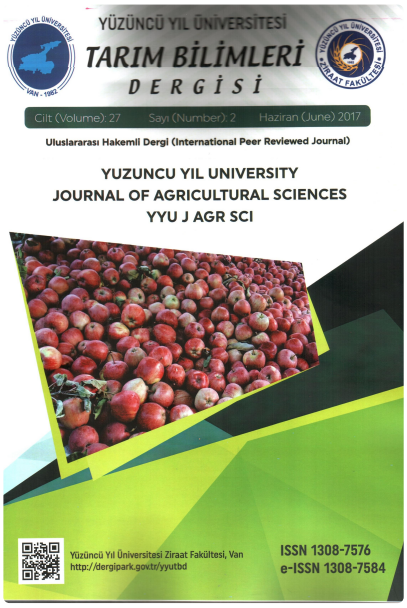
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Identification of Chemical Composition and Antibacterial Properties *Juniperus oxycedrus* L. subsp. *oxycedrus* Leaf Essential Oil

Okut, N., Yıldırım, B., Ekici, K., Terzioğlu, Ö., **Özgökçe, F.**

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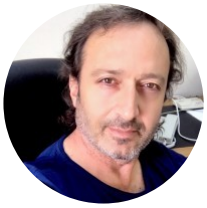
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Araştırma Makalesi/*Research Article (Original Paper)*

Identification of Chemical Composition and Antibacterial Properties *Juniperus oxycedrus* L. subsp. o*xycedrus* Leaf Essential Oil

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Abstract: The constituents of leaf essential oil of *Juniperus oxycedrus* L. subsp. o*xycedrus* (JOO) from wild flora of Gevas-Van in Turkey (Mount Artos) were studied using Gas Chromatography-Mass Spectrometry (GC-MS) and their antibacterial activities were assessed. A total of 18 compounds representing 99.98% of leaves oil were identified. The oils of the plant are all dominated by monoterpenes. The main compounds of essential oil in leaves were Limonene (45.77%), α-Pinene (23.94%), β-Phellandrene (10.83%), β-Pinene (5.68%), o-Cymene (3.30%), respectively. The essential oils were tested against *Staphylacoccus aureus, Bacillus subtilis, Pseudomanas aeruginosa*, *Enterecoccus faecalis, Salmonella typhimurium*, and *Escherichia coli* strains using the disc diffusion method. The diameter of the inhibition zones formed for bacteria were measured. The essential oils of JOO found to be active against all of the tested microorganisms and showed the susceptible inhibition zones. However, they were not as much as effective against bacterial strains when compared to ampicillin and ofloxacin. The extracts of JOO showed most significant antibacterial activity against *Escherichia coli* with inhibition zone diameter of 15 mm. The lowest inhibition zone diameter was *Staphylococcus aureus* with 11 mm.

Keywords: Antibacterial activity, Cupressaceae, Essential oil, GC-MS, *Juniperus oxycedrus* L. subsp. *oxycedrus*

Juniperus oxycedrus L. subsp. o*xycedrus* Yapraklarındaki Uçucu Yağın Kimyasal Yapısı ve Antibakteriyel Özelliklerinin Belirlenmesi

Özet: Türkiye'de Van ilinin Gevaş ilçesindeki Artos Dağı doğal ortamından toplanan *Juniperus oxycedrus* L. subsp. o*xycedrus* (JOO) (Ardıç, Dikenli ardıç) yapraklarının uçucu yağ bileşenleri Gaz Kromatografisi-Kütle Spektrometresi (GC-MS) ile araştırılmış ve sonrasında da antibakteriyel aktivitesi belirlenmiştir. Yapraklardaki uçucu yağın % 99.98'ini temsil edecek şekilde toplam 18 bileşen tespit edilmiştir. *J. oxycedrus* L. subsp. o*xycedrus* (JOO) uçucu yağının çoğunluğuu monoterpenler oluşturmuştur. Yapraklarda bulunan önemli bileşenler sırasıyla Limonen (45.77%), α-Pinen (23.94%), β-Phellandren (10.83%), β-Pinen (5.68%), o-Cymen (3.30%)'dir. Disk diffüzyon yöntemi kullanılarak, elde edilen uçucu yağlarin *Staphylacoccus aureus, Bacillus subtilis, Pseudomanas aeruginosa*, *Enterecoccus faecalis, Salmonella typhimurium* ve *Escherichia coli* üzerindeki etkisi belirlenmiştir. Bunun için bakteri oluşumunun engellendiği bölgenin çapı ölçülmüştür. Bu test sonucunda JOO yapraklarından elde edilen uçucu yağın test edilen bütün mikroorganizmalara karşı etkili olduğu ve çevresinde bakteri üremesini engelleyen bir bölge oluşturduğu ancak engelleme etkisinin ampisilin ve ofloksin ile karşılaştırıldığında bu antibiyotikler kadar etkili olmadığı görülmüştür. *J. oxycedrus* L. subsp. o*xycedrus* yapraklarının uçucu yağları *Escherichia coli'*ye karşı 15 mm çapındaki engelleyici bölge ile en yüksek antibakteriyel etkiyi göstermiştir. En düşük antibakteriyel etkiyi ise 11 mm'lik bölge ile *Staphylococcus aureus* üzerinde göstermiştir.

Anahtar kelimeler: Antibakteriyel etki, Cupressaceae, Uçucu yağ, GC-MS, *Juniperus oxycedrus* L. subsp. *oxycedrus*

Introduction

The flora of Turkey contains about 9500 species, 30% of which are endemic (Davis 1988). Eastern Anatolia in Turkey has a rich flora, because of its varied climate and several different ecological regions. As a result, the diverse in flora at East Anatolia offers a rich potential of medicinal plants, which has long been exploited by old Anatolian cultures (Ozgokce and Ozcelik 2004).

Juniperus L. (Cupressaceae) grows naturally throughout Mediterranean regions as well as northern Iran on rocky and sunny areas, on hilly and mountained, up to 1900 m level (Farjon 1998). The study showed that *Juniperus* L. is characterized with ten taxa and has two subspecies in Turkey. These are subsp. o*xycedrus* and subsp. *macrocarpa* (Coode and Cullen 1965).

Aromatic oils of fruits and leaves from *Juniperus* species have been used since ancient times for many purposes including fragrance, flavouring, medicinal, antimicrobial, insecticidal as well as cosmetic (Chalchat et al. 1991; Stassi et al. 1996; Ates and Erdorul 2003). The chemical constituents of the essential oil of wild *Juniperus* herb very much depends on the environmental conditions, plant parts and regional climate where the species grow.

J.oxycedrus L. subsp. *oxycedrus* plant samples were collected in different places (Sardinia, Italy) and leaves were hydro distilled by Angioni et al. (2003). The major compounds of essential oil in their study were α-pinene, β-pinene, δ-3 carene, sabinene, myrcene, β-phellandrene, limonene and D-germacrene. Another study on esenstial oil constituent and the antimicrobial properties of leaf have been evaluated and reviewed by Medini et al. (2013). They showed the antibacterial activities of Tunisian *J. oxycedrus* L. subsp. *oxycedrus* versus four bacteria (*Escherichia. coli*, *Staphylococcus aureus*, *Salmonella enteridis*, and *Salmonella typhimurium*). Essential oils attained by hydrodistillation were studied by GC and GC-MS. In total, fifty-five constituents were identified. Thirty four main compounds have been retained for the study of the chemical variability. The α-pinene, sylvestrene, *p*-cymene, and 13- *epi*-manoyl oxide were found to be major among them. The study carried out on antibacterial activity revealed that *Escherichia coli* was found to be the most resistant (zone diameter 0 mm) to all the oils tested, while *Staphylococcus aureus* was the considerable sensitive strain (zone diameter 13.5mm). These essential oil constituents, which they differ from different regios, were detected as α-pinene, β-pinene, δ-3-carene, sabinene, myrcene, β-phellandrene, limonene, and D-germacrene as the most abundant constituents of the leaf and fruit essential oils.

To our best knowledge, there is no previous work and no data were available on the chemical variability from the current studied area for the volatile oil of *J. oxcycedrus* L. subsp. *oxcycedrus* leaf. Hence, this research has special importance to identify the essential oil compositions and their antibacterial activity in *J. oxcycedrus* L. subsp. *oxcycedrus* leaf.

The aims of our study were: (1) to explore essential oil constituent of hydrodistilled of *J. oxycedrus* L. subsp. *oxycedrus* plants' leaves from in Eastern part of Turkey (Van region) by a GC-MS and; (2) to investigate the antibacterial activity of the obtained volatile oils components against *Staphylacoccus aureus, Bacillus subtilis, Pseudomanas aeruginosa*, *Enterecoccus faecalis, Salmonella typhimurium*, and *Escherichia coli.*

Material and Methods

Plant Material

JOO, Cupressaceae, samples were collected from wild populations growing in Gevas-Van (Mountain Artos) located in the region of Eastern Turkey. Samples collecting season was June 2010. The altitude of sampling location is 1730-1950 m. The plants were identified by Dr. Fevzi Ozgokce at Yuzuncu Yil University (YYU), Department of Biology (Van, Turkey). Aerial parts of plants were dried in shade. Then, plants leaves were detached from the stem.

Essential Oils Isolation

The essential oils were isolated by hydrodistillation (4 h) using a Clevenger type instrument and then they were dried over anhydrous sodium sulphate. Then oil samples stored into the amber vials at 4 °C in the dark prior until the analysis.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Essential oil components of JOO were identified by using GC-MS. The GC-MS consisted of a GC-MS instrument (GC-MS-QP 2010 Plus Shimadzu, Japan) fitted with a TRB Wax capillary column (30m x 0.25 mm i.d., 0.25µm film thickness). Essential oils were diluted by 1/10 in n-hexane (v/v) before the analysis done. The oil injection was performed by an auto-sampler. Column temperature was automated from 60^{o} to 240^{o}C, temperatures held for 60^{o}C for 3 min. Then temperatures gradually increased to 240^{o}C at 9^{o}C /min kept there during 10 minutes. Helium at 3 mL/min. was used as carrier gas and then mass spectra were recorded in the scan mode. The ionization voltage was established to 70 eV. The ratio for splitting was set to1:50. Ion source temperature was set to 200^{o}C and interface temperature was set to 240^{o}C and mass range was set to from 40 to 300 amu (resolution). Three minutes were set to for cutting time. For the analysis part, 0.1 µl of sample was considered.

The oil constituents were identified from their retention times (Rt) which is obtained using the composition of their mass spectra and fragmentation patterns reported at Adam 2001 and computer identical with MS-data bank (Wiley and Nist Library).

Determination of Anti-Bacterial Activity

Microorganisms

Total six bacterial species were used for testing the antimicrobial activity of JOO essential oils (Table 1 and Table 2). Strains of microorganisms were attained from Department of Biology (YYU, Van-Turkey). The microorganisms were *Staphylococcus aureus* ATCC 12600, *Bacillus subtilis* ATCC 6051, *Pseudomanas aeruginosa* ATCC 10145, *Enterecoccus faecalis* ATCC 29212, *Salmonella typhimurium* ATCC 25241, and *Escherichia coli* ATCC 11775.

Antimicrobial Assay (Disc Diffusion Method)

The technique of disc diffusion has been used to identify the antimicrobial activity of the essential oil. Agar disc diffusion method for antibacterial activity testing was performed using the standard technique proposed by Bauer et al. (1966). As a result of adding 0.5 mL of 0.048 M BaC_{12} (1.17% wt/vol BaC_{12}•$2H_2O$) to 99.5 mL of 0.18 M H_2SO_4 (1% wt/vol), the McFarland Standard was prepared (Andrews 2004). The cultures, which were frozen in Glycerol Nutrient Brotta (Oxoid), dissolved in the refrigerator. Then they activated in Tryptic Soy broth (Oxoid) for 24 h. Brain Hard Infusion Brotta (Oxoid) was activated from Tryptic Soy Agar (Oxoid) passages for 3-4 h. McFarland 0.5 tubes blur was used to adjust the density of cultures.

Activated microorganisms were spread on the surface with a known concentration of Mueller-Hinton Agar (Merck) plates. For this procedure a sterile swap was used and then incubated for 30 minutes. Filter paper discs (6mm Ø, wattman 2017-006) were soaked with essential oils then placed on the surface of these agar plates, where the surface were spread with 0.5 mL of bacterial suspencion.

Both ampicillin and ofloxacin have been used as control agents (Ozcelik et al. 2005). Microorganisms were placed to incubate in the oven along with the species and suitable temperature time (37°C for 24 hours). As colonies formed around the zones, these zones were measured using inhibition zone scale in mm. Activities of antibacterial were evaluated by measuring the inhibition zone against to the test organisms (Ponce et al. 2003). All individual assays were repeated twice.

The sizes of inhibitory zones were used to compare the sensitivity of the bacterial species to the essential oils (Ponce et al. 2003). Inhibition zones ranged between 8 mm and 20 mm and then the results were assessed as follows: If measured zones smaller than 8 mm were classified as insensitive, the zones between 9-14 mm were sensitive, zones between 15-19 mm were very sensitive, and those larger than 20 mm were extremely sensitive (Ponce et al. 2003).

Statistical Analaysis

PROC GLM in SAS 9.4 (2017) was used to compare the diffences between means of groups. The pairwise comparisons of groups were done by Tukey and $P<0.05$ was considered as Type I error for statistical significance level. SAS package program version 9.4 (2017) was used for all statistical analyses.

Results and Discussion

Essential Oil Compounds Contents

Chemical constituent of essential oil and their retention times and percentage of compounds of the collected material from study area (Mountain Artos, Van-Turkey) are illustrated in Table 1. The constituents are illustrated as order of their elution from the TRB Wax capillary column (30m x 0.25 mm i.d., 0.25µm film thickness). In total, 18 constituents were identified, representing 99.98% of the total oil fraction. Major constituents of JOO identified were Limonene (45.77%), α-Pinene (23.94%), β-Phellandrene (10.83%), β-Pinene (5.68%), o-Cymene (3.30%), respectively (Table 1).

Table 1. Essential oil composition of *J. oxycedrus* L. subsp *oxycedrus* leaves

Peak	Constituents	R_t (min)[a]	Rate (%)
1	α--Pinene	3.082	23.94
2	Camphene	3.517	0.70
3	Capronaldehyde	3.683	0.87
4	β-Pinene	4.013	5.68
5	2-Carene	4.298	0.70
6	Limonene	5.346	45.77
7	β-phellandrene	5.444	10.83
8	γ-Terpinene	5.942	0.44
9	o-Cymene	6.326	3.30
10	α-Terpinolene	6.505	1.28
11	Camphor	9.984	0.36
12	Terpinene-4-ol	11.071	0.49
13	trans-p-Mentha-2,8 dienol	11.383	0.40
14	α-Terpineol	12.275	0.50
15	Germacrene-D	12.519	0.48
16	Carvone	12.830	1.33
17	Trans-carveol	13.954	0.72
18	Manoyl oxide	19.521	2.19
	Total (%)		99.98

[a] Retention time (as minutes)

Table 2. The Grouped components of essential oils of JOO

Main Groups	Rate (%)
Monoterpenes	93.63
Aldehydes	0.87
Ketones	1.69
Sesquiterpenes	0.48
Menthane monoterpenoids	0.40
Monoterpenoid alcohol	0.72
Others	2.19
Total (%)	99.98

Studies showed that the main constituent of JOO essential oil was α-Pinene (Adams et al. 2005). Hayta and Bagci (2014) were collected samples of the leaves, bark and cones of JOO from the wild form at Bursa-Turkey. The predominant compounds have been identified in their study were α-pinene, β-pinene, β-myrcene and limonene. A comparative study carried out by Adams et al. (2005), on JOO chemical profiles of some Mediterranean countries revaled that the oils of western Mediterranean populations (Morocco, Spain, France, and Portugal) were characterized by relatively high levels of α-pinene (45.3 to 50.3%). While the oils of *J. oxycedrus* L. subsp. *oxycedrus* leaves collected at eastern Mediterranean countries (Italy, Greece and Turkey) were characterized by low concentrations of α-pinene (19.3 to 32.7%) and reasonable levels of α-phellandrene, α-terpineol, p-cymene, β-phellandrene, limonene, myrcene, α-terpineol, (E)-nerolidol, and manoyl oxide. The major compounds have been identified at two Mediterranean countries are; a) Italy (subsp. oxycedrus) limonene, α-terpinyl acetate, α-pinene, β-caryophyllene (Vidrich et al. 1992); (b) Greece, α-pinene, followed by β-phellandrene and terpinolene

(Vourlioti-Arapi, et al. 2012). Our results in terms of main components for essential oil showed strongly agreement with Adams et al., 2005 and Vidrich et al., 1992. However, our findings were not totaly confirmed by Hayta and Bagci (2014) results. Differences between results can be because of environmental conditions, plant extractions methods as well as different chemotaxonomy of the plants of juniper.

Antibacterial activity

The antibacterial activity of essential oil from JOO leaves from wild was observed in this study as well. The essential oils formed antibacterial activity inhibition zones against *Staphylacoccus aureus, Bacillus subtilis, Pseudomanas aeruginosa*, *Enterecoccus faecalis, Salmonella typhimurium* and *Escherichia coli* (inhibition zones were from 10 to 15 mm) (Figure 1).

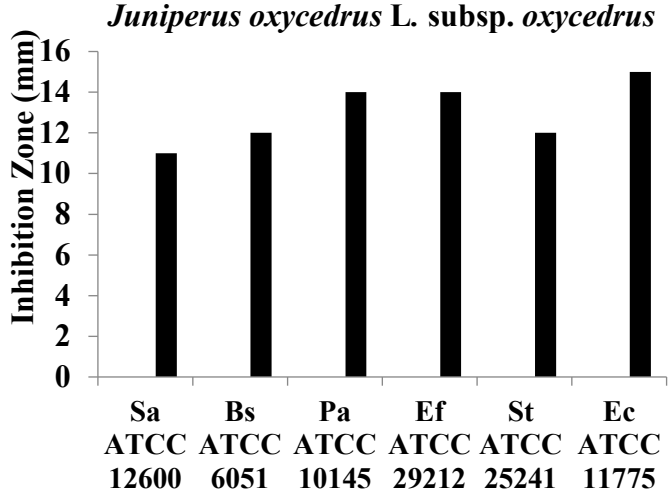


Figure 1. Screening of the antibacterial activity of essential oils of *J. oxycedrus* L. subsp. *oxycedrus* leaves against six bacterial strains. Sa, *S. aureus*; Bs*, B. subtilis*; Pa, *P. aeruginosa*; Ef, *E. faecalis*; St, *S. typhimurium*; Ec, *E. coli*

Table 3. Antibacterial activity of *J. oxycedrus* L. subsp. *oxycedrus* leaves essential oils (in µL) and two antibiotics (ampicillin and ofloxacin) against bacterias (inhibition zones in mm)*

Microorganisms	Ampicillin	Ofloxacin	*JOO* essential oil
S. aureus ATCC 12600	21.0 a**	25.0^{a}	11.0^{b}
B. subtilis ATCC 6051	25.0^{b}	30.0^{a}	11.5^{c}
P. aeruginosa ATCC 10145	22.5^{b}	27.0^{a}	14.0^{c}
E. faecalis ATCC 29212	27.0^{a}	21.0^{b}	13.5^{c}
S. typhimurium ATCC 25241	23.0^{a}	25.0^{a}	12.0^{b}
E. coli ATCC 11775	27.0^{a}	27.0^{a}	14.5^{b}

***** Not sensitive when total diameter smaller than 8 mm, sensitive when total diametre 9-14 mm, very sensitive when total diameter 15-19 mm and extremely sensitive for total diameter larger than 20 mm
** The same letter indicates no differences between means

All of the pathogens showed sensitivity (inhibition zone 9-14 mm) to essential oil except *Escherichia coli*. However, the gram-negative bacteria *E. coli* was the most sensitive (total diameter between 15-19 mm) and essential oil from leaves exhibited a relatively sensitive activity against *Staphylacoccus aureus*, *Bacillus subtilis, Pseudomanas aeruginosa*, *Enterecoccus faecalis, Salmonella typhimurium* (total diameter was between 11-15 mm) (Table 3). As illustrated in Table 3, all strains were very sensitive against control antibiotics (ampicillin and ofloxacin). Statistically, there were significant differences between oil extract with ampicillin and ofloxacin in terms of antibacterial effectiveness in the studied microorganisms. We noted some similarities and differences when our results compared with Angioni et al., (2003), Medini et al., (2013) studies. In general, the gram-positive bacteria *S. aureus* was the most sensitive and the gram-negative bacteria *E. coli* was the most resistant in Medini et al. (2013). However, Angioni et al., (2003) reported that major compounds of essential oil in their study were α-pinene, *β*-pinene, *δ*-3 carene, sabinene, myrcene, *β*-phellandrene, limonene, and D-germacrene. Conversely, *E. coli* was the most sensitive bacteria to plant essential oil in our study. Another difference between this study

and the studies performed by Angioni et al. (2003) and Medini et al. (2013) were that the essential oil constituents of study samples showed differences according to regions and plant parts.

Conclusion

The essential oils of JOO leaves were analyzed by GC-MS, and antibacterial activity were obtained by disc diffusion method. Essential oil characterized by the highest level of the major components (Limonene (45.77%), α-Pinene (23.94%), β-Phellandrene (10.83%), β-Pinene (5.68%), and O-Cymene (3.30%)) and therefore by the highest antibacterial activity. Antibacterial activity of the essential oil was somewhat lower when compared with two antibiotic effects on tested 6 bacterial organisms. Leaves essential oils displayed a comparatively sensitive activity against *Staphylacoccus aureus, Bacillus subilis, Pseudomanas aeruginosa*, *Enterecoccus faecalis,* and *Salmonella typhimurium.* However, the Gram-negative bacteria *E. coli* was the most sensitive to essential oil of test plant in our study. Results from this study recommend that essential oil of the *J.oxycedrus* L. subsp. *oxycedrus* possess can be use as for antibacterial properties.

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