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ORIGINAL ARTICLE

Biological investigations of essential oils extracted from three *Juniperus* species and evaluation of their antimicrobial, antioxidant and cytotoxic activities

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Abstract

Aims: To conduct biological investigations and to evaluate the antimicrobial and antioxidant activities of the essential oils (EOs) extracted from *Juniperus communis*, *J. scopulorum* and *J. horizontalis*; to screen their mechanisms of action by conducting the cell membrane permeability assay (CMP); and to determine the possible cytotoxicity of the three EOs against human neuroblastoma cells (SH-SY5Y).

Methods and Results: The antifungal activity was tested against four phytopathogenic fungi (*Monilinia fructicola*, *Aspergillus niger*, *Penicillium expansum* and *Botrytis cinerea*). The antibacterial activity was evaluated against two Gram-positive (G+ve) (*Bacillus megaterium* and *Clavibacter michiganensis*) and three Gram-negative (G−ve) bacterial strains (*Pseudomonas fluorescens*, *P. syringae* pv. *phaseolicola* and *Xanthomonas campestris*). Results showed that the three tested EOs have antifungal activity against *M. fructicola* and *P. expansum* and effective antibacterial activity against *P. syringae* pv. *phaseolicola* and *B. megaterium*. Moreover, the three EOs were evaluated for their ability to inhibit the growth of SH-SY5Y cells with MTT assay. *J. communis* EO was the more effective with an IC_{50} of 53·7 μg ml^{-1}. The antioxidant capacity of the three EO did not differ as measured by the DPPH assay.

Conclusions: The three tested juniper EOs showed promising antimicrobial and antioxidant activity and cytotoxic effects against human neuroblastoma cell line.

Significance and Impact of the Study: The outfindings from this research showed promising antimicrobial effects of the three oils against the majority of the tested phytopathogens with a potential to utilize them as natural alternatives to synthetic drugs, the cause of global environmental problems, pathogen resistance and difficulty to control many post-harvest plant diseases.

Introduction

Junipers are evergreen coniferous plants varying in size and shape from tall trees to columnar or low spreading shrubs with long trailing branches. *Juniperus* is the unique genus of Cupressaceae family that is widely distributed throughout the northern hemisphere (Adams 2004a; Seca and Silva 2006). The number of *Juniperus* species is still debatable; in fact, two recent studies reported different numbers of species belonging to this genus: Farjon (2000) considered 52 species while Adams (2004b) 75 species.

Juniperus communis L. (common juniper), distributed in the Boreal Hemisphere, is the most northerly among the junipers and one of the most northerly conifers in the world (Al-Snafi 2018). It grows in scrubs, pastures and cliffs, from sea level to high mountains throughout Europe, Asia and North America and it is one of the most studied juniper species (Adams 2004a; Cabral *et al.* 2012).

The Rocky Mountain juniper (*J. scopulorum* Sarg.) is an evergreen, long-lived species, diffused widely in the western of USA, Canada and Mexico (Adams 2004a; Zheljazkov *et al.* 2013a). Its leaves are palatable to domestic sheep and are a food source for elk, deer, small mammals and birds (Scher 2002). Some Indian tribes used a leaf infusion of *J. scopulorum* for the treatment of coughs and fevers and a decoction of its berries to treat colds (Lind 2005). The essential oil (EO) of the Rocky Mountain juniper has been also studied: its yield and composition are influenced by the variety (Cantrell *et al.* 2013), the age of leaf material (Adams and Hagerman 1976), the sex of the tree (Adams and Powell 1976) and the timing of sampling (Zheljazkov *et al.* 2012). However, there are few reports on the biological activities of this species.

Juniperus horizontalis Moench, known as creeping juniper, commonly considered as an ornamental species, widely distributed in the United States and Canada (Cantrell *et al.* 2014). Creeping juniper prefers hot stands and dry climate and is very tolerant to shade and poor drainage. It is slow growing, long-lived and susceptible to juniper blight (Eryiğit *et al.* 2014). Creeping juniper EOs have been used in apothecary, fragrance and pharmaceutical industries (Cantrell *et al.* 2014).

In general, juniper EOs have several medicinal properties as diuretic, antiseptic and antioxidant (Sati and Joshi 2010; Bais *et al.* 2014; Zheljazkov *et al.* 2017). The EOs from needle-like leaves, berries (galbuli) or wood of different species and varieties of juniper have been subjected to biochemical characterization in many studies (Banthorpe *et al.* 1973; Shatar 1984; Caramiello *et al.* 1995; Gonny *et al.* 2006; Zheljazkov *et al.* 2013a,b; Radoukova *et al.* 2018).

Several studies demonstrated antimicrobial activities of juniper EOs against some different pathogenic strains. The antifungal activity of the needle EO has been evaluated against yeasts, *Candida albicans*, filamentous fungi strains as *Aspergillus* sp. and dermatophytes (Cabral *et al.* 2012). The antibacterial activity has been tested against *Staphylococcus aureus, Escherichia coli*, *Shigella sonei*, *Yersinia enterocolitica* and *Streptococcus pneumoniae* (Eryiğit *et al.* 2014; Radoukova *et al.* 2018; Salamon *et al.* 2019). The EO from *J. communis* berries was more bioactive than the EO extracted from its leaves against Gram-positive (G+ve) and Gram-negative (G−ve) bacteria (Haziri *et al.* 2013; Falcão *et al.* 2018; Zheljazkov *et al.* 2018). Moreover, the possible cytotoxic activity of the EOs from different juniper species has been reported (Topçu *et al.* 2005; El-Sawi *et al.* 2007; Maurya *et al.* 2018; Vasilijević *et al.* 2018).

Zheljazkov *et al.* (2017) reported the possible antimicrobial activities against 10 human pathogen micro-organisms. Only few studies reported the cytotoxicity of *J. communis* EO but no studies are available on the possible cytotoxic effects of the EOs of *J. scopulorum* and *J. horizontalis*. In general, *J. scopulorum* and *J. horizontalis* EOs have been less studied than *J. communis* EO.

The main objectives were to (i) to evaluate the antimicrobial and antioxidant activities of the EOs from *J. communis, J. scopulorum* and *J. horizontalis*; (ii) to screen their antibacterial mechanism by the cell membrane permeability assay (CMP) and (iii) to assess the possible cytotoxicity of the three tested EOs against the SH-SY5Y cell line.

Materials and methods

Essential oils

Fresh leaves and branches from *J. communis*, *J. horizontalis* and *J. scopulorum* were collected in the Bighorn Mountains (Wyoming) in 2011. The EOs were obtained by steam distillation and the components were identified as described in Zheljazkov *et al.* (2017).

Fungal isolates

Four post-harvest phytopathogenic fungi (*Monilinia fructicola* [G. Winter] Honey, *Aspergillus niger* van Tieghem, *Penicillium expansum* Link and *Botrytis cinerea* Pers.) were used for the antifungal activity assay. The tested fungi were conserved at 4°C in pure cultures in the mycotheca of the School of Agricultural, Forestry, Food and Environmental Sciences (SAFE), Basilicata University, Potenza, (Italy) and activated on potato dextrose agar (PDA) at 24 ± 2°C. The identification of tested fungi was carried out using morphological and molecular methods.

Fungicidal assay

The possible fungicidal activity of the EOs was evaluated at two different doses (1000 and 500 ppm) (v/v). The EOs were incorporated into PDA medium at 45 ± 2°C. Fungal disks (0·5 cm) from each phytopathogenic fungi (96 h fresh culture) were deposited in the centre of each Petri dish. All plates were incubated at 22 ± 2°C for 96 h in darkness conditions. As negative control, PDA

plates without any treatments were inoculated only with each fungus. The diameter of fungal mycelium growth was measured in mm and the fungitoxicity was expressed as percentage of mycelium growth (PG%) compared to the negative control and calculated according to the formula of Zygadlo *et al.* (1994):

$$\text{PGI}\,(\%) = \frac{100\text{X}(\text{GC} - \text{GT})}{\text{GC}}$$

where PG is the percentage of growth inhibition, GC is the average diameter of fungal mycelium in PDA negative control and GT is the average diameter of fungal mycelium on the oil-treated PDA dish.

Bacterial strains

Two Gram-positive (G+ve), *Bacillus megaterium* de Bary (ITM100) and *Clavibacter michiganensis* Smith and three Gram-negative (G−ve) bacterial strains, *Pseudomonas fluorescens* Flügge (Migula), *P. syringae* pv. *phaseolicola* Van Hall and *Xanthomonas campestris* Pammel were used in this assay. The tested bacterial strains were cultured on King B (KB) (King *et al.* 1954) and were previously identified by molecular method (PCR). All bacterial strains were maintained as lyophils at −20°C in the collection present at SAFE and reactivated again using KB media for 36 h at 30°C.

Bactericidal assay

The antibacterial test was carried out following the disc diffusion method as described by Bhunia *et al.* (1988) and slightly modified by Elshafie *et al.* (2016) using the King B nutrient media (KB). A bacterial suspension of each tested bacteria was prepared in sterile distilled water adjusted at 10^6 CFU per ml (OD ≈ 0·2 nm) using a Turbidimetry instrument (Biolog, Hayward, CA, USA). Four ml of bacterial suspension mixed with soft agar (0·7%) at ratio 9 : 1 (v/v) was poured over each plate (90 mm diameter). Blank discs of 6 mm (Oxoid, Milan-Italy) were then placed over the KB-plate surfaces and about 20 µl of each tested EO at dilution of 20% was carefully applied over discs. Tween 20 was added to each oil suspension (0·2%) for accelerating the oil solubility. Tetracycline was used as a positive control. The antibacterial activity was estimated by measuring the diameter of inhibition hyaline zone (mm) around each treated disc compared to the positive control one.

Cell membrane permeability

The CMP was determined by measuring the potential of electrical current transport through water as molar conductivity or electrolytic conductivity (EC) as reported by Elshafie *et al.* (2019). This assay was performed by transferring five mycelial discs (0·5 cm diameter) from fresh culture of the tested fungi into potato dextrose broth (PDB) medium and incubated under shaking condition (180 rev min^{-1}), at 28°C for 96 h. A gram and half of dried mycelia from each fungal species was re-suspended into 20 ml of each studied EOs at 250, 500, 1000 and 2000 ppm and incubated at 22 ± 2°C. The EC values have been measured after 30, 60, 90, 120 and 150 min of incubation. The increasing percentage (IP %) of EC value was calculated as follows:

$$\text{IP}\,(\%) = \frac{\text{EC t}}{\text{EC ctrl}} \times 100$$

where EC t is the EC value of the treated sample and EC ctrl is the EC value of the PDB broth culture.

Antioxidant activity

The antioxidant activity was determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical method (DPPH) as reported by Brand-Williams *et al.* (1995) with some modifications (Lee *et al.* 1998). For preparation of the standard curve, different concentrations of DPPH in MeOH solution in the range of 10–60 µg ml^{-1} were used. One mg of each EO was solubilized in 1 ml of DPPH solution (60 µmol l^{-1}) to prepare the stock solution. Methanol (MeOH) alone was used as blank. A cuvette with 1 ml of DPPH solution (60 µmol l^{-1}) was used as control. Absorbance at 515 nm was measured on the spectrophotometer Thermo scientific Multiskan GO (Thermo Fischer Scientific, Vantaa, Finland) after 15, 30 and 45 min. The final doses of each EO were 3, 6, 12·5, 25, 50, 100 and 200 µg ml^{-1}. The DPPH concentration (µg ml^{-1}) in the reaction medium was calculated from the following calibration curve determined by linear regression (r2: 0·9993):

$$\text{Absorbance}\,(\lambda\ 515) = 0 \cdot 0008 + 0 \cdot 0118 \times [\text{DPPH}]$$

The scavenging capability of test EOs was calculated as follows:

$$\text{DPPH scavenging activity}\,(\%) = \frac{100 \times [\text{A}(\lambda 515)\text{C} - \text{A}(\lambda 515)\text{S}]}{\text{A}(\lambda 515)\text{C}}$$

where A(λ515)C is the absorbance of a control with no radical scavenger and A(λ515)S is the absorbance of the remaining DPPH in the presence of scavenger. EC_{50} value was defined as the dose of sample which reduced the initial DPPH of 50%. Ascorbic acid, one of the most effective antioxidant agent, was used as reference substance in this study.

Cytotoxic activity

Cell Cultures

Human neuroblastoma (SH-SY5Y) cancer cells were cultured in Roswell Park Memorial Institute Medium (RPMI) medium supplemented with 1% L-glutamine, 10% heat-inactivated foetal bovine serum and 1% penicillin/streptomycin (all from Sigma-Aldrich, St Louis, MO) at 37°C in an atmosphere of 95% O_2 and 5% CO_2.

MTT bioassay

Human neuroblastoma cancer cells (SH-SY5Y) were plated (5×10^3) in 96-well microplates in 150 µl of RPMI medium and incubated at 37°C in humidified 5% CO_2. The day after, a 150 µl aliquot of serial dilutions of the three EOs (500–25 µg ml^{-1}) was added to the cells and incubated for 24 h. DMSO alone was used as control. Cell viability was assessed through the 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 30 µl of MTT (5 mg ml^{-1}) was added and the cells incubated for additional 3 h. Thereafter, cells were lysed and the dark blue crystals were solubilized with 30 µl of a solution containing 50% (v/v) N,N-dimethylformamide and 20% (w/v) SDS. The optical density (OD) of each well was measured with a microplate spectrophotometer (Thermo Scientific Multiskan GO, Monza, Italy) equipped with a 520 nm filter. Cell viability for each treatment was calculated as a percentage using the following formula (Mosmann, 1983):

$$\% \text{ cell viablility} = 100 \frac{\text{OD treated cells}}{\text{OD control cells}}$$

Doxorubicin, one of the most effective anticancer agents, was used as reference drug in this study.

Statistical analysis

The results of antimicrobial and CMP assays were statistically analysed using the SPSS statistical software program (ver. 13, 2004). The significance of outfindings was detected carrying the one-way analysis of variance (ANOVA) and Tukey's *post hoc* multiple comparison test with a probability of $P < 0{\cdot}05$. The results from antioxidant and cytotoxic assays were statistically analysed using the GraphPad Prism 6.0 software followed by comparison of means (two-way ANOVA) using Tukey's *post hoc* multiple comparison test (for antioxidant assay) and Dunnett's multiple comparison test (for cytotoxic assay), both at the significance level of $P < 0{\cdot}05$.

Results

In vitro antifungal activity

The tested EOs demonstrated antifungal activity against *M. fructicola*, *A. niger* and *P. expansum*, in a dose-dependent manner and no activity against *B. cinerea* (Fig. 1). In particular, *J. scopulorum* EO showed the highest significant activity against *M. fructicola* and *P. expansum*, at 1000 ppm dose; the highest significant fungicidal activity against *A. niger* has been observed with *J. communis* EO at 1000 ppm (Fig. 1).

In vitro antibacterial assay

The tested EOs demonstrated antibacterial activity against most of tested bacteria. In general, *J. communis* EO presented highest antibacterial activity and its effect was comparable among the tested G+ve and G−ve. In fact, this EO showed significantly higher activity against *P. fluorescens* compared to the positive control and highest activity against *P. syringae* pv. *phaseolicola* compared to the other EOs and moderate activity against *B. megaterium*, *C. michiganensis* and *X. campestris* (Fig. 2). *J. horizontalis* EO showed moderate bactericidal activity against *B. megaterium* and *P. fluorescens* and low effect against *C. michiganensis* and *X. campestris* (Fig. 2). The EO of *J. scopulorum* demonstrated moderate activity against *B. megaterium* and *P. fluorescens*; however, it showed the lowest effect against *X. campestris* (Fig. 2). *J. scopulorum* EO did not have any effect against *C. michiganensis* as well as *J. horizontalis* and *J. scopulorum* EOs did not show any effect against *P. syringae* pv. *phaseolicola*.

Cell membrane permeability assay

Table 1 reports the CMP of the fungi treated with different doses of the EO of *J. communis*. The two higher treatments (1000 and 2000 ppm) caused significant higher EC value than the other two treatments in *B. cinerea*. In all cases, the increase of CMP was gradual until 150 min of incubation. The IPs% ranged between 221 and 408, after 30 min, until 328 and 578, after 150 min of incubation for all EO doses.

In the case of *M. fructicola*, the EC value after the higher treatment (2000 ppm) was dramatically increased after 60 min and then was almost stable until 150 min of incubation. On the other hand, the other three treatments (250, 500 and 1000 ppm) showed similar EC values along the incubation period. The IPs% ranged between 198 and 308, after 30 min, until 338 and 442, after 150 min of incubation for all EO doses.

Regarding *A. niger*, the higher EO dose (2000 ppm) showed a slightly higher EC value only after 150 min.

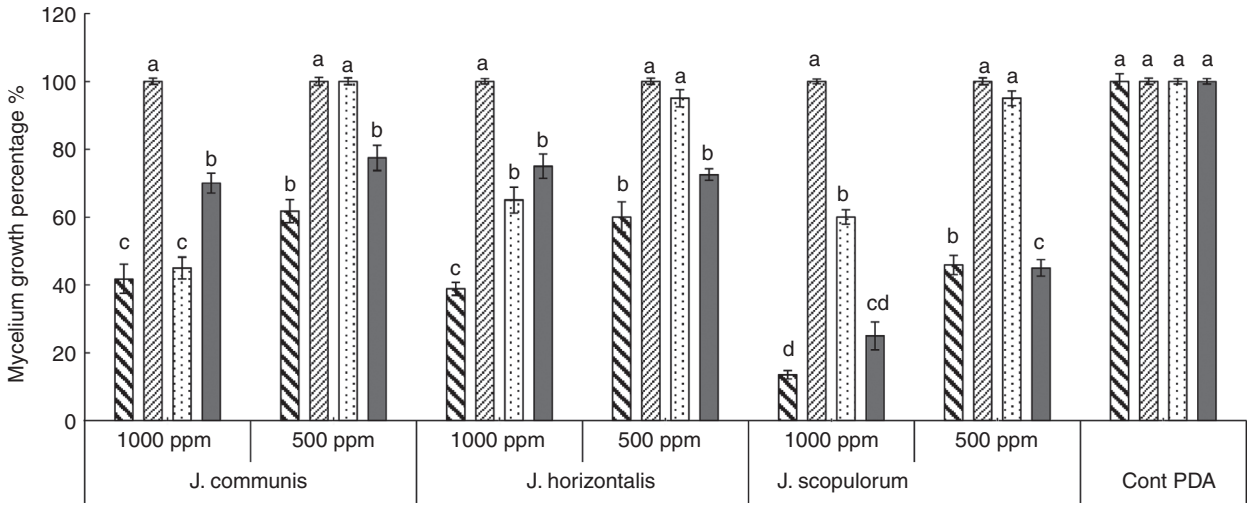

Figure 1 Antifungal activity of *Juniperus communis*, *J. horizontalis* and *J. scopulorum*. Bars with different letters indicate mean values significantly different at $P < 0{\cdot}05$ according to Tukey B test for each EO dose compared to control. Data are expressed as mean ± SDs (▧ *M. fructicola*; ▨ *B. cinerea*; ⬚ *A. niger*; ▩ *P. expansum*).

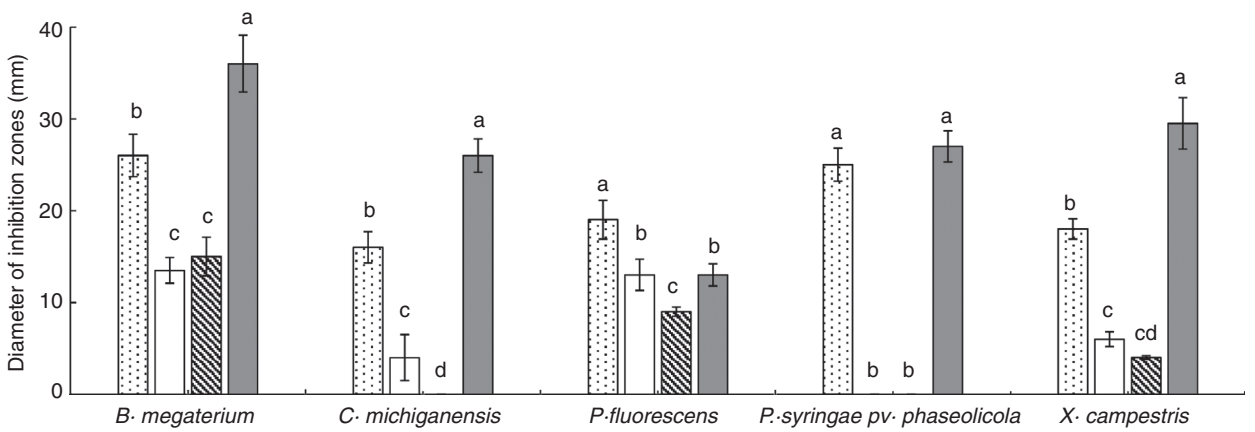

Figure 2 Antibacterial activity of *Juniperus communis*, *J. horizontalis* and *J. scopulorum*. Bars with different letters indicate mean values significantly different at $P < 0{\cdot}05$ according to *Tukey* B test for each tested bacteria compared to control. Data are expressed as mean ± SDs (▨ *J. communis*; □ *J. horizontalis*; ▧ *J. scopulorum*; ▩ tetracyclin).

The IPs% ranged between 485 and 693, after 30 min, until 404 and 523, after 150 min of incubation for all EO doses.

In the case of *P. expansum*, the CMP for the dose of 250 ppm showed the lowest significant EC value until 120 min of incubation, whereas 1000 and 2000 ppm showed the highest EC value only after 150 min. The IPs% ranged between 284 and 382, after 30 min, until 403 and 468, after 150 min for all EO doses.

Table 2 reports the CMP of the fungi, treated with different doses of the EO of *J. scopulorum*. The EC value of *B. cinerea* was generally increased over time in a dose-dependent manner. The EC values of G-ve control were almost stable along the incubation period (150 min). The EC value of the culture treated with 2000 ppm EO was significantly higher than all other treatments and was still increased even after 150 min of incubation, whereas other treatments were increased gradually until 120 min and then were slightly decreased. The IPs% ranged between 256 and 468, after 30 min, until 423 and 578, after 150 min of incubation for all doses.

In the case of *M. fructicola*, the treatment with 1000 and 2000 ppm showed higher CMP respect to the other two doses (250 and 500 ppm), even after 150 min. The IPs% ranged between 63 and 89, after 30 min, until 267 and 428 after 150 min of incubation for all doses.

Table 1 Effects of *Juniperus communis* EO on mycelium electrical conductivity of the tested fungi

	Reading (min)	Reading EC (min): *J. communis* EO				
		Js2000*	Js1000	Js500	Js250	CTRL
Botrytis cinerea	30	95	94·2	81·1	51·6	23·3
	60	97·5	91·2	88	58·4	21·7
	90	115	105	101	60·1	23·7
	120	121	116	103	63	24
	150	135·2	118·4	117·5	76·8	23·4
Monilinia fructicola	30	77·8	75·8	65·2	50·2	25·3
	60	120	82·6	77	74	27·1
	90	122	85·1	89	89	28·7
	120	122	87	90	89	26
	150	125	98·6	114	95·6	28·3
Aspergillus niger	30	114·3	116·7	94·4	80	16·5
	60	115·1	117	102	84	17·5
	90	125	127	110	95	23·3
	120	135	127	112	101	28·7
	150	146·5	132	119	113	28
Penicillium expansum	30	58·5	51·7	50·8	43·4	15·3
	60	64	60·6	59·6	44·5	17·9
	90	70	65	62·1	50·2	17·7
	120	86	75	64	61	16·5
	150	93·5	94	83	80·6	20

CTRL, control potato dextrose broth.
*Jc2000, Jc1000, Jc500 and Jc250 are the tested doses of *J. communis* EO at 2000, 1000, 500 and 250 ppm, respectively.

Table 2 Effects of *Juniperus scopulorum* EO on mycelium electrical conductivity

	Reading (min)	Reading EC (min): *J. scopulorum* EO				
		Js2000*	Js1000	Js500	Js250	CTRL
Botrytis cinerea	30	109·1	68·1	67·5	59·7	23·3
	60	112	80·5	82·5	70·2	21·7
	90	117	90	90	84	23·7
	120	130	101	103	95	24
	150	135·2	95	90	99	23·4
Monilinia fructicola	30	89·4	80·2	81	63	25·3
	60	96	94·2	83·2	64·3	27·1
	90	98	97	85	65	28·7
	120	115	101	107	91	26
	150	121	103	78·3	75·6	28·3
Aspergillus niger	30	119	111·8	104·2	92·1	16·5
	60	134	126	110	96·5	17·5
	90	132	130	122	97·5	23·3
	120	138	131	129	114	28·7
	150	140·5	139	133	125	28
Penicillium expansum	30	97·7	70·7	46·6	58·1	15·3
	60	98	73·1	61·5	59	17·9
	90	112	75	62	59·5	17·7
	120	113	106	83	76	16·5
	150	132	97	81	60	20

CTRL, control potato dextrose broth.
*Js2000, Js1000, Js500 and Js250 are the tested doses of *J. scopulorum* EO at 2000, 1000, 500 and 250 ppm, respectively.

In the case of *A. niger*, the treatment with 250 ppm showed lower CMP than the three higher doses where the maximum EC value was measured after 60 min. The IPs% ranged between 558 and 721, after 30 min, until 446 and 502, after 150 min of incubation for all doses.

In the case of *P. expansum*, the treatment with 2000 ppm showed higher CMP than the other treated doses and the maximum EC value was measured after 150 min. There are no significant differences between the two tested doses (2000 and 1000 ppm) after 120 min. The IPs% ranged between 380 and 639, after 30 min, until 300 and 660, after 150 min of incubation for all doses.

Table 3 reports the CMP of the fungi, treated with different doses of the *J. horizontalis* EO.

The EC value of *B. cinerea* treated with the highest dose (2000 ppm) was higher than the other doses. In addition, the doses 250, 500 and 1000 ppm showed similar EC values especially along the incubation from 120 to 150 min. The IPs% ranged between 179 and 490, after 30 min, until 406 and 578, after 150 min for all EO doses.

In the case of *M. fructicola*, the EC value of the highest dose (2000 ppm) was slightly higher than the other three doses. The IPs% ranged between 299 and 316, after 30 min, until 389 and 428, after 150 min of incubation for all EO doses.

In the case of *A. niger*, the lowest EC value was obtained with 250 ppm. However, the permeability was slowly increasing with the other doses along the incubation period (150 min). The IPs% ranged between 630 and 848, after 30 min, until 432 and 559, after 150 min of incubation for all EO doses.

The CMP of *P. expansum* at all treatments was increasing slowly until 90 min, and then increased dramatically after 120 and 150 min. The highest dose (2000 ppm) showed the higher EC value after 150 min. The IPs% ranged between 310 and 425, after 30 min of incubation, until 395 and 660, after 150 min of incubation for all EO doses.

Antioxidant activity

The antioxidant activity of juniper EOs was assessed by the DPPH assay. Figure 3 shows the antioxidant activity of *J. communis* EO. The inhibition of DPPH was dose-dependent: specifically, the dose of 500 μg ml^{-1} determines an inhibition of 52·03%, after 45 min experimental time. The EC_{50} values ranged between 501·3 μg ml^{-1} after 15 min and 487·1 μg ml^{-1} after 45 min of exposition. In

Table 3 Effects of *Juniperus horizontalis* EO on mycelium electrical conductivity

	Reading (min)	Reading EC (min): *J. horizontalis* EO				
		Js2000*	Js1000	Js500	Js250	CTRL
Botrytis cinerea	30	114·2	64·3	58·5	41·6	23·3
	60	116	76	62	69	21·7
	90	120	79	66	89·9	23·7
	120	130	95	84	99	24
	150	135·2	95	90	95	23·4
Monilinia fructicola	30	80	79	78·5	75·6	25·3
	60	91·5	80·1	90	81	27·1
	90	95	82	95	95	28·7
	120	111	95	100	102	26
	150	121	103	105	110	28·3
Aspergillus niger	30	140	138	130	104	16·5
	60	145	140·6	137·5	110	17·5
	90	146	142	139	116	23·3
	120	150	146	140	118	28·7
	150	156·5	139	127	121	28
Penicillium expansum	30	65	60	58	47·4	15·3
	60	67	65	61	63·9	17·9
	90	69	67	64	64·5	17·7
	120	86	80	88	71	16·5
	150	132	97	95	79	20

CTRL, control potato dextrose broth.
*Jh2000, Jh1000, Jh500 and Jh250 are the tested doses of *J. horizontalis* EO at 2000, 1000, 500 and 250 ppm, respectively.

the same figure, the antioxidant activity of the EO of *J. scopulorum* was reported: also in this case, the inhibition of DPPH was dose-dependent: specifically, the dose of 500 μg ml^{-1} determines an inhibition of 47·84, after 45 min experimental time of exposition. The EC_{50} values ranged between 531·3 μg ml^{-1} after 15 min and 527·4 μg ml^{-1} after 45 min of exposition.

The antioxidant activity of the EO of *J. horizontalis* is reported in Fig. 3, too. The highest dose tested (500 μg ml^{-1}) showed the same inhibition percentage of the highest doses of *J. scopulorum* EO, after 45 min of exposition. The EC_{50} values ranged between 547·4 μg ml^{-1} after 15 min and 533·3 μg ml^{-1} after 45 min of exposition.

Cytotoxic activity

The EOs of *J. communis*, *J. scopulorum* and *J. horizontalis* were evaluated for their ability to inhibit the growth of the human neuroblastoma cell line, SH-SY5Y. The EOs showed different cytotoxic activities. *J. communis* EO, with an IC_{50} of 53·7 μg ml^{-1}, exhibited a stronger cytotoxicity than *J. scopulorum* and *J. horizontalis* EO_S, with IC_{50} values of 278·8 and 245·5 μg ml^{-1}, respectively (Fig. 4).

Discussion

The composition of the studied EOs has been published in Zheljazkov *et al.* (2017). α-Pinene was the dominant compound in *J. communis* EO (73·0%), followed by α-terpinene (9·4%), myrcene (4·8%) and β-pinene (3·9%). Sabinene was the major component in the EOs of *J. horizontalis* (58·8%) and *J. scopulorum* (44·6%). Other components in *J. horizontalis* were as follows: α-pinene (4·9%), α-terpineol (4·8%), pregeijerene B (3·9%), γ-terpinene (3·5%), myrcene (2·3%), limonene (2·9%) and α-terpinene (2·1%). Limonene (7·6%), α-pinene (4·9%) and pregeijerene B (4·8%) were identified also in *J. scopulorum* EO. These results were in the agreement with previous studies (Zheljazkov *et al.* 2013b; Grul'ová *et al.* 2015).

The available literature reports α-pinene, β-pinene, limonene and β-myrcene as the main components of the EO of *J. communis* (Orav *et al.* 2010; Loziene *et al.* 2010; Adams *et al.* 2010; Falcão *et al.* 2018). Sabinene was identified as the dominant component in the EO of *J. communis* subsp. *alpina* (Cabral *et al.* 2012) and in the EO of *J. horizontalis* (Dambolena *et al.* 2011). α-Pinene,

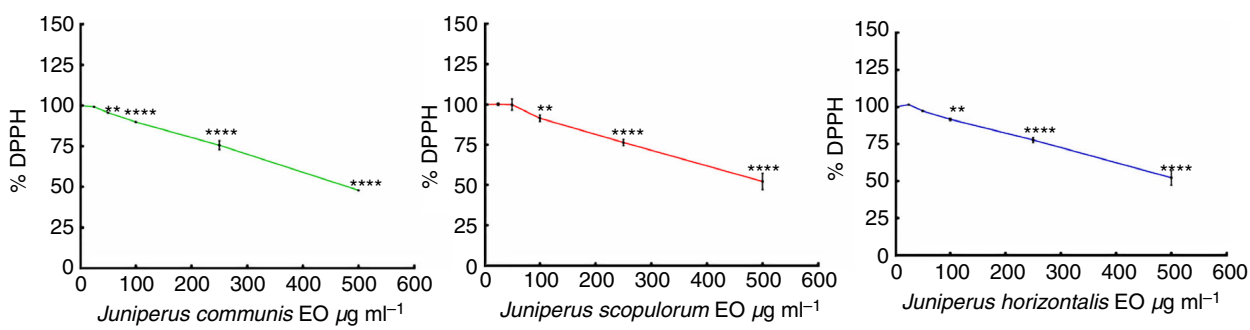

Figure 3 Antioxidant activity (percent of DPPH remaining) of *Juniperus communis, J. scopulorum* and *J. horizontalis* EOs after 45 experimental time. Results are the mean of three experiments. $^{**}P < 0{\cdot}01$; $^{****}P < 0{\cdot}0001$ *vs* CTRL DPPH (two-way ANOVA) using Tukey's test at the significance level of $P < 0{\cdot}05$. [Colour figure can be viewed at wileyonlinelibrary.com]

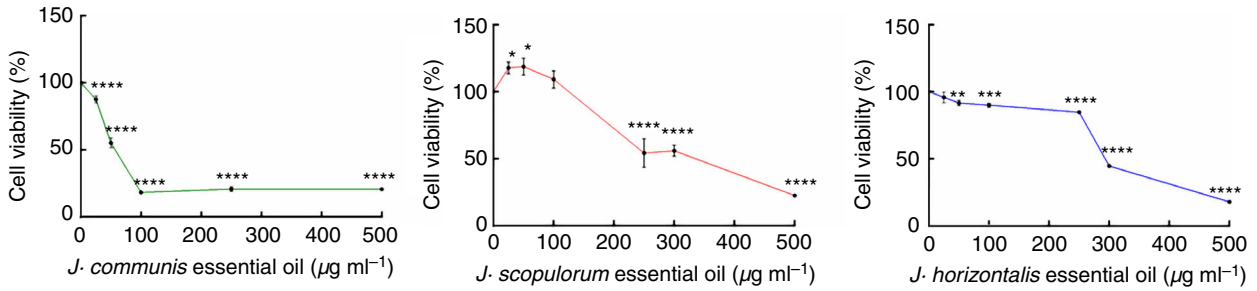

Figure 4 Percentage of cell viability after 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were treated with different concentrations (50–500 μg ml^{-1}) of three essential oils, for 24 h and solvent (DMSO, 0·1%) alone. Data are the mean ± SD of three experiments *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$, ****$P < 0·0001$ *vs* DMSO. [Colour figure can be viewed at wileyonlinelibrary.com]

sabinene, limonene and linalool have been reported as the main components of the EO of *J. horizontalis* (Cantrell *et al.* 2014; Eryiğit *et al.* 2014; Radoukova *et al.* 2018). Moreover, a long time unknown compound occurred in large amounts in numerous juniper species: it was identified as (E,E,E)-1,7-dimethylcyclodeca-1,4,7-triene or pregeijerene B (Cool and Adams 2003) with an unusual chemical structure with three E, E, E double bonds (Adams 2004b). The difference between the chemical compositions might be the results of the genetic factor as well as by the influence of environmental factors (Fejér *et al.* 2018).

The observed antimicrobial effect of the EOs is probably due to the occurrence of the dominant compounds α-pinene in the case of *J. communis* and sabinene in the case of *J. horizontalis* and *J. scopulorum*. Myrcene, α-terpinene and terpinolene, present in all three EOs, could have also an essential role in their bioactivity, in agreement with previous studies (Cavaleiro *et al.* 2006; Cabral *et al.* 2012). Other research studied the antimicrobial effects of juniper EOs on microbial strains causing problems in human (Cabral *et al.* 2012; Eryiğit *et al.* 2014). In particular, the EO from needles of *J. communis* subsp. *alpina* was evaluated against yeasts and filamentous fungi strains (*Aspergillus* sp. and dermatophytes). The majority of dermatophyte strains showed more sensitivity to this oil when compared with *Candida* sp. and *Aspergillus* sp., particularly for *Microsporum canis* and *Trichophyton rubrum* (Cabral *et al.* 2012). However, *J. oxycedrus* subsp. *oxycedrus* EO was reported as most active compared to other juniper species (Cavaleiro *et al.* 2006). In addition, the EO from *J. horizontalis* leaves was tested against three G+ve and three G−ve bacterial strains, showing high activity against the tested micro-organisms except *P. aeruginosa* (Eryiğit *et al.* 2014). Glišić *et al.* (2007) reported that α-pinene and sabinene, the main components of *J. communis* EO, showed higher antimicrobial activity than the crude EO.

In literature, there are only few reports regarding the antioxidant activities of juniper species. Antiradical activity reported in this research reveals an EC_{50} value of about 500 μg ml^{-1} for each tested EO. Lower values were reported in literature for the same or other juniper species. An EC_{50} value of 34·80 mg ml^{-1} was reported for the *J. communis* EO (Höferl *et al.* 2014); the EOs of *J. scopulorum* and *J. horizontalis* were reported for their higher antioxidant capacity, evaluated with ORAC test, in comparison to *J. communis* (Zheljazkov *et al.* 2017). Our results, obtained by DPPH test, showed that there are no significant difference in antioxidant activity between the tree oils.

The cytotoxic activity of three EOs was evaluated in the human neuroblastoma cell line, SH-SY5Y, by the MTT assay. Our results showed that *J. communis* EO has a stronger cytotoxic activity than the other two tested EOs, with 81% cell death after a treatment with 100 μg ml^{-1} for 24 h. Few studies reported the cytotoxicity of *J. communis* EO on different cell lines, but no studies have been carried out to verify the cytotoxicity of *J. scopulorum* and *J. horizontalis* EOs on neuroblastoma or other cell lines.

Maurya *et al.* (2018) demonstrated that the EO from fresh needles of *J. communis* from India was weakly cytotoxic against SiHa, A549 and A431 cell lines with IC_{50} values of 150·6, 134·4 and 98·0 μg ml^{-1}, after a treatment of 48 h, respectively. Vasilijević *et al.* (2018) reported an IC_{50} of 120 and 69·4 μg ml^{-1} against MRC-7 and A549 cell lines after a treatment of 24 h with the EO from seed cones *J. communis* var. *saxatilis* from Serbia. Furthermore, the EO from the needles of *J. communis* subsp. *alpina* was not interfered with keratinocyte cell line viability (Cabral *et al.* 2012).

Comparing the IC_{50} values, our findings indicated that the EO of *J. communis* was more cytotoxic than the EOs of the same specie tested in previous studies; probably, this was due to other components that act synergistically

with α-pinene, the main component in all researches considered (Cabral *et al.* 2012; Maurya *et al.* 2018; Vasilijević *et al.* 2018). Moreover, our EO of *J. communis* resulted more cytotoxic than *J. scopulorum* and *J. horizontalis* EOs, probably due to the presence of α-pinene (67–80%), reported as an apoptotic and antimetastatic compound (Matsuo *et al.* 2011), that instead was present in very low percentages in the other two EOs (2·3–13%). However, the studied EOs were not cytotoxic, as judged by the criterion set by the National Cancer Institute considering that their IC_{50} values were >20 μg ml^{-1} and only natural substances with IC_{50} < 20 μg ml^{-1} are considered cytotoxic (Geran *et al.* 1972).

The assays used for the evaluation of some biological activities of the tested EOs showed variable results. Heterogeneity could be explained by their different chemical composition and/or by different mode of actions against target micro-organisms. The outfindings of this research showed promising antimicrobial effects against the majority of the tested phytopathogens. Antioxidant activity was variable between all three evaluated juniper EOs, while cytotoxic activity of *J. communis* EO was significantly higher than that of *J. horizontalis* and *J. scopulorum*.

Authors' contribution

Conceptualization and project administration, D.G., V.D.F. and I.C.; antibacterial and antifungal activity, H.S.E.; cytotoxic activity, L.C., antioxidant activity, L.D.M.; data curation, H.S.E; writing original draft preparation, D.G., H.S.E., L.C. and L.D.M.; writing review and editing, I.C., H.S.E., V.J. and V.D.F. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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