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Biological Properties of *Vitex agnus-castus* Essential Oil (Phytochemical Component, Antioxidant and Antifungal Activity)

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Background: *Vitex agnus-castus* is a deciduous shrub that is native to the Mediterranean region. It has traditionally been used in Iranian medicine. In the current study, *Vitex agnus-castus* Essential Oil (EO) leaves were analyzed for their chemical component as well as antioxidant and antifungal activity.
Objectives: The aim of this study was to determine the biological properties (phytochemical component, and antioxidant and antifungal activity) of *Vitex agnus-castus* EO from an Iranian origin.
Materials and Methods: Chemical composition of the EO was determined by using Gas Chromatography (GC) and Gas Chromatography/Mass Spectrometry (GC-MS). Antioxidant activity of the *Vitex agnus-castus* EO was examined by the 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) assay, while the total phenolic content was also determined. Antifungal activity (against *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida dubliniensis*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium* species and *Alternaria* species) was performed by a broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) protocols M27-A and M38-A for yeasts and filamentous species.
Results: Thirty-two components were identified in *Vitex agnus-castus* EO. The main compound was alpha-Pinene (19.48%). The total phenolic content of EO was determined as 82.26 ± 5.94 mg Gallic Acid Equivalent (GAE)/g EO. The EO exhibited significant radical scavenging activity with IC50 value of 27.16 μg/mL. The obtained EO showed significant antifungal activity. *Aspergillus niger* was more susceptible than other fungi (MIC: 0.78 μL/mL).
Conclusions: Potent antifungal activity, make this plant an effective replacement treatment for fungal infections or fungal strains that are resistance to synthetic antifungals.

Keywords: Diphenyl-2-picrylhydrazyl; Essential oil; Phenolic Component; *Vitex agnus-castus*; Gas Chromatography-Mass Spectrometry

1. Background

During the last decade, the use of alternative medicine has increased globally. Furthermore, its use is not limited to developing countries, but also in countries where conventional medicine is predominant in the national health care system (1). Long-term use of herbal medicines offers cost-effective and potent health protection with minimum side effects (2, 3). Currently, there is a growing interest in the use of herbal medicine as a natural source of substances with antioxidant activity.

There are substantial evidences indicating the involvement of reactive oxygen species and other oxidants in the development of numerous disorders and pathological manifestations. Recently, naturally occurring antioxidant components of plant materials, such as phenolic compounds, have been widely investigated because of their effective role in scavenging free radicals and protecting health (4). They are capable of suppressing reactive oxygen formation, chelating trace elements involved in free-radical production, scavenging reactive species and up-regulating and protecting antioxidant defenses (5).

The Genus *Vitex* belongs to the *Verbenaceae* family, which grows in tropical and sub-tropical regions. This genus is an important natural source of food and medicine around the world and in Iran as well. There are approximately 270 known species of the genus *Vitex* distributed in many parts of the world (6). *Vitex agnus-castus* is a well-known medicinal plant widely distributed in the Middle East and Europe (7). Traditionally, it has been used for treatment of several female disorders

such as endometriosis, abnormal menstrual cycles, menopausal conditions, insufficient lactation and acne (8, 9). Recent studies revealed that the plant has wide pharmacological properties such as antibacterial, antihistaminic, anti-inflammatory and antioxidant activities (6). Phytochemical analysis of the of *Vitex agnus-castus* Essential Oil (EO) from an Amazon origin identified several components, mainly 1, 8-cineole, (E)-β-farnesene, sabinene, α-pinene, α-terpinyl acetate, β-caryophyllene and bicyclogermacrene (10). In other studies, the chemical composition of EO and extracts of different solvents were investigated (2, 11). Other studies have revealed that *Vitex agnus-castus* is an excellent source of phenolic compounds.

2. Objectives

Despite great advances in medicine, plants still have important roles in healthcare due to their useful natural components such as antioxidants. In the present study we investigated the potential antioxidant activity, total phenolic component and antifungal activity of *Vitex agnus-castus*.

3. Materials and Methods

3.1. Collection of Plant Material

Fresh leaves of the plant were collected from Maraghe, East Azerbaijan province, Iran, in August 2013. The plant identity was confirmed by the herbarium of the faculty of agriculture, University of Tabriz, Tabriz, Iran (under code number 16697). The leaves were dried at room temperature. They were then powdered and kept in tight containers protected from light.

3.2. Essential Oil Extraction

Powdered leaves (100 g) of *Vitex agnus-castus L.* were subjected to the hydrodistillation for 2.5 hours, using a cleavenger-type apparatus, according to the method recommended by the European Pharmacopoeia to produce oils. The obtained essential oil was kept at 4°C.

3.3. Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

The EO was analyzed by gas chromatography. The chromatograph (Agilent 6890 UK) was equipped with an HP-5MS capillary column and the data were obtained under the following conditions: initial temperature 50°C, temperature ramp 5°C/minute, 240°C/minute to 300°C (holding for three minutes), and injector temperature at 290°C. The carrier gas was helium and the split ratio was 0.8 mL/minute. For confirmation of analysis results, the essential oil was also analyzed by Gas Chromatography/Mass Spectrometry (GC/MS) (Agilent 6890 gas chromatograph equipped with an Agilent 5973 mass-selective detector; Agilent UK) and the same capillary column and analytical conditions as above (Figure 1).

3.4. Antioxidant Activity

3.4.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Assay

In this assay, hydrogen atom or electron donation ability of our EO was evaluated by measuring the bleaching of the purple-colored methanol solution of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma, Aldrich). Five milliliters of a 0.004% methanol solution of DPPH radical was mixed with 50 µL of various concentrations of the EO. The mixture was incubated at room temperature for 30 minutes, and then the absorbance was read against a blank at 517 nm using a spectrophotometer (Pharmacia, Uppsala, Sweden). Inhibition of the free radical DPPH as a percentage (I%) was calculated using the following Equation:

$$\mathrm{I\%} = \left(\frac{A_{\mathrm{blank}} - A_{\mathrm{sample}}}{A_{\mathrm{blank}}}\right) \times 100 \quad (1)$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The IC_{50} value, defined as the concentration of the essential oil, resulted in 50% inhibition (12). Vitamin C and Rutin were used as standard controls.

3.4.2. Total Phenols Assay

The total phenolic content of *Vitex agnus-castus* EO was determined employing the method described by Gharib and Silva (2013) (13). Folin-Ciocalteu reagent and gallic acid (both Sigma-Aldrich) were used as standards. Briefly, 0.1 mL of solution containing 1 mg of EO was mixed with 46 mL of distilled water, and then 1 mL of Folin-Ciocalteu reagent was added to the flask and was shaken thoroughly. After three minutes, 3 mL of 2%

Gas Chromatograph	**Agilent 6890N**
Inlet	Split/Splitless
Inlet liner	Single taper, deactivated (Agilent part no. 5181-3316)
Inlet temperature	250 °C
Split ratio	50:1
Column	20 m × 0.18 mm × 1.0 µm DB-VRX (Agilent part no. 121-1524)
Carrier gas	Helium at 1.0 mL/min constant flow
Oven temperature program	40 °C (3 min), 10 °C/min to 100 °C (0 min), 25 °C/min to 225 °C (3 min)
Mass Spectrometer	**Agilent 5973 Inert MSD**
Transfer line temperature	260 °C
Quad temperature	150 °C
Source temperature	230 °C
EM voltage	2035 volts
Scan range	35–260 *m/z*
Threshold	0
Samples	3
Solvent delay	0 min
Software	MSD Productivity ChemStation Software (Part no. G1701DA version D.01.00)

Figure 1. Gas Chromatography/Mass Spectrometry Methods

Na_2CO_3 solution was added and the mixture was incubated for two hours with intermittent shaking. Absorbance was determined at 760 nm. The same procedure was performed for all standard gallic acid solutions (0 – 1000 mg in 0.1 mL). Standard curve was obtained according to the following equation:

Absorbance = 0.0012 × Gallic acid (μg) + 0.0033

3.5. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentrations (MFC)

The tested fungi, including *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida dubliniensis*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium* species and *Alternaria* species. Fungi were cultured in Sabouraud Dextrose Agar (SDA) at 30°C for 48 hours before the experiment. The correct concentrations of prepared suspensions of fungi were adjusted by hemocytometer counts. Broth microdilution method (M27-A) as described by the Clinical and Laboratory Standards Institute (CLSI) was used to determine in vitro Minimum Inhibitory Concentration (MIC) of EO against fungi strains. The test was performed in 96-well flat-bottomed microtiter plates, using Roswell park memorial institute medium (RPMI) 1640, which had been buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (Sigma). Furthermore, 100 μL of EO (20%) was added to the first well containing 100 μL of medium, and then serially diluted by two folds. The yeast inoculum was adjusted to a concentration of 0.5×10^3 to 2.5×10^3 CFU/mL, and an aliquot of 100 μL was added to each well of the microdilution plate. The filamentous fungi inoculum was adjusted to a concentration of 0.5×10^4 to 1×10^4 CFU/mL, and an aliquot of 100 μL was added to each well of the microdilution plate. The plates were incubated at 35°C for 48 hours. The MIC was determined as the lowest concentration of EO, which inhibited the visual growth of fungi, and Minimum Fungicidal Concentrations (MFC) as the lowest concentration resulting in total growth inhibition. The test was performed twice on separate plates.

4. Results

4.1. Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

The chemical composition of *Vitex agnus-castus* EO and chromatogram is shown in Table 1 and Figure 2. The oil yield was 1.3% (v/w), calculated on dry weight basis. The gas chromatogram of the essential oil is shown in Figure 1. The major component was α-Pinene (19.48%). The combination of cyclohexenes (34.81%) had the highest percentage amongst the other constituents, this combination included, 1-methyl-4- (1-methylethenyl) (13.37%), caryophyllene (8.55%), sabinene (6.89%) and β-sesquiphellandrene (6%).

Table 1. The Most Common Components of *Vitex agnus-castus* Essential Oil

NO	Compounds	RT [a]	Percentage
1	α-Pinene	8.20	19.48
2	Sabinene	8.76	6.89
3	β-Myrcene	8.98	1.84
4	Cyclohexene, 1-methyl-4-(1-methylethenyl)	9.73	13.37
5	γ-Terpinene	10.00	1.08
6	α-Terpinolene	10.39	0.67
7	Linalool	10.51	1.07
8	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)	11.52	1.80
9	α-Terpinyl acetate	11.66	1.87
10	β-Citronellol	11.98	0.91
11	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)	12.22	0.77
12	Bicyclo [2.2.1] heptan-2-ol	12.63	0.66
13	Cyclohexene, 4-ethenyl-4-methyl	13.14	0.73
14	Camphene	13.26	3.59
15	3-Allyl-6-methoxyphenol	13.36	1.81
16	Bicyclo [3.1.1] hept-2-ene, 2, 6-dimethyl	13.62	2.65
17	Caryophyllene	14.07	8.55
18	β-Sesquiphellandrene	14.20	6.00
19	trans- β-Farnesene	14.26	1.15
20	γ-Curcumene	14.45	1.59
21	β-Cubebene	14.57	2.77
22	β-Selinene	14.63	1.09
23	7-Methanoazulene	14.70	1.66
24	Pyridinethione	14.76	1.56
25	β-Sesquiphellandrene	14.83	2.03
26	β-Bisabolene	14.97	0.92
27	Caryophyllene oxide	15.43	1.24
28	β-Eudesmol	15.95	2.46
29	α-Bisabolol	16.10	1.47
30	Phenol, bis (1,1-dimethylethyl)	18.13	4.09
31	2-Hydroxy-12-methoxy-19-norpodocarpa-1	18.28	2.09
32	Pyrrolo (3,2,1-JK) Carbazole	18.40	2.12

[a] RT: Retention Time (minute).

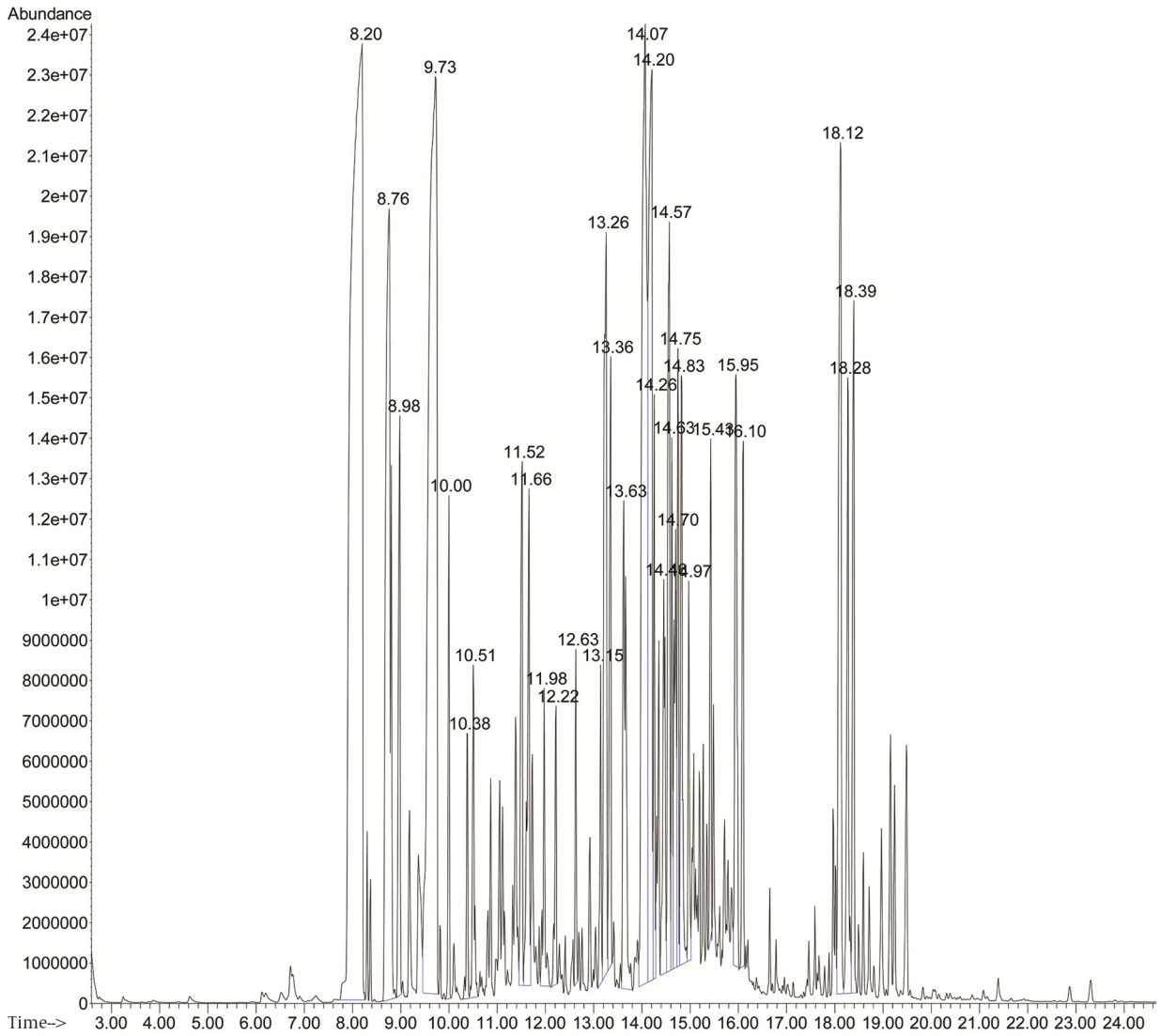

Figure 2. Chromatogram of *Vitex agnus-castus* Essential Oil

4.2. Antioxidant Activity

4.2.1. Scavenging Effect on 1, 1-Diphenyl-2-Picrylhydrazyl

The hydrogen atoms or electron donating ability of *Vitex agnus-castus* EO was determined by measurement of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. The IC50 value of the essential oil was 27.16 μg/mL.

4.2.2. Total Phenolic

In the present study, total phenolic content was determined by using the Folin-Ciocalteu reagent. It was 82.26 ± 5.94 mg/g, presented as gallic acid equivalent in milligrams per gram of EO.

4.3. Antifungal Activity

The results for the general screening for antifungal activity are shown in Table 2. Antifungal activity of *Vitex agnus-castus* EO was against all tested fungi. The EO showed highest antifungal activity against A. niger, followed by C. albicans, C. tropicalis, C. parapsilosis and C. dubliniensis, which were affected at the same level by EO. These four fungi were followed by Alternaria. Lowest antifungal activity was shown against C. krusei, A. flavus and Penicillium, which were affected at the same level by EO. According to our results, the highest MFC value was determined for C. albicans, C. tropicalis, C. parapsilosis and C. dubliniensis followed by C. krusei and Alternaria.

Table 2. Antifungal Activity of *Vitex agnus-castus* Essential Oil (MFC and MIC Value) [a,b]

Organisms	MFC	MIC
C. albicans	6.3	3.1
C. tropicalis	6.3	3.1
C. parapsilosis	6.3	3.1
C. krusei	12.5	12.5
C. dubliniensis	6.3	3.1
A. flavus	25	12.5
A. niger	50	0.8
Penicillium	50	12.5
Alternaria	12.5	6.3

[a] Abbreviations: MFC, Minimum Fungicidal Concentrations; MIC, Minimum Inhibitory Concentration.
[b] Data are presented as μL/mL.

5. Discussion

The result of the composition of EO was compared with recent reports. Stojkovic et al. found forty-six compounds in leaves with 98.4% of the oil and the essential oil yield obtained 0.56% on a dry weight basis. The main components in leaves were 1, 8-cineole (22.0%), trans-b-farnesene (9.4%), α-pinene (9.4%), trans-b-caryophyllene (8.2%) and terpine-4-ol (7.8%) (3). The GC–MS analysis of the essential oil of *V. agnus-castus* fruit detected 27 components, the main components of the EO were 1, 8-cineole (24.98%), sabinene (13.45%), α-pinene (10.60%), a-terpinyl acetate (6.66%), and (Z) - b-farnesene (5.40%) (11). The leaves EO of a North-Central Nigerian-grown *Vitex agnus-castus* had 34 compounds present in 98.5% of the essential oil and the oil yield was 0.8% v/w. The chief components in EO were β-pinene (20.0%), α-pinene (9.1%), cis-ocimene (8.4%), 1, 8-cineole (6.7%), terpinen-4-ol (4.2%), β-phellandrene (4.1%) and α-terpineol (4.1%) (14). A previous studies in Iran indicated that the most abundant components of *Vitex agnus-castus* seeds essential oil were caryophyllene oxide (24.9%), n-hexadecane (12.5%) and α-terpinyl acetate (11.6%) (2). The results showed that quantity and quality of compounds depend on geographical area and the growing season of plants. In our study the results were in agreement with this previous study however the quantity of compounds was different.

The hydrogen atoms or electron donating ability of *Vitex agnus-castus* EO was determined by measurement of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. The DPPH method is considered as an easy, rapid and sensitive way to evaluate the antioxidant activity of plant EO (13, 15). This method is based on the reduction of DPPH solution by antioxidant substances, resulting in discoloration from violet to yellow. The degree of color change is proportional to the concentration and potency of antioxidants. The IC_{50} value of the essential oil was 27.16 μg/mL. A previous study revealed that EO of leaves and fruit of *Vitex agnus-castus* has potent antioxidant activity with IC_{50} values of 0.449 ± 0.001 and 0.612 ± 0.004 mg/mL, respectively (7). Another study showed that derivatives of benzoic acids have the strongest antioxidant activity compared to other identified components of *Vitex agnus-castus* EO (7). Saglam et al. (2007) reported that EO of *Vitex agnus-castus* exhibits better antioxidant activity than n-hexane extracts (16). According to previous studies, fruits, flowers and leaves of *Vitex agnus-castus* contain tannins, iridoids and diterpenoids. In our study, *Vitex agnus-castus* contained high levels of phenolic components. The correlation between total phenolic content and antioxidant activity of the EO has been reported previously (11). Furthermore, there are many reports suggesting the antioxidant activity of polyphenols and tannins. Therefore, it can be assumed that these components are involved in antioxidant activity of essential oils. Based on our results, the *Vitex agnus-castus* EO has antioxidant activity and remarkable phenolic content, thus it can serve as an excellent natural source of antioxidant agents.

Free radicals have wide adverse effects on cells, resulting in many disease conditions such as coronary disease or cancer. Phenolic components act as free radical scavengers and cause delay or prevent oxidative stress caused by free radicals (4). In the present study, total phenolic content was determined by using the Folin-Ciocalteu reagent. It was 82.26 ± 5.94 mg/g, presented as gallic acid equivalent in milligrams per gram of EO. In a previous study, total phenolic content of leaves and fruit of *Vitex agnus-castus* EO was investigated and found as 123.9 ± 2.281 mg Gallic Acid Equivalent (GAE)/g EO and 114.5 ± 2.704 mg GAE/g EO, respectively (7). Recently, plant materials rich in phenolic components are widely exploited in the food industry because of their protective role against lipid peroxidation and enhancing food quality (4). The phenolic content of plants depends on many factors such as degree of ripeness, environmental factors, processing and storage (17). In a recent study methanolic EO of *Vitex agnus-castus* collected from Antalya-Turkey contained high levels of phenolic content (48.05 ± 1.02 mg GAE), experiments on which indicated a positive correlation between total phenolic and the antioxidant activity potential of the EO (18). The obtained data were in agreement with our findings.

The results for the general screening for antifungal activity are shown in Table 2. Antifungal activity of *Vitex agnus-castus* EO was against all tested fungi. The EO showed highest antifungal activity against *A. niger*, followed by *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis,* which were affected at the same level by EO. These four fungi were followed by *Alternaria*. Lowest antifungal activity was shown against *C. krusei*, *A. flavus* and *Penicillium,* which were affected at the same level by EO. According to our results, the highest MFC value was determined for *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis* followed *by C. krusei* and *Alternaria*. Antimicrobial activities of *Vitex agnus-castus* have been investigated previously

and results showed that fungi were more sensitive than the bacteria. The results showed that *Alternaria* with MIC of 44.5 - 130.0 μg/mL and MFC of 89.0 - 178.0 μg/mL was more sensitive than the other species (3). The antibacterial assays of the *Vitex agnus-castus* by the disc diffusion method (zone size, mm) showed its ability to inhibit *Staphylococcus aureus* (50.0 ± 0.0), *Pseudomonas aeruginosa* (41.0 ± 0.7), *Bacillus subtilis* (11.0 ± 0.7) and *Salmonella enteritidis* (9.0 ± 0.0), yet EO had no effect on *Listeria monocytogenes* and *Escherichia coli* (2). However, Maltas et al. noted the antifungal activity of *Vitex agnus-castus* EO against *Candida albicans* by the disc diffusion method (11 mm at 100 mg/mL) and broth microdilution method (MIC value was determined as 0.39 mg/mL) (18). There are many reports suggesting that antimicrobial activity depends on the contents of phenolic compounds. In conclusion, EO phytochemicals can be used against some pathogenic bacteria and fungi (3, 6, 19, 20). Our results revealed that *Vitex agnus-castus* essential oil with its potent antifungal activity can serve as an excellent natural source for use in traditional medicine.

In this study, essential oil of leaves from *Vitex agnus-castus* showed effective in vitro antifungal activity against all studied fungi species. Further investigations on components mainly responsible for these effects and their mechanism of action would be valuable.

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Authors' Contributions

Razzagh Mahmoudi and Farzad Katiraee were the guarantors, and developed the original idea and the protocol, abstracted and analyzed the data, and wrote the manuscript. Keyvan Tahapour, Seyed Jamal Emami and Gholamreza Hamidian contributed to the development of the protocol, abstracted data, and prepared the manuscript.

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