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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Determination of phenolic components of *Vitex agnus-castus* L. (Verbenaceae) from Muğla-Ula region in Turkey

Muğla-Ula yöresinde *Vitex agnus-castus* L. (Verbenaceae) fenolik bileşenlerinin belirlenmesi

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Ö Z E T / A B S T R A C T

Aims: In this research, it was aimed to determine the phenolic components of *Vitex agnus-castus* L. (Chaste) samples belonging to Verbenaceae family, which is one of the significant medicinal plants of our country.
Methods and Results: In this research, *Vitex agnus-castus* L. leaf and flower samples provided from during flowering grown under from Karabörtlen area (81m) ecological conditions. Samples were then dried in a semi-shadowy and airy place at room temperature (at 25°C) to be used in the extraction process. In this study, the phenolic composition obtained from *Vitex agnus-castus* was analyzed and determined by UPLC-ESI-MS / MS^- method. Totally, 8 phenolic components were determined in leaf and flower samples.Among the phenolic components of chasteberry (*Vitex agnus- castus* L.) obtained from leaf and flower samples during the flowering period, the most effective components; protocatechuic acid 4906.02 (mg kg^-); 4-hydroxy benzoic acid 1603.88 mg kg^-), gentisic acid 668.49 (mg kg^-) were found out.
Conclusions: Plant-derived natural products have received considerable attention in recent years due to their diverse pharmacological properties, including several compounds.Most of these bioactive compounds are plant secondary metabolites such as terpenoids, alkaloids and phenolic components found in plants. Phenolic compounds as antioxidants can prevent many diseases such as cancer, heart disease, cataracts, eye diseases, old age diseases, etc. The results of this study also showed that *Vitex agnus-castus* leaf and flower samples, were rich in phenolic components.
Significance and Impact of the Study: The results of this study indicated that *Vitex agnus-castus* possess a potential source of phenolics, and may be used in food, pharmaceutical and cosmetic industries as a protective agent to reduce oxidative damage in terms of phenolic components.This research can be used to guide current and future research.

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INTRODUCTION

The use of natural resources in therapy originated with the history of humanity. The earliest archives about use of plants in the treatment of diseases date back to written sources from Chinese, Indian and North African civilizations (Harvey, 2008; John, 2009; Phillipson, 2001; Rishton, 2008). Many medicinal plants discovered by trial and error in our country, known as medicinal plants, are applied in the treatment of diseases, just as they are in other parts of the world. The Anatolian people have benefitted from the use of medicine and food derived

from wild plants concurrently with human settlement. In developing countries, herbal medicines are an integral part of the culture and pract ices of rural communities (Yiğit and Benli 2005, Çenet and Toroğlu, 2006; Njume et al., 2009). Plant applied for the production of new and highly effective drug formulations has also been one of the pharmacology research areas due to the rich chemical structures of plants (Kulkarni and Vijiyanand, 2010; Jose et al., 2018; Mohammed et al., 2019). Considering today, despite the fact that synthetic products account for a large portion of the pharmaceutical industry, natural drug active ingredients and drugs manufactured from natural compounds account for roughly half of all drugs used today(Harvey, 2008; John, 2009; Phillipson, 2001; Rishton, 2008; Kültür, 2007).

Plants which are applied directly or indirectly as herbal medicine raw materials are called "Medicinal Plants". Most of the medicinal plants consist of aromatic plants. Nowadays, the term "medicinal" and "aromatic" plants are generally accepted together (Baydar, 2005). Many medicinal and aromatic plants are applied in a variety of fields because of the active chemical compounds present in their seeds, fruits, leaves, and roots due to their different modes of action (Maksimović et. al., 2005). Bioactive compounds that develop from a result of secondary metabolic activities of plants, cannot be consumed as food but have beneficial effects for human health are called 'phytochemicals' (Visioli et al., 2000; Sevindik, 2018). In recent years, there has been an increasing interest in phenolic compounds due to the antioxidant properties of phenolics. Antioxidants extracted from natural sources are preferred for use in food industry, pharmaceutical industry, fungicides and pesticides industry (Kähkönen et al., 1999; Willför et al., 2003). Most plants contain phenolic compounds, which are low-molecular secondary compounds. Phenolic compounds can be classified as soluble compounds such as phenolic acids, flavonoids, phenylpropanoids and quinones, and insoluble compounds such as condensed tannins, lignins, cell wall binding hydrocinnamic acid (Balasundram et al., 2006; Meral et al., 2012.). Phenolic compounds have been shown to be a preventive factor in heart disease and cancer treatment. Researches on plant phenolic components that are industrially significant are frequently carried out, and studies on alternative sources are increasing day by day (Kähkönen et al., 1999; Willför et al., 2003).

Verbenaceae family plants are defined as the family of plants generally used in traditional medicine in various countries. Verbenaceae is a family consisting of approximately 3000 species, rarely herbaceous plants in tropical and subtropical regions, and species in the form of shrubs or bushes (Gülsoy, 2011; Rahmatullah et al., 2011). It is a species that generally spreads in the Mediterranean Region, Central Asia and Southern Europe (Ono et al., 2011). *V. agnus-castus* L. (Hayıt) is known by the people with names such as 'Hayıt', 'Ayıd', 'Ayıt', 'Beşparmak Herb', 'Priest pepper' and 'Chaste tree' and it has different usage styles. *V. agnus-castus* is a deciduous, 3-6 m tall, round-crowned, upright and low-branching shrub or bushes with a thin medium texture (Brickell and Zuk, 1996; Cheifetz et al., 1999). The inflorescences are delicate, dense, fragrant, lilac-pink or rarely white colored spike or compound spike (Kayacık, 1966; Cheifetz et al., 1999). Local flowers and fruits have been applied in various cultures since ancient times (Bohnert and Hahn, 1990). It is known that the *V. agnus-castus* species has been used in the medical field for more than 2000 years (Asdadi et al., 2015). It was first mentioned in the inscriptions of Hippocrates in the 4th century BC (Odenthal, 1998). In addition, the moderate cytotoxic and pro-apoptotic effects of *V. agnus-castus* extracts on human cancer cells have also been investigated (Sezik et al., 2013). In another study, it was suggested that *V. agnus-castus* can be applied as a valuable tool in the treatment of bone resorption, benign growth of the prostate and prostate cancer in men (Ignjatović et al., 2012).

Vitex agnus-castus seeds or extracts obtained from fruit are applied among the public for therapeutic purposes, to eliminate fibroid cysts, infertility, menopause and menstrual period irregularities and troubles in women, to increase milk yield in breastfeeding mothers, acne problems, and impotence and stress in men (Odenthal, 1998; Baytop, 1999; Arokiyaraj et al., 2009; Bachrach, 2012; Ohyama et al., 2003; Stojković et al., 2011). In many studies conducted with *V. agnus-castus* samples, its chemical components were investigated and its hormonal effect was researched. For this reason, it has been stated that *V. agnus-castus* is a herbal alternative in the treatment of hormonal disorders and in relieving their symptoms (Wuttke et al., 2003). It has been stated that the estrogenic, dopaminergic and opioidergic properties of V. agnus castus are partially related to their phenolic component content of it, although their efficacy in the treatment of gynecology has not been fully investigated (Rani and Sharma, 2013; Webster et al., 2006)

It has been found out that *V. agnus-castus* leaf, flower and fruit are rich in phenolic acids and their derivatives, flavonoids, tannins, iridoids, diterpenoids and essential oil composition (Sağlam et al., 2007; Hajdú et al., 2007;

Proestos et al., 2006; Cabral et al., 2008; Stojković et al., 2011; Latovi et al., 2012; Fakir et al., 2014).

In another study, it was determined that the leaves and fruits of *V. agnus castus* contain a significant level of vitexin compound.Vitexin has many biological properties such as, antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, spasmolytic, antiviral, antithyroid and antiglication (Gökbulut et al., 2010; Peng et al., 2008; Zielińska and Zieliński, 2011). Therefore, it is very noteworthy to investigate the plants used in the treatment of diseases. The most plants are also waiting to be investigated scientifically (Karaoğul et al., 2011).The phenolic components of the *V. agnus-castus* plant, which is one of the most commonly applied natural phytochemicals today, were determined in this research.

MATERIALS and METHODS

Plant material

The material of the study consisted of *Vitex agnus-castus* (L.) that grow naturally in Muğla-Ula region Karabörtlen area (81m), May-2019 . V. agnus-castus leaf and flowers were collected from Karabörtlen area (81m), The leaves shoots and flowers of each plant were collected to be used in extraction system. The collected plants were put in plastic bags, and each bag was labeled. The data related to the collection time, place, and elevation were written on the label of each bag. These plants were then dried in a semi-shadowy and airy place at room temperature (at 25°C) to be used in extraction system. These plants samples were identified and kept at Muğla Sıtkı Koçman University Research Center Laboratory

Standards and reagents

The HPLC grade solvents such as methanol, acetonitrile, and hexane are used for extraction of phenolic compounds and were purchased from Merck (Darmstadt, Germany). Pyrogallol, homogentisic acid, 3,4-dihydroxybenzoic acid, gentisic acid, pyrocatechol, galantamine, 4-hydroxy benzoic acid, 3,4-dihydroxybenzaldehyde, catechin hydrate, vanillic acid, caffeic acid, syringic acid, vanillin, epicatechin, catechin gallate, p-coumaric acid, ferulic acid, rutin, trans 2-hydroxy cinnamic acid, myricetin, resveratrol, transcinnamic acid, luteolin, quercetin, naringenin, genistein, apigenin, kaempferol, hesperetin, chlorogenic acid and chrysin were used as the standards of phenolic compounds and purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). HPLC grade ultra-pure water was 18.2 MΩ.

Ultrasonic-assisted extraction of phenolic compounds from plant samples

Kıvrak et al., (2018) described the liquid-liquid extraction procedure by ultrasonic assisted extraction of phenolic components of *V. agnus-castus* samples which was performed with minor modifications. Approxiametly, 2.0 g of *Vitex agnus-castus* samples weighed into a centrifuge tube, and 15 mL of hexane and 30 mL of acetonitrile were added to extract the plant. The extract is mixed for 2 min then, extracted in an ultrasonic bath for 10 min followed by centrifuge at 1792 g .Then, the acetonitrile layer was separated. Those steps were applied two times more. The acetonitrile extracts were combined and washed with petroleum ether, and then evaporated to dryness using nitrogen evaporator. The residue was dissolved in water:methanol mixture (60:40, v/v), and then filtered through 0.20 µm PTFE syringe filter and 2 µL injected to UPLC-ESI-MS/MS (Waters Acquity Ultra Performance LC, Xevo TQ-S MS-MS) instrument, which stated in a previous method with slight modification(Kıvrak et al., 2018).

UPLC-MS/MS analysis of individual phenolic compounds

The phenolic profiles of the *V. agnus-castus* samples were determined according to the procedure given in (Kıvrak et al., 2018). Analysis of phenolic compounds from *V. agnus-castus* was carried out using an UPLC - ESI - MS / MS instrument. Mass spectrometry parameters, verification and quantization mass shift (m z^-) and their collision energies have been described in previous literature (Kıvrak et al., 2018).

Phenolic compounds were analyzed with using a C18 column (Acquity UPLC BEH C18 100 mm × 2.1 mm, 1.7-µm particle size). Compounds were separated by gradient elution.The mobile phases consisted of 0.5% acetic acid in water (S1) and 0.5% acetic acid in acetonitrile (S2). Elution was carried out with eluent (S1) and eluent (S2) at a flow rate of 0.650 mL min-1 at 40 ° C column oven temperature. Elution sequence from 1 minute linear gradient mode at 99% (S1), from 99% to 70% (S1) over 10 minutes, from 70% to 99% (S1) in 2 minutes, and finally a (S1) 3 minute plateau at 99% of value. Re-equilibration of the column was achieved at the end plateau. Tandem mass spectrometer parameters were ion mode elektrospray ionization nebulizer 7.0 bar, source temperature 150 ° C, desolvation temperature 500 °C.

RESULTS and DISCUSSION

In this study, the phenolic composition obtained from *V.*

agnus-castus was analyzed and determined by UPLC-ESI-MS MS⁻ method. In this study, phenolic composition of *V. agnus-castus* leaf and flower samples was examined and a total of 8 phenolic components were determined. Major components were; protocatechuic acid (4906.02 mg kg⁻); 4-hydroxy benzoic acid (1603.88 mg kg⁻), gentisic acid (668.49 mg kg⁻). Other components that we determined at high rates which are caffeic acid (84.29 mg kg⁻), vanilic acid (69.56 mg kg⁻), 3-4-dihydroxy benzaldehyde (58.63 mg kg⁻). Additionally, ferulic acid and p-coumaric acid components were detected at low rates (Table 1). The major phenolic compounds of *V. agnus-castus* are found; protocatechuic acid (4906.02 mg kg⁻); 4-hydroxy benzoic acid (1603.88 mg kg⁻), gentisic acid (668.49 mg kg⁻). Protocatechuic acid and 4-hydroxy benzoic acid are deriveties of the phenolic acid and a large variety of edible plants and possesses various pharmacological activities are found. Protocatechuic acid and 4-hydroxy benzoic acid, are valuable compounds for the synthesis of several bioproducts with potential applications in food, cosmetics, pharmacy, fungicides, etc (Vang et al. (2018).

In the studies conducted in the literature, Demirtaş and Pişkin, (2020) investigated 4 phenolic components in the samples of *V. agnus-castus* L. fruit extract, and these components and their ratios were respectively; gallic acid (126.9 (µg g⁻); caffeic acid (63.3 (µg g⁻); luteolin (344.1 (µg g⁻); p-Coumaric acid (15.6 (µg g⁻). In a study conducted in Denizli, the phenolic component content of *V. agnus-castus* seed samples were inventigated as gallic acid 0.281 (µg g⁻), 4-Hydroxy benzoic acid 21.506 (µg g⁻), caffeic acid 0.647 (µg g⁻) and ferulic acid 0.122 (µg g⁻) (Parlak et al., 2016).

Phenolic extractives detected in branch and trunk samples of *V. agnus-castus* (Chaste) plant species are gallic acid (0.02 mg g⁻), protocathic acid (0.06 mg g⁻), p-hydroxybenzoic acid (0.95 mg g⁻), chlorogenic acid (0.57 mg g⁻), epicatechin (0.02 (mg g⁻), syringic acid (0.02 mg g⁻), vanillin (0.01 mg g⁻), p-coumaric acid (0.04 mg g⁻), benzoic acid (0.21 mg g⁻), cinnamic acid (0.01 mg g⁻), quercetin (0.02 mg g⁻), luteolin (0.04 mg g⁻) and campherol (0.10 mg g⁻) (Ceviz, 2016).

In another study, they determined the amount of caffeic acid and chlorogenic acid in *V. agnus-castus* leaf and fruit samples obtained from Marmaris, Antalya and Isparta regions. They determined the highest rate of caffeic acid component (0.277 g 100 gdw⁻) in leaf samples obtained from Marmaris region and the highest rate of chlorogenic acid component in fruit samples obtained from Marmaris region (0.343 g 100gdw⁻) in the same study (Şarer and Gökbulut, 2008). Caffeic acid component one of the phenolic components we determined in the *V. agnus-castus* leaf and flower samples analyzed in this research lower than rate compared to (Parlak et al., 2016), (Demirtaş and Pişkin, 2020) and Şarer ve Gökbulut (2008) researchs .The ratio of p-coumaric acid component determined in the *V. agnus-castus* fruit extract in the study of Demirtaş and Pişkin (2020) is consistent with this study. The protocathic component of *V. agnus-castus* (Hayıt) in research of Ceviz (2016) was determined at a higher rate in this study and the p-coumaric acid component was found at lower rates in this study.

Table 1. Phenolic compounds of *Vitex agnus-castus* extracts (mg kg^{-1}) and method parameters for the phenolic compounds analysis using UPLC-ESI-MS/MS

Compounds	*Vitex agnus-castus* (mg kg⁻)	Quantification>confirmatory transition (m/z)	[a]Cone (V)	[b]CE (V)	[c]RT (Min)
4-Hydroxy benzoic acid	1603.88±0.09	136.98 > 93.03, 65.10	10	25, 14	2.75
3-4-Dihydroxy benzaldehyde	58.63±0.02	137.00 > 91.93, 107.94, 136.00	8	21, 20, 18	2.76
Trans-cinnamic acid	ND				
Vanillin	ND				
Gentisic acid	668.49±0.05	153.05 > 109.04, 108.03, 81.00	10	20, 20, 12	1.85
Protocatechuic acid	4906.02±0.08	153.06 > 108.00, 81.01, 91.01	10	20, 25, 20	1.85
p-Coumaric acid	11.35±0.04	163.01 > 119.04, 93.00, 117.01	5	27, 27, 15	4.65
Vanilic acid	69.56±0.03	166.98 > 151.97, 108.03, 123.03	20	18, 12, 14	3.61
Caffeic acid	84.29±0.07	179.10 > 135.14, 107.10, 133.9	32	23, 23, 24	3.65
Ferulic acid	8.97±0.01	193.03 > 134.06, 178.00, 149.02	20	16, 12, 13	5.36
Kaempferol	ND				
Myricetin	ND	136.98 > 93.03, 65.10			

(m/z) : Quantification>confirmatory transition, ND: not determined.

[a]Cone : Cone Voltage [b]CE : Collision Energy [c]RT: Retention time.

There are more than 8000 known types of phenolic compounds (Cartea et al., 2011). Vitex extracts showed an important diversity and variability between plant parts (Berrani et al., 2021). A lot of work have been carried out using various techniques until today in order to determine phenolic composition of V. agnus castus. Kawashty et al. (2016) identified 13 phenolic acid components in V. trifolia L. samples. These components are gallic acid (1.92 mg $100gdw^{-}$); protocatechuic acid (2.21 mg $100gdw^{-}$), chlorogenic acid (4.68 mg $100gdw^{-}$), caffeic acid (1.58 mg $100gdw^{-}$), vanillic acid (8.56 mg $100gdw^{-}$), p-Coumaric acid (2.43 mg $100gdw^{-}$), ferulic acid (6.00 mg $100gdw^{-}$), Iso-ferulic acid (2.12 mg $100gdw^{-}$), ellagic acid (9.29 mg $100gdw^{-}$), salicylic acid (9.31 mg $100gdw^{-}$), o-coumaric acid (1.24 mg $100gdw^{-}$), e-vanillic acid (35.41 mg $100gdw^{-}$), 4-hydroxy benzoic acid (16.38 mg $100gdw^{-}$). Protocatechuic acid, caffeic acid, vanillic acid, ferulic acid component ratios found out in this study were lower than those determined by Kawashty et al. (2016) in *V. trifolia* L. samples.

The content of phenolic compounds levels depend on several factors such as, growing conditions, geographical origin, processing and storage conditions, genetic factors, ripening process, as well as stress conditions such as UV radiation, wounding air pollution and exposure to extreme temperatures (Yamasaki et al., 1995; Figueiredo et al., 2008).

In the present work, the phenolic composition of *V. agnus-castus* were identified using UPLC-ESI-MS/MS instrument. The lack of information about phenolic composition of *V. agnus-castus* using UPLC-ESI-MS/MS makes this study important. In the light of results, there is some differences fenolic composition and rates among the litaratüre and our results . This indicates that the analysis methods used to determine of the *V. agnus-castus* leaves and flowers samples phenolic components can affect the phenolic component profile and rates also. In conclusions, nowadays wild plants are applied more intensely in more areas than in previous years. It is a great importance to determine some biochemical contents of medicinal and aromatic plants, such as antioxidants and phenolic substances. In terms of health, interest in phenolic compounds with natural antioxidant properties is growing every day due to the toxic and carcinogenic effects of synthetic antioxidants. Phenolic compounds as antioxidants can prevent many diseases such as cancer, heart disease, cataracts, eye diseases, old age diseases, etc. According to this research on *Vitex agnus-castus* leaf and flower samples, *V. agnus-castus* is rich in phenolic components and was determined by analysis. This research can be used to guide current and future research.

ÖZET

Amaç: Bu çalışmada ülkemizin önemli tıbbi bitkilerinden olan Verbenaceae familyasına ait, *Vitex agnus-castus* L. (Hayıt)'ın fenolik bileşenlerinin belirlenmesi amaçlanmıştır.

Yöntem ve Bulgular: Bu çalışmada, Karabörtlen (81m) ekolojik koşullarda çiçeklenme döneminde *Vitex agnus-castus* L. yaprak ve çiçek örnekleri toplanmıştır. Örnekler daha sonra ekstraksiyon işleminde kullanılmak üzere oda sıcaklığında (25 °C'de) yarı gölgeli ve havadar bir yerde kurutulmuştur. Çalışmamızda *Vitex agnus-castus*'ten elde edilen fenolik bileşiklerin kompozisyonu UPLC-ESI-MS/MS yöntemiyle analiz edilip tespit edilmiştir. Çiçeklenme döneminde elde edilen yaprak ve çiçek örneklerinde toplamda 8 fenolik bileşen belirlenmiştir. Hayıtın (*Vitex agnus castus* L.) çiçeklenme döneminde yaprak ve çiçek örneklerinden elde edilen fenolik bileşenleri arasında en etkili bileşenler; protokatekuik asit 4906.02 (mg kg^{-}); 4-hidroksi benzoik asit 1603.88 mg kg^{-}), gentisik asit 668.49 (mg kg^{-}) olarak tespit edilmiştir.

Genel Yorum: Bitki kaynaklı doğal ürünler ve bitkisel ürünlerin içeriğinde bulunan birçok bileşen çeşitli farmakolojik özelliklerinden dolayı son yıllarda büyük ilgi görmüştür. Bitkilerde bulunan bu biyoaktif bileşiklerin çoğu, terpenoidler, alkaloidler ve fenolik bileşenler gibi ikincil (sekonder) metabolitleridir. Antioksidan olarak fenolik bileşikler kanser, kalp hastalığı, katarakt, göz hastalıkları, yaşlılık hastalıkları vb. birçok hastalığı önleyebilmektedir. Bu çalışmanın sonuçları *Vitex agnus-castus* yaprak ve çiçek örneklerinin fenolik bileşenler bakımından zengin olduğunu göstermiştir.

Çalışmanın Önemi ve Etkisi: Bu çalışmanın sonuçları, *V. agnus-castus*'un potansiyel bir fenol kaynağına sahip olduğuna ve gıda, ilaç ve kozmetik endüstrilerinde fenolik bileşenler açısından oksidatif hasarı azaltmak için koruyucu bir ajan olarak kullanılabileceğini gösterdi. Bu çalışma mevcut ve gelecekteki araştırmalar için rehberlik etmek için kullanılabilir.

Anahtar Kelimeler: Verbenaceae, *Vitex agnus castus*, fenolik bileşen, Muğla-Ula, Türkiye.

CONFLICT OF INTEREST

The authors declare no conflict of interest for this study.

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