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RESEARCH ARTICLE

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Anticholinergic, Antioxidant, and Antibacterial Properties of *Vitex Agnus-Castus* L. Seed Extract: Assessment of Its Phenolic Content by LC/MS/MS

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In this current study, *Vitex agnus-castus* seed ethanol extracts were analyzed for their phytochemical component content, anticholinergic and antioxidant activities, and antibacterial properties. The phenolic compound composition of these seeds was determined by using LC/MS/MS. Antioxidant activity of the seeds was examined by the DPPH, ABTS, $Fe^{3+}-Fe^{2+}$ reducing, and CUPRAC. Also, the anticholinergic activity was measured by the inhibition of acetylcholinesterase (AChE). The antibacterial activity was performed by disc diffusion and minimum inhibitory concentration methods. The main phenolic compound was vanillic acid (22812.05 μg/L) and followed by luteolin, fumaric acid, quercetin, caffeic acid, 4-hydroxybenzoic acid, salicylic acid, kaempferol, butein, ellagic acid, resveratrol, catechin hydrate, phloridzin dehydrate, naringenin, respectively. The DPPH free radical scavenging value of ethanol extract of plant seeds was 9.41 %, while the ABTS radical scavenging activity was determined as 12.66 %. The ethanol extract of the seeds exhibited antibacterial activity on *Escherichia coli*, *Staphylococcus aureus*, and Salmonella *Typhimurium*, differently. *S. aureus* was found to be more susceptible to the extract than other bacteria. Also, the inhibition effect of seed ethanolic extract on the AChE with IC_{50} values were 36.34 ± 5.6 μg/mL. From the results, *V. agnus-castus* seed can be suggested as a promising natural antioxidant and antibacterial candidate for the preservation of foods.

Keywords: *Vitex agnus-castus*, phytochemical analysis, antioxidant, acetylcholinesterase, antibacterial.

Introduction

Medicinal plants and herbs have been used worldwide for centuries as important materials in the treatment of different diseases, due to their improved cost-effectiveness when compared to chemical remedies.[1] Also, plants play a key role in the development of plant-derived phytocomplexes in terms of medicinal properties for using health problems as curative or preventive tools.[2–3] A popular medicinal plant is *V. agnus-castus* L., belonging to the Lamiaceae family and grows as native to the Mediterranean area and diffused in Asia, Europe, and North Africa.[4–5] It is also known around the world by different names, 'chaste tree' and 'chasteberry', Sambhalu among the Urdu, Gattilier in French, and Sambha in Hindu, 'hayıt, ayıd, hayıd and beşparmak otu, acı ayıt,' in Turkish.[6–7] *V. agnus-castus* L. is a shrub of up to 5 m in height and it can be grown in humid and mild regions of central Asia and Europe. The brown or nearly black colored seeds of *V. agnus-castus* can be ripened as stony berries and give a pepper-like smell and taste. It

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belongs to the Verbenaceae family and is known as a chaste tree and monk's pepper.[4–8] The fruits of this plant have been used for more than 2500 years in ancient Egypt, Greece, Iran, and Rome for a variety of gynecologic problems.[9–11] Recently, the different parts of the plant have been used for the preparation of complementary medicine for corpus luteum insufficiency, male, and female disorders.[12–15] *V. agnus-castus* L. fruits and seeds have been traditionally used as medicine to enhance milk volume and to treat diarrhea and flatulence as well as, acne, infertility, menopause, and insufficient lactation, premenstrual syndrome, and other menstrual disorders. *V. agnus-castus* is an effective substance for emmeniopathy (menorrhea, amenorrhea, and oligomenorrhea), the long-term use of this plant reduced the symptoms of prevented estrogen hypersecretion before menstruation in premenstrual tension. During the middle age, Arabians, Salerno natives, and Anglo-Saxons recognized the *V. agnus-castus* L. plant as a remedy to treat symptoms of psychological illnesses. Also, it has been used as a sedative, energizer, tranquilizer, anticonvulsant, carminative, and for treating digestive disorders.[11–14, 16–17]

This plant has health-promoting and nutraceutical potential. There are many studies about the different health-promoting effects of this plant, including anti-mutagenic, antimicrobial, antioxidant, cytotoxic, immunomodulatory antiepileptic, anti-inflammatory, and antinociceptive properties.[18–20] Also, essential oils and extracts of *V. agnus-castus* have several antimicrobial and antifungal activities. Flavonoids extracted from Vitex also contributed to several pharmacological benefits of the plant, such as the trypanocidal activity and anti-filarial properties.[21–23]

The *V. agnus-castus* L. contains many different compounds, such as; essential oils, diterpenoids (vitexlactam, vitexilactone, viteagnusin I, and rotundifuran), flavonoids (orientin, kaempferol, penduletin, luteolin, artemetin, vitexin, apigenin, casticin, eupatorin, isovitexin, orientin, isoorientin, and luteolin-7-*O*-glucoside), iridoids (agnuside, agnusoside, agnucastosid A/B, and aucubin) ketosteroids, vitexilactone, and rolundifuran.[8,18,24,25]

The aim of this study was to investigate the phenolic compound profiles, antioxidant, anticholinergic, and antibacterial properties of the extracts from seeds of *V. agnus-castus* L. The identification and quantification of phenolic compounds in *V. agnus-castus* L. seeds ethanol extract were analyzed using LC/MS/MS. The antioxidant, anticholinergic and antibacterial effects of the seed extract were investigated by various methods. In addition, we aimed to present much innovative information about *V. agnus-castus* L. seeds with this study.

Results and Discussion

Analysis of phytochemical phenolic compound by LC/MS/MS

The analysis of the phytochemical contents of plant extracts with LC/MS/MS allows the most sensitive detection of phytochemical phenolic compounds.[26] Linear regression results and linearity ranges of standard compounds found in the library of the instrument were given in *Table 1*. The method validation analysis was performed for the determination of phenolic compounds in the literatüre. When the results were observed they found a parallel with the data of our study. Generally, several factors affect the determined phenolic compound profiles such as the sample extraction method, extraction process, using particle size of plant material, nature of phytochemicals, and phenolic compounds.[27–29]

The flavonoids and phenolic acids (phenolic compounds) profiles of *V. agnus-castus* L. seed ethanol extracts were determined compared to standard compounds. As seen in *Table 1*, vanillic acid was found as a major compound (22812.05 μg/L) in *V. agnus-castus* L. seed and it was followed by luteolin, fumaric acid, quercetin, caffeic acid, 4-hydroxybenzoic acid, salicylic acid, kaempferol, butein, ellagic acid, resveratrol, catechin hydrate, phloridzin dehydrate, naringenin, respectively in the range of 924.41 μg/L down to 14.58 μg/L. The determining components in these plant seeds contained mainly phenolic acids, flavonoid glycosides, and their derivatives. These components have an important effect as therapeutic against many diseases such as cancer, diabetes, cardiovascular disease, and other diseases associated with aging.[30] Based on this result, Aslantürk and Çelik[31] found that the extracts of V. agnus-castus seeds showed antioxidant, cytotoxic, and apoptotic properties on MCF–7 breast cancer cells.

Antioxidant properties of Vitex agnus-castus L. seed

The phenolics and flavonoids are important secondary metabolites for plants. They are important compounds for the preservation of food and the defense of the living cells against oxidative damage factors.[32–33] Otherwise, oxidative stress that occurs in the case of oxidative damage may lead to the progressive loss of

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Table 1. The phytochemical content of *Vitex agnus-castus* L. seed by LC/MS/MS method.

Standard compounds	Max Absorbance (λ_{max}, nm)	[a]MRM	[b]RSD %	[c]LOD/LOQ (µg/L)	Recovery (%)	[d]RT	[e]R^2	Equation	Concentration (µg/L)
Quercetin	254	301.1 > 151	0.0136	22.5/25.7	1.001	3.891	0.999	Y = (13.7831)X + (−146.951)	198.75
Acetohydroxamic Acid	502	76.10 > 43.10	0.0082	2.8/8.2	1.000	0.406	0.999	Y = (150.982)X + (23.1833)	42.56
Catechin hydrate	278	291.10 > 139.00	0.0236	8.2/11.4	0.994	2.532	0.999	Y = (79.2933)X + (−2406.22)	N.D.
Vanillic Acid	260	168.80 > 93.00	0.0062	125.5/142.2	1.001	2.762	0.998	Y = (48.0522)X + (−876.904)	22812.05
Resveratrol	288	229.10 > 135.00	0.0131	9.0/13.6	0.998	3.606	0.998	Y = (46.4361)X + (−1314.61)	44.49
Fumaric Acid	365	115.20 > 71.00	0.0047	25.2/31.3	0.997	0.809	0.999	Y = (20.2986)X + (−762.592)	274.58
Gallic acid	288	169.20 > 125.00	0.0136	0.90/1.6	1.000	1.278	0.999	Y = (65.3835)X + (−2699.84)	N.D.
Caffeic Acid	330	179.20 > 135.00	0.0137	6.3/10.7	1.009	2.836	0.996	Y = (124.785)X + (−487.132)	168.68
Phloridzin dihydrate	254	435.00 > 273.10	0.0564	61.0/207.0	1.000	3.594	0.999	Y = (33.4069)X + (−1396.90)	19.26
Oleuropein	278	539.10 > 377.20	0.0694	0.05/1.0	0.997	3.567	0.999	Y = (25.9240)X + (−558.916)	N.D.
Ellagic Acid	259	300.90 > 145.10	0.0856	0.101/0.333	1.002	3.681	1.000	Y = (5.25903)X + (−1167.31)	50.46
Myricetin	330	317.10 > 150.90	0.0079	55.4/59.6	0.999	3.644	0.999	Y = (37.0934)X + (2684.23)	N.D.
Protocatechuic acid	280	181.20 > 108.00	0.0129	30.3/35.4	1.011	3.556	0.994	Y = (526.954)X + (23026.1)	N.D.
Butein	378	271.10 > 135.00	0.0145	22.7/28.6	0.096	3.935	0.999	Y = (49.3543)X + (367.917)	73.91
Naringenin	288	271.10 > 150.90	0.0205	5.4/6.4	0.998	3.952	0.996	Y = (317.241)X + (33733.3)	14.58
Luteolin	330	285.20 > 132.90	0.0057	0.5/2.5	1.007	4.069	0.998	Y = (34.6668)X + (3721.79)	924.41
Kaempferol	265	285.10 > 116.90	0.0144	206.6/214.3	0.999	4.298	0.999	Y = (2.63905)X + (−206.494)	81.97
Alizarin	430	239.20 > 210.90	0.0351	65.2/77.5	0.966	4.594	0.998	Y = (3.97487)X + (1614.23)	N.D.
4-Hydroxybenzoic Acid	278	137.20 > 93.00	0.0154	30.5/40.25	0.996	3.664	0.999	Y = (735.804)X + (−498.102)	121.50
Salicylic acid	278	137.20 > 93.00	0.0124	4.2/7.6	1.009	3.558	0.999	Y = (746.369)X + (6072.41)	109.69

[a]MRM: Multiple Reaction Monitoring. [b]RSD: Relative standard deviation. [c]LOD/LOQ (µg/L): limit of detection/ limit of quantitation. [d]RT: Retention time. [e]R^2: Determination coefficient. N.D: Not detected

cellular functions, which may lead to the onset of chronic diseases and the formation of heart diseases, cancer, diabetes, and other metabolic syndromes.[34]

The antioxidant properties of phenolics are stemmed from their chelating the metal ions, free radical scavenging capability, or donating an electron to the hydrogen atom. *V. agnus-castus* L. seed might be considered an excellent antioxidant and antimicrobial due to its important properties. Several reports have related that *V. agnus-castus* contained diterpenoids, flavonoids, and some important essential oils. Phytochemical screening analysis indicated the presence of terpenes, catechic tannins, anthraquinones, and alkaloids in seeds and leaves and, in seeds.[35–37]

The DPPH test is a method used to predict the inhibition of free radical-induced oxidative cell damage over components such as DNA, protein, polyunsaturated fatty acids, and lipoproteins in metabolic

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systems. The hydrogen-donating capability of antioxidant substances can be determined with this analysis.[38–39] The free radical scavenging activities of the plant extracts were found using DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity and ABTS radical removal activity methods.[40] The ethanol extracts of *V. agnus-castus* L. seeds from Türkiye showed 9.41 ± 0.17 % DPPH radical scavenging activity and 12.66 ± 1.25 % ABTS radical scavenging activity values at 0.2 mg/mL concentration (*Table 2*). The highest DPPH activity was determined for Trolox (81.19 ± 5.63 %), while the highest ABTS activity was found at BHA (83.67 ± 5.41 %). Also, the lowest activities were found in *V. agnus-castus* L. seed extract for two methods. These results might be due to the presence of a low amount of antioxidant properties compounds. However, seeds of *V. agnus-castus* plants can be considered as total dietary natural antioxidants due to the possible health benefits. Similar results were reported for different extracts of this plant fruits From Morocco by El-Kamari et al.[29] Also, El-Kamari et al.[37] found that the ethanolic extract of *V. agnus-castus* leaves had the highest flavonoid and phenols concentrations and quite strong antioxidant capacity. Also, a very strong positive correlation was found between the total antioxidant activity of the plant extracts and their content of flavonoids, tannins, and phenols.

The CUPRAC assay is a rapid, simple, steady, selective, and effective antioxidant determination method for wide assorted polyphenols.[41] The antioxidant activity is the chelating/deactivation of transition metal elements and then these possess can catalyze hydroperoxide decomposition and Fenton-type reactions.[42] *Table 2* showed that the highest metal-reducing activity in CUPRAC assay and Fe^{3+}–Fe^{2+} reducing activity values were determined for BTH and it was followed by BHA, Trolox, and *V. agnus-castus* L. seeds, respectively. From the obtained results, it might be said that some phenolic compounds of these plant seeds showed significantly metal reduction capacity and radical removal activity. Balpınar et al.[43] found high total phenolic content in the leaf of *V. angus-cactus* leaves. In addition, Berrani et al.[44] found that this plant was a promising source of antidiabetic bioactive compounds. The previous studies relaved that the pharmaceutical, biological, antimicrobial, antioxidant, and antimutagenic activity of *V. agnus-castus* seeds caused its' use in the fields of phytomedicine and agriculture.[27,44—47]

As a result, although the antioxidant and antimicrobial activities of the methanol extract of *V. agnus castus* L. have been investigated in previous studies.[46] The phytochemical content and antioxidant activity of plant vary depending on the location where it grows. The phenolic compound composition caused by the difference in the locality is affected by environmental factors. The biochemical content of the plants changes by stressing the plant according to the period and the locality where it grows. The plant has a defense mechanism against environmental factors. As a result of this defense mechanism, it is known that the content of secondary metabolites and phenolic compounds with bioactivity in the secondary metabolites changes according to the conditions.[47,48] Considering these parameters, makes the change of phenolic compound content of the locality and harvest period meaningful. This change in phenolic content can create a unique difference in bioactivity.

Anticholinergic effect of Vitex agnus-castus L. seed

The AChE inhibitors are widely used for the symptomatic treatment of Alzheimer's disease (AD) and protect the cells from oxidative damages.[16] In a study, the AChE inhibition potentials of water and methanolic extracts of 37 Indian medicinal plants were screened. The IC_{50} values of some of the methanolic extracts of these plants were found in the range of 33.38-47.21 µg/ml.[49–51] As seen in *Table 2*, the etha-

Table 2. The antioxidant properties and inhibition capacity on AChE of *Vitex agnus-castus* L. seed.

Antioxidants	DPPH[a] (0.2 mg/mL)	ABTS[a] (0.2 mg/mL)	FRAP Assay[b] (0.2 mg/mL)	CUPRAC Assay[b] (0.2 mg/mL)	AChE IC_{50} (mg/Ml)	R^2
Vitex agnus-castus L. seed	9.41 ± 0.17	12.66 ± 1.25	0.17 ± 0.01	0.36 ± 0.03	36.34 ± 5,6	0.97 ± 0.01
BHA	71.82 ± 4.86	83.67 ± 5.41	0.45 ± 0.02	0.58 ± 0.02		
BHT	46.33 ± 2.64	48.35 ± 3.20	0.62 ± 0.02	0.64 ± 0.02		
Trolox	81.19 ± 5.63	80.06 ± 6.32	0.25 ± 0.01	0.52 ± 0.02		
Eserine hemisulfate					0.02 ± 0.01	0.96 ± 0.01

Standard antioxidants (BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene, Trolox). [a] Values are expressed as percent radical scavenging activity. [b] Values are expressed as absorbance. High absorbance indicates high metal reduction capacity.

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nolic extract of *V. agnus-castus* L. seed showed an inhibition effect on the AChE at the level of IC_{50}: 36.34±5,6 μg/mL. The phenolic compounds have neuroprotective effects in the treatment of AD and these properties are stemmed from their important antioxidant properties. There are many important approaches (with the use of AChE inhibitors) for the treatment of the disease due to the raise of the acetylcholine ratio in the brain.[50–51]

Antibacterial properties of Vitex agnus-castus L. seed

The antibacterial effects of the *V. agnus-castus* L. seed ethanol extract were found against the *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* Typhimurium (*Table 3* and *Table 4*). *S. aureus* was found to be more susceptible when compared to *E. coli* and *S.* Typhimurium (*Figure 1*). Optical density for growth curve at 600 nm (OD600) was measured and repeated twice, each microorganism in triplicates.

Table 3 showed that the 312 μg/mL *V. agnus-* inhibition zone diameter on *S. aureus* (11.0±0.30 mm) and it was followed by *S. Typhimurium* (9.0±0.25 mm) and *E. coli* (8.0±0.20 mm), respectively. The inhibition properties of *V. agnus-castus* L. seed extract on studied bacteria cultures were determined to be quite low compared to the control antibiotic (Ciprofloxacin). As seen in *Table 4*, the MIC (Minimum inhibitory concentration) results showed that the 312 μg/mL and 156 μg/mL of plant extract concentration at the volume of 10 μL had an important effect on all examined bacteria, while 78 μg/mL (10 μL) concentration showed effect only on the *Staphylococcus aureus*. However, the other four concentrations had no effect on any observed bacteria. The obtained results revealed that the seed extracts showed slightly

Table 3. Antibacterial properties of *Vitex agnus-castus* L. seed extract (Inhibition zone diameter; mm).

Sample (6.24 μg/disk)	Concentration	*Staphylococcus aureus*	*Esherichia coli*	*Salmonella* Typhimurium
Vitex agnus-castus L. seed	312 μg/mL	11.0±0.30	8.0±0.20	9.0±0.25
Control (Ciprofloxacin)	5 μg/disk	22.0±0.15	19.0±0.20	18.0±0.25

Table 4. MIC (Minimum inhibitory concentration) results of *Vitex agnus-castus* L. seed extract.

Vitex agnus-castus L. seed	Concentration (μg/mL)	Inoculum amount (μL)	S. aureus	E. coli	S. Typhimurium
	312	10	–	–	–
	156	10	–	–	–
	78	10	–	+	+
	39	10	+	+	+
	19.5	10	+	+	+
	9.75	10	+	+	+
Medium+Inoculum	0	10	+	+	+
Medium+Solvent (DMSO)	0	10	+	+	+
Medium	0	0	–	–	–

(+): Growth, (–): No Growth.

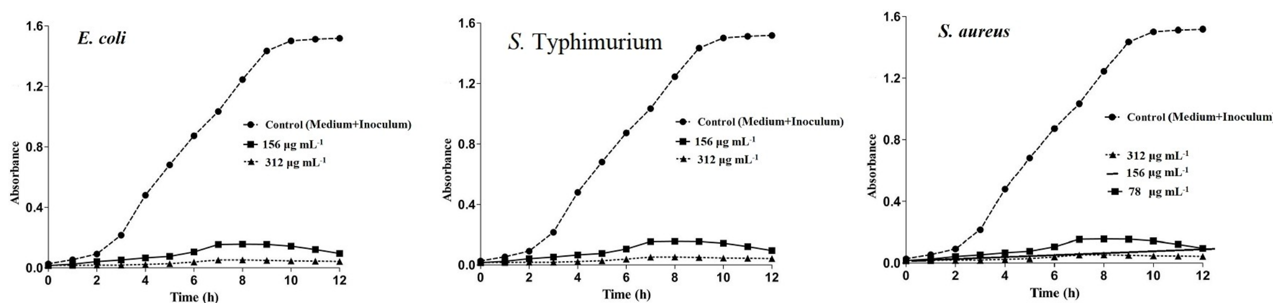

Figure 1. Growth curve (optical density at 600 nm) pattern of *E. coli*, *S.* Typhimurium *S. aureus* with *Vitex agnus-castus* L. seed ethanol extract and without the extract

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antibacterial effects on the studied bacterias. Also, Balpınar et al.[42] reported that the *V. agnus-castus* L. extracts showed an antibacterial effect on mastitis-causing *S. aureus* and Coagulase-Negative Staphylococci (CNS) strains and the extract of this plant can be used for the treatment of mastitis. Arokiyaraj et al.[22] studied the phytochemical and antibacterial properties of *V. agnus-castus.* They reported the phytochemical profiles of this plant extract consisted of steroids, flavonoids, terpenoids and also, revealed that the high antibacterial activity stems from these components. Bakr et al.[59] reported that the methanolic extract from the stem, seed, and leaf of *V. agnus-castus* L. seed extract exhibited both antibacterial and antifungal activities against *Enterobacter aeurogens*, *Staphylococcus aureus*, *Candida*, and *Rhizopus* species, mastitis pathogens, and plant contained important anti-bacterial components such as terpenoids, flavonoids, and steroids that might be used for the development of medicinal therapy for bacterial diseases.

Conclusions

This study highlighted the richness of *V. agnus-castus L.* seed in terms of phenolic compounds, and also showed the antioxidant (metal reduction and free radical scavenging activities), anticholinergic and antibacterial properties. The results of this present study demonstrated that the chemo-diversity of this species was quite promising for the treatment of important diseases of health due to the new antioxidant and antibacterial compounds. The results of this study reported that the ethanolic extract of *V. agnus-castus* seed might be used as a source of a possible food supplement and natural antioxidants in food processes and also in the pharmaceutical industry. Also, the outcomes of this study revealed the possible application of *V. agnus castus* seed extracts as the best source of bioactive molecules and antibacterial agents for health benefits.

Experimental Section

MaterialPreparation of Extracts

The extraction was made using the method reported previously by Elmastaş et al.[52] In the studies since the total phenolic and flavonoid contents of the extracts made with different solvents are higher in ethanol, that solvent was preferred for extraction.[29] The dried sample: solvent (ethanol) ratio was mixed (1:10) and extracted for 24 h using a magnetic stirrer. The extract obtained with the 5.6 percent yield was filtered through filter paper (Whatman No. 1), and it was evaporated and dried at −50 °C using a lyophilizer. The dried samples were kept in a closed container at 4 °C for analysis.

LC/MS/MS Analysis

The phenolic content of the *V. agnus-castus* L. seeds was quantified and identified using LC/MS/MS, using a Nexera model Shimadzu UHPLC attached to a tandem MS device. The validation analyzes of the using method developed for 20 phenolic substances and analysis were made in the Harran University Central Research Laboratory. The CTO-10ASVP column furnace, LC-30AD dual pumps, SIL-30AC autosampler, and DGU-20 A3R degasser were used for this study. Chromatographic separation was determined using a C18 Inertsil ODS-4 (3.0 mm × 100 mm, 2 μM) analytical column and the column temperature was set to 40 °C. The elution gradient was made using mobile phase A (0.1 % formic acid and water) and mobile phase B (0.1 % formic acid and methanol). The solvent flow rate was kept at 0.5 mL/min and the injection volume was fixed at 4 μL. The MS analysis was carried out using a mass spectrometer equipped with Shimadzu LC/MS 8040 model triple, quadrupole, and ESI source operation in both positive and negative ionization modes. The data calculations of LC/MS/MS were done using Lab Solutions software (Shimadzu, Kyoto, Japan). Multiple reaction tracking (MRM) mode was used for the measure analysis.[53] The analysis was made three times for each compound analysis, and the obtained results were reported quantitatively.

Antioxidant Activity

Fe^{3+}–Fe^{2+} Reduction Capacity

The metal reduction properties of *V. agnus-castus* L. seed in ethanol extracts were determined using the modified method reported by Elmastaş et al..[52] The 10, 20, and 40 μg/mL concentrations of seed extracts were mixed with 2.5 mL 1 % potassium ferricyanide $[K_3Fe(CN)_6]$ and 2.5 mL phosphate buffer (0.2 M, pH 6.6), and the obtained mixtures were incubated at 50 °C during 20 min. After, $FeCl_3$ (0.25 mL, 0.1 %) and trichloroacetic acid (2.5 mL, 10 %) were added to each mixture and centrifuged for 10 min at 3,000 rpm. The absorbance values of the mixture were determined at 700 nm. The reducing power values were reported as

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absorbance and the obtained results were compared to standard antioxidants.

CUPRAC Assay

The CUPRAC method based on the reduction of Cu (II) -Nc to Cu (I) -Nc chelate was determined according to Apak et al.[41] 11 mL of $CuCl_2$ (0.01 M) solution, 1 mL of neocuprin (2,9-dimethyl-1,10-phenanthroline), and 1 mL of ammonium acetate (NH_4Ac) buffer were mixed in a test tube using a vortex for the preparation of the reaction mixture. Extracts at different concentrations (10, 20, 40 μg/mL) were added to the reaction mixture and the total volume was brought to 4 mL using ultrapure water. The absorbance of extracts was measured at 450 nm after 30 min. CUPRAC method was repeatedly applied to Trolox solutions at different concentrations, and a calibration curve of Trolox (0.2-2 mM) was drawn. The results were expressed as absorbance and compared to standard antioxidants.

DPPH Removal Activity

For the determination of DPPH free radical removal activity of *V. agnus-castus* L. seed ethanol extracts and standard antioxidants that was measured using the Blois method.[54] For this purpose, 0.1 mM DPPH solution was prepared using methanol. The obtained 10, and 20, 40 μg/ml of samples were mixed with 1 mL of DPPH solution and were completed to 3 mL with ethanol. These prepared solutions were thoroughly vortexed and incubated in dark for 30 min. After, the absorbance of the samples was read at 517 nm using a spectrophotometer. The decreased absorbance results showed DPPH free radical scavenging capacity.

ABTS Radical Removal Activity

The ABTS analysis was made that the method reported by Re et al.[55] The method is stemmed from the principle of color change at the end of the treatment of colored $ABTS^{+}$ cation radical with an extract. The 2.45 mmol/L potassium persulfate ($K_2S_2O_8$) solution was mixed with ABTS (2 mmol/L) solution and this solution was incubated in dark at room temperature for 14 h. For this analysis, $ABTS^{+}$ radical solution was diluted using 0.1 mol/L sodium phosphate buffer at pH 7.4 until the desired absorbance (0.750 ± 0.025 at 734 nm) was achieved. The 10, 20, and 40 μg/mL of obtained stock solutions and phosphate buffer were completed to 3 mL. After, 1 mL of $ABTS^{+}$ solution was added to the extract samples and vortexed. The absorbance was measured at 734 nm using a spectrophotometer.

Acetylcholinesterase (AChE) Activity

The inhibitory effect of *V. agnus-castus* L. seed ethanol extracts on the AChE enzyme was found using the method of Ellman.[56] The reaction solution (50 μL AChE ($5.32x10^{-3}$ U 100 μl Tris·HCl buffer (1 M, pH 8.0) and 50 μl 5,5′-dithio-bis (2-nitro-benzoic) acid (DTNB)) was incubated at 30 °C and then stirred during the 15 min. However, the reaction was started using the substrate of 50 μL of acetylthiocholine iodide (AChI). The results of the enzymatic hydrolysis of substrate were measured at 412 nm by spectrophotometry.[49]

Antibacterial Activity

Antibacterial activity of the ethanolic extract of *V. agnus castus* L. seeds was determined by using 3 bacteria including *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Salmonella* Typhimurium (ATCC 14028) cultures as test organisms. Bacterial strains were activated for 24 h at 35–37 °C using Trypticase Soy Yeast Extract (TSYE). The densities of stock cultures turbidity were brought to $1x10^8$ CFU/mL with 0.5 McFarland standard Two different methods, disc diffusion, and Minimum inhibitory concentration (MIC) methods were employed for the determination of *the* antibacterial activity of seeds extracts.[57]

Disc Diffusion

The 20 mLMueller-Hinton Agar (MHA) medium was poured into sterile and 9 cm diameter of Petri dishes, respectively. The standard quantities of each bacterial suspension (10^8 CFU/mL) were cultivated to petries. The 6 mm in diameter discs were impregnated with 20 μl of the extracts placed on the inoculated agar. The inoculated plates were incubated at 4 °C for 1 h and then incubated for 24 to 48h at 37 °C. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms. Ciprofloxacin (5 μg/disk) was used as a positive control for the testing of activity. Each measurement in this experiment was repeated twice.[58]

Minimum Inhibitory Concentration (MIC) Method

For this purpose, *V. agnus castus* L. seed extracts were prepared at the concentration of 312 mg/mL (w/v) in 35 % Dimethyl sulfoxide (DMSO). The mix was steri-

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lized using 0.45 μm Millipore filters (France). The extracts were kept in 1.5 mL of Eppendorf tubes at 4 °C for antimicrobial activity tests. The 10 μL of each bacterial inoculum was transferred to the microplates and extracts were diluted to 312, 156, 78, 39, 19.5, 9.75 mg/mL using nutrient broth (NB) and the prepared DMSO was evaluated as a negative control in one well. The microplates were incubated for 24 h at 35–37 °C. The growth and turbidity in the microplates were accepted as positive situations. This test was made in triplicate.[58]

Statistical Analysis

The found data were evaluated by GraphPad Prism version 6 (GraphPad Software, La Jolla, California, USA). The results were shown as mean ± standard deviation (95 % confidence intervals). Differences between obtained data sets were evaluated at p-value $\leq$ 0.05, statistically. All analyzes were done in 2 parallel and 3 replicates.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Author Contribution Statement

Conceptualization, Validation, and Resources were prepared by Arzu Kavaz Yüksel, Mesut Işık, Emrah Dikici, and Mehmet Yüksel. Original-draft preparation, Supervision, and Visualization was made by Arzu Kavaz Yüksel and Mesut Işık. All authors of this manuscript have read and agreed with this version of the manuscript.

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