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Antimicrobial activity of the essential oil and different fractions of *Juniperus communis* L. and a comparison with some commercial antibiotics

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Abstract: The essential oil of common juniper (*Juniperus communis* L., from the southern part of Serbia) and its fractions of different composition, as well as commercial antibiotics were used for testing their antimicrobial activity against bacteria, yeast and fungi. The essential oil was produced by hydro-distillation in a pilot plant (130 dm^3) and then fractionated by distillation over a column, with 36 theoretical stages, under vacuum (26–66 mbar). The essential oil was also fractionated using pure CO_2 or CO_2 and methanol as co-solvent under supercritical conditions. The native oil showed weak antimicrobial activity, while the fractions with a high content of α-pinene, and mixture of α-pinene and sabinene showed the highest antimicrobial activity, especially against fungi. In comparison to the commercial antibiotics, the oil fractions showed more extensive spectra of antimicrobial activity, as well as wider inhibition zones.

Keywords: Juniperus communis L., essential oil, distillation, supercritical fractionation, antimicrobial activity, antibiotics.

INTRODUCTION

Many plants, their essential oils and extracts have potential in medical procedures and applications in the pharmaceutical, cosmetic and food industry.[1] Numerous researchers showed interest for biologically active components isolated from plants and for their influence on the elimination of pathogenic microorganisms. The resistance which certain microorganisms have developed against antibiotics initiated antimicrobial investigations and different applications of essentials oils or plants against a wide range of bacteria (Gram-negative and Gram-positive) including antibiotic resistant species,[2–4] fungal species[5] and yeast.[6]

To date, juniper essential oil has only been used in traditional medicine. The content of juniper essential oil differs depending on its origin. The amount of some

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components may significantly vary,[7] as is shown in Table I. In the studies of the essential oil of juniper fruit, it was established that the pharmacological features are derived from its constituents. Therefore, its diuretic properties were ascribed to terpinen-4-ol and pinene was found to act as a rubefaciens.[8] All terpene hydrocarbons are antiseptic, anti-inflammatory and antibacterial. They are also pain-killers, sedatives, stimulators and media for the excommunication of excrete mucus.[9,10] In addition, terpenes retard the retention of toxins in human organisms, they increase the abstraction of aggregated toxic material from the veins and liver, and act as antispasmodic agents (media for mitigating convulsions).[8] Certainly α-pinene is an acute antiseptic, while cadinene, caryophyllene, terpinene and sabinene have pronounced anti-inflammatory and antibacterial properties.[8] Myrcene acts as a sedative, an anti-inflammatory agent and as a pain-killer for peripheral organs.[8] Furthermore, myrcene stimulates the recovery of liver and it is known as a strong anti-inflammatory substance.[8,9] Limonene is known for its strong antiviral properties. Furthermore, limonene helps detoxify the liver and abstracts carcinogenic substances and can retard tumor development.[8]

Reports regarding the essential oil from juniper berry can be found in the literature. It was found that the oil derived from *Juniperus communis* did not induce skin irritation and it was not phototoxic when applied to the backs of hairless mice and swine.[11] In albino rats dosed orally, *J. communis* extract induced antifertility and abortifacient effects, but had no teratogenic effect.[11] Clinical tests showed no evidence of skin irritation or sensitization on patients with allergic reactions.[11] The acute oral and dermal LD_{50} for *J. communis* oil in rats was >5 g/kg. *J. communis* L. oil exhibited 92 % inhibition of elastase (the enzyme which degenerates dermal elastin) activity, and the IC_{50} values were 101.9 μg/ml, but limonene and α-pinene were found not to be the inhibitory components.[12] Pinene-type monoterpenes, camphor and borneol could be responsible for the total activity spectrum.[1,13] Investigation of the inhibitory action of *J. communis* essential oil against viruses has not been done yet but the extract of *Juniperus oxycedrus* berries (α-pinene 10.5 %, β-myrcene 8.1 %, geranial 5.1 %, α-caryophyllene 4.0 %, germacrene D 13.8 %, δ-cadinene 7.5 %, *trans*-calamenene 5.2 %) was active against Polio virus and Coxsackie viruses serotype B2.[14]

The mechanism of antimicrobial action of terpenes is not fully understood but it is speculated to involve membrane disruption by the lipophilic compounds.[15] All the bacterial strains demonstrated some degree of sensitivity of the plant volatile oils, although the growth of a number of bacteria was uninhibited by specific volatile oils.[16] There are many views which state that every plant volatile oil shows antimicrobial activity on some type of microorganisms, Gram-positive bacteria, Gram-negative bacteria, fungi or yeast.[17] The activity of the oils would be expected to relate to the respective composition of the plant volatile oils, the structural configuration of the constituent components of the volatile oils and their functional groups and possible synergistic interactions between the components.[16]

The aims of the present investigation were to assess the antimicrobial activities of some fractions from juniper essential oil and to compare them with the effect of commercial antibiotics on bacterial growth. Furthermore, the obtained data might be used to deduce the constituents of juniper essential oil which possess significant antimicrobial activity.

EXPERIMENTAL

Plant materials

Purplish-blue juniper berries were collected at the end of September 2002 from Leposavić, the south side of Kopaonik, Zubin Potok and the Mokra Gora Mountian and voucher specimens (16036 BEOU; Snežana Vukojčić) were deposited at the Herbarium of the Institute of Botany and Botanical Garden Jevremovac, Faculty of Biology, University of Belgrade. Needles, branches and woody parts of juniper tree as well as parts of other plants were removed manually. The berries were air-dried at room temperature. They were stored in double-layered paper bags at ambient temperature, protected from light and well air-conditioned in order to prevent fermentation.

Hydrodistillation was conducted by a standard procedure (Clevenger apparatus) with juniper berries which had previously been chopped in a domestic blender, and a pilot plant (130 m^3). The fractionation experiments were carried out continuously from July to the first half of November 2003. The yield of the obtained essential oil was about 1.15 wt %.

Fractionation

Batch distillation was performed in a pilot plant. A column of 2 m height was charged with Normag packing. It was determined experimentally that the column had 36 theoretical stages. Fractionation of the essential oil was conducted at absolute pressures of 26 and 66 mbar with a reflux ratio of 2–5. Fractions of 10 cm^3 were collected during the fractional distillation. The fractionation of essential oil with supercritical carbon dioxide was performed in an Autoclave Engineers SCE Screening System[18] in two experiments under different conditional (175 bar and 40 °C using supercritical carbon dioxide with 5 wt % of methanol as co-solvent and at 90 bar and 75 °C with pure carbon dioxide).

GC-FID Analysis

Samples dissolved in *n*-hexane were subjected to gas chromatographic analyses on a Varian 3400 gas chromatograph equipped with an FID. A fused silica DB-5 capillary column, 25 m x 0.32 mm internal diameter, and 0.25 μm film thickness, was used. The purged splitless mode of sampling was implemented. The column temperature was maintained at 50 °C for 2 min and then programmed to increase as follows: at 2 °C/min to 250 °C, and holding at 250 °C for 5 min. The flow rate of the carrier gas (nitrogen) through the column was 2 mL min^{-1} and inlet pressure was 10 psig. The injector temperature was 250 °C and detector temperature 300 °C. The quantitative presence of a compound in the samples was determined using the method of peak-area normalization, without the application of response factor corrections. Injections were repeated in triplicate.

GC-MS Analysis

Samples prepared using the same procedure as for the GC analysis were subjected to GC-MS analysis on a Varian 3400 split/splitless (1:20) gas chromatograph with a mass spectrometry detector Finningan Ion Trap ITD-705, ionization voltage of 70 eV. A fused silica Supelco column PTE-5, 30 m x 0.25 mm internal diameter and 0.25 μm film thickness, was used. The column temperature was maintained at 60 °C for 1 min and then programmed to increase as follows: at 4.3 °C/min to 286 °C and holding at 286 °C for 5 min. The linear flow velocity of the carrier gas (hydrogen) through the column was 1 mL min^{-1}. The temperatures of the injector and detector were 250 °C and 300 °C, respectively. The sample (1 μL) was injected in the split mode (1:60). The identification of the com-

pounds was based on the comparison of their retention time and GC elution sequence with literature data for juniper berry essential oil[15,16] and also by matching the mass spectra of every GC peak with those of the AMDIS Ver.2.1. Program.

Determination of antimicrobial activity

Bacterial strains and culture conditions. The following microorganisms were used as indicators: bacteria *Bacillus cereus* (isolate from the Institute of Virology and Immunology – Torlak, Belgrade, Serbia); *Escherichia coli* ATCC 8739; *Listeria monocytogenes* IM200 (isolate from the Institute for Meat Hygiene and Technology, Belgrade), *Corynebacterium* sp. 754, *Pseudomonas aeruginosa* DV5999, *Staphylococcus aureus* ATCC 6538; yeast – *Candida albicans* T (isolate from the Institute of Virology and Immunology – Torlak, Belgrade, Serbia); and fungi–*Alternaria* sp., *Aspergillus nidulans*, and *Aspergillus niger* (isolates from the Laboratory of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia). Each assay was repeated twice.

Agar well-diffusion method. The antimicrobial test was performed according to the method of Wan, Wilcock, and Coventry[19] with some modification. Briefly, 200 μL of fresh overnight cultures of the indicator strains of bacteria (~ 10^8 CFU/mL), yeast (~10^7 CFU/mL), and fungi (~10^4 spore/mL) were added in 6 mL of soft Nutrient agar (NA – Institute of Immunology and Virology, Torlak, Belgrade, Serbia) and soft Sabouraud maltose agar (SMA – Torlak, Belgrade, Serbia) medium for yeast and fungi. The soft agar was vigorously mixed and poured over Petri plates with previously dried correspondent agar medium on the surface of which the sterile tubes (7 mm diameter) were placed. After solidification of the soft agar, the tubes were removed and the obtained wells were filled with 10 μl of the oil samples. The incubation was carried out at 37 °C for the bacteria and 30 °C for the yeast and fungi. After 24–48 h of incubation, the antimicrobial activity was evaluated by measuring the width of the zone of inhibition (clear) or suppression (diffuse) of growth against the indicator organisms in comparison to a control of reference standards. To establish the nature of the inhibitory activity of the oils, samples were taken from the clear zones with a loop and surface-plated onto appropriate agar and incubated under optimal conditions for up to 48 h.

Antibiogram test. Test was performed according to the manufacturer instructions, with the application of the above appropriate agar medium. The following mass of antibiotics in the form of standard antibiogram tables (disc) were used in order to provide a control for the sensitivity of the indicator organisms in the experiments: gentamycin (30 μg/disc), clindamycin (10 μg/disc), streptomycin (30 IJ/disc), tetracycline (30 IJ/disc), erythromycin (15 μg/disc), vancomycin (30 μg/disc), ampicillin (10 μg/disc), penicillin G (6 μg/disc). All tests were performed in triplicate.

RESULTS AND DISCUSSION

The composition of the essential oil (Table I) and identification of the main components present in the essential oil were determined by GC analysis, by comparing the retention times of unkown components with the retention times of standards[20–22] as well as by GC-MS analysis.

TABLE I. The range of the composition of different juniper essential oils[7] and the content (wt %)[a] of the components of juniper oil in the samples from Zubin Potok, EO1, and from Leposavić, EO2

Rt/min	Components	Content in oil[7]/wt %[a]	EO1/wt %[a]	EO2/wt %[a]
11.5	α-Pinene	3.7–86.2	36.6±0.6	40.5±0.7
13.8	Sabinene	0.1–34.0	16.2±0.2	18.0±0.2
14.8	Myrcene	1.4–53.1	10.9±0.2	13.5±0.3
16.5	*p*-Cymene	2.4	2.2±0.1	2.3±0.1

TABLE I. Continued

Rt/min	Components	Content in oil[7]/wt %[a]	EO1/wt %[a]	EO2/wt %[a]
17.1	D-Limonene	2.0–3.8	3.9±0.1	5.1±0.1
18.8	γ-Terpinene	1.9–3.7	1.4±0.0	2.2±0.0
20.7	Terpinolene	0.9	1.2±0.1	1.2±0.1
26.4	1-Terpinen-4-ol	*	2.4±0.1	2.5±0.1
41.7	α-Cubebene	*	1.1±0.1	1.0±0.1
43.2	α-Copaene	*	1.1±0.1	1.4±0.1
44.2	β-Elemene	*	0.9±0.1	1.0±0.1
45.3	β-Caryophyllene	*	5.3±0.1	0.6±0.0
46.6	α-Humulene	*	1.2±0.2	0.6±0.1
49.2	Germacrene-D	*	1.1±0.1	0.1±0.0
51.1	Bicyclogermacrene	*	3.2±0.1	1.3±0.0
55.8	γ-Cadinene	*	0.6±0.1	0.6±0.1
56.5	δ-Cadinene	*	0.6±0.1	0.6±0.1
	Total[b]		89.90	91.4

[a]The data were calculated from the GC chromatogram peak areas. [b]The differences are unidentified peaks from the GC chromatogram. * Varies according to location

The fractional separation of juniper essential oil was performed in a vacuum distillation column with 36 stages. Several fractions of distillate were collected and their compositions were analyzed. Only α-pinene and partially sabinene were separated if the fractional vacuum distillation was performed under severe heating conditions (fast heating rates and small reflux ratio, 66 mbar absolute pressure), while the other components of the essential oil mostly decomposed. Under 26 mbar absolute pressure, a smaller heating rate and a large reflux ratio, one of the fractions contained 99 wt % α-pinene and several others mainly sabinene, myrcene and limonene. Such a distribution of the main constituents of juniper essential oil in the fractions was expected, due to the similar boiling temperatures of these chemical compounds (α-pinene 155–156 °C sabinene 163–164 °, myrcene 167–171 °C, limonene 175–177 °C).

The fractionation of juniper essential oil with supercritical carbon dioxide and methanol as co-solvent (5 wt %) at 40 °C and 175 bar, did not give larger differences in composition compared to the starting essential oil, because the extraction power of carbon dioxide under those conditions was equally good for all the chemical compounds present in the essential oil. Only terpinene-4-ol was slightly concentrated because of the presence of methanol as a co-solvent. The results of fractional separation with supercritical carbon dioxide at 75 °C and 90 bar showed that the supercritical fluid at smaller pressure and higher temperature was more selective to α-pinene, sabinene, myrcene and limonene.

Several fractions obtained by vacuum distillation of the essential oil (F) and two fractions obtained from experiment during treatment of essential oil with supercritical carbon dioxide and mixture of carbon dioxide and methanol (SCF) were used for testing their potential antimicrobial activity. The compositions of the selected fractions are shown in Table II.

TABLE II. Composition of the fractions used to determine the antimicrobial activity obtained from juniper essential oil (EO1–Table I) (with an average deviation of ±0.15 wt %)

Components	Content wt %							
	F1	F2	F3	F4	F5	F6	SCF1[a]	SCF2[b]
α-Pinene	99.5	63.1	21.9	0.7	–	–	33.9	43.4
Sabinene	–	35.7	74.9	45.0	12.0	25.4	17.8	20.4
Myrcene	–	–	2.4	26.8	24.0	39.9	12.1	14.5
p-Cymene	–	–	–	25.8	52.4	33.1	2.3	3.1
D-Limonene	–	–	–	–	–	–	3.6	5.5
γ-Terpinene	–	–	–	–	–	–	1.7	0.9
Terpinolene	–	–	–	–	–	–	1.2	1.0
1-Terpinen-4-ol	–	–	–	–	–	–	8.6	1.9
α-Cubebene	–	–	–	–	–	–	0.8	0.7
α-Copaene	–	–	–	–	–	–	0.7	0.6
β-Elemene	–	–	–	–	–	–	0.8	0.7
β-Caryophyllene	–	–	–	–	–	–	3.2	2.2
α-Humulene	–	–	–	–	–	–	0.3	0.4
Germacrene-D	–	–	–	–	–	–	0.7	0.4
Bicyclogermacrene	–	–	–	–	–	–	1.6	1.2

[a]raffinate from experiment performed at 40 °C and 175 bar by supercritical CO_2 and 5 wt % of methanol as a co-solvent; [b]extract obtained at 75 °C and 90 bar by supercritical carbon dioxide

All the tested samples (Table II) demonstrated some antimicrobial activity, which is shown in Table III. Fractions, F1, F2, F3 and F4 showed distinct antimicrobial activity with a wide spectrum and wide inhibition zones. Fractions F5, F6 and SCF1 were least effective, with a narrow spectrum (affecting only few microorganisms used in the present study as indicators for antimicrobial activity) and with small inhibition zones. Juniper essential oil showed low antimicrobial activity with respect to almost all the investigated species. *B. cereus* was susceptible to all the tested samples, while *E. coli* and *S. aureus* were resistant only to fraction F6. *Corynebacterium* sp. and *P. aeruginosa* DV5999 were the least susceptible to all the oil samples and were completely resistant to juniper essential oil.

Samples F1, F2, F3, F4 and SCF2, as well as juniper essential oil (EO1), showed strong inhibitory effects to yeast and fungi. Fractions F5, F6, SCF1 did not affect these enkaryotic indicators. Comparison between fraction F6 (sabinene 25.4 %,

TABLE III. The diameter of the inhibition zone

Substance	Bacteria					Fungi			Yeast
	Gram-negative		Gram-positive						
	E. coli ATCC 8739	*P. aeruginosa* DV5999	*Corynebac.* sp. 754	*S. aureus* ATCC 6538	*B. cereus*	*Alternaria* sp.	*A. nidulans*	*A. niger*	*C. albicans*
	Inhibition zone/mm (with an average deviation of ± 0.1 mm)								
EO1	0.5	–	–	1.0	11.0	3.0	5.0	0.1	1.0
F1 (8.75 μg of α-pinene)	16.0	6.0	8.0	13.0	14.0	17.0	12.0	20.0	17.0
F2 (5.52 μg of α-pinene and 3.23 μg of sabinene)	12.0	5.0	5.0	12.0	10.0	15.0	14.0	20.0	11.0
F3 (8.75 μg)	6.0	2.0	1.0	4.0	5.0	10.0	23.0	13.0	6.0
F4 (8.75 μg)	3.0	1.0	0.5	1.5	3.0	2.5	5.0	1.0	2.0
F5 (8.75 μg)	1.0	–	1.0	1.0	1.5	–	–	–	–
F6 (8.75 μg)	–	–	–	–	0.5	–	–	–	0.5
SCF1 (8.75 μg)	2.0	–	–	1.0	1.0	–	–	–	–
SCF2 (8.75 μg)	2.0	–	–	0.5	7.0	1.5	9.0	–	1.5
Gentamycin	11.0	3.0	–	11.0	11.0	NA	NA	NA	NA
Tetracycline (30 IJ)	9.0	8.0	2.0	8.0	9.0	NA	NA	NA	NA
Erythromycin (15 μg)	11.0	7.0	2.0	12.0	12.0	NA	NA	NA	NA
Vancomycin (30 μg)	4.0	3.0	3.0	5.0	4.0	NA	NA	NA	NA
Clindamycin (10 μg)	5.0	1.0	–	6.0	5.0	NA	NA	NA	NA
Streptomycin (30 IJ)	12.0	2.0	–	12.0	10.0	NA	NA	NA	NA
Ampicillin (10 μg)	5.0	10.0	8.0	2.0	4.0	NA	NA	NA	NA
Penicillin G (6 μg)	2.0	3.0	3.0	1.0	1.0	NA	NA	NA	NA

(NA – not analysed)

myrcene 39.9 % and *p*-cymene 33.1) and fraction F5 (sabinene 12.0 %, myrcene 24.0 % and *p*-cymene 52.4) indicated that antimicrobial activity of fraction F5 derives from the influence of *p*-cymene, which is in agreement with published data.[23] The low content of α-pinene in fraction F6 was the reason for its lower antimicrobial activity, while fraction SCF1 had almost the same composition as the starting essential oil and hence, knowing the small activity of the essential oil[24–29] the same activity of this fraction might be expected, which was the case. *A. niger* was also resistant to the SCF2 fraction, despite the high concentration of α-pinene in this fraction. From the data of the diameter of the inhibition zone in the case of fungi, it can be concluded that sabinene had a significant influence on its growth, which is in accordance with published data.[30]

Investigations of the effects of terpenoids upon isolated bacterial membranes suggest that their activity is a function of the lipophilic properties of the constituent terpenes, the potency of their functional groups and their aqueous solubility.[16, 31] Their site of action appeared to be at the phospholipid bilayer, caused by a biochemical mechanism catalyzed by the phospholipid bilayers of the cell. These processes include the inhibition of electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reactions. Although a similar tendency of water solubility was observed, specific statements on the action of single terpenoids *in vivo* have to be assessed individually, taking into account not only the structure of the terpenoid, but also the chemical composition of the cell wall.[27] The plant extracts clearly demonstrate antibacterial properties, although the mechanistic processes are poorly understood.

Fractions F1 and F2, as the fractions with the largest inhibition zones in this study, were compared with several antibiotics (Table III). It can be seen that the two fractions showed the widest spectrum of inhibiton. Moreover, they inhibited all bacterial strains at a lower concentration than the investigated antibiotics. In case of *Corynebacterium* sp. 754, almost all the investigated antibiotics showed no inhibiting effects, but both fractions of essential juniper oil had strong inhibition effects at lower concentrations than ampicillin. The fractions F1 and F2 (α-pinene and sabinene) could be used as good conservation agents, but additional investigations need to be performed in order to confirm the safety of these concentrations (MIC) for human consumption. It seems that α-pinene and a mixture of α-pinene and sabinene could be used as an additional therapy, together with the antibiotics in order to increase their efficiency.

CONCLUSION

The fractions of *Juniperus communis* L. essential oil were obtained by fractional distillation under vacuum, as well as by the fractionation using supercritical carbon dioxide and methanol as co-solvent. The obtained fractions were tested for their antimicrobial activity against certain bacteria, yeast and fungi. It was establi-

shed that the fractions containing the high concentrations of α-pinene and sabinene effectively inhibited the growth of microorganisms. The most active fractions of *J. communis* L. essential oil were compared with antibiotics commonly used therapeutically. The fractions containing pure α-pinene and a mixture of α-pinene and sabinene successfully inhibited all bacterial strains especially the growth of fungi and yeast and they showed a wider spectrum of inhibition that the investigated commercial antibiotics.

ИЗВОД

АНТИМИКРОБНА АКТИВНОСТ ЕТАРСКОГ УЉА И РАЗЛИЧИТИХ ФРАКЦИЈА *Juniperus communis* L. И ПОРЕЂЕЊЕ СА НЕКИМ КОМЕРЦИЈАЛНИМ АНТИБИОТИЦИМА

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Етарско уље плода клеке *(Juniperus communis* L. из јужног дела Србије) добијено је хидродестилацијом у пилот постројењу (130 dm^3) а затим фракционисано у дестилационој колони, са 36 теоретских подова, под вакуумом (26–66 mbar). Етарско уље је такође фракционисано коришћењем чистог CO_2 и смеше CO_2 и метанола као косолвента под наткритичним условима. Девет различитих узорака (етарско уље и његове фракције различитих састава) као и комерцијални антибиотици коришћени су у тестирању антимикробне активности и инхибиторног утицаја на развој неких бактерија, квасаца и гљивица. Етарско уље клеке је показало слабу антимикробну активност на свим сојевима а фракције са чистим α-пиненом и смешом α-пинена и сабинена нарочито на раст гљивица. У поређењу са комерцијалним антибиотицима фракције етарског уља клеке показују широк спектар деловања као и веће зоне инхибиције.

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