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Chemical composition and antimicrobial activity of hydrodistilled oil from juniper berries

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ABSTRACT

This study aimed at investigating the chemical composition and in vitro antimicrobial activity of juniper (*Juniperus communis* L.) berries essential oils (EOs), including commercial samples as well as the oil hydrodistilled from berries grown in Portugal, for which few information is available in the literature. The analysis was performed by gas chromatography coupled to mass spectrometry detection (GC/MS) allowing the identification of a total of 97 compounds. The EOs showed different chemical profiles with only one being according to the European Pharmacopoeia 8 requirements. The laboratory-hydrodistilled EO was characterized by its high content in α-pinene (41.6%), followed by β-pinene (27.6%) and limonene (6.4%), commercial EO1 by α-pinene (31.1%), β-myrcene (16.3%) and sabinene (7.5%) and commercial EO2 by *δ*-cadinene (16.0%), α-pinene (12.2%) and sabinene (9.4%). The distinct chemical profiles were also evidenced by principal components analysis (PCA), with a clear separation of the evaluated EOs. One of the commercial samples, showed the presence of propachlor, a banned herbicide in the European Union. All the EOs showed relevant antimicrobial activity as they presented microbicidal activity against *Candida albicans* and at least six of the ten tested bacteria. Commercial EO2 showed a higher biological activity, as it was active against all tested microorganisms, which could be related to its higher content in sesquiterpenes, in particular those oxygenated. Overall, results support the use of *Juniper communis* L. berries EO as an antiseptic in traditional medicine and highlight its potential as a biopreservative that could be used in different industries.

1. Introduction

Essential oils (EOs) are highly complex mixtures of volatile compounds that are biosynthesized by plants to exert diverse ecological functions, such as acting as defensive substances against microorganisms and herbivores (Bakkali et al., 2008). Since ancient times, EOs have been used in traditional medicine for their various properties including spasmolytic, anti-inflammatory, antioxidant and antimicrobial activities (Lang and Buchbauer, 2012). Additionally, due to their generally pleasant odor and/or flavour, several EOs are currently required in significant amounts by different industries such as cosmetic, perfume, pharmaceutical and food industries (Raut and Karuppayil, 2014).

Recently, consumers are becoming increasingly concerned regarding the use of synthetic preservatives to extend the shelf life of foods and cosmetics. Therefore, there has been a renewed interest regarding the possibility of using plant essential oils as biopreservatives in such products, as some have been shown to possess strong antimicrobial activity against a wide range of bacteria (Burt, 2004; Kunicka-Styczynska et al., 2009; Silva et al., 2013; Sung et al., 2013; Seow et al., 2014). Currently, according to the US Federal Regulation 21CFR182.20, several EO formulations are considered in the category of Generally Recognized as Safe (GRAS) for their intended used, among which juniper oil is included (U.S. Code of Federal Regulations, 2017). Juniper (*Juniperus communis* L.) is a plant worldwide spread belonging to the Cypress family (Cupressaceae) that has been used along the history for many purposes, including in traditional medicine, in gastronomy as a spice and as a natural ingredient in cosmetic, pharmaceutical and food industries. Juniper berries is one of the few spices

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originated from cold climates, being used as a condiment to confer a particular aroma and taste to game meat dishes traditionally cooked in some European regions, such as in northern Scandinavia and in the northeast of Portugal. They are also used in the aromatization of traditional alcoholic beverages and in the production of gin, the most popular juniper-based spirit. According to the European legislation (Council Regulation EEC 1576/89, 1989), the main flavour in the "Distilled gin" class should come from juniper berries. Additionally, since ancient times juniper berries have also been used in folk medicine for its stomachic, diuretic, antiseptic and antirheumatic properties to treat dyspepsia, cystitis, arthritis, gout and other inflammations (Yarnell, 2002; Sela et al., 2011). Its diuretic effect, in particular, has been attributed to the presence of terpinen-4-ol (EMA, 2011; Sela et al., 2011). Juniper berries are inscribed in several Pharmacopoeias, including the 8th edition of the European Pharmacopoeia (Ph. Eur. 8), and are the source of juniper oil, which is also inscribed in the same Pharmacopoeia. The characteristic composition of the essential oil obtained by steam distillation from the ripe, non-fermented berry cones of *Juniperus communis* L. is described on the monograph Iuniperi aetheroleum, which defines the following requirements: 20–50% of α-pinene, 1–35.5% of myrcene, < 20% of sabinene, 2–12% of limonene, 1–12% of β-pinene, < 7% of *trans*-(*E*)-caryophyllene, 0.5–10% of terpinen-4-ol, < 2% of bornyl acetate and < 1% of α-phellandrene. Previous studies carried out on EOs extracted by hydrodistillation from juniper berries of diverse geographical origin, including Greece, Italy, Spain, Serbia, Kosovo, Algeria, Lithuania, Estonia, Macedonia and Slovakia, showed a noteworthy variation both on the qualitative and quantitative profile (Chatzopoulou and Katsiotis, 1993; Falasca et al., 2016; Fejér et al., 2018; Foudil-Cherif and Yassaa, 2012; Glišić et al., 2007; Hajdari et al., 2015; Lo[zbreve]ienė et al., 2010; Orav et al., 2010; Sela et al., 2011; Vichy et al., 2007). While α-pinene was consistently the major compound in most EOs (although presenting a wide variation of content, ranging from 13.4% to 77.4%), a higher variability was found regarding the other compounds present at higher contents. In this regard, the second most abundant compound was most frequently sabinene or β-myrcene, although for some EOs were β-phellandrene, terpinen-4-ol, α-pinene, germacrene D or *δ*-cadinene. In addition, the variability on the chemical composition of the EOs extracted from juniper berries was also evidenced by the fact that several did not comply with Ph. Eur. 8 requirements (Angioni et al., 2003; Chatzopoulou and Katsiotis, 1993; Falasca et al., 2016; Foudil-Cherif and Yassaa, 2012; Lo[zbreve]ienė et al., 2010; Matović et al., 2011; Orav et al., 2010; Vichy et al., 2007). This noteworthy variation among the qualitative and quantitative composition can be ascribed to several factors that are known to influence the chemical composition of plant EOs, such as environmental conditions (climate, soil composition, etc), harvesting period/maturation of the berries and extraction method, among others (Fejér et al., 2018). While several reports can be found in the literature regarding the analysis of the essential oil extracted from juniper berries using a Clevenger type apparatus, few information is found on the chemical composition of commercially available oils (Filipowicz et al., 2003; Höferl et al., 2014; Falasca et al., 2016) Additionally, there is a scarcity of data regarding the chemical composition of juniper EO obtained from wild berries grown in Portugal. Therefore, in this work the chemical composition of three different juniper berries essential oils, namely one obtained on the laboratory by hydrodistillation from juniper berries grown in Portugal and two commercially acquired, was evaluated and compared. Considering that the antimicrobial/antiseptic activity is one of the main bioactive properties described for juniper berries EO, in this work, the antimicrobial activity against several pathogenic and food-spoiling bacteria and one yeast was further assessed, in view of its potential use as a biopreservative.

2. Materials and methods

2.1. Samples

Dried and mature berries of *Juniperus communis* L. were acquired in 2016 from a Portuguese supplier (Alma d'Flor, Almada, Portugal; the berries were collected in the wild, in the center region of Portugal in 2016, according to the supplier). The berries were used for essential oil extraction by hydrodistillation in a Clevenger apparatus in accordance with the description of the European Pharmacopoeia (1996). Briefly, grounded berries (50 g) were placed in a round-bottom flask with 500 mL of distilled water and the mixture was boiled during 3 h. The essential oil was separated from the water and recovered directly without adding any solvent. After being collected, the oil was dried over anhydrous sodium sulfate and stored at −20 °C until being analyzed. Additionally, two commercial essential oils from juniper berries, both labelled as being from *J. communis* berries, were tested in this study, one obtained from the same herbal shop of the berries (Alma d'Flor, Portugal; obtained by hydrodistillation according to the supplier) designated as commercial EO1, and the other from a Portuguese distributor (Dias e Beltrame, Portugal; no information was available regarding extraction method used) designated as commercial EO2.

2.2. GC–MS analysis

The GC–MS unit consisted on a Perkin Elmer Perkin Elmer system (GC Clarus® 580 GC module and Clarus® SQ 8 S MS module) gas chromatograph, equipped with DB-5 MS fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 6.1.0, Perkin Elmer, Shelton, CT, USA). Oven temperature was programmed, 45–175 °C, at 3 °C.min^{-1}, subsequently at 15 °C min^{-1} up to 300 °C, and then held isothermal for 10 min; injector temperature, 280 °C and the injection volume of 1 μL. The transfer line temperature was 280 °C; ion source temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 cm s^{-1}; split ratio, 1:40; ionization energy, 70 eV; scan range, 40–300 u; scan time, 1 s. Identifications were based on the comparison of the obtained spectra with those of the NIST 2011 mass spectral library and were confirmed using linear retention indices determined from the retention times of an n-alkane (C7–C40) mixture analyzed under identical conditions, with comparison with published data (Adams, 2007), and when possible with commercial standard compounds.

Compounds were quantified as area percentages of total volatiles using the relative values directly obtained from peak total ion current (TIC). Analysis were performed in triplicate.

2.3. Antimicrobial activity

The three essential oils were individually tested against 10 different bacterial strains and 1 yeast, namely *Bacillus cereus* (NCTC 10,320), *Bacillus subtilis* (ATCC 6633), *Enterobacter aerogenes* (ATCC 13,048), *Enterococcus faecalis* (ATCC 33,186), *Escherichia coli* (ATCC 10,536), *Klebsiella pneumoniae* (ATCC 13,883), *Proteus mirabilis* (ATCC 14,153), *Pseudomonas aeruginosa* (ATCC 27,853), *Salmonella typhimurium* (ATCC 14,028), *Staphylococcus aureus* (ATCC 29,213) and the yeast *Candida albicans* (ESAB collection). The antimicrobial activity was determined by the broth macrodilution method, based on the methodology described by the Clinical and Laboratorial Standards Institute (CLSI, 2009) with some modifications. Briefly, bacterial suspensions were prepared in Mueller-Hinton broth (MHB) for bacteria or in Yeast Extract Peptone Dextrose broth (YPD) for the yeast, from 24-hour cultures in nutrient agar for bacteria or Sabouraud dextrose agar for the yeast, by adjusting to 0.5 McFarland turbidity standard followed by dilution to approximately 5 × 10^5 CFU/mL. The essential oil was subjected to two-fold serial dilution with MHB added with 0.5% Tween 80 (v/v) in

sterile glass test tubes. Each test tube, containing the same final volume with different concentration of essential oil, was added with the same volume of bacterial suspension (1 mL), obtaining a final concentration of essential oil ranging from 25 μL mL^{-1} to 0.39 μL mL^{-1} (corresponding to 2.5%–0.039%, v/v). A blank and negative control were prepared by adding only the inoculum and culture media to MHB for the bacteria or YPD for the yeast added with 0.5% Tween 80 (v/v). Different antibiotics were used as positive control, namely ampicillin and imipenem for Gram-negative bacteria, and ampicillin for Gram-positive bacteria. After incubation at 37 °C for 24 h, the minimum inhibitory concentration (MIC) was determined as the lowest concentration of the essential oil inhibiting visible bacterial growth. To determine the minimum bactericidal concentration (MBC), a loopful of sample from each test tube was sub-cultured in nutrient agar plates and again incubated at 37 °C for bacteria or 30 °C for the yeast, for 24 h. The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) was defined as the concentration of essential oil able to kill all bacteria or yeast, respectively, in the inoculum, translated in the absence of growth after sub-culturing on a medium without antibiotics or antifungal agent. The MIC, MBC or MFC was determinate at least in duplicate for each oil and microorganism.

2.4. Statistical analysis

Principal component analysis (PCA) was applied to check the linear separability of data and detect the most important variables (chemical compounds identified in juniper berries essential oils) that are participating on the data variance. A scatter plot where the data is projected on the two principal components was further obtained. Overall, 97 variables corresponding to the essential oil components of the three evaluated samples were used in PCA. The PCA and the construction of the correspondent biplot was performed using the Python package scikit-learn: machine learning, version 0.19.1.

3. Results and discussion

3.1. Chemical composition

The results obtained for the chromatographic analysis of juniper berries EOs are presented on Table 1, where compounds are listed according to their order of elution (Fig. 1). GC–MS analysis enabled the identification of 99.2%–99.9% of the compounds, corresponding to a total of 97 identified compounds, namely 64 and 65 in the two commercial EOs and 44 in the laboratory-hydrodistilled oil. In general, the chemical composition of the three evaluated oils differed both qualitatively and quantitatively (Fig. 1). The hydrodistilled EO, extracted in the laboratory using a Clevenger apparatus, presented α-pinene as major component (41.6%), followed by β-pinene (27.6%), limonene (6.4%), β-myrcene (5.7%) and trans-pinocarveol (1.9%), therefore not being in compliance with Eur. Ph. 8 monography due to the high content of β-pinene (> 12%). In general, the sample of EO obtained from Portuguese juniper berries presented a distinct profile when compared with data reported in the literature for juniper berries with different geographical origins. One of the most marked differences concerns the content of β-pinene and sabinene, which were very high and very low, respectively, when compared with previous data (Angioni et al., 2003; Foudil-Cherif and Yassaa, 2012; Glišić et al., 2007; Matović et al., 2011). However, considering only the qualitative profile, the Portuguese berries presented some similarities with juniper berries from Kosovo, in terms of their major components (Haziri et al., 2013). By the contrary, the chemical composition was very different from the one previously reported for juniper berries collected in the central region of Portugal (Cavaleiro et al., 2006) that presented β-phellandrene and α-terpinyl acetate among the major compounds, those being absent in the herein evaluated sample. The observed differences can be attributed to a range of different factors, namely the geographical origin of the berries (with the inherent differences on climatic conditions and soil characteristics), the collection year, extraction method, but also to genetic factors since *J. communis* is known to include three closely related subspecies: *communis*, *hemisphaerica* (C. Presl) Nyman and *alpina* (Suter) Čelak (also known as *nana* or *saxatilis*) (Franco, 1986). This can be an important aspect because different chemotypes have already been described to occur inside the same species for other aromatic and/or medicinal plants (Haghighi et al., 2018).

Commercial EO1, acquired from an herbal shop, was mainly constituted by α-pinene (31.1%), β-myrcene (16.3%), sabinene (7.5%), limonene (6.2%) and β-pinene (3.7%) (Table 1) and was the only one that fully complied with the requirements of the Eur. Ph. 8 monography. Although the total number of identified compounds was similar for both commercial samples, commercial EO2 evidenced a very distinct profile, presenting as main compounds *δ*-cadinene (16.0%), α-pinene (12.2%), sabinene (9.4%), β-pinene (7.7%) and γ-cadinene (6.3%). This EO was not according to Eur. Ph. 8 since the levels of α-pinene, β-myrcene and limonene were lower than the required minimum content (Table 1). The chemical profile of this sample was somehow uncommon due to the unusual content of some compounds, such as the very low level of β-myrcene (0.9%) and the considerably high content of *δ*-cadinene (16.0%) and γ-cadinene (6.3%). However, the identification of *δ*-cadinene as one of the five major components in juniper berries EO has been previously described in oils obtained from commercial berries from Spain (Vichy et al., 2007) and from Serbia (Matović et al., 2011; Vasilijević et al., 2018), even though the reported levels were slightly lower (5.2% to 10.7%). As mentioned, this uncommon profile can be related to the origin of the berries (geographical and/or genetic). Nevertheless, considering the low content of α-pinene and β-myrcene, and slightly high of terpinen-4-ol, one cannot exclude the possibility of other juniper materials, such as the needles (leaves), being used in the production of this oil. Angioni et al. (2003) studied the chemical composition of the EO hydrodistilled from the leaves, ripe and unripe berries of *J. communis* spp. *communis* and found that the α-pinene and β-myrcene content of the leaves (6.4% and 2.6%, respectively) was much lower than that of the berries (52.3–52.9% and 8.1–15.3%, respectively), while the opposite was observed for terpinen-4-ol (10.7% in the leaves *vs*. 1.1–1.5% in the berries). Similar results were reported for the EO of *J. communis* needles from Algeria (Dahmane et al., 2016) and from the needles of the subspecies *alpina* collected in Portugal (Cabral et al., 2012). Among the three oils, commercial EO2 was the one that presented the higher content of terpinene-4-ol. As mentioned, this compound is thought to exert diuretic activity through an irritative action on the kidney tissue, therefore supporting the therapeutic value of juniper berries as a urology remedy (Stanić et al., 1998; EMA, 2011). Another relevant aspect in commercial EO2 concerns the identification of propachlor, an herbicide banned in the European Union since September 2008, although plant protection products containing propachlor could remain available until 18 months from the adoption of the decision (Commission Decision, 2008/742/EC). Despite concerns to the environment and toxicity to birds, mammals and earthworms (European Commission Health & Consumer Directorate-General SANCO/1350/08), the use of this herbicide is still allowed in China, having an approved maximum residue limit for rice of 0.05 mg/kg (China Food and Drug Administration, 2016). Recently, this herbicide was detected on pickling cucumbers produced in Brazil despite not being included in the list of approved substances by the Brazilian Agency ANVISA (Neto and Gonçalves, 2016). Regardless of being banned in Europe, a metabolite of this herbicide (propachlor oxanilic acid) was found in the urine of 8 pregnant women that participated in a EC-funded project conducted from January 2012-December 2015 in Spain and Slovakia (López et al., 2016). In the present work, neither the origin of commercial EO2 or the juniper berries used in its production is known.

To verify whether any of the evaluated samples could be grouped according to their chemical composition, PCA was applied. PCA

Table 1
Composition of *Juniperus communis* essential oils (commercial and wild berries' hydrodistilled oils).

Peak number	Compound	RT (min)	LRI[a]	LRI[b]	Juniper berries EO (relative %) [d]		
					Commercial 1	Commercial 2	Hydrodistilled
1.	Tricyclene	6.370	917	921	0.099 ± 0.003	–	0.157 ± 0.001
2.	α-Thujene	6.560	922	924	1.35 ± 0.12	0.50 ± 0.03	0.54 ± 0.07
3.	α-Pinene	6.950	934	932	31.4 ± 0.6	12.16 ± 0.27	41.64 ± 0.85
4.	Camphene	7.300	944	946	0.42 ± 0.04	0.114 ± 0.001	1.308 ± 0.003
5.	Thuja-2,4(10)-diene	7.420	947	953	0.13 ± 0.01	–	0.069 ± 0.001
6.	Sabinene	8.190	969	969	7.55 ± 0.20	9.37 ± 0.09	0.309 ± 0.001
7.	β-Pinene	8.313	973	974	3.68 ± 0.07	7.75 ± 0.15	27.63 ± 0.22
8.	Myrcene	8.940	990	988	16.5 ± 0.7	0.94 ± 0.04	5.71 ± 0.18
9.	2-Carene	9.065	994	1001	–	0.053 ± 0.001	–
10.	α-Phellandrene	9.363	1002	1002	0.07 ± 0.01	0.078 ± 0.004	–
11.	3-Carene	9.450	1004	1008	0.12 ± 0.01	0.017 ± 0.001	0.47 ± 0.03
12.	1,4-Cineole	9.730	1011	1012	–	–	0.082 ± 0.001
13.	α-Terpinene	9.783	1012	1014	0.43 ± 0.03	0.73 ± 0.02	–
14.	*p*-Cymene	10.120	1020	1020	0.60 ± 0.02	3.35 ± 0.30	1.15 ± 0.02
15.	Limonene	10.380	1026	1024	6.25 ± 0.04	1.73 ± 0.10	6.42 ± 0.31
16.	1,8-Cineole (eucalyptol)	10.448	1027	1026	–	0.229 ± 0.002	0.047 ± 0.001
17.	*trans*-β-Ocimene	10.623	1032	1032	0.16 ± 0.02	0.058 ± 0.001	0.208 ± 0.004
18.	γ-Terpinene	11.500	1052	1054	0.75 ± 0.04	2.10 ± 0.04	–
19.	*cis*-Sabinene hydrate	12.058	1065	1065	0.17 ± 0.01	0.207 ± 0.001	–
20.	Terpinolene	12.653	1079	1086	0.75 ± 0.02	0.82 ± 0.02	–
21.	α-Pinene oxide	13.231	1093	1095[c]	–	0.066 ± 0.001	1.19 ± 0.03
22.	*trans*-Sabinene Hydrate	13.406	1097	1098	–	0.169 ± 0.001	–
23.	Linalool	13.420	1097	1098 [c]	0.42 ± 0.01	–	–
24.	*trans*-2-Caren-4-ol	13.773	1105		–	–	1.39 ± 0.01
25.	*cis*-*p*-Menth-2-en-1-ol	14.438	1120	1118	0.0826 ± 0.0011	–	–
26.	α-Campholenal	14.526	1122	1122	0.64 ± 0.15	–	0.30 ± 0.01
27.	*cis*-Limonene oxide	14.771	1127	1132	–	–	0.104 ± 0.005
28.	*trans*-Pinocarveol	15.120	1135	1135	0.62 ± 0.02	0.097 ± 0.001	1.89 ± 0.04
29.	*cis*-Verbenol	15.226	1137	1137	0.25 ± 0.01	–	0.078 ± 0.001
30.	*trans*-*p*-Menth-2-en-1-ol	15.261	1138	1142 [c]	–	0.22 ± 0.01	–
31.	*trans*-Verbenol	15.440	1142	1144 [c]	0.89 ± 0.04	0.101 ± 0.002	1.07 ± 0.04
32.	*trans*-Pinocamphone	15.979	1153	1158	0.13 ± 0.01	–	0.10 ± 0.01
33.	Pinocarvone	16.066	1155	1160	0.115 ± 0.003	–	0.44 ± 0.01
34.	Terpinen-4-ol	16.990	1176	1174	2.00 ± 0.09	5.43 ± 0.07	0.16 ± 0.01
35.	*p*-Cymen-8-ol	17.326	1183	1179	0.17 ± 0.02	0.22 ± 0.01	0.217 ± 0.001
36.	Myrtenal	17.571	1188	1193 [c]	0.20 ± 0.01	0.014 ± 0.001	0.79 ± 0.06
37.	α-Terpineol	17.680	1191	1194 [c]	0.68 ± 0.05	0.50 ± 0.01	–
38.	Myrtenol	17.729	1192	1196 [c]	–	–	1.38 ± 0.03
39.	Verbenone	18.149	1201	1204 [c]	0.28 ± 0.01	0.028 ± 0.001	0.32 ± 0.01
40.	*trans*-Carveol	18.744	1214	1215	0.19 ± 0.01	–	0.220 ± 0.004
41.	Citronellol	19.234	1225	1223	0.29 ± 0.01	–	–
42.	*cis*-Carveol	19.339	1228	1228 [c]	–	–	0.050 ± 0.001
43.	Thymol, methyl ester	19.584	1233	1232	–	0.22 ± 0.01	0.03 ± 0.04
44.	Carvone	20.022	1243	1239	0.025 ± 0.003	–	0.09 ± 0.01
45.	Citronellic acid, methyl ester	20.547	1252		0.26 ± 0.01	–	–
46.	Bornyl acetate	21.614	1278	1284 [c]	0.49 ± 0.06	0.157 ± 0.002	0.149 ± 0.004
47.	Anethole	21.719	1280	1287 [c]	–	0.42 ± 0.01	–
48.	2-Undecanone	22.122	1289	1291 [c]	0.19 ± 0.01	–	–
49.	1,4-Dihydroxy-*p*-menth-2-ene	22.122	1289		–	0.170 ± 0.002	–
50.	Isocarveol	22.332	1294	1286 [c]	–	–	0.081 ± 0.002
51.	Carvacrol	22.472	1297	1298	–	0.044 ± 0.002	–
52.	δ-Elemene	23.627	1322	1325 [c]	0.096 ± 0.001	0.018 ± 0.002	–
53.	α-Cubebene	24.309	1339	1341 [c]	0.99 ± 0.05	0.32 ± 0.01	0.026 ± 0.001
54.	8-*p*-Menthen-1,2-diol	24.415	1341		–	–	0.039 ± 0.001
55.	α-Copaene	25.482	1366	1367 [c]	0.54 ± 0.04	0.49 ± 0.02	0.037 ± 0.001
56.	Propachlor	26.042	1378		–	0.15 ± 0.01	–
57.	β-Elemene	26.147	1381	1383 [c]	0.98 ± 0.08	0.36 ± 0.01	0.269 ± 0.003
58.	Longifolene	26.812	1396	1407	0.10 ± 0.01	–	–
59.	α-Cedrene	27.075	1402	1410	–	0.140 ± 0.004	0.263 ± 0.001
60.	β-Caryophyllene	27.372	1409	1417	3.60 ± 0.10	0.70 ± 0.03	–
61.	β-Cedrene	27.407	1410	1419	–	0.045 ± 0.001	0.07 ± 0.01
62.	β-Cubebene	27.757	1418	1418 [c]	0.25 ± 0.35	–	–
63.	γ-Elemene	27.880	1421	1433 [c]	2.50 ± 0.22	0.89 ± 0.03	0.028 ± 0.001
64.	*cis*-Thujopsene	27.932	1423	1425 [c]	–	0.39 ± 0.01	–
65.	α-Bergamotene	28.002	1424	1430 [c]	–	–	–
66.	α-Humullene	28.860	1445	1452	2.09 ± 0.02	0.27 ± 0.01	–
67.	β-Farnesene	28.983	1448	1456	0.81 ± 0.01	–	–
68.	epi-Bicyclosesquiphellandrene	29.122	1451		–	0.76 ± 0.03	–
69.	γ-Muurolene	29.718	1465	1465 [c]	0.351 ± 0.002	1.92 ± 0.07	0.153 ± 0.001
70.	Germacrene D	29.963	1471	1468 [c]	3.35 ± 0.46	–	–
71.	β-Selinene	30.208	1477	1475 [c]	0.39 ± 0.02	–	–

(*continued on next page*)

Table 1 (*continued*)

Peak number	Compound	RT (min)	LRI[a]	LRI[b]	Juniper berries EO (relative %) [d]		
					Commercial 1	Commercial 2	Hydrodistilled
72.	α-Muurolene	30.663	1488	1488 [c]	0.30 ± 0.03	5.10 ± 0.19	0.053 ± 0.001
73.	γ-Cadinene	31.223	1502	1508 [c]	0.364 ± 0.003	6.27 ± 0.23	0.181 ± 0.001
74.	δ-Cadinene	31.503	1508	1515 [c]	1.42 ± 0.08	16.04 ± 0.46	–
75.	α-Cadinene	32.290	1528	1537	–	1.72 ± 0.03	–
76.	Cadala-1(10),3,8-triene	32.413	1531		–	0.194 ± 0.004	–
77.	Elemol	32.710	1539	1541 [c]	0.106 ± 0.004	5.38 ± 0.21	0.334 ± 0.003
78.	α-Calacorene	33.218	1552	1553 [c]	–	0.212 ± 0.002	–
79.	*trans*-Nerolidol	33.375	1556	1554 [c]	–	0.196 ± 0.001	–
80.	Spathulenol	33.761	1565	1577	0.59 ± 0.05	0.119 ± 0.001	–
81.	Caryophyllene oxide	33.918	1569	1582	1.02 ± 0.10	0.22 ± 0.01	0.313 ± 0.004
82.	Gleenol	34.233	1577	1586	–	0.054 ± 0.001	–
83.	Cedrol	34.916	1595	1600	–	0.198 ± 0.003	1.33 ± 0.03
84.	Humullene oxide	34.986	1596		0.58 ± 0.04	–	–
85.	epi-Cubenol	35.721	1616	1621 [c]	0.19 ± 0.01	0.45 ± 0.01	–
86.	γ-Eudesmol	35.948	1622	1630	–	0.93 ± 0.03	–
87.	τ-Cadinol	36.263	1630	1634 [c]	0.095 ± 0.001	2.77 ± 0.10	0.050 ± 0.001
88.	τ -Muurolol	36.368	1633	1635 [c]	0.13 ± 0.01	2.04 ± 0.08	0.038 ± 0.001
89.	Cubenol	36.473	1636	1644 [c]	0.07 ± 0.01	–	–
90.	β-Eudesmol	36.683	1641	1645 [c]	–	–	0.077 ± 0.002
91.	δ-Cadinol	36.788	1644	1646 [c]	0.275 ± 0.004	0.68 ± 0.02	–
92.	α-Cadinol	36.893	1647	1649 [c]	0.18 ± 0.01	2.63 ± 0.09	0.17 ± 0.01
93.	5-Hydroximethyl-1,1,4,6-trimethyl-6-methylenedecahydronaphtalen-2-ol	39.676	1721		–	0.16 ± 0.01	–
94.	Labda-8(20)-12,14-triene	45.420	1916		0.065 ± 0.005	0.122 ± 0.001	–
95.	Geranyl-α-terpinene	45.784	1940		0.090 ± 0.007	–	0.77 ± 0.01
96.	Verticiol	46.292	1975		0.101 ± 0.009	–	–
97.	Abietatriene	47.115	2043	2055	0.082 ± 0.005	–	–
	Total identified				99.2	99.9	99.6
	Monoterpene hydrocarbons				70.4	40.1	85.7
	Oxygen-containing monoterpenes				7.5	7.7	10.1
	Sesquiterpene hydrocarbons				18.1	36.0	1.1
	Oxygen-containing sesquiterpenes				3.2	15.8	2.3
	Diterpene hydrocarbons				0.2	0.1	0.8
	Oxygenated diterpenes				0.1	–	–
	Others				0.2	–	–
	Herbicides				–	0.1	–

[a]LRI, linear retention index determined on a DB-5 MS fused silica column relative to a series of n-alkanes (C7–C40).
[b]linear retention index reported in literature (Adams, 2007), with the exception of those marked as [c] which refers to NIST 2011.
[d]relative % is given as mean ± SD, $n = 3$.

transforms the original measured variables (totality of chemical compounds identified in juniper berries essential oils) into a smaller number of new uncorrelated variables (principal components or factors) that adequately summarized the original information. Fig. 2 shows the obtained biplot of component loadings, where it can be observed that the juniper berries EOs were completely separated on three groups by the two principal components. The first principal component (PC1), explaining 73.0% of data variance, allowed the linear separation of the samples. Commercial EO2 was represented on the negative region of PC1, being characterized by an higher content of *δ*-cadinene and sabinene and low of α-pinene, while the opposite was verified for laboratory-hydrodistilled EO, being represented on the positive region of PC1. The second principal component (PC2), explaining 23.0% of data variance, allowed the clear separation of commercial EO1 mainly due to its higher content of β-pinene and lower of β-myrcene (Fig.2).

3.2. Antimicrobial activity

The results obtained for the antimicrobial activity of the essential oils against the tested strains are shown in Table 2. In general, all the tested EOs showed interesting bioactive properties as they presented microbicidal activity against *C. albicans* and at least six of the ten tested bacteria. The tested juniper berries EOs showed to be more active against Gram-positive (MIC, 0.08-0.63% and MBC, 0.08–1.25%) than Gram-negative bacteria (MIC, 0.16–1.25% and MBC, 0.31–2.5%, with some strains being resistant at the higher tested concentration). Among the tested strains, *B. cereus* and *B. subtilis* (MBC, 0.16%) were the most susceptible bacteria, being sensitive to all tested EOs and showing the lowest MICs (0.08-0.16% for both bacteria) and MBC (0.08-0.16% and 0.16%, respectively). On the other hand, *E. aerogenes*, *P. aeruginosa* and *S. typhimurium* were the least susceptible, with only one EO being active against these bacteria at the tested concentrations. This is in good agreement with previous studies on the antimicrobial activity of juniper berries EO (Filipowicz et al., 2003; Haziri et al., 2013), as well as with data available in the literature that describe Gram-positive bacteria has being more susceptible to essential oils than Gram-negative bacteria (Burt, 2004; Silva et al., 2013), possibly due to the presence of lipoproteins and lipopolysaccharides in Gram-negative bacteria that form a barrier to hydrophobic compounds (Mann et al., 2000).

While the laboratory-hydrodistilled EO and commercial EO1 did not evidenced bactericidal activity towards 3 and 4 bacteria strains, respectively, all tested bacteria were sensitive to commercial EO2 (Table 2). In light of the differences in chemical composition and the PCA results, that evidenced three distinct groups, it was expected that the three EOs would have different antimicrobial potential. However, commercial EO1 and the hydrodistilled EO presented similar antimicrobial activity while a stronger activity was found for commercial EO2. Table 1 shows that, compared with the other EOs, commercial EO2 presented a considerably higher content of sesquiterpene hydrocarbons (36.0%) and oxygenated sesquiterpenes (15.8%), which could

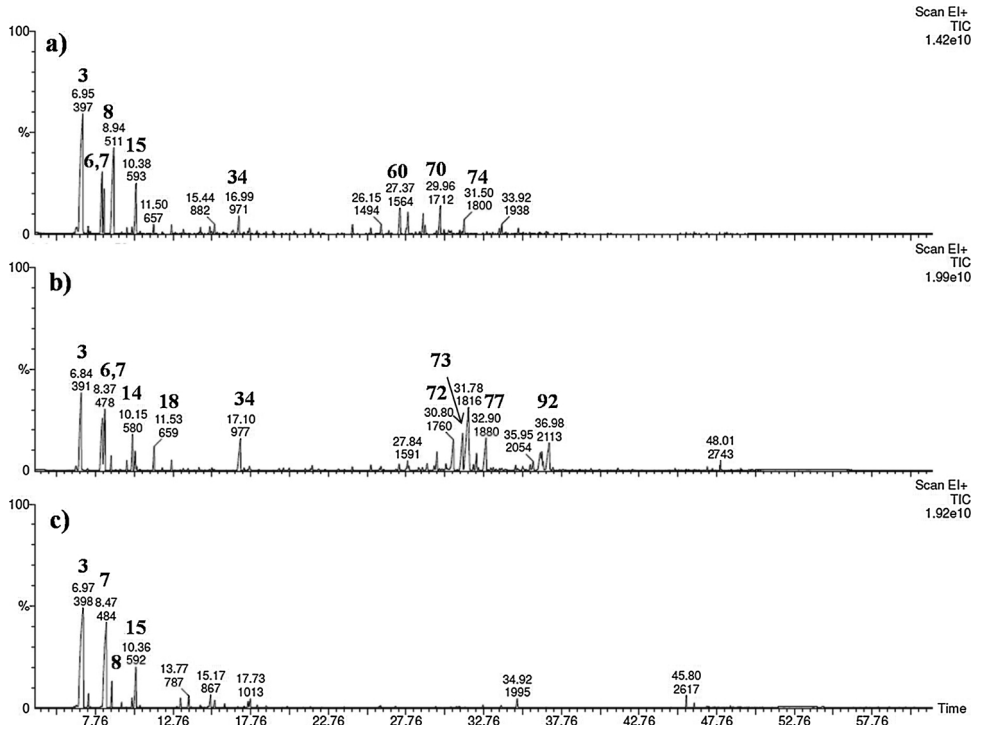

Fig. 1. Gas chromatography-mass spectrometry (GC–MS) profiles of juniper berries essential oil: (A, B) commercial samples and (C) laboratory-hydrodistilled sample. The major compounds are numbered as presented in Table 2.

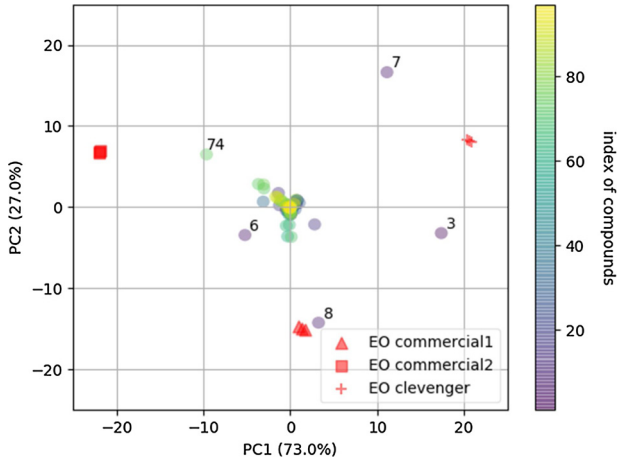

Fig. 2. Principal components analysis (PCA) biplot showing the linear separability of objects (juniper berries essential oils) and major component loadings (evaluated compounds; the index of the compounds is shown on a color scale).

explain its superior activity. However, considering that the content of sesquiterpene hydrocarbons was also much higher in commercial EO1 (18.1%) than in the hydrodistilled EO (1.1%), but both EOs showed similar biological activity, the contribution of this class to the overall activity of the oils is probably scarce. Conversely, the obtained results suggest that the class of oxygenated sesquiterpenes largely contribute to the antimicrobial activity of the assayed EOs. This is in good agreement with previous studies that associated sesquiterpene alcohols, such as elemol, α- and β-eudesmol, to enhanced antimicrobial activity (Sadgrove et al., 2014). Nonetheless, compounds of the other classes, such as monoterpenes hydrocarbons and oxygenated monoterpens, can also play an active role and synergistically contribute to the biological activity of juniper berries EOs (Glišić et al., 2007). Using transmission electron microscopy, Peruč et al. (2018) confirmed the inhibitory effect of juniper EO against *Mycobacterium* spp. by revealing significant

Table 2
Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of juniper berries essential oils.

Microorganism	Essential oil (%, v/v) [a]					
	Commercial 1		Commercial 2		Hydrodistilled	
	MIC	MBC	MIC	MBC	MIC	MBC
Gram positive						
Bacillus cereus	0.08	0.16	0.08	0.08	0.16	0.16
Bacillus subtilis	0.16	0.16	0.08	0.16	0.16	0.16
Staphylococcus aureus	0.63	1.25	0.16	0.31	0.16	0.31
Gram negative						
Escherichia coli	0.31	0.63	0.31	0.31	1.25	2.5
Enterococcus faecalis	0.31	0.63	0.31	0.31	0.31	0.63
Enterobacter aerogenes	-[b]	-[c]	1.25	2.5	-[b]	-[c]
Klebsiella pneumonia	1.25	2.5	0.16	0.63	0.31	1.25
Proteus mirabilis	-[b]	-[c]	0.16	0.16	0.63	1.25
Pseudomonas aeruginosa	-[b]	-[c]	1.25	1.25	-[b]	-[c]
Salmonella typhimurium	-[b]	-[c]	0.63	1.25	-[b]	-[c]
Yeast	MIC	MFC	MIC	MFC	MIC	MFC
Candida albicans	0.16	0.31	0.039	0.08	0.16	0.31

[a] Essential oils were tested in the concentration range of 2.5% to 0.039% (v/v).

[b] No inhibition was visually observed for the maximum tested concentration (2.5%).

[c] Growth was obtained for the maximum tested concentration (2.5%).

morphological changes in the cell membrane and cytoplasm, and leakage of intracellular material. This effect could be due to monoterpene hydrocarbons that can easily pass through lipid bilayers and cause damages in the cell. Also, according to previous studies, antimicrobial activity has already been described for some oxygenated monoterpenes, such as terpinen-4-ol and 1,8-cineole (Carson and Riley, 1995). Thus, the higher content of these compounds in commercial EO2 can also contribute to explain its higher antimicrobial activity.

4. Conclusions

The three EOs from juniper berries studied in this work showed distinct profiles and only one presented a composition that fulfilled the requirements of Ph. Eur. 8. The identification of a banned herbicide in one commercial EO together with the large variation on the chemical composition observed between the two commercial oils highlights the importance of quality control/monitoring of EO composition.

Overall results also showed that juniper berries EO have antimicrobial activity against several pathogenic bacteria and *C. albicans*, with one commercial EO presenting microbicidal activity against all tested microorganisms, including *P. aeruginosa*. This is particularly interesting considering the increasing levels of resistance of this bacteria towards multiple classes of antibiotics. The stronger activity of commercial EO2 can possibly be related to a higher amount of oxygenated sesquiterpenes, such as elemol. The obtained results support the use of the essential oil from *Juniper communis* L. berries in traditional medicine for its antimicrobial activity and highlight its potential as an interesting biopreservative for different industries.

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