

Radical Scavenging Activity and Vitexin Content of *Vitex agnus-castus* Leaves and Fruits

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Summary

The goal of this study was to determine some of the important phenolic constituents of the methanolic extracts of the leaves and fruits of *Vitex agnus-castus* by RP-HPLC with regard to the results obtained from radical scavenging activity and total phenolic content assays. 1,1-diphenyl-2-picrylhydrazyl (DPPH) test was used to assess the radical scavenging potential of the extracts. Both leaf and fruit extracts exhibited significant radical scavenging activity with IC₅₀ values of 0.449±0.001 mg/mL and 0.612±0.004 mg/mL, respectively. By using Folin-Ciocalteu's method, the total phenolic content of leaves and fruits of the plant was determined as 123.9±2.281 mg GAE/g extract and 114.5±2.704 mg GAE/g extract, respectively. In continuation of our ongoing studies on the phenolics of *Vitex agnus-castus*, a simple and accurate HPLC method was used to determine some phenolic compounds such as vitexin, ferulic acid, rutin, and luteolin qualitatively and quantitatively. Contrary to the previous literature, ferulic acid and rutin were determined neither in leaf nor in fruit extracts, while luteolin was determined in trace amount only in fruit extract. Vitexin, one of the important flavonoid constituents of *Vitex agnus-castus*, was determined in significant amount as 0.252±0.0089% and 0.342±0.0153% in the leaves and fruits, respectively. The results showed that vitexin is partially responsible for the antioxidant potential of the extracts due to its significant amount and the standardization of the extracts should be performed on vitexin by this suitable RP-HPLC method.

Key Words: *Vitex agnus-castus*, Antioxidant activity, Vitexin, DPPH, RP-HPLC, Total phenolic content.

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Vitex agnus-castus Meyva ve Yapraklarının Radikal Süpürücü Aktivitesi ve Vitexin İçeriği

Özet

Bu çalışmanın amacı, radikal süpürücü aktivite ve total fenol miktar tayini deneylerinden elde edilen sonuçlara dayanarak *Vitex agnus-castus* yaprak ve meyvalarının metanollü ekstrelerinin bazı önemli fenolik bileşenlerini TF-YBSK ile tayin etmektir. Ekstrelerin radikal süpürücü aktivite potansiyelleri 1,1-difenil-2-pikrilhidrazil (DPPH) testi kullanılarak değerlendirilmiştir. Sırasıyla, 0,449±0,001 mg/mL ve 0,612±0,004 mg/mL IC₅₀ değerleriyle, yaprak ve meyva ekstrelerinin her ikisi de belirgin derecede radikal süpürücü etki göstermiştir. Bitkinin yaprak ve meyvalarının total fenol içeriği Folin Ciocalteu yöntemi kullanılarak, sırasıyla, 123,9±2,281 mg GAE/g ekstre ve 114,5±2,704 mg GAE/g ekstre olarak tayin edilmiştir. *Vitex agnus-castus*'un fenolik bileşikleri üzerindeki çalışmalarımızın devamı olarak, vitexin, ferulik asit, rutin ve luteolin gibi bazı fenolik bileşiklerin kalitatif ve kantitatif tayini için basit ve hassas bir YBSK yöntemi kullanılmıştır. Literatür verilerinin aksine, hem yaprak hem de meyva ekstrelerinde ferulik asit ve rutine rastlanmazken, luteolin sadece meyva ekstrelerinde eser miktarda saptanmıştır. *Vitex agnus-castus*'un önemli flavonoid bileşenlerinden biri olan vitexin, yaprak ve meyvalarda sırasıyla %0,252±0,0089 ve %0,342±0,0153 olarak belirgin miktarlarda saptanmıştır. Sonuçlar, vitexinin bitkideki yüksek miktarına bağlı olarak ekstrelerin antioksidan potansiyelinden kısmi olarak sorumlu olduğunu ve ekstrelerin standardizasyonunun vitexin üzerinden bu elverişli TF-YBSK yöntemi ile gerçekleştirilebileceğini göstermektedir.

Anahtar Kelimeler: *Vitex agnus-castus*, Antioksidan aktivite, Vitexin, DPPH, TF-YBSK, Total fenol miktarı

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INTRODUCTION

Concerning the analytical studies on *Vitex agnus-castus* L. (VAC), too little information can be found in previous investigations. Bioactive and marker compounds such as casticin, aucubin, *p*-hydroxy benzoic acid, rutin, ferulic acid have been determined by validated analytical methods (1-4). Contrary to the restricted number of analytical studies, researchers mostly focused on the pharmacology of the plant; especially the effect of the plant on female troubles such as premenstrual syndrome and menstrual disorders has been well investigated (5-7). On the other hand, antioxidant activity studies were performed on *Vitex* species and positive results were obtained in dose dependent manner (8-10). VAC contains iridoids, flavonoids, diterpenoids and steroids as the liable phytochemical principles of above mentioned bioactivities (11-15). In this study, the radical scavenging activity and the total phenolic content of leaves and fruits of VAC collected from Antalya, Turkey were investigated. With regard to the prominent activity results, current work is mainly focused on the quantification of some phenolic compounds which are known as potent antioxidants. Among the investigated compounds, only vitexin was found in significant amount in both leaves and fruits of VAC and the quantification of vitexin was carried out for the first time in VAC. Vitexin is a flavon-C-glucoside with a wide range of pharmacological effects including antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, spasmolytic, antiviral, antithyroid and anti-glycation activities and so the quantification of such an important compound should contribute considerable interest in the standardization procedures of VAC extracts (16-23).

MATERIAL and METHODS

Plant Material

Vitex agnus-castus L. was collected from Antalya during its flowering period. The leaves and fruits of the plant were dried at room temperature away from sunlight. Voucher specimen of the plant was deposited in the herbarium of the Ankara University Faculty of Pharmacy (AEF 24676).

Chemicals and Standarts

Chromatographic grade double-distilled water, HPLC grade methanol, acetonitrile and analytical

grade trifluoroacetic acid were used for HPLC analysis. Folin Ciocalteu reagent, DPPH (D9132) and the following phenolic compounds were purchased from Sigma-Aldrich (Germany): gallic acid (G7384), vitexin (49513), ferulic acid (128708), rutin (R5143) and luteolin (L9283). All the other chemicals were analytical grade and were obtained from either Sigma-Aldrich or Merck.

Extraction

For HPLC analysis, dried and milled flowers and leaves of plant (200 mg) were extracted with methanol using a magnetic stirrer, for 6 h (50°C, 400 rpm). The extracts were then filtered and completed to 10.0 mL in a volumetric flask with methanol, passed through 0.45 µm filter and injected into the HPLC system. For the determination of radical scavenging potential and the total phenolic content, flowers and leaves of the plant (2 g) were extracted with the same method mentioned above.

Analysis of Phenolic Compounds by RP-HPLC

HPLC Conditions

An Agilent 1100 Series HPLC system with a quaternary solvent delivery system, an online degasser, an autosampler, a diode array detector (DAD) were used for the analysis. The column was Phenomenex Luna C₁₈ (5 µm, 250 mm X 4.6 mm). The analysis was performed by isocratic elution with a flow rate of 1 mL/min. Column temperature was set to 30°C. The mobile phase was a mixture of trifluoroacetic acid 0.1% in water (A), trifluoroacetic acid 0.1% in methanol (B), trifluoroacetic acid 0.1% in acetonitrile (C) with a ratio of 60:20:20 (A:B:C). All the solvents were filtered through a 0.45 µm Millipore filter before use and degassed in an ultrasonic bath. 10 µL volumes of each standard solution and sample were injected into the system 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 the detection of these phenolics with maximum sensitivity. Qualitative and quantitative analyses were performed by measuring at 335 nm for vitexin, 330 nm for ferulic acid, 360 nm for rutin and 340 nm for luteolin using DAD. The chromatographic run time was 25 minutes and the column void volume

was 2 minutes. The system was controlled and data analysis was performed by Agilent Chemstation software. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of the peak areas.

Calibration

Five different concentrations of vitexin were prepared in methanol ranging between 2.75-110 µg/mL. Triplicate 10 µL injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak areas obtained from injections were plotted against the concentrations to establish the calibration graph.

Limits of Detection and Quantification

Limit of detection (LOD) was established at a signal to noise ratio (S/N) of 3. Limit of quantification (LOQ) was established at a signal to noise ratio (S/N) of 10. LOD and LOQ were experimentally verified by the nine injections of vitexin in LOQ concentrations.

Precision

The precision of the method (within-day variations of replicate determinations) was checked by injecting nine times of vitexin at the LOQ level. The area values were recorded and RSD% was calculated.

Recovery

The spike recovery was carried out by the standard addition method. For the determination of the recovery from the methanol extract, three different concentrations of reference compound were added prior to the extraction. In each additional level, three determinations were carried out and the mean value of recovery percentage was calculated.

Determination of Total Phenolic Content

The total phenolic content of the extracts was determined spectrophotometrically using a modified Folin Ciocalteu method (24). The reduction of the reagent, which resulted in the formation of blue colour, was recorded at 765 nm. 100 µL of the methanol extracts of each plant part (2 mg/mL) were mixed with 7.9 mL of distilled water. 500 µL of Folin Ciocalteu reagent was added and the content of the

flask was shaken vigorously. After 8 min, 1.5 mL of 20% Na₂CO₃ was added. After 2h incubation at room temperature, the absorbance was measured at 765 nm with a Shimadzu spectrometer. Gallic acid was used as a standard. All the measurements were performed in three parallels and the average values were used to express the mg of gallic acid equivalents (GAE)/g dry extract.

DPPH (1,1-Diphenyl-2-picrylhydrazyl) Radical Scavenging Activity

The capacity to scavenge the stable free radical DPPH was monitored according to the modified method of Barros et al (25). Various concentrations of extracts (0.25 mL) were mixed with 2.75 mL of methanolic solution containing DPPH radical. The mixture was shaken vigorously and left to stand for 10 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm. The radical scavenging activity (Inh%) was calculated as a percentage of DPPH discoloration using the equation: $\text{Inh}\% = [(A_{\text{DPPH}} - A_s) / A_{\text{DPPH}}] \times 100$, where A_s is the absorbance of the solution when the sample extract was added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage against extract concentration. Caffeic acid from Sigma-Aldrich (Germany) was used as a standard.

RESULTS and DISCUSSION

As known to all, dietary antioxidants may play a role in the prevention of various diseases. Numerous plant constituents have been known to show free radical scavenging or antioxidant activity. Flavonoids and other phenolic compounds of plant origin have been reported as antioxidants and as scavengers of free radicals (16,23). In this study, VAC was investigated for its phenolic constituents and radical scavenging activity. First of all, the total phenolic content of leaves and fruits of VAC was determined and found as 123.9 ± 2.281 mg GAE/g extract and 114.5 ± 2.704 mg GAE/g extract, respectively. The yields of the extracts and the amounts of the total phenolics are given in Table 1. Afterwards, radical scavenging activity of the plant extracts was investigated. IC_{50} values obtained

Table 1. Total phenolic content, radical scavenging activity and vitexin amount in the leaves and fruits of VAC

	Total Phenolic Content		Radical Scavenging Activity	Vitexin Amount %
	Extract Yield %	mg GAE/g extract	IC ₅₀ (mg/mL)	
VAC leaves	29.5	123.9±2.281	0.449±0.001	0.252±0.0089
VAC fruits	30	114.5±2.704	0.612±0.004	0.342±0.0153
Caffeic acid	-	-	0.052±0.0001	-

Results were expressed as mean±SD (n=3)

from DPPH assay were determined as 0.449±0.001 mg/mL and 0.612±0.004 mg/mL for leaf and fruit extracts, respectively (Table 1). The leaf extract with higher total phenolic content exhibited higher radical scavenging activity with lower IC₅₀ value compared to the fruit extract. In a previous study, the antioxidant activity of water, ethanol and *n*-hexane extracts of VAC collected from Kuşadası-Turkey, was investigated by ABTS assay and water and ethanol extracts were found to possess stronger activity (8). This finding supports our results in terms of the strong antioxidant activity obtained by methanol extracts. In the current study, due to the significant antioxidant activity of the plant, some phenolic compounds such as vitexin, ferulic acid, rutin and luteolin which were partially supposed to cause this antioxidant potential were analyzed. In previous studies, some other phenolics were analyzed and results revealed that especially casticin, caffeic acid and chlorogenic acid have been found in high amount in VAC as the liable antioxidant agents (1,3). Our results showed that vitexin was determined in significant amount as 0.252±0.0089% and 0.342±0,0153% in the leaves and fruits, respectively as one of the important antioxidant constituents of VAC (Table 1).

As there is an increasing interest in Agni-casti preparations, the quality of the plant material should be well determined. For this reason, the quantitative

characterization of the plant sample is an important requirement. In previous studies, casticin and *p*-hydroxy benzoic acid were shown to be suitable phenolic compounds for standardization studies. In the current work, as a part of our progressive studies on the phenolic constituents of VAC, vitexin, ferulic acid, rutin, and luteolin which could be more efficiently used for the standardization of VAC preparations, were investigated by HPLC-DAD. According to the results of qualitative analysis, the presence of these compounds in Turkish sample was evaluated and contrary to the previous literature (4), ferulic acid and rutin were determined neither in leaf nor in fruit extracts while luteolin was determined in trace amount only in fruit extract. Vitexin is one of the main constituents of VAC and should be the most suitable compound for the quantitative determination of both leaf and fruit extracts. For the quantification of vitexin, a simple and accurate HPLC-DAD method was generated. Fine linearity results were obtained within a wide concentration range with R²= 0.9999. The precision of the method (within-day variations of replicate determinations) was checked by injecting nine times of vitexin at the LOQ level and expressed as RSD%=5.346. LOD and LOQ values were determined as 0.0149 µg/mL and 0.0499 µg/mL, respectively. No other effects were determined as it was ensured by the recovery values fall within the ranges from 97.29 to 98.27%. Linearity

Table 2. Linearity results and validation values for quantification of vitexin

	Regression line	R2	LOD(µg/mL)	LOQ(µg/mL)	Recovery(%)	Precision(RSD%)
Vitexin	y=22396x-19.671	0.9999	0.0149	0.0499	97.29-98.27	5.346

results and validation values for the quantification of vitexin are given in Table 2. The HPLC chromatograms of standard vitexin, fruit and leaf extracts and the UV spectra are given in Figure 1-5. A simple and feasible extraction procedure was applied and an isocratic elution profile was chosen for the stability of the media. Due to the findings above, this method should be used for the quantitative determination of vitexin in VAC samples.

CONCLUSIONS

In the current work, the radical scavenging activity and the total phenolic content of the plant were

investigated in Turkish sample, and the vitexin content of *Vitex agnus-castus* was determined with an easy and accurate RP-HPLC-DAD method for the first time. Both leaf and fruit extracts of the plant exhibited significant radical scavenging activity, and the leaf extract with higher total phenolic content exhibited higher radical scavenging activity compared to the fruit extract. Vitexin was determined in significant amount in the plant parts as one of the liable antioxidant constituents and should be used for the standardization of VAC preparations.

Figure 1. HPLC chromatogram of vitexin

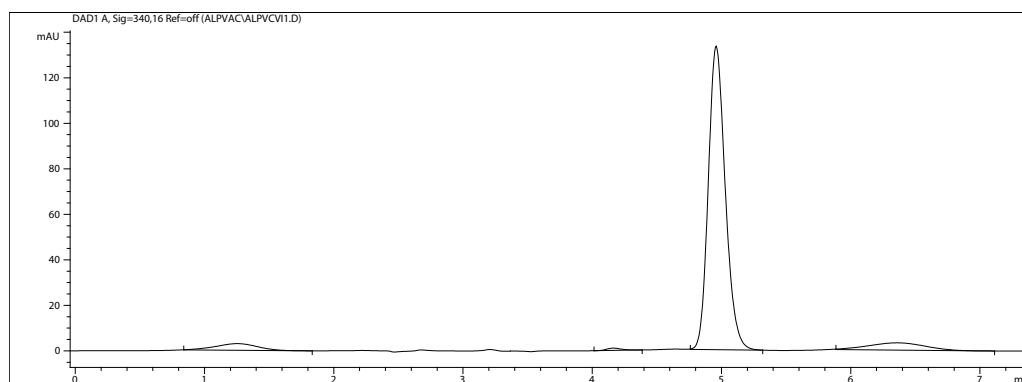


Figure 2. HPLC chromatogram of fruit extract

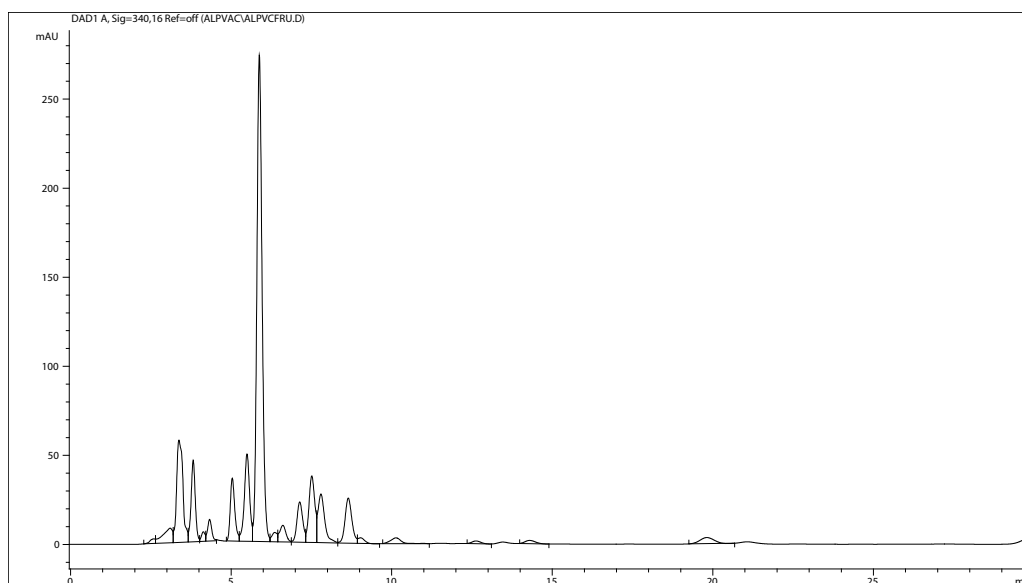


Figure 3. HPLC chromatogram of leaf extract

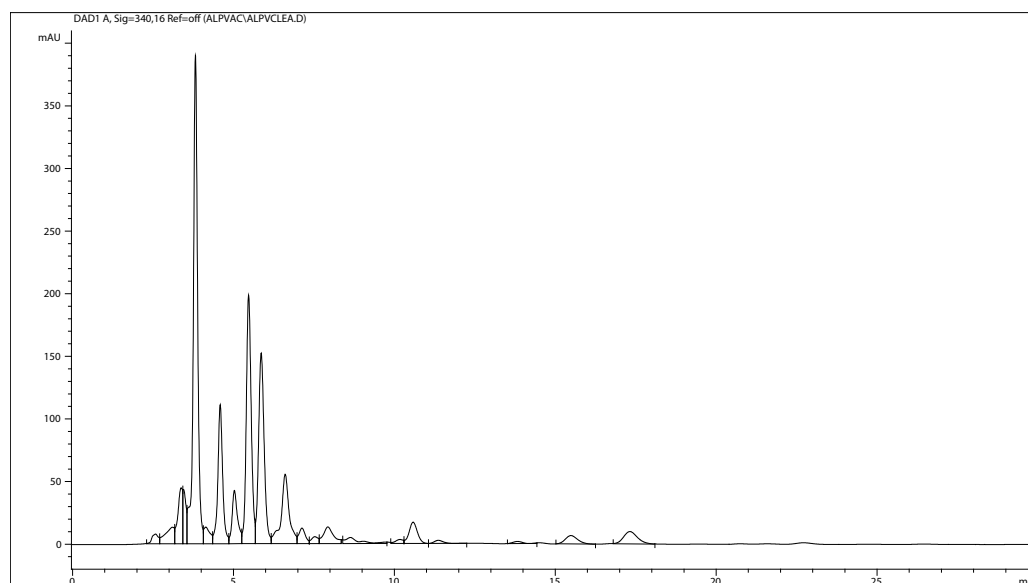


Figure 4. UV spectrum of vitexin

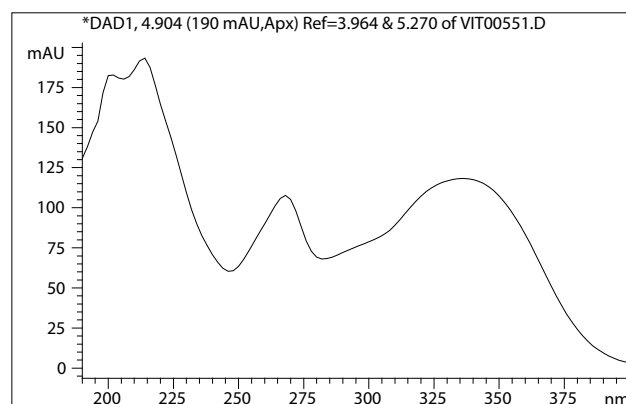
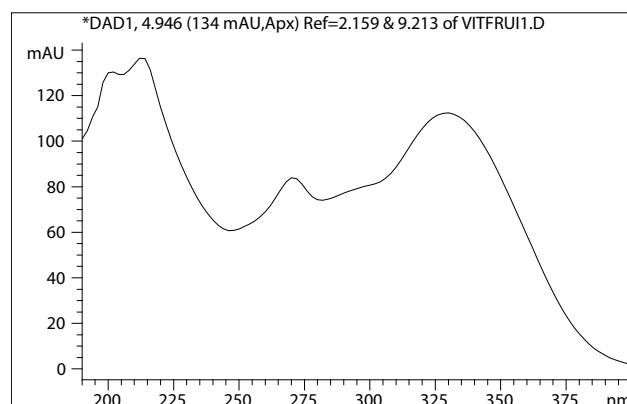


Figure 5. UV spectrum of vitexin in fruit extract



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