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Original Article

DETERMINATION OF CAFFEIC AND CHLOROGENIC ACIDS IN THE LEAVES AND FRUITS OF *VITEX AGNUS-CASTUS*

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Abstract

A high performance liquid chromatographic method was applied to the determination of caffeic and chlorogenic acids in the leaves and fruits of Vitex agnus-castus collected from three different region of Turkey. Caffeic and chlorogenic acids were known to be potent antioxidants and the two phenolic acids were investigated in Turkish Vitex agnus-castus samples for the first time. The qualitative and quantitative analysis of six extracts of the plant were performed by RP-HPLC method. The linear range of detection for caffeic and chlorogenic acids were between 1.02-400 (r^2 0.9999) and 1.08-476 (r^2 0.9948) µg/mL, respectively. Marmaris and Antalya leaf samples display the highest content of caffeic acid as 0.277%(w/w) and 0.266%(w/w) while Marmaris fruit and Isparta fruit samples were rich in chlorogenic acid as 0.343%(w/w) and 0.303%(w/w), respectively.

Key words: *Vitex agnus-castus, phenolic acids, RP-HPLC*

Vitex agnus castus Yaprak ve Meyvalarında Kafeik Asit ve Klorojenik Asit Tayini

Türkiye'nin üç farklı yöresinden toplanan Vitex agnus castus bitkisinin yaprak ve meyvalarındaki kafeik ve klorojenik asit tayini yüksek basınçlı sıvı kromatografisi yöntemi uygulanarak gerçekleştirilmiştir. Kafeik ve klorojenik asit potansiyel antioksidanlar olarak bilinmektedir ve Türkiyede yetişen Vitex agnus castus örneklerinde bu iki fenolik asit ilk defa incelenmiştir. Bitkiden elde edilen altı ekstrenin kalitatif ve kantitatif analizleri RP-HPLC yöntemi ile yapılmıştır. Kafeik asit ve klorojenik asit için sırasıyla 1.02-400 (r^2 0.9999) ve 1.08-476 (r^2 0.9948) µg/mL konsantrasyon aralıklarında mükemmel doğrusallık elde edilmiştir. Marmaris ve Antalya yaprak ekstrelerinde kafeik asit miktarları en yüksek seviyede olup sırasıyla %0.277(a/a) ve %0.266(a/a) oranında iken Marmaris meyva ve Isparta meyva ekstreleri klorojenik asit açısından zengindir ve sırasıyla % 0.343(a/a) ve %0.303(a/a) oranında klorojenik asit içermektedir.

Anahtar kelimeler: *Vitex agnus-castus, fenolik asitler, RP-HPLC*

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INTRODUCTION

Vitex agnus-castus-chaste tree (Verbenaceae) is an important medicinal plant that grows naturally in Mediterranean area and widespread in South, West and North Anatolia [1]. Chaste tree is particularly used as a popular remedy for disorders of the menstrual cycle [2,3]. It was reported that fruit extracts of *Vitex agnus-castus* possess cytotoxic activity against certain kinds of human cancer cell line resulting in the induction of apoptosis [4]. In Turkey, the plant is used in folk medicine as a diuretic, carminative, sedative and an anaphrodisiac [5].

Phenolic acids, especially hydoxycinnamic acid derivatives are rather widespread in plants and they have attracted a great interest because of their various biological and pharmacological activities including antioxidative, antiviral and antilisterial activities [6-10]. Corresponding to this activities, determination of phenolic acids both qualitatively and quantitatively is very important. For the seperation and determination of these compounds several methods were applied. Since the derivatisation is not required prior to analyses, most of these methods were based on a high performance liquid chromatography (HPLC) technique with UV spectrophotometry [11-14]. A few studies were performed on the phenolic acids of *Vitex agnus-castus* [15] and there is not any study on the phenolic acid content of Turkish *Vitex agnus-castus* samples. In this study, we aimed to determine the two phenolic acids both qualitatively and quantitatively with RP-HPLC in *Vitex agnus-castus* growing naturally in three different region of Turkey.

EXPERIMENTAL

Materials

Vitex agnus-castus L. was collected from Marmaris (South-West Anatolia) (AEF 24674) in July 2006, Antalya (South Anatolia, close to the coast) (AEF 24676) and Isparta (South Anatolia, far to the coast) (AEF 24675) during their flowering period in July 2007. The leaves and fruits of the plants were dried at room temperature away from sunlight. Voucher specimen of the plants have been deposited at the Herbarium of Ankara University, Faculty of Pharmacy.

Chemicals and Standarts

Chromatographic grade double distilled water, HPLC grade methanol (Merck-1, 06007), 2-propanol (Merck-101040) and analytical grade *o*-phosphoric acid (Merck-563) were used for the HPLC analysis. Caffeic acid (SC0625) and chlorogenic acid (SC3878) were purchased from Sigma.

Extraction

400 mg of dried and powdered leaves and fruits of *Vitex agnus castus* were macerated with 10 ml *o*-phosphoric acid 0.085 % in water, *o*-phosphoric acid 0.085 % in methanol, *o*-phosphoric acid 0.085 % in 2-propanol (80:10:10 v/v/v) in a 25 ml erlenmayer and sonicated in an ultrasonic bath for 20 minutes at room temperature. The extracts were then filtered and added to 10.0 ml volumetric flask with mobile phase. Finally, the extracts were passed through 0.45 μm filter and injected into the HPLC column [16].

Apparatus

An Agilent 1100 Series HPLC system with a quaternery solvent delivery system, an online degasser, an autosampler, a DAD dedector was used for the analysis. The column was Phenomenex Luna C_{18} (5 μm, 250 mm X 4.6 mm) and column temperature was maintained at 30 °C. The system was controlled and data analysis were performed by Agilent Chemstation

Software. All the calculations concerning the quantitative analysis were performed with external standardization by the measurement of peak areas.

Stock and Standart Solutions

Caffeic acid (10.90 mg) and chlorogenic acid (11.90 mg) were accurately weighed into a 10 ml volumetric flask, dissolved in the mobile phase and filled up to volume for preparing stock solutions. Standard solutions were prepared in mobile phase with combination of each phenolic acid at five different concentration levels in 10 mL volumetric flasks for the establishment of calibration curves (Table 1).

Table 1. Calibration curves of two phenolic acids

Analyte	R T(min)	Standard curve	r^2	LOD (µg/mL)	LOQ (µg/mL)
Chlorogenic acid	4.82	y=15.157x-69.262	0.9948	0.36	1.08
Caffeic acid	7.83	y=24.108x+60.232	0.9999	0.34	1.02

y: peak area; x: concentration of analyte (µg/mL)
(LOD) S/N:3; (LOQ) S/N:9

Procedure

Chromatographic Conditions

The analysis were performed by gradient elution with a flow rate of 1 mL/min. Column temperature was set to 30 °C. The mobile phase was the mixture of *o*-phosphoric acid 0.085 % in water (solution A), *o*-phosphoric acid 0.085 % in methanol (solution B), *o*-phosphoric acid 0.085 % in 2-propanol (solution C). All solvents were filtered through a 0.45 µm Millipore filter before use and degassed in an ultrasonic bath. A gradient system was used for the HPLC analysis (Table 2). To obtain the chromatograms all parameters (mobil phase, flow rate, column temperature and wavelength) were investigated in our previous study and a gradient system with *o*-phosphoric acid 0.085 % in water (A), *o*-phosphoric acid 0.085 % in methanol (B) and *o*-phosphoric acid 0.085 % in 2-propanol (C) was found to be the efficient one for phenolic acids [16].

Table 2. Gradient system for the HPLC analysis

Minutes	A %	B %	C %	Flow Rate
0	80	10	10	1.0 ml/min
10	70	15	15	1.0 ml/min
15	60	20	20	1.0 ml/min
20	60	20	20	1.0 ml/min

Calibration

Mixed standard solutions containing caffeic acid (1.02-400 µg/mL) and chlorogenic acid (1.08-476 µg/mL) were prepared in the mobile phase. Triplicate 5 µl injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak area of each drug was plotted against the concentration to obtain the calibration graph.

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 a signal to noise ratio (S/N) of 9. LOD and LOQ were experimentally verified by nine injections of caffeic and chlorogenic acids at the LOD and LOQ concentrations. The LOD was calculated to be 0.34 and 0.36 µg/mL and the LOQ was calculated to be 1.02 and 1.08 µg/mL for caffeic and chlorogenic acids, respectively (Table 1).

Precision

The precision of the method (within–day variations of replicate determinations) was checked by injecting nine times of caffeic and chlorogenic acid at the LOQ level. The precision of the method, expressed as the RSD % at the LOQ level were 1.087 %, 2.376 % for caffeic and chlorogenic acid, respectively (Table 3).

Table 3. Precision of the method at the LOQ level (n=9)

Compound	λ (nm)	Peak Area (Mean)	RSD %
Chlorogenic acid	330	25.121	2.376
Caffeic acid	330	54.389	1.087

RSD % = (SD / Mean) X 100
SD = Standart Deviation

RP-HPLC Analysis

Volumes of 5 µL of each prepared solutions of samples were injected into the column and the chromatograms were recorded from 200 to 400 nm. Standard solutions were analyzed and three-dimensional chromatograms (wavelength; time; absorbance) were obtained to select the optimum wavelength for detection of these phenolic acids with maximum sensitivity. Quantification was performed by setting the detection wavelength as 330 nm for caffeic and chlorogenic acids using photo-diode array detector. The results were obtained as a mean value of three separate injections by using external standard method. The standard solutions of caffeic and chlorogenic acids were added respectively to extracts and injected. The areas of peaks corresponding to standards were increased to prove the presence of these compounds. The peaks in the chromatograms were identified by comparing the retention times and UV-spectra with two standards.

RESULTS AND DISCUSSION

Since there is an increasing interest in Agni casti preparations, it is well understood that determining the antioxidant principles in the plant is very important. The chemical composition of *Vitex agnus castus* exhibit differences according to the plant origin. In this study, qualitative and quantitative determination of caffeic and chlorogenic acids in the leaves and fruits of *Vitex agnus castus* from different origin were carried out by RP-HPLC. The chromatogram of the standard mixture of caffeic and chlorogenic acids and of Antalya leaf extract are given in Figure 1, 2.

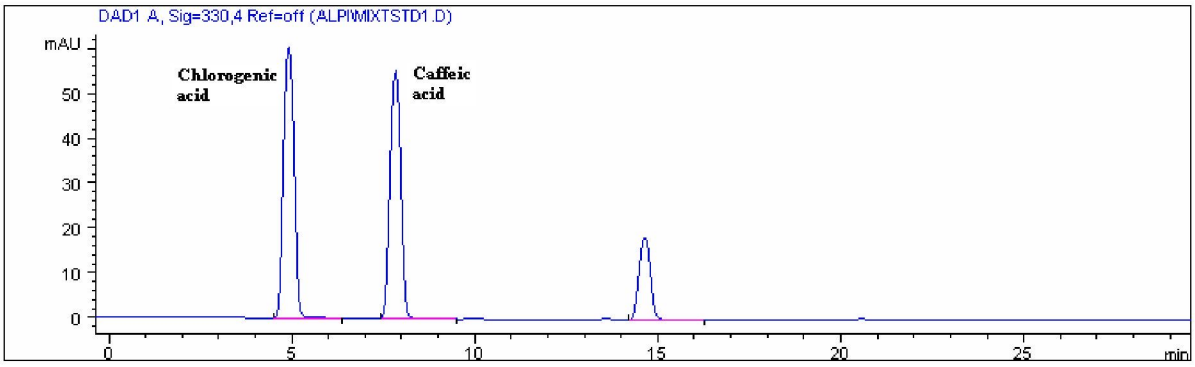

Figure 1. Chromatogram of the standard mixture

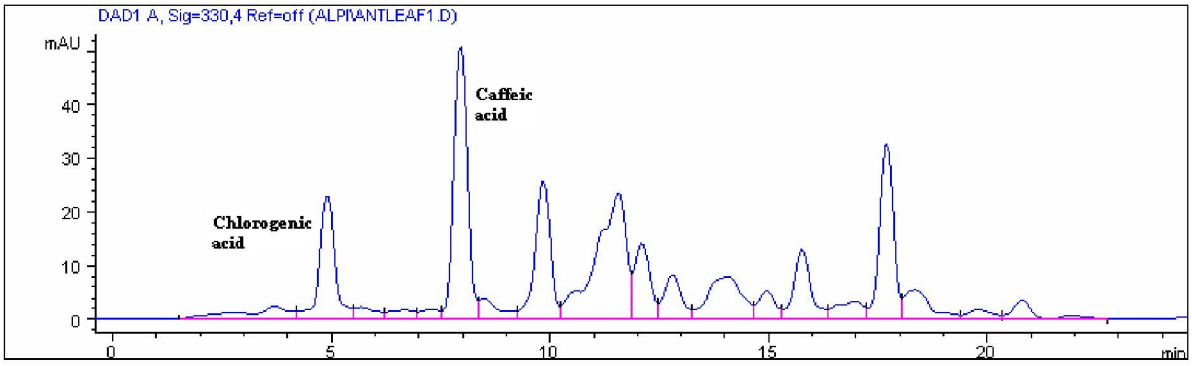

Figure 2. Chromatogram of the VAC Antalya leaves

The results of the contents of these two phenolic acids in *Vitex agnus castus* samples were summarized in Table 4. It was found that all leaf samples contain much more caffeic acid compared with the fruit samples. Marmaris and Antalya leaf samples have higher amounts of caffeic acid as 0.277 g/100g dry weight and 0.266 g/100g dry weight, respectively; on the other hand Marmaris and Isparta fruit samples were rich in chlorogenic acid as 0.343 g/100g dry weight and 0.303 g/100g dry weight, respectively. We could not meet any study about the content of phenolic acids in *Vitex agnus castus* except Proestos et al. study in which RP-HPLC was used and some phenolic acids were determined. Chlorogenic acid was not investigated and the caffeic acid content was given as 0.93 ± 0.02 mg/100g dry weight which was a very low amount when we compare with our all leaf and fruit samples [15].

In conclusion, It is clear that *Vitex agnus castus* samples from Turkey have caffeic and chlorogenic acids which can be used for the standardization of the extracts in significant amounts.

Table 4. Contents of Caffeic and Chlorogenic acids in *Vitex agnus castus* (VAC)

Sample	Caffeic acid (g/100gdw) n=3, mean Mean±SD	Chlorogenic acid (g/100gdw) n=3, mean Mean±SD
VAC Marmaris Leaves	0.277 ± 0.0013 (0.470)*	0.206 ± 0.0006 (0.292)*
VAC Marmaris Fruits	0.031 ± 0.0009 (3.015)*	0.343 ± 0.0050 (1.470)*
VAC Antalya Leaves	0.266 ± 0.0012 (0.460)*	0.190 ± 0.0072 (3.807)*
VAC Antalya Fruits	0.089 ± 0.0002 (0.192)*	0.130 ± 0.0008 (0.622)*
VAC Isparta Leaves	0.133 ± 0.0003 (0.241)*	0.089 ± 0.0003 (0.321)*
VAC Isparta Fruits	0.031 ± 0.0004 (1.302)*	0.303 ± 0.0033 (1.105)*

*RSD % values are given in the parenthesis
RSD % = (Standart Deviation / Mean) X 100
SD = Standart Deviation, dw = dry weight

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