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Pinus sylvestris var. *hamata* Uçucu Yağlarının Fitokimyasal Bileşimi, Antioksidan ve Antimikrobiyal Aktiviteleri

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ÖZ

Çamgiller familyasına ait olan *Pinus sylvestris* L., değerli orman ağaçlarından biridir. Çam yağları; içecek ve yiyeceklerde tatlandırıcı olarak ve kozmetikte koku olarak kullanılmaktadır. *Pinus sylvestris* var. *hamata* Türkiye'nin Orta Karadeniz Bölgesi'nden toplanmış ve iğneleri gölgede kurutulmuştur. Uçucu yağlar (EO'lar) hidrodistilasyonla üretilmiştir ve EO bileşiklerini tanımlamak için gaz kromatografisi/kütle spektrofotometrisi (GC-MS/MS) kullanılmıştır. β-pinene (%18,70), α-pinene (%15,62), Germacrene (%12,53), Karyofilen (%11,35) ve Limonen (%3,62) ana bileşenlerdir. EO'ların antioksidan analizi için DPPH$^{\bullet}$, ABTS$^{\bullet+}$ süpürme ve FRAP testleri yapılmıştır. Ayrıca antibakteriyel etki; *Bacillus cereus*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* ve *Staphylococcus aureus* ve kullanılarak disk difüzyon testi ile araştırılmıştır. EO'lar, *Salmonella typhimurium* dışında test edilen bakterilere karşı iyi antibakteriyel aktiviteler sergilemiştir. Ek olarak, EO'lar, test edilen deneylerde önemli aktiviteler ortaya çıkarmıştır. Sonuç olarak, *P. sylvestris* var. *hamata* esansiyel yağının ilaç ve gıda endüstrisinde kullanılması mümkündür.

Phytochemical Composition, Antioxidant and Antimicrobial Activities of Essential Oils of *Pinus sylvestris* var. *hamata*

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ABSTRACT

Pinus sylvestris L. belongs to the Pinaceae family and is one of the valuable forest trees. Pine oils are used as a flavoring in beverages and food and as a fragrance in cosmetics. *Pinus sylvestris* var *hamata* was collected from Turkey's Central Black Sea Region, and its needles were dried in the shade. Essential oils (EOs) were generated by hydro-distillation, and Gas chromatography/mass spectrophotometry (GC-MS/MS) was used to identifiy the EOs compounds. pinene (18.70%), α-pinene (15.62%), Germacrene (12.53%), Caryophyllene (11.35%), and Limonene (3.62%) were major constituents. DPPH$^{\bullet}$ ABTS$^{\bullet+}$ scavenging and FRAP tests were performed for antioxidant analysis of EOs. Moreover, antibacterial effect was investigated by disk diffusion assay using *Bacillus cereus*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*. EOs exhibited good antibacterial activities against the tested

bacteria, except for *Salmonella typhimurium*. In addition, EOs revealed the significant activity on tested assays. As results, *P. sylvestris* var. *hamata* essential oil has possible to be utilized in the pharmaceutical and food industries.

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1. Introduction

Plants have been employed for food and medicinal roles for years since they contain secondary metabolites with critical biological activities (Ertas et al., 2016; Heyem et al., 2022). Essential oils (EOs) are compounds obtained from plants' leaves, flowers, seeds, and roots, often fragrant, also called essential or ethereal oils. It is possible to obtain EOs by distillation, most commonly by boiling water from vegetable sources or passing water vapor through the material (Packiyasothy and Kyle, 2002; Greathead, 2003; Al-Breiki et al., 2018). A large number of compounds in the composition of essential oils are identified by separating them from each other by an effective analytical technique called Gas Chromatography/Mass Spectrometry (GC/MS) (Krone et al., 2010).

EOs are used as herbal tea and are preferred in preparing sauces and ready meals. They are also suitable in the cosmetic industry, weed control, and organic agriculture due to their odor properties. EOs obtained from plants are classified as safe additives regarding their chemical structure. It has been determined that there is no harm in terms of health when consumed by humans and animals (Inouye et al., 2009; Soković et al., 2009). It has significant benefits in medicine as a treatment for colds, appetite, indigestion, diarrhea, toothache, inflammation, and gout. In recent years EOs have been used instead of synthetic antioxidants (such as butyl hydroxy and hydroxy anisole) that are harmful to health in storing foods and extending their shelf life. Some EOs exhibit high biological activities such as antimicrobial and antioxidant (Chao et al., 2000; Botsoglou et al., 2002; Burt, 2004). It was reported that thymol in thyme essential oil has antioxidant activity by reducing the formation of hydroxy peroxide (Youdim and Deans, 2000; Keawsa-ard and Kongtaweelert, 2012). The main components of Eos, such as carvacrol, cinnamaldehyde, ionone, and eugenol, obtained from thyme, rosemary, and cinnamon, and plants have antimicrobial, antiviral, antifungal, and antiparasitic properties (Wei A and Shibamoto, 2004 Hong et al., 2012). In another study, it was determined that clove oil inhibited *Pseudomonas aeruginose*, *Klepsiella pneumoniae*, *Clostridium albicans*, *Escherichia coli*, and *Staphylococcus aureus* microorganisms by 99.9% (Tekce and Gul, 2016).

Pinus sylvestris L., an important forest tree species, belongs to the Pinaceae family in the conifer class of Gymnospermae. *Pinus sylvestris*, *Pinus brutia*, *Pinus pinea*, *Pinus nigra*, and *Pinus halepensis* are pine species registered in Turkey, and only *P. nigra*, *P. brutia*, and *P. sylvestris* are employed commercially (Sezik et al., 2010). Apart from perfumery and cosmetics, essential pine oils exhibit pharmacological activities such as expectorant, diaphoretic, diuretic, antimicrobial, and antispasmodic.

P. sylvestris is a very complex species with many subspecies, variants, and forms, and there needs to be more literature regarding critical biological activity studies such as antimicrobial and antioxidant. Our study aimed to analyze the antimicrobial and antioxidant activities by obtaining EOs from *P. sylvestris* var. *hamata*.

2. Material and Methods

2.1. Plant material

P. sylvestris var. *hamata* was collected from the Central Black Sea Region, Tokat-Turkey (40º 19' 21" N, 36º 28' 03" E) in June 2022, and it was identified by Dr. Bedrettin Selvi from Tokat Gaziosmanpasa University (TOGU). A voucher specimen was deposited in the Herbarium of Tokat Gaziosmanpasa University (Herbarium No: 8249).

2.2. Isolation of essential oils

Air-dried aerial parts of the plant (300 g) was mixed with distilled water (900 mL) and the mixture was subject to hyro distillation for 5 hours using a Clevenger-type apparatus to produce oil in 0.24% (w/w) (Karan et al., 2018).

2.3. Gas chromatography/mass spectrophotometry

Chemical analyses of the EOs were performed using a divided-mode (50:1) Perkin Elmer Clarus 500 Series GC-MS/MS equipped with a flame ionization detector (FID). The essential (20 mg) oils were dissolved in acetone (1.2 mL). The BPX5 column was used. 2.0 L injection volume at a 250°C injection temperature were the parameters. Ionization energy was set to 70 eV, and the total program time was 60 minutes (Erenler et al., 2018).

2.4. Antimicrobial test

The EOs was assayed for antibacterial effect against *Salmonella typhimurium* (ATCC®14028), *Bacillus cereus* (ATCC®10876), *Staphylococcus aureus* (ATCC®25923), *Escherichia coli* (ATCC®25922), *Klebsiella pneumoniae* (ATCC®13883) and *Pseudomonas aeruginosa* (ATCC® 27853) by Mueller Hinton Agar (MHA) plate disc diffusion method. The microorganism concentrations were adjusted by 0.5 McFarland standard (0.5×10^5 cfu/mL). Chloramphenicol (50 µg) and methanol were used as the positive and negative control. 35 µl of EOs was impregnated on the discs, then the plates were allowed for incubation (37 °C, 16 h). After incubation, the inhibition zone diameters (mm) were measured (Usta et al., 2018).

2.5. DPPH free radical scavenging activity

The antioxidant activity of EOs of *P. sylvestris* var. *hamata* was examined using a 1.1-diphenyl-2-picryl-hydrazil ($DPPH^{\bullet}$) assay. DPPH solution (1.0 mL, 0.1 mmol/L in methanol) was added to the

samples at various concentrations. The absorbance measurement was performed at 517 nm with a spectrophotometer. The equation was used for free radical scavenging activity (Karan, 2018).

2.6. ABTS free-radical scavenging activity

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) cation radical ($ABTS^{\bullet+}$) is based on reducing the ABTS radical cation at 737 nm compared to the standard. (Karan and Cadırci, 2018a).

2.7. Reducing power activity

In 1 mL of deionized water, different quantities of samples (2.5-10 g/mL) were combined with sodium phosphate buffer (1.25 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1.25 mL, 1%). The reaction liquid was completely vortexed, and absorbance at 700 nm was measured using a spectrophotometer (Erenler et al., 2017).

2.8. Statistical analysis

GraphPad Prism (8.0.1) with ANOVA was used for the statistical analysis. The multiple-compassion analysis was executed by Tukey test. The results were stated as mean values ± SDs of three independent assays ($P < 0.05$) and considered significant.

3. Results and Discussion

3.1 Phytochemical composition of P. sylveris var. *hamata*

EOs compounds were defined by GC-MS/MS analysis. The mass spectra of the components were defined by comparing them with the standards in the Adams and NIST library database. 66 components were identified, representing 97.12% of the oil and β-Pinene (18.70%), α-Pinene (15.62%), Germacrene D (12.53%), Caryophyllene (11.35%) and Limonene (3.62%) were the chief products. In addition, our study revealed the presence and amount of many previously undetected components in *P. sylvestris* essential oils. The results determined that monoterpene hydrocarbons (43.98%) were dominated group, and sesquiterpenes hydrocarbons (35.52 %) described the second largest group (Table 1).

The EOs of P. sylvestris collected from different regions of Turkey have been reported by previous studies. There is a coherence between this study and the reported study. In the former study, α-pinene, camphene, β-pinene were the major compounds. The studies also detected the presence of essential components such as Camphene, β-myrcene, γ-terpinene, Limonene, and Bornyl acetate. (Ustun et al., 2006; Tumen et al., 2010). There are many studies on the EOs of *Pinus* sp., but the chemical content of EOs varies due to the geographical, seasonal, genotypic, and environmental conditions in which the plant lives (Barnola and Cedeño, 2000; Koukos et al., 2000).

Essential oils obtained from plants have been employed for treating diseases and as a preservative and flavoring in food products. Valuable essential oil source coniferous species such as *P. sylvestris* are

used in many industrial areas such as food and pharmaceuticals. Compounds α-pinene, β-pinene, caryophyllene, limonene and germacrane D shown in Figure 1 have high biological activities, including antioxidant, antimicrobial, and apoptotic effects (Doughari and Bazza, 2010; Silva et al., 2012; Zheljazkov et al., 2012; Dahham et al., 2015; Zheljazkov et al., 2018).

Table 1. EOs contents of *P. sylvestris* var. *hamata*

No	RT(min)	Compounds	Content (%)
1	10.31	α-thujene	0.32
2	10.69	α-pinene	15.62
3	11.19	Camphene	0.59
4	12.53	β-pinene	18.7
5	13	β-myrcene	1.73
6	13.57	α-phellandrene	0.05
7	13.83	3-carene	1.13
8	14.11	α-terpinene	0.08
9	14.47	p-cymene	0.08
10	14.69	Limonene	3.62
11	15.07	β-ocimene	0.05
12	15.55	α-ocimene	0.81
13	16.03	γ-terpinene	0.15
14	17.39	Isoterpinolene	1.01
15	17.91	Linalool	0.04
16	18.1	Nonanal	0.05
17	18.55	Fenchol	0.14
18	19.13	α-campholenal	0.15
19	19.72	Pinocarveol	0.35
20	19.98	Camphor	0.5
21	20.17	2-norbornanol	0.27
22	20.72	Pinocamphone	0.1
23	20.82	Pinocarvone	0.14
24	20.97	endo-borneol	0.25
25	21.01	α-phellandren-8-ol	0.17
26	21.5	4-terpineol	0.3
27	22.15	α-terpineol	1.69
28	22.38	Myrtenol	0.48
29	23.78	Citronellol	0.13
30	24.09	Methyl thymol ether	0.3
31	26.39	Bornyl acetate	1.79
32	26.99	trans-pinocarvyl acetate	0.59
33	28.62	Elemene isomer	0.04
34	29.13	Copaene	0.13
35	30.07	Ylangene	0.07
36	30.27	Copaene-isomer	0.18
37	30.66	β-bourbonene	0.5
38	30.95	β-elemene	0.23
39	31.46	Methyleugenol	0.25
40	32.23	Caryophyllene	11.35

41	32.51	β-copaene	0.79
41	33.1	Isogermacrene D	0.33
43	33.49	Humulene	2.56
44	34.45	γ-muurolene	0.94
45	34.66	Germacrene D	12.53
46	34.86	Isovaleric acid phenethyl ester	1.33
47	35.01	γ-amorphene	0.42
48	35.16	α-muurolene	0.26
49	35.46	γ-cadinene	0.2
50	35.72	β-cadinene	0.64
51	36.04	δ-cadinene	1.59
52	36.68	Bisabolene	2.4
53	38.09	Caryophyllene oxide	1.16
54	39.93	tau-muurolol	0.73
55	40.05	δ-cadinol	0.32
56	40.32	α-cadinol	1.52
57	40.82	6.7-dihydrofarnesol	0.54
58	41.94	Pentadecanal	0.6
59	42.19	trans-farnesol	0.3
60	45.42	Farnesol acetate	0.4
61	45.98	Platambin	0.27
62	49.37	Thunbergol	0.37
63	50.73	trans-geranylgeraniol	0.33
64	52.18	Sclareol	0.27
65	57.37	Neoabietinal	0.13
66	57.58	11-methyltricosane	0.21
	Monoterpene hydrocarbons (Sr. No. 1-15)	43.98	
	Oxygenated monoterpenes (Sr. No. 17-20, 22-29)		4.54
	Sesquiterpenes hydrocarbons (Sr. No. 33-38,40-45,47-52)		35.52
	Oxygenated sesquiterpenes (Sr. No. 53-59, 61)		5.44
	Oxygenated diterpenes (Sr. 62-65)		1.1
	Others (Sr. No. 16,21,30-32,39,46, and 3)		6.54
	Total identified		97.12

RT: Retention times (min), % calculated from FID data.

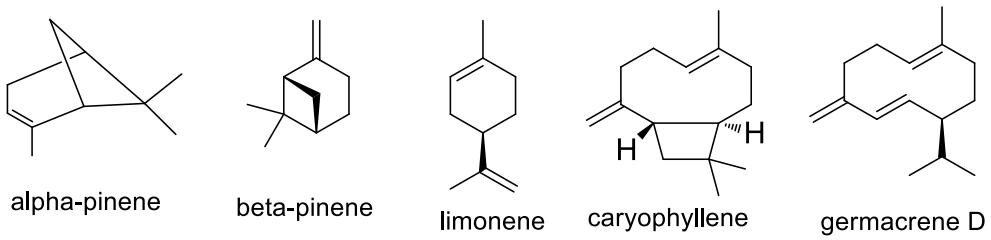

Figure 1. Major bioactive compounds of *P. sylvestris* EOs (Ayad and Akkal, 2019).

3.2 Antioxidant analyses of essential oils

The antioxidant activities of *P. sylvestris* var. *hamata* EOs were determined by $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging and FRAP assays. $DPPH^{\bullet}$, radical scavenging activity was determined to be more effective than the BHT standard with the value of 9.19 (IC50, μg/mL), but it was observed to have lower activity than BHA (4.35 μg/mL) and trolox (4.54 μg/mL). When ABTS cation radical scavenging activity was examined, *P. sylvestris* was found to be high (6.11 μg/mL) compared to all standards. In the FRAP assay, EOs displayed the excellent activity with a value of 6.61 ± 0.02 (μ mol TE/mg EOs) (Table 2).

Table 2. Antioxidant activities of *P. sylvestris* var *hamata*

Samples	*$DPPH^{\bullet}$ scavenging	*$ABTS^{\bullet+}$ scavenging	**FRAP
Pinus sylvestris var. *hamata*	9.19 ± 0.32^{b}	6.11 ± 0.04^{c}	6.61 ± 0.02^{c}
BHT	10.23 ± 0.12^{c}	7.28 ± 0.19^{d}	5.34 ± 0.07^{b}
BHA	4.35 ± 0.10^{a}	4.25 ± 0.12^{a}	7.52 ± 0.04^{d}
Trolox	4.54 ± 0.14^{a}	5.52 ± 0.22^{b}	nt

* IC_{50} (μg/mL), ** (μ mol TE/mg EOs) Values represent the mean of the average of experiments ± SD, nt: not tested. Different letters in each column pointed out the statistically different ($p < 0.05$).

When the recent literature studies are examined, the fact that the essential oil contents of Pinus species are very diverse and enormous. Antioxidant tests were carried out on *P. sylvestris* L. var. *hamata* Steven, *P. pinaster* Aiton subsp. *pinaster*, and *P. pinea* L. bark extracts and found that *P. pinea* showed higher activity (1.643 μg/mL) than standard BHT and trolox (Karacelik et al., 2022). Studies have shown that the antioxidant capacity of *Pinus* sp. is remarkable (Kačániová et al., 2017). Russian Siberia, the northeastern region of China, and Mongolia are the native habitats of *Pinus sylvestris*. var. *mongolica*, also known as Mongolian Scots pine. The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenger's half-inhibition concentration was 14.36 ± 0.28 mg/mL (Namshir et al., 2020). The EOs and antioxidant analyses of five different species of *Pinus* (*P. holdreichii*, *P. peuce, P. mugo, P. nigra,* and *P. sylvestris*), all grown wild in Southern Kosovo, were investigated, and the most activity was reported in *P. mugo* EOs (Kurti et al., 2019). In the antioxidant study performed with EOs of *Pinus* taxa (*P. tabulaeformis, P. tabulaeformis* f. *shekanensis, P. tabulaeformis* var. *mukdensis, P. tabulaeformis* var. *umbraculifera, P. henryi* and, *P. massoniana*) endemic in China, the highest activity was determined in *P. tabulaeformis* var. *mukdensis* (Xie et al., 2015). In another study, *P. canariensis, P. attenuata*, and *P. nigra* var. *caramanica* EOs were found to have significant antioxidant activities (Koutsaviti et al., 2021).

3.3 Antibacterial effect

According to our results, the growth of all six bacterial strains tested were inhibited by EOs of *P. sylvestris* var. *hamata* in different degrees. *P. aeruginosa* showed higher diameters of growth inhibitions in 1.65 mm zone. *K. pneumonia* and *S. aureus* had the same inhibition as 1.55 mm. *S. tphimurium, B. cereus* and *E. coli* showed growth inhibition diameters of 0.15, 1.35, and 1.45 mm respectively. Both the gram-negative and gram-positive strains showed their sensitivities with varying inhibition zone diameters, as can be seen (Table 3).

Table 3. Inhibition zones of *P. sylvestris* var *hamata* EOs

Bacteria	**Inhibition zones (mm)**	**C_p**	**C_n**
Staphylococcus aureus	1.5 ± 0.07^a	1.9^a	0.1
Salmonella typhimurium	0.1 ± 0.2^b	1.4^b	0.1
Escherichia coli	1.4 ± 0.1^a	1.5^b	0.1
Bacillus cereus	1.3 ± 0.08^a	2.3^c	0.1
Klebsiella pneumoniae	1.5 ± 0.07^a	2.1^c	0.1
Pseudomonas aeruginosa	1.6 ± 0.3^a	1.7^a	0.1

C_p: Positive control (Chloramphenicol); Cn: Negative contol (methanol). Values represent the mean of the average of experiments ± SD. Same letters in each column indicated the no significant differences ($p < 0.05$).

In a study, it was determined that the main components of EOs obtained from *Pinus caribaea* needles, phelandrene (67.9%), caryophyllene (10.2%) and a-pinene (5.4%) had good antibacterial activity by disc diffusion method (Sonibare and Olakunle, 2008). It has been reported by disc diffusion method that *Pinus halepensis* EOs is effective against *Staphylococcus aureus* and *Bacillus cereus*. (Abi-Ayad etal., 2011). In another study, *Pinus roxburghaii* EOs were also found to be effective on *S. aureus* and *B. subtilis* (Hassan and Amjid, 2009). As a result of the disc diffusion test, *Pinus pinaster* EOs were reported to have moderate activity against *S. aureus*, *B. subtilis*, and *E. coli* (Mimoune et al., 2013*).*

EOs from *Pinus* sp. consist of a complex mixture of terpenes, sesquiterpenes, and diterpenes compounds (Allenspach et al., 2020). The bioactive chemicals in EOs are effective against various pathogenic microorganisms (Hong et al., 2004).The presence of different types of aldehydes, terpenes, phenolics, and other phytoactive compounds particularly may alter some metabolical pathways and structural parts in bacteria, fungi, and viruses. For example, some studies in the literature presented that some delocalized electrons and the hydroxyl group of the phenolic terpenoids have been accepted

among the antimicrobial determining factors (Thapa et al., 2015). The antimicrobial activities of terpenoids were determined according to their functional groups (Mahizan et al., 2019).

In the present study, α-and β-pinenes in the EOs may interact with the cell membranes of both gram-negative and gram-positive bacterial cells. *P. sylvestris* showed different sensitivities against the gram-negative bacterial strains, *P. aeruginosa* (1.65 mm) and *K. pneumonia* (1.55 mm). This differential response may be possibly related to the fact that the contents of the sample *P. sylvestris* EOs. The positive bacterial strains, *B. cereus* and *S. aureus*, showed different susceptibilities at 1.35 mm and 1.55 mm, respectively. This might indicate that different reaction mechanisms could take place in the bacterial cells regardless of the cell wall types. EOs components may have other targets in bacterial cells, such as the cell membrane and embedded proteins. Thus, the lipophilic nature of the terpenoid compounds eventually disrupts the cell membrane integrity and leads to cell fragmentation (Kačániová et al., 2017). The results may also suggest that the composition of each essential oil may vary in effectiveness against bacterial cell structures and cellular metabolic reactions. Reports indicate that some interactions between the components EOs lead to synergistic or additive effects (Burt, 2004). Even though additive effects have been observed in some studies, it was also suggested that the other minor components are essential to the synergistic activity (Bassolé and Juliani, 2012).

4. Conclusions

Essential oils that do not pose any risk to human and animal health are important because of their biological activities. EOs from the *P. sylvestris* var. *hamata*, which have good antimicrobial properties, has prospects that can be used against bacterial pathogens, primarily gram negatives. It has been revealed that the EOs exhibits excellent activity close to the standards in the screening of antioxidant activity using various tests. EOs generated from *P. sylvestris* var. *hamata* may be a favorable agent for cosmetic, food, and pharmaceutical industries. Further research in vivo is required to confirm the antimicrobial and antioxidant activities of *P. sylvestris* var. *hamata*, which may be used for the preservation and/or extension of the shelf life of raw and processed foods, as well as pharmaceuticals and natural therapies for infectious diseases in humans.

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Authors' Contributions

TK designed the study, wrote the manuscript and did the statistical analysis. CU executed the antibacterial assays. AK and SB work the lab experiments.

Conflicts of interest

The authors declare that they have no conflict of interest.

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