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Research Article

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Evaluation of the phenolic compounds and the antioxidant potentials of Vitex agnuscastus L. leaves and fruits

https://doi.org/10.1515/tjb-2021-0208 Received September 10, 2021; accepted November 15, 2021; published online December 29, 2021

Abstract

Objectives: In this study, we aim at deciphering the phenolic content of Vitex agnus-castus L. leaf and fruit extracts prepared with different methods and relate it to their antioxidant activity.

Methods: In this study, phenolic compounds and the antioxidant potential of the ethanol fruit and leaf extracts of V. agnus-castus L. (Chaste tree) were evaluated spectrometrically. Furthermore, selected polyphenols, i.e., chlorogenic acid and rutin, were determined by the HPLC-DAD method qualitatively and quantitatively.

Results: The results obtained from leaf and fruit extracts were compared with a commercial product (CP) containing the fruit extract of V. agnus-castus. Leaf extract was found to be richer in flavonoids when compared to the fruit counterparts. Accordingly, they also showed higher antioxidant activity.

Conclusions: Extracts prepared here can be considered as promising antioxidant agents for future therapeutic formulations.

Keywords: chaste tree; HPLC-DAD; Lamiaceae; Vitex agnus-castus.

Introduction

When considering the prolonged life expectancy of humankind, natural sources rich in phenolic components

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have gained significant interest against chronic diseases. Oxidative damage is one of the most important factors in developing and progressing many chronic diseases, including cardiovascular disorders and cancer [1]. During this inevitable process, phenolic compounds step forward due to their superior effects, including free radical scavenging, enzyme regulation, and antiallergic and antiinflammatory properties [2]. Furthermore, antioxidants have proven to delay the oxidation of molecules by inhibiting the initiation and/or propagation of oxidizing chain reactions by free radicals. Therefore, they are crucial in reducing oxidative damage in living organisms [3]. To date, the presence of these compounds in medicinal plants has been strongly related to their antioxidant activity [4]. Therefore, natural antioxidants rich in phenolic components are widely investigated in healthcare, active pharmaceutical ingredient research, and the food industry.

Vitex agnus-castus L. is a plant that belongs to the Lamiaceae family. V. agnus-castus L. is a rarely small tree, 1-3 m in height, much-branched, shortly tomentosecanescent. The leaves of the plant can be defined as digitately 5(-7)-partite, leaflets are usually entire, $3.5-15 \times 0.5-$ 2.8 cm, and occasionally broader or distinctly dentate, acute, narrowed to both ends, sessile or at least terminal petiolulate, dull green above, white-tomentose beneath; petioles long, and those of lower leaves to ~4 cm. Inflorescence of the plant is relatively dense. Cymes are compact, frequently subglobose, sessile, or sub-sessile. Drupes are 3–4 mm in size, globose, and black or reddish [5]. In Turkey, it is also known with different regional names such as "hayıt, acıayıt, ayıd, hayıd and beşparmakotu" [6]. In the regions that it naturally grows (e.g., middle Asia, southern Europe, Mediterranean countries), it is widely used as a treatment against premenstrual syndrome, lactation difficulties, low fertility, and the regulation of menstrual cycles. However, the active principles responsible for such therapeutic effects have not been fully identified yet [7]. Traditionally, the seeds of *V. agnus-castus* are also used as lactagog and hormone regulators [8]. In addition, it is indicated in the literature that the extract of V. agnus-castus

fruits has an antiaging effect in the reproductive system *in vivo* [9]. Moreover, antimicrobial, antifungal, fracture healing activities of *V. agnus-castus* extracts prepared in different methods have been reported previously [10, 11]. Phytochemical screening analyses performed with *V. agnus-castus* revealed that the plant is rich in phenolic compounds, glycosides, iridoids, flavonoids, diterpenes, and essential oils [12, 13]. Casticin and agnuside are determined to be major compounds of its fruits, and the analytical determination of these compounds is studied well [14–16].

This study investigated the phenolic components of ethanolic fruit and leaf extracts of *V. agnus-castus*. This is the first multi-comparative analysis that evaluates the effect of different extraction techniques on antioxidant potential, total phenolic and flavonoid content of chaste tree fruits and leaves to the best of our knowledge. In detail, we determined the total phenolic and flavonoid content of the extracts, then investigated the rutin and chlorogenic amounts in selected preparations qualitative and quantitatively using high-performance liquid chromatography diode array detector system (HPLC-DAD). Finally, we determined the antioxidant activity of the extracts and related the results to their phenolic contents.

Materials and methods

Plant materials

The leaves and fruits of *V. agnus-castus* L. were collected from Urla, Izmir, Turkey (May-leaves; and July-fruits, 2019). The plant was identified by Dr. Hüsniye Kayalar from Ege University, Faculty of Pharmacy, Department of Pharmacognosy. A voucher specimen is conserved in the Herbarium of the Faculty of Pharmacy, Department of Pharmacognosy, Ege University (No. 1262/2).

Preparation of the plant extracts and tinctures

The collected leaves and fruits of the plant were air-dried at room temperature (RT) by avoiding any light exposure. Before extraction, the dried material was reduced to a coarse powder using an electrical grinder (Retsch GmbH, diameter: 1 mm). The powdered materials were subjected to two different extraction methods. To perform the first set of extraction, powdered plant material (5 g) was dispersed in ethanol solution (96% v/v), and the solid-liquid extraction was performed with Soxhlet apparatus for 4 h (samples were denoted as VL S and VF S for leave and fruit prepares, respectively). As a second extraction method, the maceration technique was selected. Here, 5 g of powdered material was placed into 50 and 60% v/v of 100 mL ethanol solution and gently stirred overnight (denoted as VL50 and VL60 for leave preparations; VF50 and VF60 for fruit preparations). Next, the dispersions were placed in an ultrasonic bath for 4 h to avoid any light exposure. Finally, obtained dispersions were filtered and evaporated to dryness

Table 1: Methods applied to obtain plant samples and their abbreviations.

| Source | Sample | Solvent | Method |
|--------|--------|-------------|--------------------|
| Leaves | VL50 | 50% ethanol | Maceration |
| | VL60 | 60% ethanol | Maceration |
| | VL S | 96% ethanol | Soxhlet extraction |
| | VL70 | 70% ethanol | Tincture |
| Fruit | VF50 | 50% ethanol | Maceration |
| | VF60 | 60% ethanol | Maceration |
| | VF S | 96% ethanol | Soxhlet extraction |
| | VF70 | 70% ethanol | Tincture |

VL, leaf extract of *Vitex agnus-castus*; VF, fruit extract of *Vitex agnus-castus*.

in vacuo. The obtained extracts were weighed, and the extraction yield was calculated by means of the initial and final weight difference.

The tinctures were prepared by placing the samples (5~g) in 70% ethanol (100 mL) under shaking at RT for five days and subsequent filtering. The final solutions were VL70 and VF70 for leaf and fruit tinctures, respectively. All the prepared samples were coded as described in Table 1.

Determination of the total phenolic compounds

The total phenolic content of V. agnus-castus extracts was assessed by applying the Folin-Ciocalteu method as described in Singleton et al. [17]. In brief, 0.1 mL of each sample (extract=10 mg/mL) was mixed thoroughly with 2.8 mL of ddH_2O and $2\,mL$ of Na_2CO_3 (2% w/v). Then, 0.1 mL of 0.1 N of Folin-Ciocolteau reactive agent was added to the mixtures. The obtained mixture was shaken thoroughly and incubated for 30 min by avoiding any light exposure. Next, the absorbance of the mixtures was detected at 750 nm against a reference mixture that does not contain any plant extract. The total phenolic content of the extracts was calculated by converting the absorbance of the samples to gallic acid equivalent by using a gallic acid standard curve, which is obtained by measuring the absorbance values of serially diluted gallic acid solutions (GA, Sigma Aldrich, Schnelldorf, Germany) at 750 nm. All tests were carried out as triplicates.

Total flavonoid analysis

The total flavonoid content of V. agnus-castus extracts was determined spectrophotometrically using aluminum chloride colorimetric method as described in Zhishen et al. [18]. Briefly, 0.5 mL of the plant extracts (extract=10 mg/mL) were mixed with 1.5 mL of ethanol, 0.1 mL of 10% aluminum chloride, and 2.8 mL of ddH₂O. The mixture was incubated at RT for 40 min by avoiding any light exposure. The solution was mixed carefully, and the absorbance was measured against ethanol at 415 nm. Here, quercetin was used as the standard for a calibration curve. The flavonoid content was calculated using a linear equation based on a serial dilution of quercetin (QE, \geq 98% HPLC-grade, Sigma Aldrich) and described as quercetin equivalents (µg QE/mg) [19]. All tests were carried out as triplicates.

Analysis of chlorogenic acid and rutin by HPLC-DAD

The plant extracts' quantification of chlorogenic acid and rutin amounts was carried out using a high-performance liquid chromatography diode array detector system (Agilent 1100 HPLC-DAD). Before experiments, standard curves for chlorogenic acid and rutin were prepared as follows: To detect rutin in the plant extracts, 8.2 mg of rutin hydrate (Sigma, R5143) was dissolved in 2 mL of methanol. Twenty microliter of the prepared rutin solution was then mixed with 980 µL of methanol and scanned in the wavelength range of 345-580 nm (concentration range of 20-80 µg/mL). The maximum absorbance was obtained at the wavelength of 355 nm. To detect the chlorogenic acid content of the plant extracts, chlorogenic acid solution (Sigma Aldrich, 1 mg/mL in methanol, concentration range of 5-20 ug/mL) was scanned in the wavelength range of 200-400 nm by taking methanol as a reference. The maximum absorbance was detected at 330 nm. The calibration curves forrutin and chlorogenic acid were prepared by injecting serially diluted solutions of the chemicals. The calibration curve equations for rutin and chlorogenic acid was determined as y=1,395.3x ($R^2=0.93$) and y=609.47x $(R^2 = 0.99)$, respectively.

Plant extracts were dissolved in corresponding solvents to a 100 mg/mL concentration and diluted in methanol to a concentration of 10 mg/mL. Next, the extract was centrifuged at 5,000 rpm for 20 min and filtered (diameter: 0.45 µm) before injection to the column. Inertsil[®] ODS-3 (25 cm \times 4.6 mm \times 5 μ m) column was used, and the analyses were carried out at isocratic conditions. The mobile phase consisted of acetonitrile and water with a ratio of 15:85, including 0.1% phosphoric acid. The column temperature was set to 30 °C. For each test, 20 µL of the sample was fed to the system with a 1 mL/min flow rate. The identification of chlorogenic acid and rutin was held using retention time and standard internal methods, and the quantification was performed using the abovementioned regression curves. Finally, the obtained results were compared to the CP that contains 4 mg of V. agnus-castus fruit extract. To do so, the product was dissolved in methanol (1 mg/mL), centrifuged, filtered, and injected into the HPLC-DAD system and analyzed as described above.

For the method validation, performance parameters such as linearity, the limit of detection (LOD), the limit of quantitation (LOQ), and precision were determined. Linearity was defined by drawing a five-point calibration curve. Based on the signal-to-noise ratio of S/N=3/1, the measurement limit and the signal-to-noise ratio S/N=10/1, the detection limitwas calculated [20]. Repeatability and relative standard deviation percentages were determined to determine method accuracy. The repeatability was tested by performing five repetitions on the same day and three repetitions on three different days. Using Microsoft Office Excel, a descriptive statistical analysis (correlation coefficient, mean \pm SD, and % relative standard deviation) was calculated.

Determination of the antioxidant activity

The antioxidant activities of the fruit and leaves extracts of the V. agnus-castus were determined by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability based on the method as described in Esmaeili et al. [21]. The absorbance of the serially diluted solutions was measured at 517 nm using a spectrophotometer. One thousand microliter of 1 mg/mL extracts were appended to 4 mL of 0.004% DPPH in methanol. The absorbance of samples at 517 nm was assessed after incubation for 30 min (Optima SP 3000 Nano UV-Vis, Tokyo, Japan). Then, the relative inhibition of the tested samples was evaluated by comparing with control. The DPPH inhibition value was calculated as described in Eq. (2.1).

DPPH Inhibition [%] =
$$[A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}})] \times 100$$
 (2.1)

where $A_{\rm blank}$ refers to absorbance observed for 1 mL methanol diluted with 4 mL of DPPH radical stock solution, and A_{sample} refers to the sample absorbance. The relative DPPH radical scavenging activity (%) values were converted to α -tocopherol equivalent values using a standard curve relating the DPPH inhibition (%) to equivalent α -tocopherol concentration. To do so, serially diluted α -tocopherol solutions (0.50-10 µg/mL) were prepared, and their free radical scavenging activities were determined as described above. The IC₅₀ values are calculated by detecting the extract amount required to obtain 50% DPPH radical scavenging.

Statistical analysis

The data were reported as the mean \pm standard deviation. Linear regression coefficient (R²) for phenolic and flavonoid content with antioxidant activity was analyzed by Graph Pad Prism for Windows, Version 7 (GraphPad Software, San Diego CA, USA). A p-value<0.05 was considered significant.

Results

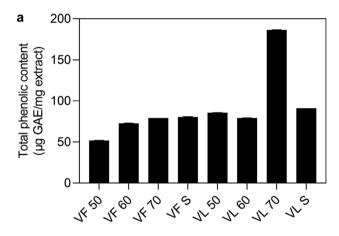
It is known that the phenolic compounds of a plant have a detrimental impact on the antioxidant activity, which is responsible for the essential medicinal effects of the plants. Here, a conceptual phytochemical analysis was performed with the extracts and tinctures obtained from V. agnus-castus leaves and fruits to detect rutin and chlorogenic acid, as well as total phenolic contents. Notably, several parameters affecting the phenolic content of the extracts were evaluated using different techniques (i.e., maceration, Soxhlet extraction, and tincture preparation) and two separate parts (i.e., leaves and fruits) of the plant. For all groups, extracts were prepared with ethanol since it was previously found that the ethanolic extracts of *V. agnus-castus* show superior antioxidant activity when compared to their counterparts prepared with n-hexane [22]. As shown in Table 2, an extraction yield of 11–38% was obtained for the different preparations. Overall, extraction of the leaf samples resulted in a higher vield when compared to the fruit samples.

Next, the total phenolic contents of the extracts were determined spectroscopically and described as gallic acid equivalent per unit mass of extract (Figure 1A). The regression coefficient obtained by the gallic acid standard curve (R²>0.99) indicated a good precision of the method used here. Among all preparations, the tincture of V. agnuscastus leaves (VL70) showed the highest total phenolic

Table 2: Extraction yield.

| Group | Extraction yield, % |
|-------|---------------------|
| VL50 | 33.39 |
| VL60 | 36.00 |
| VL S | 38.10 |
| VF50 | 14.82 |
| VF60 | 13.23 |
| VF S | 10.55 |

VL50, leaf extract of *Vitex agnus castus* prepared by maceration method with 50% ethanol; VL60, leaf extract of *Vitex agnus castus* prepared by maceration method with 60% ethanol; VL S, leaf extract of *Vitex agnus castus* prepared by Soxhlet extraction method with 96% ethanol; VF50, fruit extract of *Vitex agnus castus* prepared by maceration method with 50% ethanol; VF60, fruit f extract of *Vitex agnus castus* prepared by maceration method with 96% ethanol; VF S, fruit extract of *Vitex agnus castus* prepared by Soxhlet extraction method with 96% ethanol.



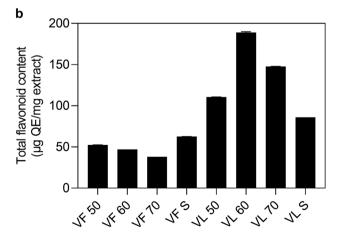


Figure 1: Total phenolic (A) and flavonoid contents (B) of the extracts obtained from V. agnus-castus fruits and leaves represented as μg GAE/mg extract and μg QE/mg extract, respectively. Data represents mean values; error bar represents standard deviation as obtained n=3 samples (p<0.05).

amount (\approx 190 µg GAE/mg extract). All the other preparations exhibited a similar level of phenolic content, which is in the range of 50–90 µg GAE/mg extract. However, when the same extraction method and conditions were applied to leaf and fruit samples, leaf extracts showed significantly higher phenolic content than fruit extracts (p<0.05). In line with the total phenolic content determination, VL70 exhibits comparably higher levels of flavonoid contents (\sim 150 µg QE/mg extract). Surprisingly, the highest flavonoid content was determined for the preparate VL60 (\sim 190 µg QE/mg extract). Here also, the leaf extracts showed significantly higher flavonoid contents when compared to fruit extracts (p<0.05).

Next, the antioxidant activity of the extracts was assessed by DPPH radical inhibition tests. Among fruit extracts, the group VF60 showed the highest antioxidant activity (17.86 \pm 0.021 μg α -tocopherol equivalent/mL) at a concentration of 1,500 $\mu g/mL$. The leaf extracts showed significantly higher antioxidant activity when compared to fruit extracts (p<0.05). Accordingly, the calculated IC50 values obtained from leaf extracts were lower (161.9–396 $\mu g/mL$) than those obtained for fruit extracts (626.4–798.3 $\mu g/mL$).

Discussion

Among the natural phenolics, flavonoids represent one of the most important compounds responsible for a wide range of biological and chemical properties. For example, such secondary metabolites of the plants can scavenge reactive oxygen species that are harmful to cells by inducing oxidative damage in essential macromolecules such as proteins, nucleic acids, and lipids [23, 24]. Additionally, their preventive role in cancer and coronary heart diseases was underscored [25]. Therefore, we have evaluated the total flavonoid content of the extracts using the aluminum chloride colorimetric method. Overall, all the samples prepared with the leaves of *V. agnus-castus* showed higher levels of flavonoid contents when compared to fruit extracts. In a study by Gökbulut et al. [26], the total phenolic content of the methanolic extracts of the V. agnus-castus leaves and fruits was found as 123 and 114 µg GAE/mg extract, which is in line with our findings. Similarly, in another study, the phenolic content of the essential oil (EO) obtained from *V. agnus-castus* leaves was found as 82 µg GAE/mg EO [27]. Previously, Maltas et al. [28] determined the total flavonoid and phenolic content of methanolic extract of V. agnus-castus leave as 27 mg QE and 48 mg GAE per dry extract respectively.

Previously, *V. agnus-castus* extracts were found to be rich in casticin, aucubin, *p*-hydroxybenzoic acid, rutin,

Table 3: Detection and comparison of rutin and chlorogenic acid in extracts and a commercial product (CP) using HPLC-DAD analysis.

| Sample | Rutin amount, mg/mg extract | Chlorogenic acid amount, mg/mg extract |
|--------|--------------------------------|-------------------------------------------|
| VF60 | 0.068×10^{-2} | 0.17×10^{-2} |
| VL60 | 0.330×10^{-2} | 0.45×10^{-2} |
| CP | Not detected | 0.025×10^{-2} |

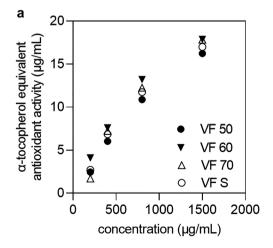
and ferulic acid [15, 16, 29, 30]. Indeed, among those, rutin is one of the flavonoid compounds that has a wide range of pharmacological activities [31]. It has been reported that rutin has a positive effect during the treatment of chronic diseases such as hypercholesterolemia, diabetes, and hypertension [32]. Therefore, we analyzed the extracts VF60 and VL60 for their content of rutin and chlorogenic acid by using high-pressure liquid chromatography with a diode array detector (HPLC-DAD). Rutin was detected for the samples at a retention time of 34.9 min at the maximum wavelength of 355 nm. As summarized in Table 3, it was found that extracts constituted rutin concentrations of 0.68×10^{-3} and 3.3×10^{-3} mg rutin/mg extract at fruit and leaf samples, respectively. Previously, Proestos et al. [30] identified the rutin content of methanolic extract of V. agnus-castus as a 1.58 mg/100 g dry sample.

As another substance, chlorogenic acid is an important antioxidant in plants, which limitslow-density lipid oxidation [33]. Furthermore, findings indicate that chlorogenic acid-supplied diets support protection against degenerative, agerelated diseases *in vivo* [34]. Previously, the chlorogenic acid amount in *V. agnus-castus* fruits and leaves was determined and found in the range of 0.103–0.343 and 0.089–0.206%

w/w, respectively [29]. Additionally, it was indicated that the chemical composition differs according to plants' region. Here, chlorogenic acid was observed at a retention time of 9.3 min at a maximum wavelength of 330 nm and detected for VF60 and VL60 samples as 0.17×10^{-2} and 0.45×10^{-2} mg/mg extract, respectively. These two groups were selected for these experiments due to their identical extraction conditions, which further helps to obtain a better comparison. In line with previous findings, leaf samples were richer in both rutin and chlorogenic acid.

The widely used CP, which contains fruit extracts of *V. agnus-castus*, was proven to have anti-inflammatory potential, and this finding was related to its antioxidant activity and potential to reduce inflammatory cytokines *in vivo* [35]. Therefore, we compared the performance of this product with the obtained extracts here in terms of chlorogenic acid and rutin amounts. Surprisingly, although rutin is one of the components identified in *V. agnus-castus*, it was not detected in CP. Furthermore, the chlorogenic acid amount was also relatively low compared to VF60 and VL60 samples.

Previous studies focused on *V. agnus-castus* showed that the high antioxidant activity of the extracts obtained from this plant is strongly related to their total phenolic content [36]. Therefore, it is crucial to perform phytochemical analyses and biological activity studies in parallel. To relate the analyses performed for phenolic compound detection, the antioxidant activity of the extracts was determined by the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical in the last step scavenging method. As observed in Figure 2, free radical scavenging activity of both fruit and leaf extracts of *V. agnus-castus* followed a dose-dependent response. It was found that the leaf extracts showed higher antioxidant potential when compared to fruit extracts. VL70 showed the most potent



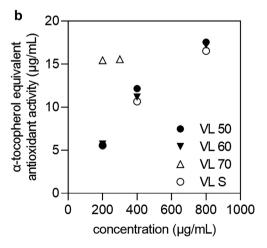


Figure 2: α-tocopherol equivalent antioxidant activity values of the fruit (A) and leaf (B) extracts of *V. agnus-castus* that are obtained with different extraction methods.

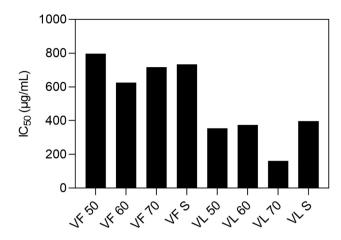


Figure 3: IC_{50} values representing the radical scavenging activity of the ethanolic extracts of V. agnus-castus leaves and fruits.

radical scavenging activities among all preparations, followed by VL50, VL60, and VLS. This trend in antioxidant activity possibly stems from phenolic compounds in these extracts. In line with these findings, previously, *V. agnuscastus* methanolic extracts of leaves were found to show higher antioxidant activity when compared to fruit extracts. However, rutin was not detected in any of these samples [26].

The IC₅₀ values calculated according to the findings was depicted in Figure 3. The lowest IC₅₀ value was detected for the VL70 sample and calculated as $132 \,\mu\text{g/mL}$. The findings related to the antioxidant activity of the *V. agnus-castus* extracts and essential oils show a wide range of results, which shows that not only the extraction method but also the drying process, solvent selection, and the sample region has an important effect on the results [26, 37, 38]. In a study, the IC₅₀ value for methanol, chloroform, and water extracts of *V. agnus-castus* leaves was calculated as 127, 179, and 224 $\,\mu\text{g/mL}$ [39], which shows some similarities with the findings presented here.

Conclusions

This study shows that the *V. agnus-castus* fruit and leaf extracts prepared with different methods and solvent concentrations contain different amounts of phenolic and flavonoid components, which are projected to impact their antioxidant activity. Overall, samples obtained from leaves showed higher content of phenolics and flavonoids when compared to the fruit samples. Furthermore, both of the groups (VF60 and VL60) exhibited higher chlorogenic acid and rutin content than the commercial product as detected with HPLC-DAD. Furthermore, in line with these findings, leaf extracts showed higher antioxidant activity when

compared to fruit extracts. Therefore, although the medicinal part of this plant is widely defined as its fruits [40], leaf extracts displayed here very promising results that could be evaluated for future formulations. Of course, the plant materials used in this study were collected from one particular location, and variations in locations may vary the concentrations of active phytochemicals. Thus, comparing the extracts of plant materials collected at different locations in future studies can help obtain well-standardized preparations.

Quantification and activity studies with the extracts prepared by different methods are very important for preparing standardized products. Furthermore, to benefit from such therapeutic effects of these extracts, some strategies can be considered to maximize their bioavailability while considering safety and acceptable dosages in the downregulating body environment. The results show that extracts from chaste tree leaves and fruits are rich in phenolic components and flavonoids and should be considered an antioxidant raw material for therapeutic preparations.

Research funding: None declared.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest. **Informed consent:** Not applicable. **Ethical approval:** Not applicable.

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Supplementary Material: The online version of this article offers supplementary material (DOI: https://doi.org/10.1515/tjb-2021-0208).