (2), diluted sample of *Primulae extractum fluidum* (3) and sample of Primulae extractum fluidum observed at 254 nm.

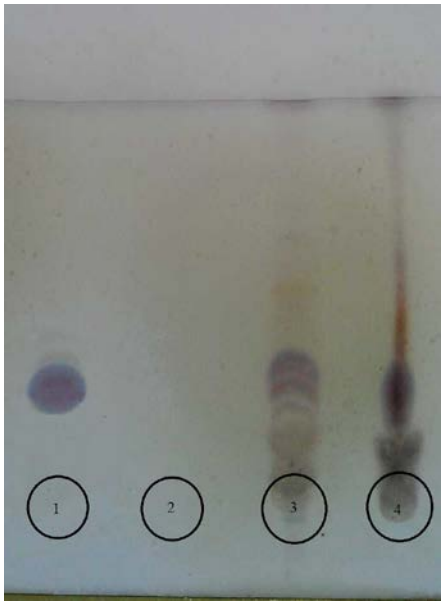

Figure 4. Chromatogram of standard of primula acid 1 (1), placebo (2), sample of *Primulae extractum fluidum* (3), sample of *Primulae extractum fluidum* (4) observed at daily light after spraying the plate with vanillin/ sulfuric acid reagent.

In this case, HPLC method was optimized and developed for identification and quantification of primula acid 1 in the *Primulae extractum fluidum.*
Validation of the analytical method was carried out by examining the following validation parameters: specificity, linearity, accuracy, repeatability, detection limit, quantification limit. (Figure 5. and Figure 6.) (ICH, 2005; Ivanović, Zečević, Malenović, 2000).
For the **selectivity** it was injected 20 μl of primrose extract (*Primulae extractum fluidum*) and 20 μl of placebo. Both solutions were recorded five times. Figure 5. and Figure 6. show a chromatogram of the primrose extract sample and a placebo chromatogram that clearly shows the retention time and area below the signal of the primula acid 1.

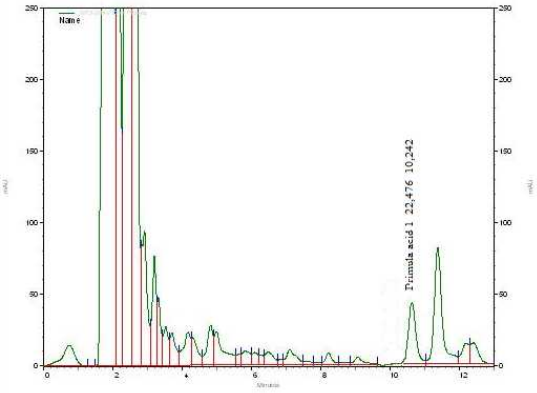

Figure 5. Chromatogram of sample of *Primulae extractum fluidum* detected at 195 nm

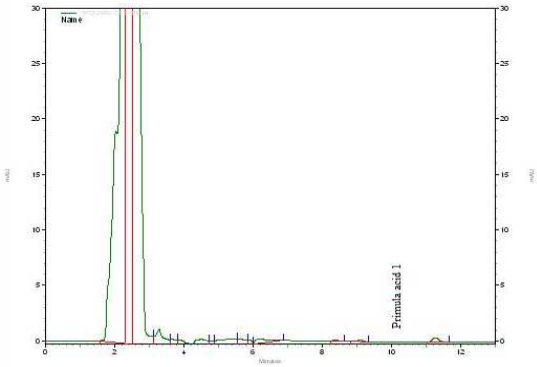

Figure 6. Chromatogram of placebo of solvents used in preparation of *Primulae extractum fluidum* detected at 195 nm

The **linearity** of the primula acid method in a wide range of concentrations of 50-150 µg/ml was determined. The calibration curves were constructed, the coefficient of correlation was calculated for primula acid R^2=0.9924, while the equation of direction was y=765.17x+1102.5 (Table 1, Figure 7.).

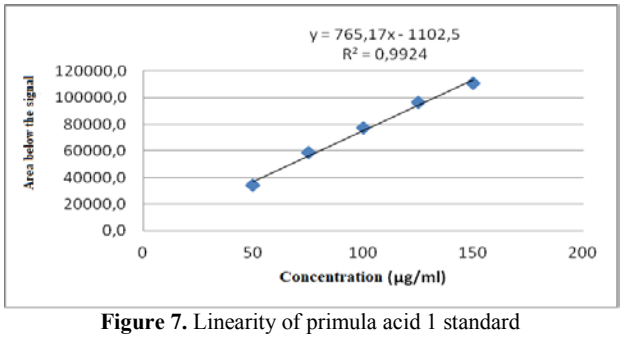

Figure 7. Linearity of primula acid 1 standard

Based on the linearity validation parameter, the values for the detection limit and quantification limit were obtained:
The detection limit was LD=10.41 µg/ml, and **the quantification limit** was LQ=34.69 µg/ml.

The **accuracy** of the test method was determined for primula acid concentrations 80; 100; 120 µg/ml representing 80, 100, 120% of the stock standard concentration. Tables 1 and 2 give the values for areas below the signal, concentration, recovery *(R%),* standard deviation (SD), relative standard deviations (RSD) and reliability coefficient (tα).

Repeatability of the test method was determined for primula acid concentrations 80; 100; 120 µg/ml representing 80, 100, 120% of the stock standard concentration. Tables 3. and Table 4 give the values for *recovery (R%)*, standard deviation (SD) and relative standard deviations (RSD%).

Intermediate precision
Intermediate precision for primula acid was also assessed, with three analysts separately performing three sample analysis in two different days (Table 5.).

Sample analysis
The chromatogram of the prepared extract solution (preparation was previously explained) can be seen in Figure 5., with associated retention times and area below the signal (10.108 minutes and area was 22 476).
The calculation shows that the solution contains 27.93 µg/ml of primula acid 1. This concentration is below quantification limit, but above detection limit, so it can be determined in the sample. Considering the extract preparation and dillution, the content of primula acid 1 per gram of extract is 0.2793 mg per gram (0.02793%) of Primulae extractum fluidum. The process of extract preparation has to be standardized so that the exact contents of the active components of primula acid coming from a different herbal material can be guaranteed by the manufacturer. These preparations are an important part of herbal medicines which have to pass strict quality control so that they could be registered on the market. This is a very fast, reliable and low-cost analysis. Due to the simple preparation of samples, this method can be used in the regular control of the content of primula acid in the final products and in semiproducts (*Primulae extractum fluidum*).

Table 1. The areas below the signals of chromatogram for different concentrations of primula acid 1 standard

Concentration (µg/ml)	P1	P2	P3	$\bar{X}$	LD (µg/ml)	LQ (µg/ml)
50	34084	34155	34061	34100.00		
75	58516	58863	58653	58677.33		
100	76919	77409	76905	77077.67	10.41	34.69
125	96372	94805	94616	95264.33		
150	111175	110100	111583	110952.67		

Table 2. Validation parameter accuracy for primula acid 1

	P1	P2	P3	$\bar{X}$	<ɣ>	SD	<R%>	RSD (%)	t_α
80% (80 µg/ml)	62194	62535	62124	62284.33	82.84	0.29	103.55	0.4	1.31
100% (100 µg/ml)	77059	77109	77907	77358.33	102.54	0.62	102.54	0.6	1.34
120% (120 µg/ml)	91859	91356	92368	91861	121.49	0.66	101.24	0.5	1.45

Table 3. Validation parameter repeatability for primula acid 1

Number of measure	**concentration 1**			**concentration 2**			**concentration 3**		
	Area	Conc. (µg/ml)	*R (%)*	Area	Conc. (µg/ml)	*R (%)*	Area	Conc. (µg/ml)	*R (%)*
1	61194	81.42	101.77	77059	102.15	102.15	91859	121.49	101.24
2	62535	83.17	103.96	77109	102.21	102.21	91356	120.83	100.69
3	62124	82.63	103.29	77907	103.26	103.26	92368	122.16	101.80
4	61931	82.38	102.97	77721	103.01	103.01	92652	122.53	102.11
5	61907	82.35	102.93	77398	102.59	102.59	91768	121.37	101.14
6	61582	81.92	102.40	77532	102.77	102.77	92579	122.43	102.03
$\bar{X}$	61878.83	82.31	102.89	77454.33	102.67	102.67	92097	121.80	101.50
SD		0.60	0.75		0.44	0.44		0.67	0.61
RSD (%)		0.7	0.73		0.4	0.43		0.6	0.60

Table 4. Validation parameter intermediate precision for primula acid 1 and (First day)

	Analyst 1/ Sample 1			**Analyst 2/ Sample 2**			**Analyst 3/ Sample 3**		
Number of measure	*Area*	*Conc. (µg/ml)*	*R (%)*	*Area*	*Conc. (µg/ml)*	*R (%)*	*Area*	*Conc. (µg/ml)*	*R (%)*
1	91615	121.17	100.98	88984	117.73	98.11	89175	117.98	98.32
2	90586	119.83	99.86	89629	118.58	98.81	89915	118.95	99.13
3	92423	122.23	101.86	89113	117.90	98.25	90007	119.07	99.23
$\bar{X}$	91541.33	121.08	100.90	89242.00	118.07	98.39	89699.00	118.67	98.89
SD		1.20	1.00		0.45	0.37		0.60	0.50
RSD (%)		0.99	0.99		0.38	0.38		0.50	0.50

Table 5. Validation parameter intermediate precision for primula acid 1 and (Second day)

	Analyst 1/ Sample 1			**Analyst 2/ Sample 2**			**Analyst 3/ Sample 3**		
Number of measure	*Area*	*Conc. (µg/ml)*	*R (%)*	*Area*	*Conc. (µg/ml)*	*R (%)*	*Area*	*Conc. (µg/ml)*	*R (%)*
1	89632	118.58	98.82	89633	118.58	98.82	90924	120.27	100.22
2	90196	119.32	99.43	88518	117.12	97.60	89733	118.71	98.93
3	89316	118.17	98.47	88082	116.56	97.13	90817	120.13	100.11
$\bar{X}$	89714.67	118.69	98.91	88744.33	117.42	97.85	90491.33	119.70	99.75
SD		0.58	0.49		1.05	0.87		0.86	0.72
RSD (%)		0.49	0.49		0.89	0.89		0.72	0.72

CONCLUSIONS

The results of HPLC analysis of *Primulae extractum fluidum* showed that the concentration of primula acid 1 was 0.2793 mg per gram (0.02793 %) of the extract. The proposed HPLC method was validated through validation parameters and all results of the statistical analysis were within the reference range recommended by the ICH guidelines. Besides that, this method proves to be very fast, reliable and low-cost. Considering all of this and the fact that this method does not require a complicated sample procedure, it can be used in the regular control of the content of primula acid 1 in the final products and in semiproducts (*Primulae extractum fluidum*).

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Summary/Sažetak

Primula veris L. (Primulaceae) je dobro poznata ljekovita biljka čiji se korijen u službenoj upotrebi koristi za liječenje kašlja kod prehlada. Ostale prijavljene indikacije odnose se na respiratorne, torakalne i medijastinalne poremećaje. Ovi efekti su rezultat visokog sadržaja triterpenskih saponina i fenolnih glikozida. Primula kiselina 1 (PA 1, također primulasaponin 1) je glavna aktivna komponenta u *Primula elatior* L. U ovom radu je razvijena metoda tečne hromatografije pod visokim pritiskom (HPLC) za određivanje sadržaja primula kiseline 1 u ekstraktu jagorčevine. Za utvrđivanje prisustva supstance od interesa prethodno je korištena hromatografija na tankom sloju. Određivanje je provedeno na obrnutoj fazi C18, a za razdvajanje je korištena mobilna faza 0.2% H_3PO_4 i acetonitril. Navedena metoda validirana je kroz različite parametre. Limit detekcije za primula kiselinu 1 iznosio je LD=10.41 µg/ml, a limit kvantifikacije LQ=34.69 µg/ml. Za određivanje sadržaja primula kiseline 1 konstruisana je kalibraciona kriva, te je potom na osnovu jednačine pravca određen sadržaj primula kiseline 1 u ekstraktu jagorčevine koji je iznosio 0.2793 mg po gramu ekstrakta. Rezultati i jednostavnost pripreme uzorka ukazuju na to da je HPLC metoda izbora za ovakvu vrstu analize.