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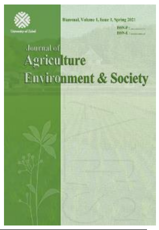

The first report of essential oil composition of *Vitex agnus-castus* L. growing in the Sistan region and its antibacterial activity

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ABSTRACT

This study aimed to investigate the chemical compounds in the essential oil extracted from the flowering branch of *Vitex agnus-castus* L., grown in the Sistan region (Sistan and Baluchestan Province), and its antibacterial effects. Samples were hydrodistilled in a Clevenger–type apparatus and analyzed with gas chromatography mass spectrometry (GC-MS). In vitex essential oil, 30 bioactive compounds were identified. The most important compounds are 1,8-Cineole (39.16%), Sabinene (8.78%), β-Myrcene (6.44%), Sclareol (4.3%), and *trans*-Caryophyllene (3.17%). The composition of the essential oil of *Vitex agnus-castus* was described for the first time from Sistan region. *Bacillus cereus* (ATCC 11778) was the most sensitive strain against this essential oil, and *Pseudomonas aeruginosa* (ISIRI 275) was the most resistant strain. The monoterpenes and sesquiterpenes confer the chemical profile of the analyzed essential oil of vitex causing antibacterial effects. Further studies are required to explain the oil mechanism of action of this species involved in antimicrobial activities.

1. Introduction

Since 2000 years ago, the vitex plant has been used in the traditional medicine of different countries. The vitex genus of the Verbenaceae family grows wild in Iran and had four species of shrubs in the flora of Iran, including *V. trifolia* L., *V. negundo* L., *V. agnus-castus* L. and *V. psendoneggundo* is often observed in tropical and subtropical regions of the country, especially Sistan and Baluchestan, Khuzestan, Alborz (Karaj), Tehran, Qom, Khorasan, Hormozgan (Bandar Abbas), Lorestan provinces and different areas of the Persian Gulf (Nasri et al., 2005). VAC (*Vitex agnus-castus*), has been used since ancient Greek times as a treatment for menstrual problems (Webster et al., 2011). In addition, it has been used to treat pain, swelling, inflammation, headaches, rheumatism, and sexual dysfunction (Webster et al., 2006).

The insecticidal property of the plant has also been reported (Tandon et al., 2008). New research shows the therapeutic effect of chaste extract in premenstrual syndrome (PMS), elimination of menopausal complications and increased milk production in lactating mothers (Eryigit et al., 2015). Vitex, Chaste tree, chaste berry, monk's pepper, and Abraham's balm are the latin names by which this plant is known. This plant is a tiny deciduous shrub that grows to be 1-2 meters tall, with a strong black pepper scent emanating from all parts. The plant has compound claw leaves (5 - 7 leaflets), dark greyish-green, long peduncles, slender flower clusters, purple bluish flowers and small berry fruits like black pepper. The leaves, flowers, and fruits of this plant contain active ingredients such as iridoid glycosides (acobin and agnoside), alkaloids (viticin), flavonoids (vitexin and castin), steroid hormones (progesterone and testosterone), and essential oil (1, 8- cineole and linalool) (Ghorbani Ghouzhdi et al., 2021). Two glycoside compounds in the leaves of this plant have been identified as vitexin and vitexinin (Maltaş et al., 2010).

One of the most important secondary metabolites produced by this plant is essential oil. Essential oils are generally extracted using distillation, and often using

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steam. Essential oils extracted from different parts of a plant may differ in physical, chemical and odor properties. The constituents of essential oils change under different conditions. Therefore, it is not possible to comment definitively on the essential oils of a plant without considering specific environmental conditions. Essential oils are directly related to the biosynthesis, metabolism and biological activities of the plant which are a function of the climatic conditions of the plant environment. These oils are produced by plants as secondary metabolites for protection against bacteria, viruses, fungi and pests (Rota et al., 2008; Ghannadi et al., 2012). The antimicrobial properties of vitex essential oils and their constituents have been evaluated and reviewed (Malesand and Blazevic, 1998; Hamid et al., 2010; Küçükboyaciand and Sener, 2010; Ghannadi et al., 2012). Since the identification of the chemical components of plant essential oils from aromatic plants is needed to take better advantage of their opportunities, this study aimed to identify the secondary metabolites in the essential oil of the vitex grown in Sistan climatic conditions and to evaluate its antimicrobial properties.

2. Materials and methods

2.1. Plant materials

The aerial parts of *Vitex agnus-castus* L. were collected during their flowering stage in 2021 June from the Chah Nimeh (medicinal plants collection, Institute of Agriculture, University of Zabol, Iran) (Figure 1). Collected plant samples were air-dried in shade and under room temperature conditions.

The aerial parts of vitex (leaves, stems, flowers, and seeds) were collected from the Sistan region (the medicinal plants Collection, Institute of Agricultural reaserch, Zabol, Iran), during June- July 2021 (Figure 1). Collected plant samples were air-dried under shade and room temperature conditions.

Figure 1. The aerial parts of vitex grown in Zabol climate

2.2. Essential oils isolation

One hundred grams of dried plants were crushed into smaller pieces, and hydrodistilled in a Clevenger–type apparatus for 4 h. The oils were stored in dark glass vials at 4 ºC until the analysis time.

2.3. Gas Chromatography-Mass Spectrometry (GC-MS)

The compounds were analyzed on a 6890 N Agilent gas chromatograph coupled with a 5975 C Agilent mass-selective detector (Agilent Technologies, Avondale, PA, USA). A 7683 Agilent autosampler and 2 µL of the sample were injected in splitless mode at 250ºC into a 30 m × 0.25 mm × 0.5 µm DB-5 MS capillary column and were operated by MSD Chemstation Software (Agilent Technologies). The temperature program used for the chromatographic separation was as follows: 50ºC for 2 min, the temperature increase at 25ºC/min to 100ºC and hold for 2 min, then temperature increased at 5ºC min−1 to 290ºC where it was finally held for 5 min. The carrier gas was helium (99.999%) and was kept at a constant flux of 1.0 ml /min. The mass spectrometer operated in electronic impact ionization mode and the energy of the electrons was kept at 70 eV. The interface was kept at 290ºC. The mass spectrums were obtained at a mass ratio can range from 100 to 400 m/z to determine the appropriate mass.

2.4 Identification of components

The linear retention indices for all the compounds were determined by co-injection of the sample with a solution containing the homologous series of C8–C22 n-alkanes (van den Dool and Kratz, 1963). The individual constituents were identified by their identical retention indices, referring to the known compounds taken from the literature and by comparison of their mass spectra either with the known compounds or with the Wiley mass spectral database.

2.5. Antimicrobial assay

Antibacterial activity of essential oils, isolated from *vitex* aerial parts was analyzed using Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests (Habbab et al., 2016). In microbiology, MIC is the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after overnight incubation. MIC were defined as the lowest concentration of essential oil inhibiting visible growth of the bacteria (Owuama, 2017).

A collection of six organisms, including three Gram-positive and three Gram-negative bacterial strains, was used. The groups included one organism of American Type of Culture Collection (ATCC), one organism of Institute of Standards and Industrial Research of Iran (ISIRI) and four organisms of Plat Total Colony Count (PTCC). Table 1, shows the source of the bacterial strains. In this study, we used three Gram-negative bacterium, *E. coli* (PTCC 1533), *Pseudomonas aeruginosa* (ISIRI 275), *Salmonella typhi* (PTCC 1609), and three Gram-positive bacterium, *Listeria monocytogenes* (PTCC 1163), *Bacillus cereus* (ATCC 11778), and *Staphylococcus aureus* (PTCC 1112).

Serial dilutions of *vitex* essential oil were prepared in microdilution tubes with concentrations ranging between (1/2) 156.25µg/ml and (1/128) 20,000 µg/ml.

Bacterial suspensions were adjusted to the logarithmic-phase growth to match the turbidity of a 0.5 McFarland standard, yielding approximately 106 CFU/mL. The same amounts of bacteria were added to all tubes and the tubes were incubated at 37 ºC for 24 h.

Each tube was examined for growth and was compared with the control. Without adding bacteria, medium with no essential oil, medium containing DMSO and different essential oil concentrations were used as a control for each mentioned component, respectively. The absence of growth was defined as antibacterial activity.

Bacterial inoculum was prepared by suspension of freshly grown bacteria in sterile saline (0.85% NaCl w/v) and was adjusted to a 0.5 McFarland standard. The MBC is the lowest concentration of antibiotics required for killing a particular bacterium. The eight dilutions were run in duplicate for the MBC test. At the end of 24 h of incubation, the tubes were read for the MIC and then the MBC by Spectro photometrical method using ELISA reader (Bio-Tek Instruments) at 580 nm.

Table 1. The source of the bacterial strains

Gram-positive bacterium		Gram-negative bacterium	
Listeria monocytogenes (PTCC 1163)	Plat Total Colony Count	*E. coli* (PTCC 1533)	Plat Total Colony Count
Bacillus cereus (ATCC 11778)	American Type of Culture Collection	*Pseudomonas aeruginosa* (ISIRI 275)	Institute of Standards and Industrial Research of Iran
Staphylococcus aureus (PTCC 1112)	Plat Total Colony Count	*Salmonella typhi* (PTCC 1609)	Plat Total Colony Count

3. Results and discussion

The results of identifying the active compounds in the essential oil of vitex aerial parts are reported in Table 2. In vitex essential oil, 30 bioactive compounds were identified based on retention time and mass spectra.

The most important compounds are 1,8-Cineole (39.16%), Sabinene (8.78%), β-Myrcene (6.44%), Sclareol (4.3%), and *trans*-Caryophyllene (3.17%). In other words, this sample is a 1,8- Cineole chemotype (Table 2).

Table 2. Chemical composition of essential oil from flowering branch of *Vitex agnus-castus*

No	Compounds	Percentage of compounds	Retention time
1	α-Pinene	1.09	7.729
2	β- Pinene	2.33	8.03
3	Sabinene	8.78	9.602
4	β-Myrcene	6.44	9.8
5	Isoborneol	2.21	9.090
6	α-Terpinene	2.11	9.981
7	Limonene	1.43	10.049
8	2-Cyclopenten-1-one, 3-ethyl-2-hydroxy	0.87	10.267
9	1-Cyclohexene-1-methanol, α,α,4-trimethyl	1.15	10.687
10	1,8-Cineole	39.16	11.045
11	*trans*-Sabinene Hydrate	0.93	11.169
12	*trans*-Caryophyllene	3.17	11.356
13	β-Farnesene	1.34	11.704
14	α-Humulene	1.17	11.922
15	α-Terpinyl Acetate	1.02	12.441
16	Bicyclogermacrene	1.09	12.518
17	Caryophyllene Oxide	1.93	12.57
18	Retinol Acetate	1.78	12.638
19	Spathulenol	1.97	12.679
20	*tau*-Cadinol	0.82	13.266
21	α-Bisabolol	2.26	13.406
22	4-(4-Hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)-2-butanone	2.34	13.541
23	2,6-Bis(aminomethyl)-2,6-adamantanediol	1.18	14.36
24	Sclareol	1.14	14.495
25	4-Bromo-1-Naphyhalenamine	0.93	14.521
26	β-n-Methylionone	1.84	14.713
27	Dihydroselarene	2.57	14.947
28	Sclareol	4.3	15.222
29	Thunbergol	1.86	15.595
30	7-a-Hydroxymintlactone	0.79	18.086
	Total	**100**	

Other studies have identified these compounds as the most important compounds in vitex fruit essential oil grown in Turkey (Sarikurkcu et al., 2009; Eryigit et al., 2015; Tin et al., 2017). Another study that looked at the chemical composition of essential oils over time found that the following components are the most important: α-pinene (26.99%), 1,8-cineole (14.20%), trans-caryophyllene (9.13%), sabinene (8.29%), germacrene-B (8.20%), limonene (6.53%), 1,6,10-dodecatriene (6.37%), while main components of the essential oils obtained in fruit maturity period were detected as 1,8-cineole (28.34%), α-pinene (26.96%), sabinene (16.36%), and limonene (9.08%) (Tin et al., 2017).

A study on vitex growing in Nigeria considered the most important active compounds in its leaves were β-pinene (20.0%), viridifloral (9.8%), α-pinene (9.1%), cis-o-cymene (8.4%), 1,8 cineole (6.7%) and β-farnesene (5.4%) (Hamid et al., 2010).

In the Southern-West Algeria region, the major components in the oil of flowers were 1,8-cineole

(17.16%), caryophyllene (12.94%) and terpinen-4- ol (10.22%), while the dominant compounds in the oil of the seeds were 1,8-cineole (14.92%), cedrelanol (13.95%) and 7a-isopropenyl-4,5-dimethyloctahydroindene-4-carboxylic acid (13.90%) (Habbab et al., 2016).

The major components of vitex essential oil from Brazil were identified as 1,8-cineole (47.9%), terpinyl α-acetate (11.6%), sabinene (11.2%) and caryophyllene oxide (9.7%) (Ricarte et al., 2020).

Changes in the active ingredients of medicinal plants vary depending on climate conditions, geographical conditions, medicinal organs, and the method of extracting essential oils (Mazandarani et al., 2013, Yaldiz and Sekeroglu, 2013).

Previous studies have examined the antimicrobial effects of vitex fruits essential oil. Their results indicate the sensitivity of gram-positive and gram-negative bacteria to this essential oil compared with chemical antibiotics (ampicillin and ofloxacin) (Eryigit et al., 2015). The researchers reported that 1,8-cineole and α-Pinene showed very high antimicrobial potency as well (Stojkovic´ et al., 2011).

Table 3. Antibacterial activity of *vitex* essential oil

Bacteria	**Type of bacteria**	**MIC (μg/ml)**	**MBC (μg/ml)**
Escherichia coli (PTCC 1533)	Gram negative	5000	5000
Pseudomonas aeruginosa (ISIRI 275)	Gram negative	20000	20000
Salmonella Typhi(PTCC 1609)	Gram negative	10000	10000
Listeria monocytogenes (PTCC 1163)	Gram positive	625	625
Bacillus cereus (ATCC 11778)	Gram positive	312.5	312.5
Staphylococcus aureus(PTCC 1112)	Gram positive	5000	5000

MIC= Minimum Inhibitory Concentration: i.e., the lowest concentration of a particular antibiotic needed to kill bacteria.
MBC= Minimum Bactericidal concentration; i.e., the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by ≥99.9%.

Significant antibacterial activity of essential oil was recorded against that of the examined multi-resistant pathogenic bacteria, *E. coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*.

Bacillus cereus (ATCC 11778) was the most sensitive strain against this essential oil (Table 3). On the other hand, in our experiment, gram-positive bacteria were more sensitive than gram-negative bacteria.

Eryigit et al., (2015) reported that, after *Enterococcus faecalis*, *Bacillus subtilis* was one of the sensitive species with a 12 mm inhibition zone. In other studies, the essential oils of *vitex* seeds and leaves showed antibacterial potency against *Klebsiella pneumonea*, *E. coli*, and *Pseudomonas aeruginosa*, respectively.

While the oil of leaves and flowers showed high antifungal activity against *Penicillium escpansum*, *Aspergillus flavus* dominant compounds, respectively (Habbab et al., 2016).

4. Conclusion

Vitex's healthy and beneficial effects should be further analysed. Considering the essential oils obtained from areal parts of vitex, it seems that 1,8-cineole and sabinene were highly present in this plant. The study revealed significant antibacterial activity of the investigated essential oils. The examined oil exhibited high resistant pathogenic bacteria, which was found to be in correlation to the content of mainly monoterpenes and sesquiterpenes. These results indicate that essential oils could be served not only as flavor agents but also as safe antiseptic supplements in preventing the deterioration of foodstuff, beverage products, and pharmaceuticals.

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