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Chemical Composition of Different Parts of the *Vitex agnus-castus* L. Essential Oils and Their *In-Vitro* Cytotoxic Activities

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Abstract: The essential oil (EO) compositions and chemotypes of the important ethnomedicinal plant *Vitex agnus-castus* L. flowers, leaves, and fruits collected from Türkiye (Balıkesir and İstanbul) were identified in the present study. Different parts of the *V. agnus-castus* EO's *in-vitro* cytotoxic effects on the MCF-7 (human breast adenocarcinoma) and A549 (human lung carcinoma) human-origin cell lines were anaylzed in the current study. The composition of hydrodistiled EOs extracted from flowers, leaves, and fruits of *V. agnus-castus* were analyzed by GC–FID/MS. Monoterpene hydrocarbons and oxygenated monoterpene compounds were detected as the predominant component class of the *V. agnus-castus*. EOs extracted from Balıkesir region were defined as the "*α*-pinene-1,8-cineole" chemotype, while EOs extracted from İstanbul region were defined as the "sabinene-1,8-cineole" chemotype. Sesquiterpene hydrocarbons constituted more than 20% of the compounds in the EOs extracted from the flowers. To the best of our knowledge, this study is the first to analyze the *in-vitro* cytotoxic effects of flowers. This study is also the first to show the *in-vitro* cytotoxic effects of fruit, the most commonly used part of the plant, EO on the MCF-7 cell line. Balıkesir region's EOs were observed as more potent -especially the purple flower's IC_{50} is about 4.68 µg/mL on the MCF7 cell line- than İstanbul regions, which might be attributed to the higher amount of *α*-pinene, caryophyllene, and limonene content. Our results indicated that the *V. agnus-castus* EOs, which contain *α*-pinene, 1,8-cineole, caryophyllene, and limonene as major components, showed relatively high cytotoxic effects compared to the control groups on the MCF7 and A549 cell lines.

Keywords: *Vitex agnus-castus* L.; chasteberry; essential oil; cancer; cytotoxicity.

1. Introduction

The genus *Vitex* includes 214 species worldwide, and only one species, *Vitex agnus-castus,* grows naturally in the Türkiye [1, 2]. *Vitex agnus-castus* L., also known as a chaste tree and monk's pepper, belongs to the family Lamiaceae [2, 3]. It was reported by the famous Roman historian Plinius that

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chaste branches were used in making baskets in ancient times. For this reason, it is thought that the *Vitex* genus comes from the word Vitalis-made by knitting because the branches can be braided. The epithet "*agnus-castus*" is derived from the ancient Greek word "ágnos" meaning holy, pure, or chaste, and is thought to have been pronounced later as "agnus." The second part of the epithet "*castus*," is derived from the Latin word "castitas," meaning chastity, because the plants have been associated with anaphrodisiac properties in men [3]. Also, the other common name of the plant, "monk's pepper," comes from its frequent use in churches in the Middle Ages, as the plant is thought to have libido-lowering activity in men [3-5].

Vitex agnus-castus is a large shrub or a small tree with an aromatic smell, and with palmate shape leaves with green-gray above and white tomentose beneath. The flowers are about 8 mm in diameter and white-pale lilac to dark purple, which bloom from June to early fall. The fruit is a black-reddish fleshy drupe, 3-4 mm in diameter, with a unique smell and slightly bitter taste [1, 4].

The plant is found in Central Asia, the Mediterranean, and Southern Europe [1, 5]. *V. agnus-castus*, also known as "hayıt, beş parmak out," is a popular plant in Türkiye. There are even some villages named Karacahayıt, Karahayıt, and Hayıtlı because the plant is widely found [6]. Chaste berries are popularly used as a diuretic, carminative, and sedative agent in Turkish folk medicine by infusion of 5%. Fruits and leaves are said to protect woolen fabrics against moths, and the plant branches are used to weave baskets and dye fabrics [7]. The plant is also famous for its use in female reproductive disorders such as mastalgia, menopausal disorders, pre-menstrual syndrome, menstrual cycle irregularities, and fertility disorders. European Union herbal monograph recommended 20 mg of dry fruit extract, equivalent to 180 mg of the herbal substance or 40 drops of the liquid extract, as a daily dose for premenstrual syndrome in women [8].

When the ethnobotanical studies conducted in different settlements of Türkiye were examined, it was determined that *V. agnus-castus* was used mainly for gynecological diseases and lung health. The decoction of fruits and flowering branches of *V. agnus-castus* used 2-3 times a day for two weeks for menstrual pain, vaginal itching, and respiratory tract diseases in Alaşehir-Manisa (Türkiye) [9]. The fruits can be used to treat asthma, bronchitis, cold, flu, and hormonal disorders if they are combined with honey and consumed 1-2 times per day for two weeks or as an infusion tea as it was observed in a study from Bozyazı-Mersin [10]. The decoction of fruits is used for infertility for women in Datça-Muğla [11]. An infusion prepared from *V. agnus-castus* fruits has a menstrual-regulating effect when used for 15-30 days by drinking 2-3 cups a day in İzmir [12]. The decoction prepared from the stem of the *V. agnus-castus* is used to treat cough and bronchitis in Marmaris-Muğla [13]. MCF-7 (human breast adenocarcinoma) and A549 (human lung carcinoma) cell lines were selected according to the use of *V. agnus-castus* among the people in ethnobotanical studies of Türkiye.

The studies confirmed that *V. agnus-castus* contains flavonoids, diterpenoids, iridoid glycosides, ecdysteroids, and essential oils [14-15]. The studies generally focused on EO extracted from *V. agnus-castus* leaf and fruit; however, there are fewer studies that identified the chemical composition of the EO extracted from the flower. Previous studies have identified a wide range of major components of the fruit, leaf, and flower EOs, including 1,8-cineole, *α*-pinene, *β*-caryophyllene, sabinene, *α*-terpinyl acetate, *β*-phellandrene, (Z)-*β*-farnesene, limonene, bicyclogermacrene, *β*-selinene and *α*-terpineol [16-19]. Two dominant chemotypes, *α*-pinene and *α*-terpinyl acetate, were mentioned in the first study to determine the chemotypes of *V. agnus castus* fruit and leaf essential oils. However, it was observed that EO chemotypes of the plant were generally made on binary compounds such as "*α*-pinene-1,8-cineole", "(Z)-*β*-farnesene-bicyclogermacrene" and "sabinene-1,8-cineole" in the later research [20-23]. Biological activities of *V. agnus-castus* leaf EO were mainly focused on by the studies. The acaricidal, larvicidal, antioxidant, antibacterial, antifungal, phytotoxic, and cytotoxic activities of *V. agnus-castus* leaf EO have been described. However, in the few studies, anti-oxidant, acaricidal, antibacterial, and antifungal activities of the fruit and flower essential oils also have been described [17, 21-24].

Cancer is among the diseases that cause the most deaths worldwide. Lung, liver, stomach, colorectal, breast, prostate, and oesophageal cancer types have a very high rate of incidence. [24]. For this reason, many resources are being researched to develop novel anti-cancer drugs. Because of their elevated rate of cell division mechanism and ensuing potential side effects of conventional chemotherapy drugs, new treatment agents have been researched. Especially the elevated rate of cell division mechanism causes problems with the tumor cell specificity and related cytotoxicity to healthy

cells. Natural ingredients are excellent sources in the development of anti-cancer drugs. EOs have shown anti-cancer properties with various mechanisms. They can prohibit cancer due to their antioxidant, detoxifying, antiproliferative, and antimutagenic properties on the tumor cell or interaction with the microenvironment. The essential oils' different chemical components serve targeted treatment chance and increase specificity to cancer and healthy cells [26]. For instance, 1,8-cineole which is one of the significant components of *V. agnus-castus* flower, leaf, and fruit EOs‘’anti-cancer activity on the skin carcinoma (A431), osteosarcoma (MG-63) and human keratinocytes (HaCaT) cell lines were detected in a dose depending manner by Sampath et al.[27]. The other major compound of the *V. agnus-castus* flower, leaf, and fruit essential oils, the monoterpene α-pinene, was observed to trigger cell death on the A549 (human lung carcinoma) and HepG2 (hepato-blastoma) cell lines by inflecting oxidative stress connected signaling pathways and the sesquiterpene compound which also found in the *V. agnus-castus* essential oil, *β*-caryophyllene’s cytotoxicity in different human tumor cells (MCF-7, DLD-1 and L-929 cells) was discovered by researchers, too. Since chaste tree essential oil contains these compounds as a mixture, it is expected to have a more severe effect [28, 29].

Breast cancer, the most common fatal cancer in women, ranks second after lung cancer in cancer-related deaths [25]. The present study was carried out to examine and compare the essential oil compositions of *V. agnus-castus* fruits, leaves, and flowers collected from Türkiye (Balıkesir and İstanbul) and analyze the cytotoxic effect on the MCF-7 (breast adenocarcinoma) and A549 (lung carcinoma) human origin cell lines. Before performing in-vivo culture studies, it is essential to perform *in-vitro* tests for toxicity research [30]. As far as is known, this study is the first to analyze the cytotoxicity of flowers. Different colored white and purple flowers found in nature were distilled, and the chemical contents and cytotoxicity of essential oils were compared. This study is the first to examine fruit essential oils’ cytotoxic effect on the MCF-7 cell line. Essential oils extracted from different parts of the *V. agnus-castus* collected from its natural habitat (Balıkesir, Burhaniye) and the cultivated area (İstanbul, Zeytinburnu Medicinal Plants Garden) were compared in this research. The chemotypes and significant components were also determined.

2. Materials and Methods

2.1. Cell Culture and Chemicals

Human breast adenocarcinoma cells (MCF-7, ATCC®, HTB-22) and human lung carcinoma cells (A549, ATCC®, CRM-CCL-185) were used to analyze the cytotoxicity of *Vitex agnus-castus* essential oils. Dimethyl sulfoxide (DMSO), 2,5-diphenyl-2H-tetrazolium bromide (MTT), and n-hexane were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Fetal bovine serum (FBS), Phosphate buffer solution (PBS), high glucose Dulbecco’s Modified Eagle Medium (DMEM), F12 cell culture medium, and Trypsin/EDTA solution were purchased from Wisent INC (Quebec, Canada).

2.2. Plant Material and Essential Oil Isolation

Vitex agnus castus L. plants with white and purple flowers, from their natural habitat, were collected from Balıkesir (Burhaniye) during the initial flowering period in July and the post-flowering period in September. They were identified by Dr. Ebru Özdemir Nath, Altınbaş University, Türkiye. A herbarium specimen of the plants was deposited in the Altınbaş University Herbarium (Herbarium number: HERA1057, HERA1061)

The cultivated *Vitex agnus castus* L. plant was collected from İstanbul (Zeytinburnu Medicinal Plants Garden) during -the initial flowering period- in July and -the post-flowering period- in October. It was identified by Dr. Ebru Özdemir Nath, Altnbaş University, Türkiye. A herbarium specimen of the plant was deposited in the Altınbaş University Herbarium (Herbarium number: HERA1058).

The flower, leaf, and fruit parts of each plant were separated and air-dried. 300 g of each plant material were mechanically and coarsely grounded, which were cut between 20 to 40 mm in size before being distilled. Therefore, each plant material was subjected to hydrodistillation for 3h in triplicate using a Clevenger-type apparatus according to the European Pharmacopoeia 7.0 procedure.

Anhydrous Na_2SO_4 was used to dry the isolated EOs, and samples were stored at 4°C in amber vials before the analysis.

2.3. Physical Properties of the EOs

The relative densities (d^{20}) of the EOs were determined by the means of three different experiments done at 20 ºC, using a pycnometer (1 mL).

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

V. agnus-castus EOs were analyzed by using GC-FID/MS analysis. The compounds of EOs were detected and measured using Agilent 7890B GC-FID (Santa Clara, CA, USA) connected to Agilent 5977E electron impact mass spectrometer (Santa Clara, CA, USA) through a two-way capillary splitter. An Agilent G4513A (Santa Clara, CA, USA) auto-injector was used to inject 1 µL of samples. DB-WAX column (60m, 0.25mm, 0.25µm) was used with the following temperature program; 70 ºC for 15 minutes and raised to 180 ºC at a rate of 2 ºC/min. After maintaining an isothermal temperature of 180 °C for 5 minutes, the column temperature was increased to 230 °C at a rate of 5°C/min. Then the isothermal column temperatures of 230 °C for 15 minutes were set. 100 minutes were assigned for the analysis. As a carrier gas, helium was employed (constant flow rate of 1.5 mL/min). A split ratio of 1:50 was chosen. Injector port, quadrupole, MSD transfer line, ion source, and FID temperatures were, in order, 220 °C, 150 °C, 250 °C, 230 °C, and 220 °C. Mass spectra were recorded at 70 eV. H_2 flow was arranged to 30 mL/min while FID air flow was 400 mL/min. Mass range was 45-450 *m/z* [31].

Identification of the EO compounds was performed by comparing the mass spectra of compounds obtained from the Wiley Registry of Mass Spectral Data, 9th edition (April 2011) with the NIST 11 Mass Spectral Library (NIST11/2011/EPA/NIH). Three separate simultaneous auto-injections were conducted for the same operational conditions on GC-FID/MS. The identification method based on the relative retention indices (RRI) of compounds on the DB-WAX column was calculated against saturated n-alkane series (C_7-C_{40}) from the FID data. The results were appraised with NIST online webbook and previous studies from the literature. The relative percentages were calculated using an external standard approach based on calibration curves derived from GC-FID analyses of representative components.

2.5. MTT assay

Both human breast adenocarcinoma cells (MCF-7, ATCC®, HTB-22) and human lung carcinoma cells (A549, ATCC®, CRM-CCL-185) cell lines were maintained in DMEM: F12 (1:1) cell culture medium was supplied with FBS (10%) and antibiotic (100 U/mL penicillin and 100 mg/mL streptomycin). Cells were incubated at 37 °C, 5% CO_2, and in an environment with 90% humidity.

Cells were assessed by seeding $1x10^4$ cells/100µL well in a 96-well plate. The cells were allowed to be attached overnight, then the medium was removed, and a new fresh medium containing different concentrations was added. Cells were exposed to the samples for 24 hours. After that, 20 µL/ well of MTT (0.5 mg/mL) was added and incubated for additional 3 hours. Finally, the solutions were removed carefully, and 100 µL of DMSO was added to each well. The absorbance (OD) of the dissolved formazan crystals was measured by a Thermofisher microplate reader (Massachusetts, USA) at 590 nm. The viability and death rate were calculated compared to the solvent group that was exposed to 1% DMSO. The unexposed cells and cells that were exposed to 1% triton-x 100 were accepted as negative (growth) and positive controls, respectively. The assay was applied in triplicates and repeated on three separate days (n=3x3). The cell death ratios (%) at concentrations were expressed as the mean± standard deviation (SD), and the results were expressed as the half maximal inhibitory concentration (IC_{50}) which is the concentration that caused the death in one-half of the cells [32].

3. Results and Discussion

3.1. Physical Properties

The EOs extracted from the fruit of the *V. agnus-castus* have the highest yield in both Balıkesir and Istanbul regions (1.37±0.04 mL/100 g and 1.09±0.06 mL/100 g). Additionally, the EOs extracted from the flower have the lowest yield (0.38±0.07 mL/100 g, 0.44±0.19 mL/100 g, and 0.50±0.00 mL/100 g) of all. Table 1 presents the *V. agnus-castus* plant's collection regions, relative densities, and yields of essential oils on average of three hydrodistillation.

Table 1. Collection regions, relative densities, and yields of the *Vitex agnus-castus* EOs

Part of the plant	Region	Name of the sample	Relative density (g/L)	% Yield (mL/100 g)
Flower	Balıkesir (Burhaniye)	BWFL (white flowered plant)	0.84±0.08	0.38±0.07
	Balıkesir (Burhaniye)	BPFL (purple flowered plant)	0.90±0.09	0.44±0.19
	İstanbul (Zeytinburnu)	IPFL (purple flowered plant)	0.86±0.05	0.50±0.00
Leaf	Balıkesir (Burhaniye)	BPLE (purple flowered plant)	0.87±0.12	0.59±0.02
	İstanbul (Zeytinburnu)	IPLE (purple flowered plant)	0.86±0.04	0.63±0.12
Fruit	Balıkesir (Burhaniye)	BPFR (purple flowered plant)	0.85±0.02	1.37±0.04
	İstanbul (Zeytinburnu)	IPFR (purple flowered plant)	0.86±0.05	1.09±0.06

3.2. Chemical Composition of EOs

A total of 54 compounds in the *V. agnus-castus* flower, leaf, and fruit EOs were quantified. The characterized components indicate 96.8-100% of the essential oils. Constituents in terms of the retention indices order are demonstrated in Table 2. The main components of the white-flowered *V. agnus-castus* essential oil obtained from the Balıkesir region (BWFL)'s major four components were analyzed as caryophyllene (17.5±0.1%), 1,8-cineole (9.3±0.1%), *α*-pinene (7.4±0.1%), and bicyclogermacrene (6.3±0.1%). Also, while the main components of the purple-flowered *V. agnus-castus* essential oil obtained from the Balıkesir region (BPFL)'s major compounds were detected as *α*-pinene (15.7±0.1%), caryophyllene (12.3±0.1%), 1,8-cineole (9.3±0.1%) and sabinene (8.1±0.1%), the main components of the İstanbul region (IPFL)'s major compounds were analyzed as sabinene (20.1±0.1%), caryophyllene (9.7±0.1%), 1,8-cineole (9.5±0.1%) and (Z)-*β*-farnesene (8.6±0.1%) respectively. In the leaf essential oil obtained from Balıkesir region (BPLE): 1,8-cineole (17.6±0.1%), *α*-pinene (13.1±0.1%), sabinene (11.4±0.1%) and *α*-terpinyl acetate (7.5±0.1%) were detected as major components. The other leaf essential oil (IPLE) contains 1,8-cineole (26.6±0.1%), sabinene (23.5±0.1%), *α*-terpinyl acetate (8.3±0.1%), and bicyclogermacrene (6.3±0.1%) as the major compounds. The fruit essential oil obtained from the Balıkesir region (BPFR)'s main components were detected as *α*-pinene (21.8±0.1%), sabinene (16.6±0.1%), 1,8-cineole (16.0±0.1%) and limonene (7.3±0.1) respectively when the main components of the İstanbul region (IPFR)'s, major components were detected as sabinene (37.3±0.1%), 1,8-cineole (17.3±0.1%), bicyclogermacrene (6.0±0.1%) and *α*-terpinyl acetate (5.8±0.1%).

Monoterpene hydrocarbons and oxygenated monoterpene compounds were observed as the predominant component class of the *V. agnus-castus* EOs. The sesquiterpene hydrocarbons, oxygenated sesquiterpenes, diterpene hydrocarbons, oxygenated diterpenes, and classes of fatty acids were followed by them, respectively. Essential oil of BPFR contains the highest amount of monoterpene hydrocarbons

(60.0%), while IPLE has the highest number of oxygenated compounds (41.6%). Compared to BPFL essential oil, BWFL essential oil contains less amount of monoterpene hydrocarbons (25.3%) and is richer in sesquiterpene hydrocarbons (32.1%). Sesquiterpene hydrocarbons constituted less than 20% of the compounds in both the leaf and fruit essential oils, while they constituted more than 20% of the compounds in the essential oils extracted from the flowers. BWFL is the richest essential oil in terms of sesquiterpene hydrocarbons. On the contrary, oxygenated monoterpenes constituted less than 20% of the compounds in the flower essential oils, while they constituted more than 20% in both the leaf and fruit essential oils. According to these results, it is possible to easily differentiate between the EOs of *V. agnus-castus* flowers and the EOs extracted from the leaves and fruits. Both the leaf EOs (BPLE, IPLE) contain 1,8-cineole as the most abundant component, distinguishing them from the fruit and flower essential oils.

Table 2. Composition of different parts of the *Vitex agnus-castus* EOs

No	Components	KI[a]	RRI[b]	Relative Percentage Amounts						
				BWFL[g]	BPFL[h]	IPFL[i]	BPLE[j]	IPLE[k]	BPFR[l]	IPFR[m]
1	α-Pinene	1018-1032[c]	1026	7.4±0.1	15.7±0.1	7.4±0.1	13.1±0.1	4.6±0.1	21.8±0.1	0.9±0.1
2	α-Thujene	1020-1035[c]	1028	-	-	-	-	-	-	0.8±0.1
3	β-Pinene	1102-1118[c]	1113	0.5±0.1	0.8±0.1	0.7±0.1	1.1±0.1	1.2±0.1	1.2±0.1	1.1±0.1
4	Sabinene	1115-1132[c]	1125	4.1±0.1	8.1±0.1	20.1±0.1	11.4±0.1	23.5±0.1	16.6±0.1	37.3±0.1
5	Myrcene	1155-1169[c]	1160	1.9±0.1	3.0±0.1	2.4±0.1	2.7±0.1	2.2±0.1	3.5±0.1	1.9±0.1
6	α-Phellandrene	1160-1176[c]	1168	0.4±0.1	0.9±0.1	0.6±0.1	0.6±0.1	0.3±0.1	1.3±0.1	-
7	α-Terpinene	1170-1188[c]	1181	-	0.5±0.1	0.8±0.1	0.9±0.1	1.1±0.1	0.6±0.1	0.8±0.1
8	Limonene	1190-1204[c]	1203	5.3±0.1	7.3±0.1	2.6±0.1	6.0±0.1	1.6±0.1	7.3±0.1	0.9±0.1
9	1,8-Cineole	1203-1220[c]	1213	9.3±0.1	9.3±0.1	9.5±0.1	17.6±0.1	26.6±0.1	16.0±0.1	17.3±0.1
10	β -Phellandrene	1202-1218[c]	1217	3.5±0.1	4.6±0.1	3.2±0.1	4.4±0.1	-	5.1±0.1	-
11	γ-Terpinene	1238-1255[c]	1247	0.5±0.1	0.9±0.1	1.5±0.1	1.6±0.1	2.1±0.1	1.0±0.1	1.4±0.1
12	β-Ocimene, (E)-	1244-1257[c]	1254	0.6±0.1	0.4±0.1	-	0.7±0.1	-	0.4±0.1	-
13	*p*-Cymene	1264-1280[c]	1275	0.6±0.1	0.5±0.1	-	0.3±0.1	-	0.3±0.1	-
14	Terpinolene	1277-1290[c]	1284	-	0.3±0.1	0.4±0.1	0.4±0.1	0.5±0.1	0.3±0.1	0.4±0.1
15	3-methyl-1-pentanol	1313-1341[d]	1336	-	-	-	-	-	0.1±0.1	-
16	trans-Sabinene Hydrate	1474[e]	1464	-	-	-	-	0.3±0.1	-	0.9±0.1
17	α-Gurjunene	1523-1538[c]	1524	0.6±0.1	0.3±0.1	0.5±0.1	0.3±0.1	0.4±0.1	0.4±0.1	0.3±0.1
18	Linalool	1537-1553[c]	1545	1.0±0.1	0.8±0.1	0.2±0.1	0.5±0.1	0.3±0.1	0.4±0.1	-
19	cis-Sabinene Hydrate	1556[e]	1549	-	-	-	-	-	-	0.5±0.1
20	Terpine-1-ol	1562-1589[c]	1568	-	-	0.2±0.1	0.2±0.1	0.2±0.1	0.1±0.1	0.2±0.1
21	trans- α-Bergamotene	1568-1583[c]	1572	0.5±0.1	0.4±0.1	0.2±0.1	-	-	-	-
22	Caryophyllene	1585-1612[c]	1591	17.5±0.1	12.3±0.1	9.7±0.1	4.7±0.1	4.5±0.1	4.5±0.1	3.5±0.1
23	Terpinen-4-ol	1592-1611[c]	1602	3.3±0.1	3.4±0.1	4.8±0.1	4.9±0.1	5.7±0.1	3.0±0.1	3.7±0.1
24	Alloaromadendrene	1638-1661[c]	1639	1.4±0.1	1.8±0.1	1.8±0.1	1.2±0.1	1.0±0.1	0.9±0.1	0.6±0.1
25	Citronellyl acetate	1650-1666[c]	1663	0.6±0.1	0.6±0.1	0.4±0.1	0.3±0.1	-	0.2±0.1	0.4±0.1
26	(Z)-β-Farnesene	1638-1667[c]	1667	4.8±0.1	2.6±0.1	8.6±0.1	0.7±0.1	3.7±0.1	1.4±0.1	5.3±0.1
27	α-Terpineol	1682-1706[c]	1684	-	-	-	0.7±0.1	-	0.4±0.1	1.2±0.1
28	α-Terpinyl Acetate	1685-1709[c]	1697	5.8±0.1	4.3±0.1	4.2±0.1	7.5±0.1	8.3±0.1	4.1±0.1	5.8±0.1
29	Germacrene-D	1699-1726[c]	1703	0.2±0.1	-	2.6±0.1	-	0.7±0.1	0.1±0.1	1.8±0.1
30	β-Bisabolene	1718-1741[c]	1741	0.3±0.1	0.4±0.1	-	-	-	-	-
31	Bicyclogermacrene	1723-1751[c]	1747	6.3±0.1	3.9±0.1	7.9±0.1	4.8±0.1	6.3±0.1	4.9±0.1	6.0±0.1
32	Geranyl acetate	1743-1764[c]	1757	0.3±0.1	0.4±0.1	-	-	-	-	-
33	δ-Cadinene	1746-1772[c]	1771	0.3±0.1	-	0.4±0.1	-	-	-	-
34	Citronellol	1756-1774[c]	1767	0.3±0.1	0.2±0.1	-	0.3±0.1	-	0.1±0.1	0.2±0.1

Table 2 Continued…

35	*β*-Sesquiphellandrene	1766-1783[c]	1784	-	0.2±0.1	-	-	-	-	-
36	Geraniol	1830-1857[c]	1853	-	-	-	0.2±0.1	-	-	-
37	Palustrol	1931-1938[c]	1937	0.2±0.1	-	-	0.2±0.1	-	-	-
38	Caryophyllene oxide	1970-2008[c]	1988	0.7±0.1	0.6±0.1	-	0.5±0.1	-	0.2±0.1	-
39	Ledol	2025-2057[c]	2042	0.8±0.1	0.7±0.1	0.6±0.1	1.0±0.1	0.6±0.1	0.3±0.1	0.6±0.1
40	Germacrene-D-4-ol	2000-2070[c]	2044	1.6±0.1	1.5±0.1	0.6±0.1	0.8±0.1	0.4±0.1	-	0.2±0.1
41	Globulol	2070-2098[c]	2088	0.5±0.1	0.2±0.1	0.2±0.1	0.3±0.1	0.2±0.1	-	-
42	Viridiflorol	2083-2104[c]	2097	0.4±0.1	-	-	0.1±0.1	-	-	-
43	Spathulenol	2117-2144[c]	2119	1.0±0.1	0.5±0.1	0.5±0.1	1.3±0.1	0.4±0.1	0.6±0.1	0.8±0.1
44	*β*-Bisabolol	2090-2189[c]	2154	0.2±0.1	-	0.2±0.1	0.3±0.1	-	-	-
45	*τ*-Cadinol	2134-2191[f]	2165	2.0±0.1	-	2.0±0.1	-	0.7±0.1	0.1±0.1	0.3±0.1
46	*α*-Bisabolol	2204-2232[c]	2231	0.5±0.1	1.0±0.1	-	0.4±0.1	-	-	-
47	trans-*α*-bergamotol	2241-2247[f]	2244	0.2±0.1	-	-	0.2±0.1	-	-	-
48	*α*-Cadinol	2218-2255[c]	2251	0.9±0.1	-	-	0.4±0.1	-	0.1±0.1	0.3±0.1
49	*β*-Eudesmol	2222-2256[c]	2256	0.6±0.1	0.9±0.1	0.4±0.1	0.3±0.1	-	0.1±0.1	0.4±0.1
50	Caryophylladienol I	2316-2320[f]	2316	0.2±0.1	-	-	-	-	-	-
51	Manoyl oxide	2335-2376[c]	2354	0.2±0.1	-	-	-	-	-	0.3±0.1
52	Abietatriene	2476-2530[c]	2524	3.9±0.1	3.6±0.1	1.4±0.1	2.3±0.1	0.9±0.1	0.3±0.1	1.5±0.1
53	Phytol	2510-2633[c]	2536	0.8±0.1	0.7±0.1	0.2±0.1	0.4±0.1	-	-	-
54	Manool	2370-2628[c]	2542	0.9±0.1	1.0±0.1	0.1±0.1	0.6±0.1	0.2±0.1	-	0.2±0.1
55	Tetradecanoic acid	2670-2713[c]	2700	1.6±0.1	1.4±0.1	0.4±0.1	1.1±0.1	0.3±0.1	-	0.5±0.1
56	Pentadecanoic acid	2720-2840[c]	2786	0.3±0.1	-	-	-	-	-	-

Total Identified	96.8	97.9	98.9	99.0	100.0	99.4	99.6
Monoterpene hydrocarbons (1-8, 10-14)	25.3	43.5	40.1	43.8	37.4	60.0	45.9
Oxygenated monoterpene (9, 16, 18-20, 23, 25, 27, 28, 32, 34, 36)	20.9	19.4	19.4	32.5	41.6	24.7	30.5
Sesquiterpene hydrocarbons (17, 21, 22, 24, 26, 29-31, 33, 35)	32.1	22.2	32.1	11.9	16.8	12.4	17.7
Oxygenated sesquiterpenes (37-50)	10.5	5.8	4.9	6.2	2.5	1.6	2.7
Diterpene hydrocarbons (52)	3.9	3.6	1.4	2.3	0.9	0.3	1.5
Oxygenated diterpenes (51, 53, 54)	2.0	1.8	0.4	1.1	0.2	-	0.5
Fatty acids (55,56)	1.9	1.4	0.4	1.1	0.3	-	0.5
Others (15)	-	-	-	-	-	0.1	-

Abbreviations

[b]RRI: Relative retention indices calculated against n-alkanes; % calculated from the FID data.

[g]BWFL: white flowers collected from Balıkesir.

[h]BPFL: purple flowers collected from Balıkesir.

[i]IPFL: purple flowers collected from İstanbul.

[j]BPLE: purple-flowered plant leaves collected from Balıkesir.

[k]IPLE: purple-flowered plant leaves collected from İstanbul.

[l]BPFR: purple-flowered plant fruits, collected from Balıkesir.

[m]IPFR: purple-flowered plant fruits collected from İstanbul.

[a]KI were given from literature mostly with confidence intervals 50% of RI data ranges for each compound [16[e], 33[c], 34[d], 35[f]].

3.3. Cytotoxicity Analysis in Cancerous Cell Lines

MTT assay was used to assess the cytotoxic potential of *V. agnus-castus* EOs. The IC_{50} values of essential oil extracted from the white flower, purple flower, leaf, and fruit from Balıkesir region were calculated to be 39.39, 48.62, 49.77, and 55.85 µg/mL respectively, in A549 (Figure 1A) and 45.93, 4.68, 39.2, and 32.43 µg/mL respectively in MCF-7 cells (Figure 1B). The essential oil obtained from İstanbul region of the purple flower, leaf, and fruit were analyzed, and IC_{50} values were calculated to be 63.84, 75.16 and 55.37 µg/mL, respectively, in A549 (Figure 1C) and 57.77, 65.08, and 58.11 µg/mL, respectively in MCF-7 cells (Figure 1D). Cytotoxic activities of the *V. agnus-castus* EOs which were collected from İstanbul and Balıkesir regions and extracted from different parts of the plant were represented in Table 3 and Figure 1. The cell death in negative control was negligible (≤1%), and in the positive control, it was 97%.

Table 3. Cytotoxicity (IC_{50}) µg/mL values of the *V. agnus-castus* EOs

Part of the plant	Name of the sample	Region	Cell lines	
			MCF-7	A549
Flower	BWFL	Balıkesir (Burhaniye)	45.93	39.39
	BPFL	Balıkesir (Burhaniye)	4.68	48.62
	IPFL	İstanbul (Zeytinburnu)	57.77	63.84
Leaf	BPLE	Balıkesir (Burhaniye)	39.2	49.77
	IPLE	İstanbul (Zeytinburnu)	65.08	75.16
Fruit	BPFR	Balıkesir (Burhaniye)	32.43	55.85
	IPFR	İstanbul (Zeytinburnu)	58.11	55.37

The previous studies that showed the cytotoxic effect of *V. agnus-castus* mostly focused on the extract of the plant. Also, while there are a few studies that analyzed the cytotoxic effect of *V. agnus-castus* EO, they primarily focused on the leaf or the aerial parts of the plant. EOs extracted from the flowers, fruits, and leaves of the *V. agnus-castus* collected from İstanbul and Balıkesir were analyzed in the current study. Our results determined that MCF-7 cells are more sensitive than A549 cells to EOs. Essential oils obtained from Balıkesir region appeared more potent in the MCF-7 and A549 cells. The *in-vitro* cytotoxic effects of *V. agnus-castus* leaf and aerial parts EOs' were analyzed in previous studies (Table 4).

Different cell lines such as MCF-7, A549, pro-myelocytic leukemia (HL-60), rat glial cell line (C6), human lung cancer cell line (H6AR), human carcinoma cell line (NCI-H292), human embryonic kidney cell line (HEK-293), human cervical adenocarcinoma cell line (HEP-2), Hela, and colon adenocarcinoma (LS180) were used to analyze *V. agnus-castus* essential oil's cytotoxicity. The IC_{50} values of the essential oils are arranged between 3.8 to 879.0 µg/mL with differences according to the used parts and the cell type (Table 4). In a study by Zhelev et al., the cytotoxicity of *V. agnus-castus* fruit EOs was analyzed in Hela, A549, and LS180 cancer cell lines, compared to Amniotic cell normal cells. Their results indicated cytotoxicity in all tested cell lines with differences in sensitivity, as HeLa cells appear to be the more sensitive ones. They calculated the IC_{50} to be 0.118 – 0.252 % (v/v). In the A549 cell line, the IC_{50} of EO extracted from the aerial parts of fruity *V. agnus-castus* was 90.0 µg/mL, while in the same study, the one extracted from fruitless plant-induced cytotoxicity with IC_{50} was about 157 µg/mL. Similarly, in the MCF-7 cell line, the IC_{50} of EO extracted from fruity *V. agnus-castus* was

70.0 µg/mL, and fruitless plant EO was calculated to be 111.7 µg/mL. Ricarte et al. reported the IC_{50} of EO extracted from fresh leaves and evaluated in MCF-7 was 32.4 µg/mL [21, 36-38].

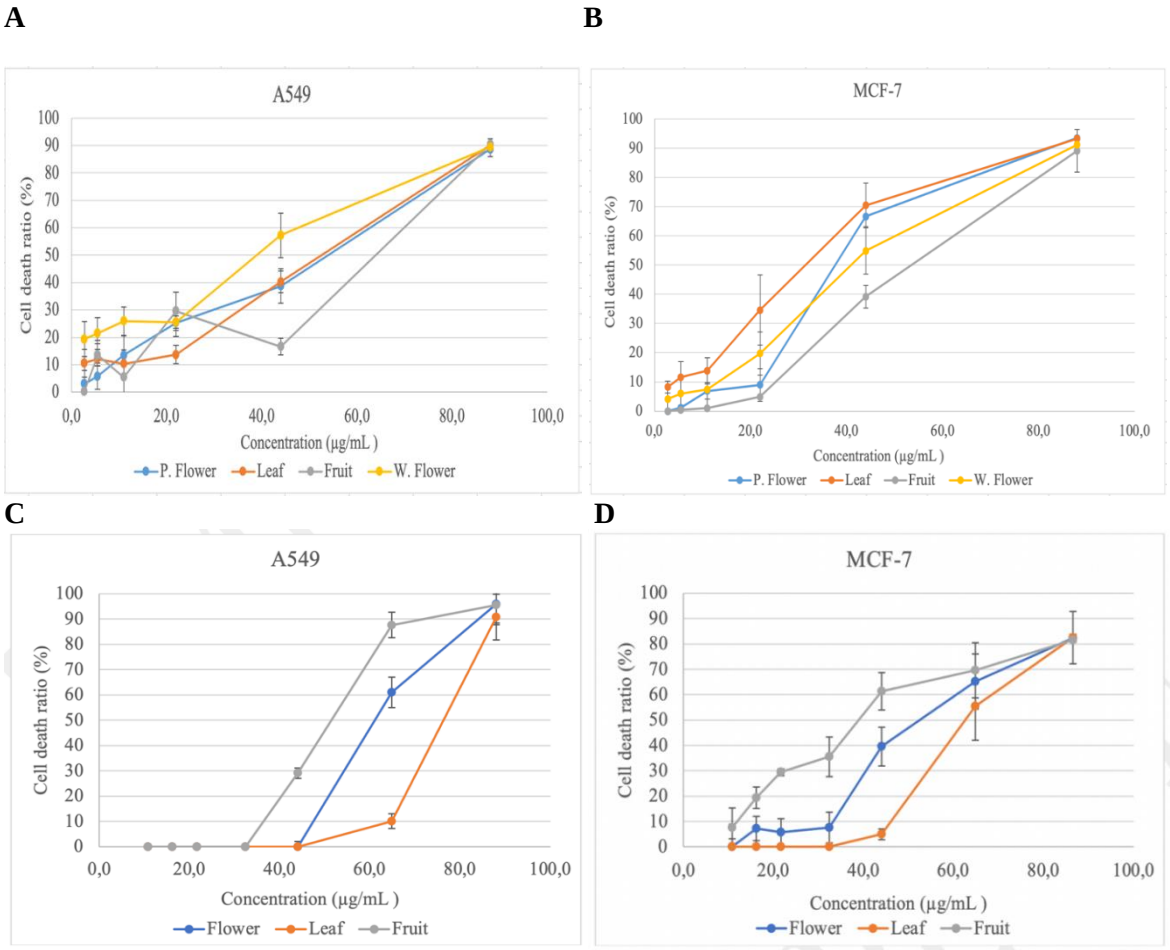

Figure 1. The cytotoxic effect of *V. agnus-castus* essential oils. (**A**) from Balıkesir region in A549 cells, (**B**) from Balıkesir region in MCF-7 cells, (**C**) from Istanbul region in A549 cells, and (**D**) from Istanbul region in MCF-7 cells.

MCF-7 and A549 cells' cytotoxicity was higher when the essential oils were extracted separately from the plant's leaf, fruit, and flower parts instead of the total aerial parts according to Table 3 and Table 4. To the best of our knowledge, this study is the first to examine *V. agnus-castus* fruit EOs' cytotoxic effect on the MCF-7 cell line. In the MCF-7 cell line, the IC_{50} of fruit EO extracted from Balıkesir region was calculated as 32.43 µg/mL while the fruit EO extracted from İstanbul region was calculated as 58.11 µg/mL. As far as we know, this study is also the first to analyze the cytotoxicity of the white and purple flowers of the *V. agnus-castus*. In MCF-7 cells, the EO of purple flower collected from Balıkesir (BPFL) was observed as the most potent among the EOs of both Balıkesir and İstanbul regions with IC_{50} about 4.68 µg/mL which is 10-times more potent than the EO of a white flower (BWFL). However, the EO of BWFL was detected as more potent in A549 cells with no big difference from the other EOs from Balıkesir region's potency (IC_{50} 39.39 to 48.62 µg/mL). The EOs extracted from İstanbul region (IPFL, IPLE, IPFR) were detected to be markedly less potent than BWFL in A549 cells. The results showed that *V. agnus-castus* had high cytotoxicity potential and highlighted the importance of the collecting region [21, 36-38].

Table 4. Previous *in-vitro* cytotoxicity studies of *Vitex agnus-castus* EO

Part of the Plant	Cell line	IC_{50} concentration	Effects	Reference
Leaves	MCF7 NCI-H292 HL-60 HEP-2	32.4 µg/mL 46.5 µg/mL 3.8 µg/mL 41.7 µg/mL	Cytotoxicity in all of the cells.	[21]
Aerial parts of fruitless plant	C6 A549 MCF-7	280.0 µg/mL 156.7 µg/mL 111.7 µg/mL	Cytotoxicity in all of the cells with induction of apoptosis in A549 and MCF7 cells.	[36]
Aerial parts of fruity plant	C6 A549 MCF-7	169.7 µg/mL 90.0 µg/mL 70.0 µg/mL	Cytotoxicity in all of the cells with induction of apoptosis in A549 and MCF7 cells.	[36]
Leaves	H6AR	42.03 µg/mL	Induced apoptotic cell death by activating caspase pathways.	[37]
	HEK-293	879 µg/mL	No cytotoxicity.	
Fruits	HeLa A549 LS180 Aminotic cell (Normal)	0.118% 0.212% 0.252% 0.213%	Cytotoxicity in the tested cells. HeLa is sensitive.	[38]

In the current study, our results are confirmed by the literature which elucidates the EO composition of *V. agnus-castus*. For instance, sabinene, 1,8-cineole, caryophyllene, bicyclogermacrene, (Z)-*β* -farnesene, and α-pinene were analyzed as the major components of the *V. agnus castus* fruits EOs extracted from different regions in Türkiye by Gulsoy Toplan et al. [16]. 1,8-cineole (25.0%) and sabinene (13.3%) were detected as the major compounds of leaf EO extracted from *V. agnus-castus* by Karakoti et al. [39]. Also, 1,8-cineole (24.38%), sabinene (22.77%), *α*-pinene (7.14%), trans-*β*-farnesene (8.50%), *β*-caryophyllene (6.49%) and terpinene-4-ol (5.23%) were detected as major compounds of the leaf EOs extracted from *V. agnus-castus* by Ulukanli et al. [22]. In studies of the *V. agnus-castus* leaf EO, 1,8-cineole was observed as the most abundant compound which distinguish itself from the fruit and flower EOs just as in our results. 1,8-cineole (21.6%), caryophyllene (17.1%), sabinene (14.7%), terpinene-4-ol (8.7%) and (E)-*β*-farnesene (5.7%) were analyzed as the main components of the *V. agnus-castus* flowers EO by Senatore et al. [18]. Although *α*-pinene and *α*-terpinyl acetate were detected in the EOs from Balıkesir region (BWFL, BPFL, BPLE, and BPFR) and İstanbul region (IPFL, IPLE, and IPFR)~~;~~, monoterpene hydrocarbons α-pinene with oxygenated monoterpene 1,8-cineole were detected majorly in BWFL, BPFL, BPLE and BPFR. Therefore, they were defined as the "*α*-pinene-1,8-cineole" chemotype. IPFL, IPLE and IPFR essential oils were defined as the "sabinene-1,8-cineole" chemotypes due to their main components. The current study did not support

Novak et al.'s supposition that the presence of two distinct chemotypes -α-pinene and *α*-terpinyl acetate- in the *V. agnus-castus* leaf and fruit essential oils [19]. But the recent studies confirm our results that the *V. agnus-castus* fruit and leaf essential oil chemotypes of the plant were made up of binary compounds-"sabinene-1,8-cineole", "*α*-pinene-1,8-cineole" and "(Z)-*β*-farnesene-bicyclogermacrene"- [21-23].

In the case of eliciting the EO component's cytotoxic activities~~,~~, the monoterpene hydrocarbon *α*-pinene was observed to have an inhibitory effect on tumor invasion in highly metastatic MDA-MB-231 human breast cancer cells. Anticancer activity of natural killer cells was induced using ERK/AKT pathway and cell death of the A549 (human lung carcinoma) and HepG2 (hepatoblastoma) cell lines was triggered by *α*-pinene [28, 40, 41]. In the current study, the amount of the *α*-pinene in the EOs obtained from İstanbul region was calculated as considerably lower than the EOs obtained from Balıkesir region. Especially, α-pinene was detected in the EO of IPFR in the amount of 0.9±0.1%. As a result of the MTT assay, Balıkesir region EOs were detected as more potent in both MCF-7 and A549 cells which might be due to the presence of higher amount of *α*-pinene than İstanbul region. Also, in the EO of BWFL, *α*-pinene was detected about twice less than the EO of BPFL. Parallel to this, in the results of the MCF-7 cell line, EO of BPFL was observed as more potent than the EO of BWFL. On the other hand, in the A549 cell line, the EO of BWFL was observed as more potent than the EO of BPFL with no big difference. The sesquiterpene hydrocarbon caryophyllene's cytotoxic activities on the NSCLC, A549, MCF-7, DLD-1, L-929 and NCI-H1299 cell lines have been proved. Studies have also drawn attention to caryophyllene's enhancing activities on anti-tumor agents such as α-humulene, isocaryophyllene, paclitaxel, and cisplatin [29, 42, 43]. In our study, the amount of the caryophyllene compound in the flower EOs were detected as higher than in leaf and fruit EOs. Likewise, MTT results showed that in the MCF7 cell line, the EOs of the flowers collected from Balıkesir and İstanbul regions appeared as the most potent among all the EOs. Especially, the caryophyllene (12.3±0.1%) and α-pinene (15.7±0.1%) compounds were detected in the BPFL essential oil which is almost 10-times more potent than the other EOs. Therefore, higher amount of the α-pinene and caryophyllene compounds of the BPFL essential oil may have caused this result. The monoterpene hydrocarbon limonene's anti-cancer activities on the MCF-7, A549, and H1299 were discovered by researchers, too [44-46]. In the current study~~,~~, the limonene content of the white flower (5.3±0.1%), purple flower (7.3±0.1%), leaf (6.0±0.1%), and fruit (7.3±0.1%) EOs obtained from Balıkesir region were detected in higher amounts than the EOs which obtained from Istanbul region (IPFL, IPLE, and IPFR). In view of our MTT results, in which the essential oils obtained from Balıkesir region were higher than Istanbul region might have been due to the contribution of the limonene compound.

4. Conclusion

In-vitro cytotoxicity of the *V. agnus-castus* purple and white flowers was analyzed for the first time in our research. Our results proved that the *V. agnus-castus* leaf, flower, and fruit EOs can be differentiated by their range of total monoterpene hydrocarbon, oxygenated monoterpene, sesquiterpene percentages, and their major compounds. EOs obtained from Balıkesir region were defined as the "*α*-pinene-1,8-cineole" chemotype, while EOs obtained from İstanbul region were defined as the "sabinene-1,8-cineole" chemotype. Balıkesir region's EOs were observed as more potent -especially the purple flower's $IC_{50,}$ about 4.68 µg/mL on the MCF7 cell line- than İstanbul region's, which might be attributed to the higher amount of α-pinene, caryophyllene, and limonene content. We want to investigate whether the difference in the chemical composition and activity of white and purple flowers of *V. agnus-castus'* also exists in leaves and fruits in the future. The usage potential of the different parts of *V. agnus-castus* essential oil and most dominant components in cancer treatment will be investigated by using both *in-vitro* and in silico methods.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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