

HPLC method for the analysis of chlorogenic acid of *Viburnum tinus* L. and *Viburnum orientale* Pallas

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A simple and sensitive method for separation and determination of chlorogenic acid has been developed. Chlorogenic acid was separated using a Supelcosil LC 18 column (250 x 4.6 mm, 5 µm) by gradient elution at the flow rate of 1.2 mL/min. The composition of mobile phase consisted of *o*-phosphoric acid, bidistilled water (0.2%) and acetonitrile. Spectrophotometric detection was carried out at 330 nm. The linear range of detection for chlorogenic acid was between 0.7237-500 µg/mL. The method described was suitable for the determination of chlorogenic acid in the leaves, branches and fruits of *Viburnum tinus* L. and *Viburnum orientale* Pallas. It was observed that *V. orientale* fruit sample has the highest chlorogenic acid content (0.5069%), while *V. tinus* branches (0.0141%) have the lowest chlorogenic acid content as w/w (%) in our case. This study is the first application of High Performance Liquid Chromatography (HPLC) method to the determination of chlorogenic acid content of *V. tinus* and *V. orientale* in Turkey.

Key words: *Viburnum tinus*, *Viburnum orientale*, HPLC, Chlorogenic acid

Viburnum tinus L. ve *Viburnum orientale* Pallas'de YPSK ile Klorojenik Asit Analizi

Çalışmada, klorojenik asidin ayrımı ve tayini için basit ve hassas bir yüksek performanslı sıvı kromatografisi (YPSK) yöntemi geliştirildi. Ayrım Supelcosil LC 18 kolon (250 x 4,6 mm, 5µm) kullanılarak, 1.2 mL/dak akış hızında, *o*-fosforik asitli bidistile su (%0,2) ve asetonitrilden oluşan gradient çözücü sistemi ile gerçekleştirildi. Spektrofotometrik ölçüm 330 nm dalga boyunda yapıldı. Klorojenik asit için doğrusallık aralığı 0.7237-500 µg/mL olarak bulundu. Geliştirilen yöntem, *Viburnum tinus* L. ve *Viburnum orientale* Pallas yaprak, dal ve meyvelerinde klorojenik asit tespiti için uygun bulunmuş ve analiz sonucunda, en yüksek klorojenik asit içeriği *V. orientale* meyvelerinde (%0.5069) görülürken, en düşük miktarın *V. tinus* dallarında (%0.0141) olduğu tespit edilmiştir. Bu çalışma, Türkiye'de yetişen *V. tinus* ve *V. orientale* türlerinde klorojenik asit miktar tayini için yapılan ilk YPSK uygulamasıdır.

Anahtar kelimeler: *Viburnum tinus*, *Viburnum orientale*, YPSK, Klorojenik asit.

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INTRODUCTION

The genus *Viburnum* (Caprifoliaceae) comprises more than 230 species distributed from South America to South-East Asia, the majority of them being endemic (1). However, recent classifications based on molecular phylogeny have put them in the family Adoxaceae (2). The plant is represented by four species in Flora of Turkey; *Viburnum opulus* L., *Viburnum lantana* L., *Viburnum orientale* Pallas and *Viburnum tinus* L. (3, 4).

In inner Anatolia, a traditional drink named gilaburu has been prepared from the fruits of *V. opulus*. The fruit has a dark-red color and is edible. The barks of *V. lantana* have been used in folk medicine as rubefiant and analgesic (5). On the other hand *V. tinus* has been used neuroprotective, hepatoprotective, sedative and spasmolytic activities (6,7). *Viburnum grandiflorum* Wall ex D.C. has been used in wound healing treatments, diuretic and in treatment of malaria (8). *Viburnum punctatum* Buch.-Ham. ex D. Don leaves have been traditionally used for the treatment of fever, stomach disorder (9). The bark and root of *Viburnum prunifolium* L. (black haw) is used for complaints of dysmenorrhea in folk medicine (10). The preventive effect of *Viburnum dilatatum* Thunb. on oxidative damages was reported in rats subjected to stress (11) and streptozotocin-induced diabetic rats (12). In addition, the effect of *V. dilatatum* on antioxidant enzymes in plasma, liver, stomach was examined and the results suggested that ingestion of the fruits of this plant might contribute to reduce the consumption of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and glutathione (13). Some iridoid aldehydes isolated from *V. Luzonicum* Rolfe exhibited moderate inhibitory activity against He La S3 cancer cells (14). The genus *Viburnum* is known to contain triterpenoids (15,16) diterpenoids (17,18) sesquiterpenes (19), iridoids (14,20-23) and polyphenols (24-26).

The aim of this study was to determine the appropriate HPLC method. The HPLC procedure was optimized according to the experimental conditions in our laboratory. Quantitative evaluation was performed at 330

nm. The HPLC method was selective, precise, accurate and successfully applied to determine the chlorogenic acid contents of the leaves, branches and fruits of the two species of *Viburnum* in Turkey.

EXPERIMENTAL

Plant material

V. tinus was collected from Aydın, Turkey and *V. orientale* was collected from Artvin, Turkey. Voucher specimens were deposited at the Herbarium of Ankara University, Faculty of Pharmacy (AEF) with the herbarium numbers of AEF 25891, AEF 25988, respectively.

Chemicals

Chlorogenic acid (Sigma; C-3878) was obtained from Sigma Chemicals. Chromatographic grade-double distilled water, HPLC grade acetonitrile (Merck 100030) and HPLC grade *ortho*-phosphoric acid (Merck 100565) were used.

Extraction

Five grams of dried and powdered samples (leaves, branches and fruits) of *V. tinus* and *V. orientale* were macerated with methanol (MeOH) at room temperature for 8 h. The resulting extracts were filtered, and diluted to 100.0 mL with MeOH. Solution was passed through a 0.45 µm filter and 10 µL of sample was directly injected into the HPLC. The assay results were obtained from the mean value of three separate injections.

Apparatus

The chromatograms of standards and samples were plotted by HPLC system consisting of a Hewlett Packard Agilent 1100 Series G1315 DAD. UV-DAD detector was set at 330 nm and peak areas were integrated automatically by computer using Agilent software. The chromatograms were plotted and processed by using the above mentioned software. Separation was carried out using a Supelcosil LC 18 column (250 x 4.6 mm, 5 µm) at the flow rate of 1.2 mL/min in a gradient elution. The mobile phase was prepared daily, filtered through a 0.45 µm membrane filter. All the calculations

concerning the quantitative analysis were performed with external standardisation by measurement of peak areas.

Standard working solution

Chlorogenic acid (5 mg) was accurately weighed into a 10 mL volumetric flask and dissolved in MeOH and filled up to volume with MeOH. The standard stock solution containing 500 µg/mL chlorogenic acid was prepared.

Calibration

Standard stock solution was prepared as chlorogenic acid. Eight different concentration levels (0.7237, 1.5, 10, 25, 50, 100, 200, 500 µg/mL) were prepared by diluting the stock solution. Triplicate 10 µL injections were performed for each standard solution. Peak area of each solution was plotted against the concentration to obtain the calibration curves.

Chromatographic conditions

HPLC analysis was performed by gradient elution at the flow rate of 1.2 mL/min. The mobile phase composition consisted of *o*-phosphoric acid, bidistilled water and acetonitrile. All solvents were filtered through a 0.45 µm milipore filter before use and degassed in an ultrasonic bath. Volumes of 10 µL each prepared solutions and samples were injected into the column.

Method development

In our study, several chromatographic conditions were tested for the separation and determination of chlorogenic acid in samples. Good separation and determination of chlorogenic acid in the extracts of leaves, branches and fruits of *V. tinus* L. and *V. orientale* Pallas were performed by using the mobile phase consisting of *o*-phosphoric acid bidistilled water (0.2%) and acetonitrile and a Supelcosil LC 18 column (250 x 4.6 mm, 5 µm), at the flow rate of 1.2 mL/min at 25°C. The mobile phase was made up of 0.2% phosphoric acid in water (A), acetonitrile (B) and in gradient elution: initially 0 min, A–B (94:6, v/v); then 0–25 min, linear change from A–B (94:6, v/v) to A–B (70:30); 25–30 min the linear isocratic elution is from A–B

(70:30). Chromatograms were plotted by UV-DAD detector at the wavelength 330 nm. Detector responses were measured as peak areas. The injection volume was 10 µL and triplicate injections were used for each sample. At the flow rate of 1.2 mL/min the retention times for chlorogenic was observed to be 7.68 min. The maximum absorption of chlorogenic acid was found to be at 330 nm and this wavelength was chosen for the analysis.

Linearity

Table 1 presents the equation of the regression line, correlation coefficient (r^2), relative standard deviation (RSD) values of the slope and intercept for each compound. Excellent linearity was obtained for compounds between peak areas and concentrations of 0.7237–500 µg/mL with $r^2=0.9996$.

Limits of detection and quantification

Limits of detection (LOD) were established at a signal-to-noise ratio (S/N) of 3. Limits of quantification (LOQ) were established at signal-to-noise ratio (S/N) of 10. LOD and LOQ were experimentally verified by six injections of chlorogenic acid at the LOD and LOQ concentrations. The LOD was calculated to be 0.2171 µg/mL and the LOQ was calculated to be 0.7237 µg/mL for chlorogenic acid (Table 1).

Precision

The precision of the method (within-day variations of replicate determinations) was checked by injecting nine times of chlorogenic acid at the LOQ level. The precision of the method, expressed as the RSD% at the LOQ level were 1.154% for chlorogenic acid (Table 2).

Chlorogenic acid analysis

Quantitative determination of chlorogenic acid in the leaves, branches and fruits of *V. tinus* and *V. orientale* were carried out by RP-HPLC using external standard method.

RESULTS and DISCUSSION

The assay results of two *Viburnum* species are shown in Table 3. The standard solutions of chlorogenic acid was added to plant extracts and injected at each time. The area of peak corresponding to standards was increased to prove the presence of these compounds. Their percent mean and standard deviation values are summarized in the same table (Table 3).

The present HPLC method was applied to the *V. tinus* and *V. orientale* extracts and allowed the efficient separation of chlorogenic acid in the extracts (Figure 1). HPLC analysis results of chlorogenic acid contents in different parts of *V. tinus* and *V. orientale* are given in Table 3. The chlorogenic acid contents of *V. tinus* were found to be 0.2413% for leaves, 0.0141% for branches and 0.0741% for fruits. For *V. orientale*, these values were found as 0.3811%, 0.2328% and 0.5069%, respectively.

Chlorogenic acid content of *V. orientale* fruit and leaf extracts (0.5069%, 0.3811% respectively) was higher than other *Viburnum* extracts. The quantity of the substance found in the *V. tinus* branch and fruit was low (0.0141%, 0.0741% respectively). This indicates that *V. orientale* fruits and leaves, may be considered as the most favourable parts as a source of chlorogenic acid (Table 3). In our previous study, the MeOH extracts of branch, leaf and fruit of *V. opulus* were analyzed by high-performance liquid chromatography for their chlorogenic acid contents and *V. opulus* fruits were found to contain the highest chlorogenic acid amounts (1.24%). In the same study, *V. lantana* fruit were found to have 0.01% chlorogenic acid as the same in *V. tinus* branch (27).

Table 1. Linearity Results, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Compound	λ , nm	Equation ^a	r^2	Slope (RSD% ^b)	Intercept (RSD% ^b)	LOQ $\mu\text{g}/\text{mL}$	LOD $\mu\text{g}/\text{mL}$
Chlorogenic acid	330	$y=26.9492x-41.2420$	0.9996	0.497	5.804	0.7237	0.2171

^a Linear regression equation $y = ax + b$, in which x is the concentration as $\mu\text{g}/\text{mL}$ and y is the peak area at the selected wavelength.

^b $\text{RSD}\% = (\text{Standard Deviation} / \text{Mean}) \times 100$

Table 2. Precision of the developed method at the LOQ level (n= 9)

Compound	λ , nm	Peak Area (mean)	RSD%
Chlorogenic acid	330	14.38589	1.154

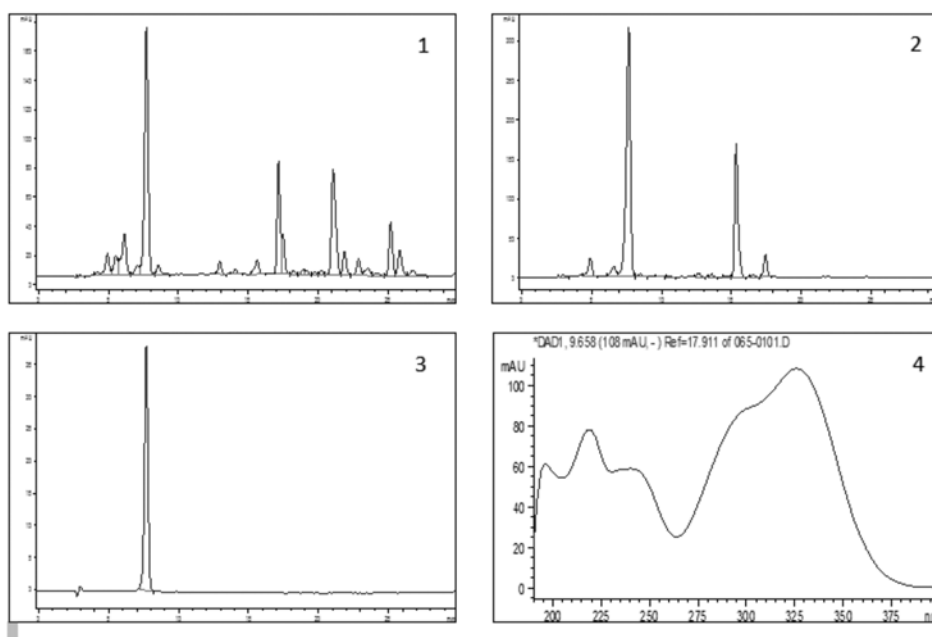
Table3. Contents of chlorogenic acid in *Viburnum* species

Species	Chlorogenic acid % (n= 3) Mean \pm SD ^a
<i>V. tinus</i> leaves	0.2413 \pm 0.0010 (0.4089) ^b
<i>V. tinus</i> branches	0.0141 \pm 0.0001 (0.4104) ^b
<i>V. tinus</i> fruits	0.0741 \pm 0.0002 (0.2062) ^b
<i>V. orientale</i> leaves	0.3811 \pm 0.0036 (0.9340) ^b
<i>V. orientale</i> branches	0.2328 \pm 0.0001 (0.0430) ^b
<i>V. orientale</i> fruits	0.5069 \pm 0.0049 (0.9738) ^b

^a SD= Standard Deviation

^b RSD% values are given in the parenthesis, $\text{RSD}\% = (\text{Standard Deviation} / \text{Mean}) \times 100$

Figure 1. HPLC chromatograms of *V.tinus* leaves (1), *V. orientale* fruits (2), chlorogenic acid (3) and UV spectrum of chlorogenic acid (4).



CONCLUSION

Chlorogenic acid is a common polyphenol and is contained in various food and beverages. It has been reported that chlorogenic acid has potent antioxidative and free radical-scavenging activities *in vitro*. Chlorogenic acid also increases the resistance of LDL to lipid peroxidation and inhibits DNA damage. Furthermore, chlorogenic acid inhibits lipopolysaccharide induced cyclooxygenase-2 expression in RAW264.7 cells.

These antioxidative and anti-inflammatory effects suggest that chlorogenic acid could aid in the prevention of cardiovascular diseases (28). *Viburnum* species show similar effects (29). Besides, *Viburnum* species have antitumor and antibacterial activities due to its polyphenolic content (30). According to our results, *V.orientale* fruits also can be evaluated as a good chlorogenic acid source. Hence it is not productive to use *V.tinus* branches to obtain chlorogenic acid.

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