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GC-MS analysis of the essential oils of *Juniperus communis* L. berries growing wild in the Molise region: Seasonal variability and *in vitro* antifungal activity

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ABSTRACT

Juniperus communis L., also known as the common juniper, is a dioecious aromatic evergreen shrub and has been traditionally used in many countries as a diuretic, antiseptic, and digestive and as a flavor to aromatize certain alcoholic beverages. We analyzed the chemical variability in the volatile profiles from berries of *J. communis,* harvested in one of the oldest European parks, the National Park of Abruzzo, Lazio, and Molise (PNALM, Central Italy). We examined the berries in different phases of the biological cycle for 1 year (at six ripening stages). Hydrodistilled essential oils from the fresh berries were analyzed by gas chromatography–flame ionization detection (GC-FID), gas chromatography–mass spectrometry (GC-MS) and principal component analysis (PCA). A total of 90 components were detected, and remarkable qualitative and quantitative differences were observed in the chemical components during the ripening stages, from the green unripe berries to the bluish-black berries harvested at full maturity. The essential oils were an α-pinene (13.43–32.34%) chemotype. The monoterpene hydrocarbons decreased during the ripening with a progressive increase in sesquiterpenes such as germacrene D (12.29–17.59%) and β-caryophyllene (7.71–8.51%), which are the major components in ripe berry essential oils. The sesquiterpene hydrocarbon fraction (65.3–47.9%) also contained α-humulene, germacrene B, δ-cadinene, bicyclogermacrene, and eudesma 4(14),11 diene. Germacrene D and β-caryophyllene in high concentrations may be considered as marker components of the genus *Juniperus* from the Molise region. This particular chemical composition has been reported for the first time. It is interesting to note the presence of β-caryophyllene (7–11%), whose inhalation has been reported to affect anxiety and depression in a rat model. An *in vitro* antifungal assay showed that the essential oil from green and ripe berries inhibits the growth of *Sclerotium rolfsii*, a phytopathogen fungus that causes post-harvest diseases in many fruits and vegetables.

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1. Introduction

The genus *Juniperus* comprises about 70 species that are widely distributed throughout the northern hemisphere. *Juniperus communis* L. is a dioecious aromatic evergreen shrub belonging to the Cupressaceae family with simple stiff green leaves arranged in whorls of three and berry-like seed cone fruits. These fruits have straight peduncles and a globose to ovoid shape (of 6–13 mm); the berries contain elongated tubercles, which act as reservoirs of volatile oils. Common juniper berries take 2 years to ripen; thus, the green berries of the first year and the blue berries of the second year occur on the same plant (Acta Plantarum, 2007).

Currently, the berries and leaves are widely used in many countries as diuretics, antiseptics, and digestives in knowledge-based medicine systems. The essential oils, infusions, decoctions, and alcoholic extracts exhibit diverse pharmacological features, including anti-inflammatory, hypoglycemic (Sanchez de Medina et al., 1994), antioxidant (Miceli et al., 2009), antimicrobial (Angioni et al., 2003), antifungal (Filipowicz et al., 2003), and memory-enhancing effects (Cioanca et al., 2014). Most of the studies reported in the literature are focused on the determination of the chemical composition of the essential oils from different plant organs of *J. communis* (e.g., the needles, berries, and wood) (Angioni et al., 2003; Filipowicz et al., 2003; Lawrence, 2001). *Juniperus* berries are also widely used as a raw material as a flavor to aromatize certain alcoholic beverages; in the perfumery, cosmetics, and pharmaceutical industries, because of their characteristic rich, fresh coniferous odor with a faint “fruitiness” and balsamic aroma; and as a spice, because of their slightly bitter taste.

The chemical composition of *J. communis* essential oils has a wide range of variability depending on several factors: the geographical origin of the plants, the maturity stage, age of the shrub, the climate conditions, the seasonal variations, and the amount of exposure to sunlight (Angioni et al., 2003).

In Mediterranean countries, unripe and ripe berry essential oils are mostly of an α-pinene chemotype (Angioni et al., 2003; Lawrence, 2001; Adams, 2000) with an α-pinene content ranging from 27% in samples from Greece (Koukos and Papadopoulou, 1997) 38% in samples from Montenegro (Damjanovic et al., 2006), 43% in samples from Macedonia (Sela et al., 2011) to 62% in samples from Estonia (Orav et al., 2010). *J. communis* from Sweden showed a high concentration of α-pinene (56.8%) but lacked diterpenes (Adams, 2000). A sabinene chemotype has been found in leaf (40.7%) and berry (36.8%) oils from Iran (altitude of 2000 m) (Shahmir et al., 2003) and India (48.8% in leaf oils) (Pande and Manthela, 2000). A comparative study was conducted in Greece on essential oils from berries (collected at altitude above 1000 m) by headspace analysis and with the classic hydrodistillation procedure. The distilled oils contained mainly α-pinene and were rich in sesquiterpenes, mostly germacrene D (10.4%), β-caryophyllene (2.6%), and α-humulene (2.1%) (Chatzopoulou and Katsiotis, 2006). Previous studies conducted in Italy analyzed *J. communis* essential oils from Sardinia (Angioni et al., 2003) and the Northwestern Italian Alps (Caramiello et al., 1995). The unripe berry oils from Sardinia contain, predominantly, α-pinene (52%), followed by sabinene (13%), myrcene (8%), and D-germacrene (6%) (Angioni et al., 2003), while sabinene was the main component (41.4%), followed by α-pinene (13.4%) and terpinen-4-ol (8.7%) in the leaves from the Northwestern Italian Alps (Caramiello et al., 1995).

Any variation in the content of the secondary metabolites is very important, especially in plants of commercial significance, where the chemical composition clearly affects the quality of consumer products. Recently, the effects of juniper berry oil inhalation on anxiety and depression levels in a beta-amyloid (1–42) rat model have been examined (Hritcu et al., 2016), and it has been hypothesized that the oils could be used to reduce nervous tension and ameliorate stress-related conditions (Cioanca et al., 2015). In the last few years, the biocide action of plant essential oils has also been evaluated and, in terms of pest prevention, they can be considered as an alternative to the synthetic chemical pesticides (Angioni et al., 2003; Sacchetti et al., 2005).

To the best of the authors’ knowledge, there are no published studies that address the composition of essential oils obtained from *J. communis* growing wild in the Molise region. Therefore, we selected an area in one of the oldest European parks, the National Park of Abruzzo, Lazio, and Molise (PNALM, Central Italy), with a long history of nature preservation and an ecological diversity that is unique to the mountainous areas of the central Italy. Because essential oils are very sensitive to intrinsic or extrinsic factors, we evaluated whether the different phases of the biological cycle might affect the composition of the berry essential oils.

The principal aims of this study are as follows: *(a)* to perform a detailed analysis of the chemical composition of the essential oils obtained at various stages of ripeness in a mountain ecosystem, *(b)* to determine the evolution of volatiles during the ripening, and *(c)* to assess the antifungal activity *in vitro* against *S. rolfsii* by a dose–response relationship analysis. The antifungal activity was tested on *Sclerotium rolfsii*, a soilborne polyphagous phytopathogen fungus that causes diseases in most agricultural and horticultural crops (Sharma et al., 2002).

2. Materials and methods

2.1. Plant materials

The collection site is located in a natural open woodland habitat characterized by a temperate oceanic bioclimate. The *J. communis* formations are included in the Directive Habitats 92/43/EEC Annex I Habitat 5130, and the site is in a very good state of conservation. Unripe (green) and ripe berries (bluish-black) berries of *J. communis* were harvested (18 samples) in May, July, September, and November 2010 and January 2011, while the fully ripe (bluish-black) ones were harvested in

January 2011 from three shrubs in the same area. At each maturation/ripening stage, a large amount of homogenous berries were treated. The samples were collected in the Molise region of the PNALM in Central–Southern Italy at an altitude of 620 m a.s.l. (lat. 41.64331914, long. 14.081). The Molise region is located on the eastern side of the Apennine and has a typical climate of interior lands in South-central Italy.

The voucher specimens were preserved in the Herbarium of DiBT, University of Molise, with the numbers: JC–49–2010(a1), JC–49–2010(b2), and JC–49–2010(c3). Juniper fresh berries were frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Essential oil isolation

Regular and homogeneous berries were hand selected, cleaned, and then individually crushed. An aliquot of 20 g was steam-distilled in triplicate and duplicate (January green and January black) with a Clevenger-type apparatus according to the European Pharmacopoeia standards for 4 h. The essential oils were dried over anhydrous sodium sulfate until the last traces of water had been removed and then stored in dark vials at 4 °C prior to the gas chromatography–mass spectrometry (GC-MS) analysis.

2.3. GC-FID and GC/MS analyses

The composition of the essential oil samples was determined with a gas chromatography system *GC 86.10 Expander (DANI)*, equipped with an FID detector, *Rtx®-5 Restek* capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) (diphenyl-dimethyl polysiloxane), a split/splitless injector heated to 250 °C, and a flame ionization detector (FID) heated to 280 °C. The column temperature was maintained at 40 °C for 5 min, then programmed to increase to 250 °C at a rate of 3 °C/min and held, by using an isothermal process, for 10 min; the carrier gas was He (1.0 ml/min); 1 μl of each sample was dissolved in n-hexane and injected; and the split mode was 1:50.

The GC-MS analyses were performed on a *Trace GC Ultra (Thermo Scientific)* gas chromatography instrument equipped with a *Rtx®-5 Restek* capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and coupled to a *Polaris-Q (Thermo Scientific)* mass spectrometer. The ionization voltage was 70 eV; the source temperature was 250 °C, full scan mode; and the mass range was 35–350 Da. The GC conditions were the same as those described above for the gas chromatography–flame ionization detection (GC-FID) analyses.

2.4. Identification of components

The components in the essential oils were identified by comparing their GC relative retention times [KI (Kovats, 1965), RI (linear retention indices, van den Dool and Kratz, 1963)], and MS fragmentation pattern of a single compound with those from the NIST (2005) and Adams (2007) mass spectra libraries. A mixture of a homologous series of aliphatic hydrocarbons C_9–C_{25} was directly injected into GC under the abovementioned conditions to calculate the retention indices of the peaks in the chromatogram. The standards used in the analysis were as follows: α-pinene, myrcene, α-phellandrene, *p*-cymene, limonene, γ-terpinene, terpinen-4-ol, bornyl acetate, and Juniper berry oil from Sigma-Aldrich (W260401K-FCC). The relative contents of the sample components were computed as the average of the GC peak areas obtained in triplicate without any corrections (Grob and Kaiser, 2004).

2.5. Antifungal activity assay

The mold strain of *S. rolfsii* was isolated in the Campobasso area of mid-south Italy by Dr F. De Curtis and identified by the observation of the macroscopic and microscopic characteristics of the mycelium cultivated in a potato dextrose agar (PDA) medium, followed by amplification of the nuclear ribosomal ITS (internal transcribed spacer) region with the oligonucleotides ITS1-F (5′-CTTGGTCATTTAGAGGA AGTAA-3′), ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), and ITS4-B (5′-CAGGAGACT TGTA-CACGGTCCAG-3′). The amplified DNAs were purified and sequenced. Pure essential oils from the three samples [Juniperus berry oil standard (**JSt**), January (green, unripe berries) (**Jg**), and January (black-bluish, ripe berries) (**Jb**)] were dissolved in a final volume of 200 μl in ethanol, and then added to 19 ml PDA to obtain the different final concentrations. Mycelial plugs (4 mm in diameter) from the edges of the *S. rolfsii* culture were incubated in the center of each PDA plate (90 mm diameter). The cultures were incubated in the dark at 25 °C and 70% relative humidity (RH) for 2 days. The tests were conducted in triplicate. The antifungal activity was determined by measuring the diameter (in mm) of radial growth. The positive controls for the antifungal activity were carried out using PDA plates added with mancozeb (Mancozeb plus 80 WP, powder, Manica) at the final concentration in the ranges of 0.025–0.05% and 0.2–1%. The antifungal activity was determined in triplicate by measuring the diameter (in mm) of the mycelia radial growth at 25 °C and 70% RH for 2 days. The negative control was prepared without essential oils and the growth of *S. rolfsii* mycelia was monitored on the PDA plate.

2.6. Data analysis

Principal component analysis (PCA), using the SIMCA-P+ 12 package (Umetrics, Umeå, Sweden), was performed to extract and show the systematic variation in the data matrix to identify trends and clustering in an unsupervised manner. Mean-

centering and Pareto scaling were used as data pretreatment procedures. Each variable was scaled to $(1/s_k)^{1/2}$, where s_k is the standard deviation for variable k, increasing the contribution of lower concentration components in the generated models compared with models where no scaling is used. In this case, all the variables were given an equal weighting, so that the model was not biased toward the higher magnitude variables (as these generally have larger variances). The quality of the PCA model was evaluated using the correlation coefficient R^2 and a cross-validated correlation Q^2.

3. Results

The hydrodistillation of *J. communis* fresh berries provided essential oils and, by using the GC and GC-MS techniques, 18 juniper oil samples from May to January were analyzed; 90 different components were detected, of which 70 were identified representing 88.87–98.11% of the total oils (Table 1). The yields (%, w/w) ranged from 1.02 in May to 0.22 in July. The essential oils showed a light amber color with an aromatic warm, herbaceous smell, and a pleasant long-lasting woody note. The yields of the essential oils (%, w/w) decreased from May (1.02%) to September (0.64%) and November (0.60%) and finally in January (the unripe green berries, 0.57%), while in July (0.22%), we observed a strong reduction in the yield associated with a marked change in the chemical composition.

The most abundant monoterpene hydrocarbon was α-pinene (13.43–32.34%), while sabinene (2.13–3.41%), β-pinene (0.72–2.68%), myrcene (0.96–3.79%), and limonene (0.51–1.23%) were identified in lower amounts. A sesquiterpene prevalence was evidenced in all months analyzed, from 47.9% to 65.3% of the total components, higher than the monoterpene fraction (18.9–45.2%) (Fig. 1 and Table 1). Oxygen-containing sesquiterpenes were present from 2.3% (September) to 6.2% (January ripe, bluish-black), while oxygenated monoterpenes appeared in all months ranging from 0.32% (January unripe, green) to 1.10% (September). Besides the monoterpenes and sesquiterpenes, small amounts of a few non-terpene components were identified, such as tricyclene and 2-undecanone.

The main components in the sesquiterpene hydrocarbon fractions were germacrene D (12.29–20.65%), β-caryophyllene (7.72–11.77%), δ-cadinene (3.86–5.47%), α-humulene (5.21–7.87%), germacrene B (4.84–9.33%), bicyclogermacrene (2.50–4.18%), γ-cadinene (0.84–1.99%), α-selinene (0.69–1.56%), and eudesma-4(14),11-diene (1.16–3.89%) (Fig. 2). Among the oxygenated sesquiterpenes worthy of mention are α-cadinol (0.88–2.84%) and germacrene D-4-ol (0.23–2.68%). The relative abundance of the components observed during these months has allowed us to represent the change trend of the metabolite level throughout the ripening.

To obtain statistically relevant biochemical information from the GC-MS analysis, each sample profile was fully analyzed, and pattern recognition based on the multivariate data analysis was applied. The PCA of all the GC-MS profiles led to an accumulative percentage of 82% in the first two principal components (PC1 58%, PC2 24%). The PCA score plot (18 samples, 90 volatile components) clearly indicated that the first two principal components (PCs) could separate the different developmental and ripening stages (Fig. 3). The score plot showed a clear intergroup separation revealing significant seasonal variations in the chemical composition.

Score plot 1 shows marked differences between the samples harvested in May and September and the samples collected in the other months along the first principal component PC1 (labeled t[1]). The samples harvested in July, November, and January showed negative t[1] values, but they differed in their t[2] (PC2) values: the July and November samples showed negative t[2] values, whereas essential oils obtained in January showed positive t[2] values. The samples harvested in May and September and the samples collected in July and November correctly cluster according to the month, but the groups are close to each other along the two components, suggesting that they share a similar essential oil composition. In score plot 1, the green and bluish-black berries collected in January were not separated because of their similar essential oil profiles.

4. Discussion

J. communis populations growing wild in the PNALM are located in mountainous areas characterized by a temperate oceanic bioclimate.

In terms of the chemical composition detected, PCA evidenced that the essential oil obtained in July is similar to the sample of November. This unusual behavior could be interpreted as a result of the biochemical and physiological modifications that occurred in the late autumn/winter due to the low temperature and the reduction of the photosynthetic yield. It has been reported that at sites with summer drought, the woody species experiences lower stress during winter due to the milder temperatures and, therefore, summer would be the season when there is a reduction in physiological functions (Ogaya and Peñuelas, 2003; Gulías et al., 2009). However, any change in environmental conditions such as temperature, light, nutrient status, or water availability in summer or in winter can potentially cause a metabolic imbalance, which may affect the biosynthetic pathways (Granda et al., 2014). The data obtained from this study should be correlated with the trend of the physiological performance of *J. communis* during the seasons and we suggest that, in the temperate ecosystem of the PNALM, the changing of the seasons from autumn to winter (November samples) and from spring to summer (July samples) generates similar stress conditions.

In light of the GC-MS and PCA analyses, the volatile metabolites were grouped according to their alteration pattern as follows: volatile compounds associated with the unripe berries (immature stage, May and September, *stage I* and immature stage, July and November, *stage II*) and volatile compounds associated with ripening (January unripe, green and January ripe, bluish-black berries).

Table 1
Chemical composition of essential oils isolated from berries of *Juniperus communis* L. from May to January.

No.	Compounds[a]	RI_{La}[b]	RI_a[c]	ID method[d]	Content [%][e]						
					May	July	September	November	January (unripe berries)	January (ripe berries)	*J. communis* standard oil
1	Tricyclene	921	919	KI, RI, MS	0.05 ± 0.01	0.02 ± 0.01	0.06 ± 0.00	0.02 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.12
2	α-Thujene	924	926	KI, RI, MS	0.27 ± 0.00	0.19 ± 0.03	0.37 ± 0.00	0.13 ± 0.02	0.22 ± 0.00	0.24 ± 0.01	1.01
3	α-Pinene	932	931	KI, RI, MS, CoI	29.62 ± 1.86	15.43 ± 2.97	32.34 ± 1.61	13.43 ± 1.39	18.58 ± 3.29	17.29 ± 1.99	41.38
4	Camphene	946	945	KI, RI,MS	0.18 ± 0.01	0.11 ± 0.02	0.22 ± 0.01	0.08 ± 0.01	0.11 ± 0.02	0.09 ± 0.01	0.40
5	Verbenene	961	965	KI, RI,MS	0.35 ± 0.03	0.10 ± 0.02	0.08 ± 0.01	0.06 ± 0.01	0.17 ± 0.01	0.14 ± 0.02	–
6	Sabinene	969	972	KI, RI,MS	3.41 ± 0.51	2.82 ± 0.38	3.81 ± 0.42	2.13 ± 0.28	2.90 ± 0.35	2.53 ± 0.23	5.01
7	β-Pinene	974	974	KI, RI,MS	2.31 ± 0.23	1.45 ± 0.29	2.68 ± 0.11	1.08 ± 0.05	0.88 ± 0.09	0.72 ± 0.10	2.78
8	Myrcene	988	992	KI, RI, MS, CoI	1.44 ± 0.10	0.96 ± 0.16	1.82 ± 0.04	0.72 ± 0.13	3.79 ± 0.33	2.85 ± 0.28	8.43
9	α-Phellandrene	1002	1003	KI, RI,MS, CoI	0.02 ± 0.01	0.02 ± 0.01	0.05 ± 0.02	tr	tr	tr	0.41
10	δ-3-Carene	1008	1008	KI, RI,MS	tr	tr	0.02 ± 0.00	tr	0.03 ± 0.00	0.03 ± 0.00	0.11
11	α-Terpinene	1014	1015	KI, RI,MS	0.29 ± 0.14	0.33 ± 0.05	0.66 ± 0.21	0.11 ± 0.01	0.11 ± 0.02	0.11 ± 0.00	0.87
12	*p*-Cymene	1020	1023	KI, RI,MS, CoI	0.02 ± 0.00	0.07 ± 0.01	0.07 ± 0.01	0.03 ± 0.01	0.17 ± 0.01	0.21 ± 0.02	3.52
13	Limonene	1024	1027	KI, RI,MS, CoI	0.93 ± 0.06	0.73 ± 0.09	1.23 ± 0.07	0.51 ± 0.09	0.73 ± 0.04	0.60 ± 0.04	5.97
14	γ-Terpinene	1054	1058	KI, RI,MS, CoI	0.45 ± 0.17	0.51 ± 0.07	0.85 ± 0.22	0.17 ± 0.02	0.29 ± 0.02	0.31 ± 0.01	2.47
15	Terpinolene	1086	1088	KI, RI,MS	0.58 ± 0.02	0.52 ± 0.05	1.06 ± 0.11	0.46 ± 0.08	0.37 ± 0.02	0.34 ± 0.01	1.55
16	*p*-Cymenene	1089	1096	KI, RI,MS	0.02 ± 0.01	tr	–	tr	–	–	–
17	Linalool	1095	1101	KI, RI,MS	tr	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.00	–	–	–
18	Terpinen-4-ol	1174	1177	KI, RI,MS, CoI	0.15 ± 0.05	0.30 ± 0.09	0.30 ± 0.02	0.19 ± 0.05	0.10 ± 0.01	0.18 ± 0.00	1.73
19	α-Terpineol	1186	1191	KI, RI,MS	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	tr	0.02 ± 0.00	0.13
20	*trans*-Chrysanthenyl acetate	1235	1236	KI, RI,MS	0.12 ± 0.00	0.08 ± 0.01	0.09 ± 0.01	0.05 ± 0.00	tr	tr	–
21	*cis*-Myrtanol	1250	1253	KI, RI,MS	0.04 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	tr	tr	–
22	Methy citronellate	1257	1264	KI, RI,MS	–	–	–	–	tr	tr	0.02
23	Bornyl acetate	1287	1287	KI, RI,MS, CoI	0.35 ± 0.04	0.44 ± 0.01	0.49 ± 0.02	0.35 ± 0.02	0.21 ± 0.02	0.18 ± 0.00	0.17
24	2-undecanone	1293	1295	KI, RI,MS	0.22 ± 0.03	0.20 ± 0.00	0.22 ± 0.02	0.17 ± 0.01	0.05 ± 0.00	0.04 ± 0.00	0.02
25	*trans*-Pinocarvyl acetate	1298	1301	KI, RI,MS	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	tr	–
26	Myrtenyl acetate	1324	1333	KI, RI,MS	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	tr	tr	tr	–
27	δ-Elemene	1335	1339	KI, RI,MS	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.02 ± 0.00	tr	tr	0.04
28	α-Cubebene	1345	1350	KI, RI,MS	0.13 ± 0.00	0.07 ± 0.00	0.11 ± 0.01	0.12 ± 0.01	0.05 ± 0.00	0.06 ± 0.00	0.79
29	Citronellyl acetato	1350	1356	KI, RI,MS	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	–	–	tr
30	α-Copaene	1374	1376	KI, RI,MS	0.15 ± 0.01	0.10 ± 0.00	0.09 ± 0.01	0.08 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.87
31	β-Cubebene	1387	1386	KI, RI,MS	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	–
32	β-Elemene	1389	1393	KI, RI,MS	0.94 ± 0.06	0.68 ± 0.06	0.89 ± 0.08	0.59 ± 0.13	1.02 ± 0.23	0.58 ± 0.08	0.88
33	1		1398	–	–	–	–	–	0.06 ± 0.02	0.04 ± 0.00	–
34	Sibirene	1400	1402	KI, RI,MS	0.54 ± 0.04	0.28 ± 0.02	0.09 ± 0.01	0.12 ± 0.03	–	–	0.23
35	Longifolene	1407	1405	KI, RI,MS	–	–	–	–	0.06 ± 0.00	0.07 ± 0.00	0.40
36	β-Caryophyllene	1417	1420	KI, MS	7.72 ± 0.32	9.14 ± 0.18	8.71 ± 0.23	7.56 ± 0.60	11.06 ± 0.33	11.77 ± 0.42	3.88
37	2		1424	–	0.17 ± 0.08	0.12 ± 0.01	0.19 ± 0.08	0.06 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	tr
38	Thujopsene	1429	1431	KI, RI,MS	0.21 ± 0.04	0.12 ± 0.02	0.11 ± 0.02	0.08 ± 0.02	–	–	0.38
39	γ-Elemene	1434	1434	KI,MS	0.08 ± 0.03	0.06 ± 0.01	0.10 ± 0.02	0.04 ± 0.00	0.06 ± 0.02	0.03 ± 0.01	0.14
40	Aromadendrene	1439	1439	KI, RI,MS	0.05 ± 0.02	0.05 ± 0.01	0.07 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.04
41	α-Humulene	1452	1452	KI, RI,MS	5.21 ± 0.29	6.57 ± 0.34	5.62 ± 0.12	5.76 ± 0.28	7.36 ± 0.32	7.87 ± 0.31	1.59
42	(*E*)-β-Farnesene	1454	1458	KI, RI,MS	0.09 ± 0.01	0.09 ± 0.01	0.13 ± 0.00	0.15 ± 0.01	0.08 ± 0.01	0.08 ± 0.00	0.15
43	*trans*-Cadina1,(6),4-diene	1475	1475	KI, RI,MS	0.26 ± 0.14	0,31 ± 0.01	0.24 ± 0.05	0.16 ± 0.05	0.10 ± 0.01	0.12 ± 0.01	0.19
44	γ-Muurolene	1478	1478	KI, RI,MS	0.40 ± 0.12	0.47 ± 0.03	0.50 ± 0.06	0.37 ± 0.02	0.31 ± 0.01	0.34 ± 0.04	0.72
45	Germacrene D	1484	1484	KI, RI,MS	17.59 ± 1.93	18.40 ± 1.59	12.29 ± 0.82	19.82 ± 0.56	20.38 ± 0.44	20.65 ± 0.06	3.02
46	β-Selinene	1489	1489	KI, RI,MS	0.86 ± 0.34	0.93 ± 0.06	1.06 ± 0.22	0.78 ± 0.04	0.56 ± 0.01	0.64 ± 0.04	0.38
47	*trans*-Muurola-4(14), 5-diene	1493	1493	KI, RI,MS	0.22 ± 0.01	0.25 ± 0.02	0.16 ± 0.01	0.14 ± 0.03	0.08 ± 0.01	0.10 ± 0.01	0.09
48	α-Selinene	1498	1496	KI, RI,MS	1.44 ± 0.63	1.06 ± 0.03	1.56 ± 0.35	1.05 ± 0.21	0.84 ± 0.04	0.69 ± 0.00	0.48
49	Bicyclogermacrene	1500	1498	KI, RI,MS	4.18 ± 0.26	4.56 ± 0.27	2.50 ± 0.28	4.27 ± 0.10	3.68 ± 0.31	3.56 ± 0.11	0.24
50	α-Muurolene	1500	1502	KI, RI,MS	0.68 ± 0.10	0.95 ± 0.03	0.65 ± 0.06	0.73 ± 0.05	0.41 ± 0.01	0.48 ± 0.01	0.81
51	Eudesma-4(14),11-diene	1506	1506	KI, RI,MS	1.16 ± 0.51	2.83 ± 0.43	1.42 ± 0.34	3.04 ± 0.11	3.38 ± 0.01	3.89 ± 0.17	0.25
52	γ-Cadinene	1513	1516	KI, RI,MS	1.52 ± 0.29	1.18 ± 0.22	1.10 ± 0.14	1.99 ± 0.34	0.91 ± 0.22	0.84 ± 0.07	0.83
53	δ-Cadinene	1522	1526	KI, RI,MS	5.39 ± 1.01	7.61 ± 0.38	4.89 ± 0.42	5.47 ± 0.28	3.86 ± 0.58	3.91 ± 0.25	3.93
54	*trans*-Cadina-1,4-diene	1533	1534	KI, RI,MS	0.12 ± 0.05	0.12 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.02 ± 0.00	0.03 ± 0.00	0.12
55	3		1536	–	0.08 ± 0.08	0.10 ± 0.02	0.18 ± 0.08	0.03 ± 0.00	tr	tr	0.08
56	α-Cadinene	1537	1539	KI, RI,MS	0.35 ± 0.22	0.30 ± 0.05	0.32 ± 0.11	0.32 ± 0.11	0.14 ± 0.02	0.15 ± 0.01	0.16

Table 1 (*continued*)

No.	Compounds[a]	RI_{La}[b]	RI_a[c]	ID method[d]	Content [%][e]						
					May	July	September	November	January (unripe berries)	January (ripe berries)	*J. communis* standard oil
57	Selina-3,7(11)-diene	1545	1543	KI, RI,MS	0.08 ± 0.06	0.11 ± 0.03	0.17 ± 0.08	0.05 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.09
58	Elemol	1548	1551	KI, RI,MS	0.04 ± 0.01	0.09 ± 0.01	0.04 ± 0.02	0.10 ± 0.01	0.09 ± 0.02	0.06 ± 0.01	tr
59	Germacrene B	1559	1559	KI, RI,MS	4.82 ± 0.46	7.20 ± 0.51	4.98 ± 0.40	7.26 ± 0.37	8.75 ± 0.68	9.33 ± 0.18	1.49
60	Germacrene D-4-ol	1574	1577	KI, RI,MS	0.70 ± 1.08	0.27 ± 0.23	0.23 ± 0.23	2.32 ± 0.71	2.68 ± 0.20	1.56 ± 0.04	–
61	Spathulenol	1577	1579	KI, RI,MS	0.03 ± 0.03	0.15 ± 0.04	0.10 ± 0.07	0.29 ± 0.01	–	0.37 ± 0.03	0.08
62	Caryophyllene-oxide	1582	1585	KI, RI,MS, CoI	0.05 ± 0.01	0.14 ± 0.01	0.07 ± 0.02	0.18 ± 0.01	0.06 ± 0.00	0.21 ± 0.03	0.12
63	Humulene epoxide II	1608	1612	KI, RI,MS	tr	0.04 ± 0.01	0.02 ± 0.00	0.05 ± 0.01	0.02 ± 0.00	0.08 ± 0.01	–
64	1-*epi*-Cubenol	1627	1631	KI, RI,MS	0.19 ± 0.02	0.28 ± 0.03	0.07 ± 0.01	0.19 ± 0.03	0.04 ± 0.00	0.08 ± 0.02	0.11
65	γ-Eudesmol	1630	1634	KI, RI,MS	0.03 ± 0.02	0.09 ± 0.01	0.04 ± 0.01	0.07 ± 0.02	0.03 ± 0.00	0.06 ± 0.01	0.02
66	*epi*-α-Cadinol + *epi*-α-Muurolol	1640	1644	KI, RI,MS	0.70 ± 0.05	1.85 ± 0.26	0.68 ± 0.14	1.82 ± 0.40	0.79 ± 0.10	1.25 ± 0.25	0.20
67	α-Muurolol	1644	1649	KI, RI,MS	0.08 ± 0.02	0.21 ± 0.04	0.08 ± 0.02	0.22 ± 0.02	0.07 ± 0.00	0.13 ± 0.02	0.04
68	α-Cadinol	1652	1657	KI, RI,MS	0.88 ± 0.23	2.84 ± 0.44	0.96 ± 0.27	2.79 ± 0.35	1.44 ± 0.26	2.34 ± 0.43	0.14
69	4		1672	–	–	–	–	0.02 ± 0.01	0.02 ± 0.00	tr	–
70	5		1693	–	0.22 ± 0.26	0.27 ± 0.10	0.13 ± 0.12	1.05 ± 0.19	0.82 ± 0.11	0.59 ± 0.03	–
71	Eudesm-7(11)-en-4-ol	1700	1698	KI, RI,MS	0.03 ± 0.01	0.06 ± 0.00	0.03 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	tr
72	6		1932	–	0.11 ± 0.02	0.25 ± 0.02	0,18 ± 0.03	0.37 ± 0.05	0.21 ± 0.02	0.19 ± 0.01	–
73	7		1955	–	0.02 ± 0.00	0.04 ± 0.00	0,03 ± 0.00	0.06 ± 0.01	0.02 ± 0.00	tr	0.64
74	Sandaracopimara-8(14),15-diene	1968	1963	KI, RI,MS	–	0.03 ± 0.00	tr	0.03 ± 0.00	–	–	–
75	8		1989	–	0.33 ± 0.06	1.05 ± 0.13	0.63 ± 0.10	1.50 ± 0.29	0.27 ± 0.04	0.23 ± 0.03	0.21
76	9		2014	–	0.07 ± 0.01	0.20 ± 0.03	0.12 ± 0.02	0.25 ± 0.03	0.03 ± 0.00	0.02 ± 0.00	–
77	10		2021	–	tr	0.02 ± 0.00	tr	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	–
78	Abietatriene	2058	2055	KI, RI,MS	0.17 ± 0.02	0.60 ± 0.08	0.30 ± 0.05	0.75 ± 0.07	0.28 ± 0.04	0.27 ± 0.07	0.02
79	Abietadiene	2087	2082	KI, RI,MS	–	0.02 ± 0.00	tr	0.02 ± 0.00	–	–	–
80	11		2138	–	0.12 ± 0.02	0.38 ± 0.05	0.22 ± 0.05	0.78 ± 0.12	0.23 ± 0.05	0.26 ± 0.07	–
81	12		2193	–	0.60 ± 0.09	2.31 ± 0.36	0.91 ± 0.23	5.04 ± 0.95	0.43 ± 0.05	0.57 ± 0.17	tr
82	13		2220	–	0.09 ± 0.01	0.32 ± 0.05	0.14 ± 0.04	0.59 ± 0.07	0.03 ± 0.01	0.03 ± 0.01	–
83	14		2237	–	tr	0.03 ± 0.01	tr	0.08 ± 0.01	tr	0.02 ± 0.00	–
84	Dehydro-Abietal	2274	2271	KI, RI,MS	–	0.04 ± 0.01	tr	0.06 ± 0.00	0.03 ± 0.00	0.04 ± 0.02	–
85	15		2278	–	tr	0.09 ± 0.03	0.03 ± 0.03	0.39 ± 0.04	0.03 ± 0.02	0.05 ± 0.02	–
86	16		2285		–	0.04 ± 0.01	0.02 ± 0.01	0.17 ± 0.01	tr	tr	–
87	17		2334		tr	0.09 ± 0.03	tr	0.55 ± 0.09	–	–	–
88	18		2347		–	–	–	–	0.03 ± 0.00	0.06 ± 0.02	–
89	19		2362		–	0.03 ± 0.01	–	0.15 ± 0.02	–	–	–
90	20		2369		–	–	–	–	tr	0.02 ± 0.01	–
	Total				**98.11**	**94.64**	**97.14**	**88.87**	**97.60**	**97.74**	**97.97**
	Monoterpene hydrocarbons				39.89	23.24	45.26	18.91	28.35	25.46	73.31
	Oxigenated monoterpenes				0.77	0.97	1.09	0.74	0.33	0.38	2.06
	Sesquiterpene hydrocarbons				54.28	63.53	47.89	60.11	63.24	65.30	21.00
	Oxigenated sesquiterpenes				2.73	6.02	2.32	8.09	5.26	6.19	0.7
	Total				**97.67**	**93.76**	**96.56**	**87.85**	**97.18**	**97.33**	**97.13**

Values are mean ± standard deviation of two [January (unripe berries), January (ripe berries)] or three (May, July, September, November) different experiments.
tr: trace (<0.02%); -: not found.
[a] Compounds are listed in order of elution from *Rtx®-5 Restek* capillary column.
[b] Retention index from literature on the apolar column (RI_{La}) according to Adams, 2007.
[c] Retention index experimentally determined on the apolar *Rtx®-5 Restek* capillary column (RI_a).
[d] Identification method adopted for each compound (KI: comparison of Kovats' retention index; RI (linear retention indices, van den Dool, & Kratz, 1963); MS, comparison of mass spectra CoI: co-injection with authentic standard).
[e] Relative content expressed as percentage of the total oil composition obtained by GC-MS.

4.1. Volatile metabolites associated with the unripe berries (immature stages I and II)

Several volatile metabolites appear at a high level at immature *stage I* (May and September), and then progressively decrease during the development toward maturity (Table 1, Figs. 1 and 2). Monoterpene hydrocarbons account for approximately 40–46% at this stage, but total sesquiterpenes make the highest contribution, representing 57.01% (May) and 50.21% (September) of the essential oils. The α-pinene (29.63–32.36%) reaches a higher level than in the other months analyzed, followed by germacrene D (12.29–17.59%), β-caryophyllene (7.71–8.51%), δ-cadinene (4.89–5.39%), α-humulene (5.22–5.62),

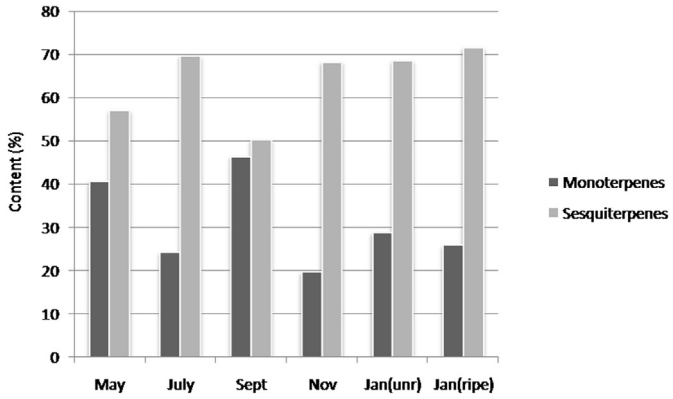

Fig. 1. -Variability of two principal component groups (monoterpenes and sesquiterpenes) from *J. communis* L. essential oils.

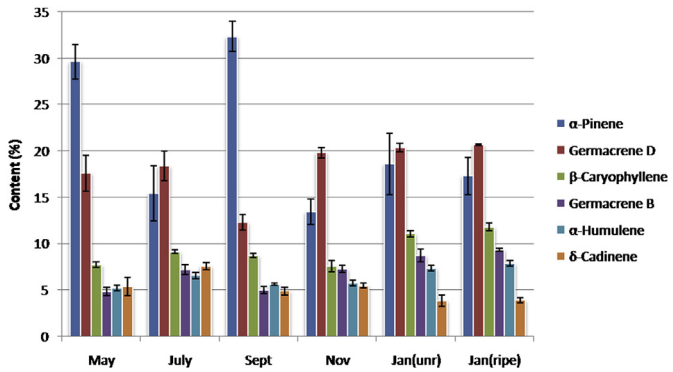

Fig. 2. −Percentages of the main representative compounds (>5%) in the essential oils from *J. communis* L. The data are shown as mean concentrations ± SD of three different experiments for each month.

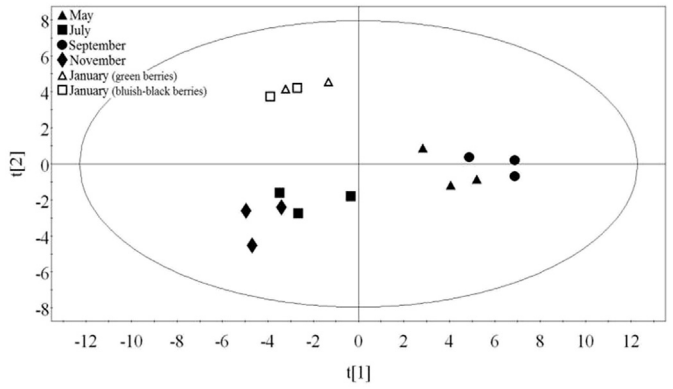

Fig. 3. - Score plot of the two-component PCA model. $R^2 = 0.820$, $Q^2 = 0.699$.

germacrene B (4.81–4.98%), and bicyclogermacrene (2.20–4.18%), and γ-cadinene, α-selinene, and eudesma-4(14)11 diene are obtained as minor components. On average, sabinene, β-pinene, myrcene, limonene, and terpinolene were identified at <4%, while volatile oxygenated components were detected in concentrations of <1%.

In July and November (*stage II*), a progressive increase in the level of several sesquiterpenes was observed, while an opposite trend was found for the monoterpenes. Compared with May and September, the concentration of monoterpenes such as α-pinene, sabinene, β-pinene, myrcene, limonene, and terpinolene decreased. At this stage, germacrene D (18.41–19.86%) was found to be the major component, the second main constituent being α-pinene (13.4–15.4%), followed by

β-caryophyllene (7.56–9.15%). The other sesquiterpene hydrocarbons found with increased concentration were δ-cadinene (5.47–7.61%), germacrene B (7.21–7.26%), α-humulene (5.76–6.58%), bicyclogermacrene, and eudesma-4(14)11 diene. The alcohols epi-α-cadinol + epi-α-muurolol, and α-cadinol were the only oxygenated components reaching their highest level in July, while germacrene D-4-ol increased dramatically in November.

4.2. Volatiles associated with ripening

Essential oils from ripe (bluish-black) and unripe (green) berries harvested in January showed a quite similar chemical composition to that observed from the PCA. Germacrene D (20.38–20.65%) was the major constituent, followed by α-pinene (17.3–18.6%) and β-caryophyllene (11.06–11.77%). Myrcene (3.79–2.8%) and sabinene (2.5–2.9%) were the main components among the monoterpenes. The amount of myrcene increased during the ripening, while α-pinene and sabinene were found in concentrations lower than those in the previous months. The total level of sesquiterpenes increased to 65% as the process progressed. Germacrene D, β-caryophyllene, eudesma-4(14),11-diene, α-humulene, germacrene B (8.75–9.33%), and the alcohol germacren D-4-ol reached the maximum concentration at this stage.

Several components were constantly present, and their relative abundance did not change significantly throughout maturation. Particular relevance should also be given to the presence of volatile minor compounds (<1.0%) detected in all samples, which contribute to the differences in aroma and flavor.

In our study, we also analyzed the composition of a juniper essential oil commercially available from Sigma-Aldrich as the standard reference (Table 1).

Comparison of our data with the literature data shows that the essential oils of juniper berries harvested in the PNALM presented remarkable qualitative and quantitative differences compared with the other studied juniper oils from various regions in Europe and other Mediterranean countries. Essential oils from unripe and ripe berries of *J. communis* growing wild in the Molise region (altitude 620 m) were of α-pinene chemotype, which is the major component (13.4–29.6%), followed by germacrene D (12.3–20.6%) and β-caryophyllene (7.6–11.8%). The concentration of alcohols was low in ripe and unripe berries and a low level (<1%) of a few esters was found. The amount of α-pinene decreased during the ripening with a corresponding increase in the level of several sesquiterpenes, mainly germacrene D and β-caryophyllene, which were the first and third main components at the immature *stage II* and in the ripe berries, respectively.

In previous papers, the content of germacrene D and β-caryophyllene was found to be high (3–10%), only in the leaf essential oils (Angioni et al., 2003; Caramiello et al., 1995; Orav et al., 2010), while the concentration of these sesquiterpenes is reported as low (0–3%) in the berry oils (Adams, 2000; Lawrence, 2001; Angioni et al., 2003). Our data are in complete agreement with the previous papers that show a great variation in the volatile components from *J. communis* essential oils and are in partial agreement with the results concerning the chemical composition of berry oils obtained from plants growing in mountainous areas. Previous studies revealed that the essential oil from berries harvested in Montenegro (altitude 950 m, whole berries) contained germacrene D (12.1%) and β-caryophyllene (9.2%); the berry oil from Greece (above 1000 m) contained germacrene D (10.4%) and β-caryophyllene (2.6%) (Chatzopoulou and Katsiotis, 2006); that from Iran (about 2000 m) contained germacrene D (8.1%) and β-caryophyllene (1.5%) (Shahmir et al., 2003); and that from Sardinia (the mountainous areas of Laconi) contained germacrene D (6.6%) and β-caryophyllene (0.78%) (Angioni et al., 2003).

In the berry essential oils from the PNALM, the concentration of germacrene D (ripe berries, 20.6%) is double and the concentration of β-caryophyllene (ripe berries, 11.8%) is about fourfold higher than the concentration of the same components reported by Chatzopoulou and Katsiotis, (2006) from the Greek samples (10.4% and 2.6%, respectively). In addition, in our samples, we found a lower rate of α-pinene (32.3% vs. 40.3%) and myrcene (3.8% vs. 10.6%) than that in the corresponding data reported by these same authors.

Next, the high content of germacrene D and β-caryophyllene can be considered a characteristic of the essential oils of the *J communis* berries from the Molise region (PNALM); these sesquiterpenes reach their maximum concentration in the Molise ripe berries, while they are reported in low amounts in juniper berry oils from other countries.

Particularly relevant is the rate of increase of β-caryophyllene in our samples, in light of new biological studies. Recently, it has been demonstrated that different plants contain components that interact with the endocannabinoid system. This system regulates important functions in mammals through cannabinoid receptors. Beta-caryophyllene, although a sesquiterpene, can selectively bind the type-2 cannabinoid receptor with a significant anti-inflammatory activity without any psychotomimetic effects (Klauke et al., 2014) and may ameliorate the symptoms of anxiety and depressive disorders in a rat model (Hritcu et al., 2016). The inhalation of the juniper volatile oil could protect the brain from oxidative stress (Cioanca et al., 2015) and may improve memory deficits in a rat model of Alzheimer's disease (Cioanca et al., 2014). Beta-caryophyllene is usually found in high concentrations in many plants and spices, such as oregano, rosemary, thyme, cinnamon, and black pepper, and the United States Food and Drug Administration (USFDA) has approved it as food additive (approval reference no. 21CFR172.515) because of its weak aromatic taste.

4.3. Antifungal activity against S. rolfsii

Molds are a large group of taxonomically diverse fungal species that are responsible for the decay or deterioration of a variety of foods with consequent quantitative and qualitative losses. Natural antimycotics from plants are an ideal alternative to traditional chemical additives to improve the food quality in fresh fruits, vegetables, and grains. *S. rolfsii* can survive and

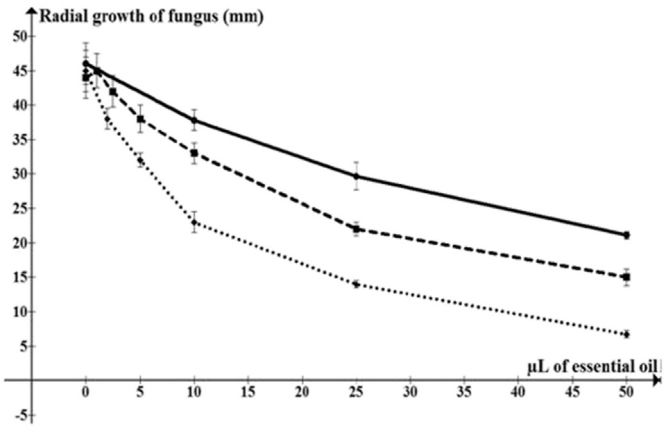

Fig. 4. Toxic effect with different concentrations of standard – **JSt** (●), ripe berry – **Jb** (■), and unripe berry – **Jg** (◆) pure essential oils of *J. communis* on the radial growth of *S. rolfsii*. The standard errors are indicated. Tests were conducted in triplicate.

thrive within a wide range of environmental conditions and in a broad pH range; it has been isolated in tropical, subtropical, and temperate regions with warm climates (Ekundayo et al., 2015). In Italy, *S. rolfsii* is typically present in southern regions, but, following warm seasons, infections are occasionally also reported in the north.

The antifungal activity of the *Juniperus* berry oil standard (**JSt**) (from Sigma-Aldrich), January (green, unripe berries) (**Jg**), and January (black-bluish, ripe berries) (**Jb**) was evaluated *in vitro* at various concentrations against the phytopathogenic fungus *S. rolfsii*. The three samples presented significant differences (Table 1) in terms of their qualitative and quantitative composition. (**JSt**) has a higher concentration of monoterpenes (73.31%) than sesquiterpenes (21%) in contrast to the (**Jg**) and (**Jb**) oils, in which the sesquiterpenes were at a higher level (63.2% and 65.3%, respectively). Fig. 4 shows the reducing fungal growth (expressed as a reduction of mycelium growth) in a dose-dependent manner with the green berry (**Jg**) essential oil being the most effective. The fungal toxic effect decreased in the ripe berry essential oil (**Jb**), while a lower activity was detected in the standard essential oil (**JSt**). This behavior is certainly due to the variation of phytochemicals in the samples analyzed. Some authors have reported that the sesquiterpene components of the oil are more effective as antifungals than the monoterpene components (Jing et al., 2014). The (**Jg**) and (**Jb**) essential oils have a quite similar chemical composition as shown also by the PCA However, the fungal toxic effect is greater in the (**Jg**) oil than in the (**Jb**) oil in our samples, where myrcene, β-elemene, and germacrene-D-4-ol being the only components that slightly differ in their concentrations. Therefore, we suggest that the antifungal activity can certainly be correlated with the sesquiterpene concentration and, in addition, may be produced by the synergistic or antagonistic effects of different terpenoid components, as reported by several authors (Jing et al., 2014). Some mechanisms have been proposed, such as the possibility that the terpenes may increase the concentration of lipidic peroxides and result in cell death (Lucini et al., 2006), or that they may act on the hyphae of the mycelium, inducing leakage of components from the cytoplasm, and therefore, the death of the mycelium (Sharma and Tripathi, 2008).

The positive control of the antifungal activity was carried out in triplicate and revealed a "no growth, mycelium-free" result at 0.2–1% and 0.05% of mancozeb on PDA plates. At lower concentration (0.025%), there was a reduction (30%) of the mycelial growth of *S. rolfsii* comparable with the results obtained in the experiment using 6 µL of "pure" essential oil (unripe berries, **Jg**).

The results obtained in this study against *S. rolfsii* suggest that *J. communis* essential oil could be considered as a potential natural fungicide for the control of fungal pathogens in post-harvest diseases, instead of chemical additives. It would, therefore, seem worthy of further investigation.

In summary, *J. communis* is an economically important crop with a medicinal and commercial value. Therefore, the results of this study may be useful in terms of increasing knowledge on changes in the composition of essential oils resulting from environmental factors, seasonal variations, and harvesting time.

In this study, the average level of β-caryophyllene is related to the harvesting time. As a result, if the essential oil should be used for inhalation and/or as a biocide, it is appropriate to collect fully ripe berries (in January) when the concentration of β-caryophyllene is high (11%). Conversely, the essential oils for pharmacological purposes require a low concentration of β-caryophyllene, and the collection, therefore, should occur in May or November (when the concentration is <7%) as required by the European Pharmacopoeia, 2015 standards (8th Edition).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2016.07.026.

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