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Chemical composition and biological activity of juniper (*Juniperus sabina* **L.) essential oil growing in the aegean region of Türkiye**

Chemische Zusammensetzung und biologische Aktivität des ätherischen Öls von Wacholder (Juniperus sabina L.) aus der ägäischen Region der Türkei

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Summary Microorganisms and the problems they cause are the most important problems in the meat and meat products industry, as in all branches of the food industry. Utilizing natural additives in food is the emerging trend for preventing microbial growth. For this reason, studies on the antimicrobial properties of natural herbal products have increased in recent years. In this study, it was aimed to determine the potential antimicrobial activity of the chemical components of Juniperus sabina berry essential oil (EO) on the most isolated bacteria and molds from meat products. Sabinene (31.13 %), α -pinene (27.14 %), limonene (16.14 %), β -myrcene (9.28 %) were found to be the major components of *J. sabina* berry EO. The EO showed the highest antifungal activity on *R. nigricans* with a zone of 22.57 in mm diameter, followed by *A. flavus* (22.45 mm), *P. chrysogenum* (20.20 mm) and *M. racemosus* (17.69 mm), respectively. The minimum inhibitory concentration (MIC) values of *J. sabina* EO on the mold species used in the study varied between 70.32 and 468.75 mg/L, and the minimum fungicidal concentration values ranged from 78.13 to 312.50 mg/L. The EO showed the highest antibacterial activity on *S. aureus* with a zone of 20.27 mm in diameter and the lowest antibacterial activity on *S. dysenteria* (13.98 mm). The MIC values of *J. sabina* EO on the bacteria species used in the study varied between 54.69 and 281.25 mg/L, and the value of minimum bactericidal concentration values ranged from 39.06 to 187.50 mg/L.

Keywords: sabinene, *A. flavus, S. aureus,* antifungal, antimicrobial

Introduction

Meat and meat products are nutritious, especially proteinrich products. However, if proper preservation methods are not used, it can be spoiled very easily. It is the first option as an animal protein source for many individuals around the world. Global consumption of meat increases every year. However, in parallel with this increase, a significant portion of meat and meat products deteriorate every year. Microbial deterioration is responsible for a significant portion of this loss (Heinz and Haut zinger, 2007). In the meat industry, synthetic chemical preservatives are widely used to delay and prevent spoilage caused by microbial growth. owever, due to the potential health hazards (cardiovascular diseases, neurodegenerative diseases, cancers, and others) caused by synthetic preservatives in meat, consumers are increasingly sensitive to purchasing meat products containing such additives. For this reason, there is growing interest in the use of natural food preservatives in the meat industry.

As a natural food preservative; Plants rich in polyphenolics are the best alternative. They can be obtained from natural sources such as olives, fruits, grapes, vegetables, spices, herbs and algae and others. The presence of one or more -OH groups and one or more aromatic rings, which are required to exhibit antimicrobial activities, is a common characteristic of these phenolic compounds (Beya et al., 2021).

Juniperus L. (Cupressaceae), a genus of evergreen aromatic shrubs or trees, is distributed in temperate and cold regions of the northern hemisphere and includes roughly 68 species and 36 varieties (Lohani et al., 2010).

EO's obtained from different aromatic and medicinal plants possess a significant antimicrobial and antioxidant potential and therefore they are progressively used as natural additives in the modern food industry (Šojic et al., 2019). They attract attention as natural food additives (antioxidants and/or antimicrobials), as they are commonly in "generally recognized as safe" (GRAS) status and have a wide range of customer acceptance (Tomović et al., 2020).

Juniper EO is obtained from its fruit, leaves, and woody parts. Juniper EO obtained from the fruit has superior properties compared to the oil obtained from the other parts and it is stated to have a less woody, sweet, fresh aroma. In the food chemicals codex (FCC), juniper oil is defined as a slightly green or yellow liquid with a characteristic odor and aroma and a bitter taste (Attokaran, 2007). Juniper berry is commonly used in the liquor (it is best known as the unique flavoring agent of gin) and food industries as a spice, while its EO is used in pharmacy, aromatherapy, and in perfume industry (Alçay et al., 2008). Juniper berry EO has diuretic, gastrointestinal, irritant, antiseptic, carminative, diuretic, and antirheumatic properties. Juniper EO has been found to be effective against yeast, fungi, and dermatophytes (Pepeljnjak et al., 2005). Although the antimicrobial activity of the EO obtained from juniper berries is known, it has been observed that it has a stronger antifungal activity than its antibacterial effects (Bagıs, 2019). In particular, *J. sabina* L. is a medicinal plant which is used in folk medicine as an agent for abortion. Its lignanes have antiviral activity and antineoplastic activity. Its EO has shown antibacterial and antifungal activity (Asili et al., 2013).

In this study, it was aimed to determine the potential antimicrobial activity of the chemical components of Juniperus sabina berry EO on the most isolated bacteria and molds from meat products.

Materials and methods

Plant material

J. sabina L. berries (5 kg) were hand-picked in Turkey/ Afyonkarahisar (38° 48' 58'' North–30° 32' 00'' South, 1011 m above sea level) in October and November 2021. The berries of plants were dried in the shade for 15 days. Species, subspecies, and diversity were identified by Dr. Mustafa Kargıoğlu (Afyon Kocatepe University, Faculty of Science and Literature, Department of Molecular Biology and Genetics, Afyonkarahisar, Turkey), and a voucher specimen has been kept at the Afyon Kocatepe University herbarium of the Faculty of Science and Literature with the registration number of AKU712.

Mold and bacteria species used in the study

In the study, *Escherichia coli* O:157 H:7 (ATCC 43895), *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (ATCC 14579), *Listeria monocytogenes* (ATCC 51774), *Salmonella pullorum* (ATCC 19945), and *Shigella dysenteriae* (ATCC 13313) bacteria were used. Strains obtained from the American Type Culture Collection (ATCC, Rockville, MD, US), *Aspergillus flavus* (ATCC 204304), *Penicillium chrysogenum* (ATCC 10106), *Rhizopus nigricans* (ATCC 6227b), *Mucor racemosus* (ATCC 42647), *Geotrichum candidum* (ATCC 62218), and *Closporium cladosporioides* (ATCC 16022) were also employed.

Hydrodistillation

The hydrodistillation procedure of *J. sabina* berries was carried out using a Clevenger apparatus according to French Pharmacopoeia features for approximately 6 hours until all of the EO was recovered. The distillation process was done ten times separately (10 x 500 g). Each time, an average of 500 g of berries was put into the glass vessel of the distillation unit, and the distillation unit was placed on the heater. The cooling water was turned on and each unit was boiled for approximately 2 hours. The upper phase (EO) collected from the device was separated from the water. The collected EO's were then dried with anhydrous sodium sulfate. The EO's obtained were stored in capped brown glass bottles at 4°C until the analysis process was completed (Simard et al., 1998).

GC/MS analysis of EO

The chemical composition analysis of EO in *J. sabina* berries was carried out by using a GC-MS (Agilent 7890A GC, Agilent 5975C Inert XL EI / CI MSD) according to Ogundajo et al. (2014) and Akarca (2019). After the EO samples were diluted with n-hexane (1:10, v/v), they were injected into the GC-MS under the specified conditions. The injector and MS transfer line temperatures were set to 250°C, and the injection split ratio for GC and GC-MS analyses was determined as 1:50. In the analysis, the FSC column (Innowax, 60 m x 0.25 mm , 0.25 \mu) and helium, as a carrier gas (1 mL/min) were used. The oven temperature program was set to be at 60°C for 1 minute, then the temperature was increased to 240°C at 3°C / minute. The GC-MS, analysis was set to be used under the following conditions: carrier gas He; flow rate of 0.8 mL min–1; split ratio 1:20; injection volume 0.1 µL; injection temperature 250°C; and the oven temperature between 60 and 250°C at 2°C / min. The values of the mass spectra were recorded at m/z 45–425 to 70 eV at 70 eV EI (electronic pulse) at 1 scan/s–1 using full scan mode. The identification of EO components was performed by comparison of the relati-

ve retention indices (RRI) obtained with the n-alkane (C_s) $-C_{30}$) series using the calculated arithmetic indexes (RI lit) with values reported in the literature. The mass spectra recorded were identified using the onboard computer mapping with a library search in the Wiley 275 L GC/MS database (Adams, 2007). Quantification was done by an external standard method using calibration curves generated by running GC analysis of representative compounds.

Determination of EO antimicrobial activity by the disc diffusion method

Antibacterial and antifungal properties were determined by the disc diffusion technique. For this analysis, the techniques specified by Alastruey-Izquierdo et al. (2015) were modified. The colonies of microorganisms were transferred into peptone water using a sterile loop (Merck, 115525, Germany) and adjusted according to the 0.5 McFarland standard with a densitometer (8.17 Log cfu/mL) (Biosan 1B, Turkey). Using sterile pipettes, $0.1 \text{ mL} (10^6 - 10^7 \text{ cft})$ mL) of prepared inoculums was placed on the surface of a Sabouraud 2% Dextrose Agar (Merck, Germany, 1.07315) (SDA) for the antifungal tests and a Mueller Hinton Agar (MHA) (Merck 1.05437) for the antibacterial tests.

Then, 200 µl of *J. sabina* EO was soaked into blank discs (6 mm, Bioanalyse). The discs were placed in different areas of the medium at a distance where the growth zones would not touch each other.

Yeasts and molds were incubated at 25 ± 0.1 °C for 72–96 h, whereas bacteria were incubated at 37±0.1°C for 16–20 h (*L. monocytogenes* in a 5% CO2 atmosphere) (Incucell, MMM, Germany) (EUCAST, 2018). The zones formed in the post-incubation disc circles were measured in millimeters in a well-lit setting using digital calipers (Mitutoyo, Ip-67 0-150, Japan) (Bauer et al., 1959; Bauer et al., 1966).

Determination of minimum inhibitory, bactericidal, and fungicidal concentrations

Minimum inhibitory concentration (MIC) values of *J. sabina* EOs were obtained by the macro dilution method of de Castro et al. (2015) with some modifications. After the incubation, sediment, turbidity, and membrane on the surfaces of the tubes were evaluated as development positive. The MIC value was calculated by taking half the sum of the concentrations of the first tube with positive growth and the tubes with no growth before (CLSI, 2015).

The method specified by Owuama (2017) was modified to determine the minimum fungicidal (MFC) and bactericidal (MBC) concentration values. In the determination of the MIC value, $1 \mu L$ was taken from the first tube with microbial growth and the tubes in each concentration afterwards with a sterile pipette, and plated on the surface of an SDA for MFC value and MHA for MBC value. Then, the SDA was incubated at $25 \pm 0.1^{\circ}$ C for 72–96 h and MHA was incubated at 37±0.1°C for 16–20 h (*L. monocytogenes* at 5% $CO₂$ atmosphere) (Incucell, MMM, Germany) in the incubator. At the end of the incubation, concentrations with the absence of microbial growth were accepted as MFC for yeast and mold, and MBC for bacteria.

Statistical analysis

The results were obtained using two replicates, and experiments were done in two repetitions in the study. The variance analysis of the V 23.0.0 version of the SPSS program was used. The significant levels of difference were determined using Duncan's multiple range tests $(P<0.05)$.

Results and discussion

The average yield of *J. sabina* berries' EO was determined as 0.30 g / 100 g (average EO density was 0.887 g mL $^{-1}$) and average oil yield was calculated as the percentage by weight (1.02 % w/w) of *J. sabina* berries. The main components identified in the EO of *J. sabina* berries are sabinene (31.13%) , β -pinene (27.14%) , limonene (16.14 %) and α -mrycene (9.28 %) (Table 1). The volatile compound groups of the EO are monoterpene hydrocarbons and oxygenated monoterpenes, and their proportions are 71.8 % and 18.12 %, respectively.

The main components of *J. sabina* berry EO were specified as follows: The major components in the EO's were

TABLE 1: *Chemical composition of J. sabina L. berries EO.*

No.	Compound	RT ^a	RI b	RI (lit) ^c	(%) ^d
1	α -Pinene	13.01	933	932	27.14
2	Camphene	14.34	944	946	0.06
3	B-Pinene	15.66	957	974	0.17
4	Sabinene	16.13	965	969	31.13
5	β-Myrcene	17.40	993	988	9.28
6	α -Terpinene	18.17	1010	1014	0.71
7	Limonene	18.96	1018	1024	16.14
8	B-Phellandrene	19.33	1021	1025	0.16
9	γ -Terpinene	20.69	1042	1054	1.19
10	p-Cymene	21.75	1080	1082	0.49
11	α -Terpinolene	22.21	1085	1086	1.15
12	Sabinene <trans></trans>	29.54	1090	1098	0.68
13	Linalool	30.12	1087	1088	0.41
14	Camphor	30.20	1139	1141	0.13
15	Terpineol $<\!\alpha\!>$	30.35	1179	1186	1.83
16	Bornyl acetate	30.47	1252	1254	0.38
17	Copaene	31.00	1368	1374	0.05
18	Isoledene	33.38	1373	1374	0.22
19	β-Cubebene	33.76	1386	1387	0.79
20	B-Elemene	34.80	1385	1389	0.13
21	Caryophyllene	35.28	1394	1408	1.88
22	α -Humulene	36.11	1448	1452	0.15
23	Germacrene B (CAS)	36.59	1476	1480	0.10
24	Cadinene	37.02	1533	1537	0.63
25	Nerolidol	37.04	1562	1567	0.07
26	Caryophyllene oxide	37.10	1574	1582	0.20
27	Naphthalene	37.19	1601	1608	0.11
28	1-Naphthalenol (Junenol)	38.05	1612	1618	0.22
29	2,6-Octadienoic acid	38.72	1644	1650	0.27
30	Eudesmol <7-epi-α->	51.03	1656	1662	0.11
31	Cyclohexanemethanol	51.13	1676	1680	1.08
	Total				97.11
	I (No: 1-16, 29)				91.37
	II (No: 17-26, 30)				4.33
	III (No: 27-28, 31)				1.41

^a) Retention time; ^b) Retention indices measured relative to n-alkanes (C-8 to C-40) using capillary column (⁰ DB 5; Retention indices calculated on DB-5column. 6) Literature²⁰ retention indices on DB-5 column. ^a Quantification was done by external standard method using calibration curves generated by running GC analysis of representative authentic compounds Percentage is given as the average of 2 independent measurements. I) Monoterpene hydrocarbons, II) Sesquiterpenes, III) Polycyclic aromatic hydrocarbon.

TABLE 2: *Antifungal effect J. sabina L. EO on foodborne mold species (zone diameter, mm).*

^{a–d} (↓): values with the same capital letters in the same column for each analysis differ significantly (p<0.05), 6–8(–): resistance, 8–9(+): intermediate sensitive, 9–11(++): sensitive, 11≥ (+++): multi sensitive.

monoterpene hydrocarbons. The main monoterpenes of the oils of the fruits were sabinene (29.65 %), β -pinene (16.36 %), a-pinene (8.13 %), limonene (5.52 %) by Emami et al (2009). In similar studies, the main components of *J. sabina* fruit EO were specified as follows: Nikolic et al. (2016) (18 component identified) sabinene (5.3 %), α -pinene (2.7 %), β-mrycene (4.3 %), limonene (3.1 %), 4-terpineole (6.6 %).

Asili et al. (2013) (30 component identified) sabinene (48.6 %), myrcene (10.8 %), a-pinene (8.1 %), limonene (2.7 %). Khani et al. (2017) (40 component identified) sabinene (12.57 %), a-pinene (12.02 %), limonene (9.25 %), and β -mrycene (2.46 %).

Table 2. shows the antifungal effects of EO obtained from J. sabina berries on some food borne pathogenic and saprophyte molds according to the disc diffusion method. The J. sabina EO showed the highest antifungal effect on R. nigricans (22.57 mm), followed by A. flavus (22.45 mm) and P. chrysogenum (20.20 mm) $(P<0.05)$.

Juniper EO showed strong inhibitory effects on yeast and molds (Glišić et al., 2007). From the data of the diameter of the inhibition zone in the case of molds, it can be concluded that the sabinene had a significant influence on mold growth, which is in accordance with the published data (Santoyo et al., 2006).

The antifungal activities of the *J. sabina* EO occur as a result of the presence of volatile compounds and phenolic compounds that cause serious damage to cell membranes of microorganisms, leakage of the cell contents, and finally cell death. In particular, juniper berries are rich in phenolics and oxygenated monoterpenes, which promote their antifungal activity (Sevik and Akarca, 2021).

The antifungal activity of sabinene, pinene $(\alpha$ and $\beta)$ and limonene was particularly strong. Mechanism of antifungal action of these compounds was explained as the deterioration of the cellular and cellular membrane integrity, causing cellular content to leak out of the cell and disrupt

TABLE 3: *Antibacterial effect of J. sabina L. EO on food borne pathogen bacteria species (zone diameter, mm).*

a–c (↓): values with the same capital letters in the same column for each analysis differ significantly (p < 0.05), ≤7(–): resistance, 8–16(+): intermediate sensitive, ≥17(++): sensitive.

transportation and the deterioration of the protein synthesis mechanism (Cai et al., 2019).

The antibacterial effects of *J. sabina* berries EO on some food borne and water borne pathogenic bacteria are shown on Table 3. The *J. sabina* EO showed the highest antibacterial effect on *S. aureus* 20.27 (mm in diameter). *L. monocytogenes* (19.23 mm), *S. pullorum* (17.63 mm) and *B. cereus* (17.53 mm) (P<0.05).

Asili et al. (2013) stated that the EO of *J. sabina* berries showed antibacterial effects on all pathogenic bacteria used in the study, except for *P. aeroginosa.* The results obtained in the study are in line with our findings. The antibacterial effect manifests itself as irreversible cell wall damage, cell content leakage, altered cell wall permeability, and effects on the structure of bacteria nucleic acids (Lin et al., 2018).

Gram-negative bacteria are more resistant to plant EOs and extracts than gram-positive bacteria, due to the presence of an additional layer of polysaccharides in their cell walls (although there are exceptions) (Yashaswini and Arvind, 2011). These findings support our findings.

In our study, it was determined that the MIC values of *J. sabina* EO on mold species, ranged from 70.32 to 468.75 mg/L, while the lowest MIC values and MFC values varied between 78.13 and 312.50 mg/L (P<0.05; Table 4). Similarly, the lowest MIC and MFC values were obtained with

TABLE 4: *MIC, MFC (µg/mL) and MIC/MFC ratio for foodborne mold as affected by J. sabina L. EO.*

Mold species	MIC	J. sabina L. MFC	MIC/MFC
Aspergillus flavus	140.63±48.87	93.75 ± 31.25	1.5
Penicillium chrysogenum	187.5 ± 0.00	156.25±93.75	1.2
Rhizopus nigricans	70.32±23.44	78.13±46.87	0.9
Mucor racemosus	281.25±93.75	187.50±62.50	1.5
Geotrichum candidum	468.75±281.25	312.50±187.50	1.5
Cladosporium cladosporioides	281 25+93 75	93.750±31.25	3,0

R. nigricans at 70.32 and 78.13mg/mL respectively. The highest MIC and MFC values were obtained with *G. candidum* with 468.75 mg/mL and 312.50 mg/mL, respectively.

Similar to our research results; Nikolic et al. (2016) reported that the MIC values ranged from 6.25 to 50 (mg/ mL) in their research. In the same study, the researchers determined that the lowest MIC value of *J. Sabina* berry EO was 6.25 mg/mL for *A. hydrophila* and *B. subtilis.* Asili et al. (2013) reported that the MIC value of *J. sabina* berry EO on *C. albicans* was 3.125 mg/mL.

The MIC/MFC ratio of *J. sabina* EO on different mold species varies between 0.9 and 3.00 mg/L. If the MIC/MFC ratio is less than 2, the extract or EO should be evaluated as fungicidal, whereas if it is between 2–5, then it should be evaluated as fungistatic (Sevik et al. 2021). According to this; *J. sabina* EO showed a fungicidal effect on all mold species used in our research, except for *C. cladosporioides.*

It was determined that the MIC values of *J. sabina* EO on six foodborne pathogenic bacteria, ranged from 54.69 to 281.25 mg/L and MBC values varied between 39.06 and 187.50 mg/L. (P<0.05; Table 5). The lowest MIC and MBC values were determined against *S. aureus* at 54.69 and 39.06 mg/mL, respectively. Also, the highest MIC and MBC values were determined against *S. dysenteria* at 281.25 mg/mL and 187.50 mg/mL, respectively.

TABLE 5: *MIC, MBC (µg/mL) and MIC/MBC ratio for foodborne pathogen bacteria as affected by J. sabina L. EO.*

a^{→b} (↓): values with the same capital letters in the same column for each analysis differ significantly $(p < 0.05)$

Nikolic et al. (2016) reported that *J. sabina* berry EO was effective on *B. subtilis* at 6.25 mg/mL, followed by *A. hydrophila* at 12.5 mg/mL, which is consistent with our findings.

The MIC/MBC ratio of *J. sabina* EO on different bacteria species varies between 1.4 and 2.4 mg/L. When the extract or EO has a MIC/MBC of 4, it is considered bactericidal, whereas MIC/MBC>4 is considered bacteriostatic (Djeussi et al., 2013). Accordingly, *J. sabina* EO showed a bacteriaocidal effect on all pathogen bacteria used in the research.

Conclusions

Thirty-one components were identified in the *J. sabina* EO. Sabinene and α -pinene were the most abundant compounds. The *J. sabina* EO showed high antifungal and antibacterial effects. It was determined that the most antifungal effect and the lowest MIC and MFC values were obtained against *R. nigricans.* Additionally, the most antibacterial effects and the lowest MIC and MBC values were obtained against *S. aureus.* The *J. sabina* EO had a fungucidal effect on all mold and a bactericidal effect on all bacteria species used in the study. As a result, it should be revealed that the *J. sabina* EO berries are safe to be used and do not have any toxic effects. In future research on the subject, it would be appropriate to investigate the opportunities to use *J. sabina* EO or natural and GRAS additives produced with the addition of this oil, especially in foods with rapid spoilage, microbial contamination risk, foodborne diseases, and its effects on mycotoxin production.

Conflict of Interest

The authors of the manuscript declare no conflict of interest.

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