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Original Article

Effect of Short and Long Term Irrigation with Treated Wastewater on Chemical Composition and Herbicidal Activity of *Eucalyptus camaldulensis* Dehn. Essential Oils

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Abstract

Water shortage throughout the world, especially in arid regions in the later decades has led to search for alternatives to save potable fresh water. Treated wastewater (TWW) appears to be an opportunity for irrigation. However, it could represent a stress factor for plants, and influence their metabolism, changing their secondary metabolites and, consequently, their biological properties. *Eucalyptus camaldulensis* essential oil (EO) had been reported to possess phytotoxic activity. The main objective of this work was to compare the chemical composition and herbicidal activity of *E. camaldulensis* EO obtained from leaves of young plants and old trees irrigated with well water (WW) and TWW. Germination tests were performed *in vitro* against *Amaranthus hybridus, Chenopodium album, Echinochloa crus-galli* and *Lolium perenne.* The EOs composition was analyzed by gas chromatography and gas chromatography/mass spectrometry. A high percentage of oxygenated monoterpenes, with 1,8-cineole as main compound, was found in the EOs from leaves of young plants irrigated with both types of water. The EO from leaves of old trees irrigated with WW contained a main fraction of monoterpene hydrocarbons (45.17%) with *p*-cymene as principal compound. The highest herbicidal potential was shown by the EO from young plants irrigated with TWW. It completely inhibited *A. hybridus* and *L. perenne* germination, and nearly blocked the others at all concentrations assayed. It also showed strong phytotoxic activity on seedling length. The results suggest the possible use of TWW to irrigate *Eucalyptus* crops as it enhances the EOs herbicidal potential that could be used as natural herbicides.

Keywords: germination; natural products; phytotoxicity; seedling growth; weed control

Introduction

The agricultural system is exposed to several threats that can influence crop yields and sustainability such as diseases and pests especially weeds (Vyvyan, 2002). Weeds take benefit of the advantageous conditions that occur in agricultural fields and present a good adaptability to different environments. There are many methods for weed management (cultural, mechanical, chemical, biological and biotechnological) but in most cases control relies in the use of synthetic herbicides. Their indiscriminate use can cause

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serious ecological and environmental problems such as the degradation of soil and water, the development of resistant weed biotypes and health problems for the living organisms (Verdeguer *et al*., 2011).

Allelopathy is a biological phenomenon which offers potential for weed management through the production and release of allelochemicals. This compounds can be released from the leaves, flowers, seeds, stems and roots of living or decomposing plant materials (Weston, 1996). Allelopathy can have positive or negative effects on the target organisms (Ben Ghnaya *et al*., 2015).

A variety of allelochemicals have been identified in aromatic plants known to be rich in active principles including essential oils that inhibit seed germination and plant growth (Arminante *et al*., 2006).

Eucalyptus is a large genus of aromatic trees of the Myrtaceae family which allelopathic effects have been studied. In California, the vegetation adjacent to naturalized stands of *E. camaldulensis* is often inhibited severely by the release of allelochemicals (Del Moral and Muller, 1970). The EO of this species showed phytotoxic effects on seed germination and seedling growth of some weeds (Verdeguer *et al*., 2009). *Eucalyptus* species are used for many purposes including forestry and forest products and in ornamental plantings and floriculture as well as in arts and crafts. Its leaves contain oil glands which produce oils with different allelochemicals, such as 1,8-cineole, limonene or α- and β-pinene, employed in medicine, cosmetic and pharmaceutical industries (Verdeguer *et al*., 2009). In Tunisia, 117 species of the genus *Eucalyptus* have been introduced since 1957. They were essentially used as fire wood, for the production of mine wood, and in the fight against erosion (Khouja *et al*., 2000).

Allelochemicals are a subset of secondary metabolites (Stamp, 2003). The synthesis and accumulation of secondary plant products can be influenced by environmental factors and growing conditions as temperature, light regime and nutrient supply (Gershenzon, 1984; Falk *et al*., 2007). Studies on the effects of treated wastewater irrigation focused on its impact on biomass growth, nutrient cycling and accumulation of trace metals in tree plantations and agricultural crops (Farahat and Linderholm, 2013) but there are few studies about the substitution of fresh water with TWW for irrigation of aromatic plants. Bernstein *et al*. (2009) demonstrated that oregano and rosemary were suitable as industrial crops for EO and antioxidant production under irrigation with TWW because their yield quantity and quality were not affected. They affirmed that cultivation of aromatic plants for EOs is suitable for irrigation with treated effluents because the heat applied during EO extraction eliminates human bacterial pathogens originated in the effluents and alleviates health concerns. Additionally, the EO, which is extracted mainly by steam distillation, will be free of inorganic ion contaminants such as heavy metals originating from the effluents, which may accumulate in the plant tissues and the soil.

The aim of this work was to evaluate the effect of short and long-term irrigation with TWW on *E. camaldulensis* EO composition and therefore, on its herbicidal potential.

Materials and Methods

Plant material and treatment conditions

To study the effects of long-term irrigation with TWW on *E. camaldulensis* EO composition, leaves of *Eucalyptus camaldulensis* Dehnh were collected from two different plots. The first was located in the waste water treatment station in the city of El Hamma-Gabes (33° 53′ 0″ N, 9° 47′ 0″ E, south of Tunisia), with an average annual potential evaporation of 1200 mm combined with the low rainfall and high temperatures. Trees were irrigated since 2006 with treated wastewater (TWW). The second was considered as a control plot, it was located in the same city, next to the waste water treatment plant of El Hamma-Gabes. In this plot the trees were irrigated with well water (WW) since 2006.

On the other hand, to study the effects of short-term irrigation with TWW on *E. camaldulensis* EO composition, a plantation of *E. camaldulensis* was carried out in experimental pots in a greenhouse at the regional station of the National Institute of Research on Rural Engineering, Water and Forest (INRGREF) - Gabes (Tunisia), from May 2, 2014 to September 28, 2015. The seeds of *E. camaldulensis* used for this experiment were obtained and identified in the laboratory of INGREF-Gabes (Tunisia). They were sown in 50 pots (30 × 60 cm) each containing experimental soil (3/4 sand and ¼ manure). One individual was kept per pot. Two treatments were carried out: (T1) irrigation with WW and (T2) irrigation with TWW obtained from waste water treatment station El Hamma-Gabes. Each treatment comprised 25 plants. Irrigation was done twice a week with 300 ml in summer and 200 ml in winter.

Mature seeds of the annual weeds *Amaranthus hybridus* L., *Chenopodium album* L. and *Echinochloa crus-galli* (L.) P. Beauv were collected from parent plants growing in agricultural fields in Valencia province, Spain. The plants were dried for 15 days at room temperature. Afterwards the seeds were extracted. Uniform healthy seeds were selected and stored at room temperature until germination tests. *Lolium perenne* L. seeds were purchased to Dalmau semillas.

Isolation of essential oils

Air-dried leaves were submitted to hydro distillation (100 g of each sample in one liter of distilled water) using a Clevenger-type apparatus for 3 h. The EOs were dried over using anhydrous sodium sulfate and stored in sealed glass vials at 4 °C until they were tested and analyzed. Yield was calculated (w/w %) based on dried weight of the sample (mean of three replications). The oil yields were higher after irrigation with TWW and varied between 1.06% and 1.58% for leaves of *E. camaldulensis* cultivated in pots for one year and between 1.23% and 2.95% for leaves of *E. camaldulensis* trees cultivated in plots for 10 years.

Analysis of the essential oils: Gas chromatography-mass spectrometry

For the EOs analysis 1 µl of diluted EO extract (1:100 in hexane) was injected in a Hewlett Packard model HP6890 gas chromatograph (Agilent Technologies. Palo Alto. CA. USA) equipped with DB-5MS capillary column (30 m ×

0.25 mm i.d; film thickness 0.25 µm; Agilent Technologies. USA) and coupled to a HP model 5973 mass selective detector. Injector temperature was 270 °C and initial column temperature was 50 °C. It was held at this temperature for 5 min, then heated to 280 °C at 4 °C min^{-1} and finally kept at 280 °C for 5 min. Helium was used as the carrier gas with a flow rate 1 $mLmin^{-1}$. The split ratio was 100:1, MS electronic was set at 70 eV, mass range was from m/z 40 to 500. Quadrupole temperature was 150 °C and ion source temperature 230 °C. Retention Indices (RI) of each constituent were calculated relative to a standard mix of n-alkanes (C_8-C_{26}, Sigma-Aldrich Co.) analyzed under identical experimental conditions. The identification of the constituents was performed by comparison of RI and MS spectra with those reported in the literature (Adams, 2007) and by computer matching with standard reference databases (NIST98, Wiley275 and CNRS libraries).

In vitro germination bioassay

The phytotoxic effect of four EOs (EO1, EO2, EO3 and EO4) was tested *in vitro* on seeds of *A. hybridus*, *C. album*, *E. crus-galli* and *L. perenne*.

EO1: Obtained from leaves of *E. camaldulensis* cultivated in pots and irrigated with well water for one year.

EO2: Obtained from leaves of *E. camaldulensis* cultivated in pots and irrigated with treated wastewater for one year.

EO3: Obtained from leaves of *E. camaldulensis* trees cultivated in plot and irrigated with well water for 10 years.

EO4: Obtained from leaves of *E. camaldulensis* trees cultivated in plot and irrigated with treated wastewater for 10 years.

For the *in vitro* phytotoxicity tests, sets of 20 seeds were placed between two layers of filter paper 73 g/cm^2 thick, wetted with the corresponding solution, in Petri dishes 9 cm^2 of diameter, with 5 replications per treatment for dicotyledon weeds *A. hybridus* and *C. album*. Sets of 10 seeds with 10 replications were prepared in the same way for monocotyledon weeds *L. perenne* and *E. crus-galli*. For *A. hybridus* only one test was performed, for the other weeds two, with higher concentrations. The treatments applied are summarized in Table 1. The Petri dishes were incubated at 30.0 ± 0.1 °C 16 h in light and 20.0 ± 0.1 °C 8 h in dark. To evaluate the phytotoxic potential of the EOs photos of the Petri dishes were recorded after 3, 5, 7, 10 and 14 days. The photos were processed with the software Digimizer and the germination and seedling length were obtained.

Statistical analysis

Tests were conducted in a randomized design. Data were submitted to analysis of variance. Previously, homoscedasticity was checked with Levene's test. Percentage values were arc sin transformed. Length values were transformed to y = log (x + 1). Means were compared using Fisher's least significant difference (LSD) test ($p < 0.05$).

Results

Essential oils chemical composition

The composition of the EOs tested is reported in Table 2. Chemical composition was different for each EO: 39 compounds were identified in *E. camaldulensis* EO from young plants irrigated with well water (EO1), accounting for 96.01% of the total EO composition, while 35 compounds were identified for *E. camaldulensis* EO from young plants irrigated with treated waste water (EO2), being 94.11% of the total EO. Both EOs (EO1 and EO2) contained a high percentage of oxygenated monoterpenes (58.05% and 58.33%, respectively). The main compound in the two EOs was 1,8-cineole (50.0 ± 2.35 and 52.26 ± 4.16), but in EO1 was followed by α-pinene (10.2 ± 1.3), p-cymene (8.4 ± 0.7) and globulol (6.46 ± 1.53) and in EO2 instead of p-cymene (1.4 ± 0.3), the third most abundant compound was the oxygenated sesquiterpene globulol (7.46 ± 0.59), followed by limonene (5.24 ± 1.52). In *E. camaldulensis* EOs from old trees irrigated with well water (EO3) 35 compounds were identified, representing 91.83% of the total EO composition, compounds were identified in *E. camaldulensis* EOs from old trees irrigated with treated waste water (EO4) defining 96.42% of the total EO. The composition of EO3 was the most diverse as compared with the other EOs studied, as it was characterized by a high fraction of monoterpene hydrocarbons (45.17%) with a prevalence of *p*-cymene (32.78 ± 0.27) instead of 1,8-cineole (21.64 ± 0.17). It also contained a greater fraction of oxygenated sesquiterpenes than the other EOs analyzed, being globulol (10.37 ± 0.09) the most abundant compound after 1,8-cineole. The composition of EO4 was similar to EO1 and EO2, with the oxygenated monoterpenes (60.34) as main fraction and also 1,8-cineole was detected as the major compound (55.47 ± 9.17), followed by α-pinene (17.54 ± 3.02) and limonene (5.95 ± 0.35).

Table 1. Treatments applied on the phytotoxicity *in vitro* tests

Weed species	EO concentrations tested
Amaranthus hybridus (AM)	Control (0), 0.125, 0.25, 0.5, 1µl ml^{-1}
Chenopodium album (CHE)	Test 1: Control (0), 0.125, 0.25, 0.5, and 1 µl ml^{-1} Test 2: Control (0), 1, 2, 4 and 8 µl ml^{-1}
Echinochloa crus galli (ECH)	Test 1: Control (0), 0.5 and 1 µl ml^{-1} Test 2: Control (0), 1, 2, 4 and 8 µl ml^{-1}
Lolium perenne (L)	Test 1: Control (0), 0.5 and 1 µl ml^{-1} Test 2: Control (0), 1, 2, 4 and 8 µl ml^{-1}

Table 2. Constituents of essential oils from *Eucalyptus camaldulensis* by GC and GC-MS analysis

Compounds	KI	EO1	EO2	EO3	EO4
Monoterpene hydrocarbons		25.70	19,46	45,17	26,71
α-Thujene	924	0,12 ± 0,04	0,04 ± 0,00	0.24 ± 0.01	0.03 ± 0.01
α-Pinene	931	10.25 ± 1.33	11.62 ± 0.68	3.35 ± 0.04	17.54 ± 3.02
β-Pinene	975	0.30 ± 0.04	0.37 ± 0.02	1.25 ± 0.01	0.56 ± 0.02
Myrcene	988	0.16 ± 0.01	0.11 ± 0.05	0.19 ± 0.00	0.46 ± 0.01
α-Phellandrene	1005	2.16 ± 0.51	0.17 ± 0.00	2.43 ± 0.04	0.05 ± 0.01
α-Terpinene	1015	0.08 ± 0.02	--	0.13 ± 0.01	0.04 ± 0.01
p-Cymene	1023	8.43 ± 0.73	1.40 ± 0.33	32.78 ± 0.27	0.73 ± 0.09
Limonene	1028	3.30 ± 0.17	5.24 ± 1.52	1.62 ± 0.06	5.95 ± 0.35
Y-Terpinene	1057	0.34 ± 0.03	0.25 ± 0.02	2.92 ± 0.04	0.40 ± 0.04
Terpinolene	1084	0.17 ± 0.04	0.17 ± 0.16	0.10 ± 0.02	0.80 ± 0.06
p-Cymenene	1088	0.13 ± 0.01	0.09 ± 0.01	0.16 ± 0.01	0.11 ± 0.01
α-Phellandrene epoxide	1201	0.26 ± 0.02	--	---	0.04 ± 0.01
Oxygenated monoterpenes		58.05	58.33	30.08	60.34
1,8-Cineole (Eucalyptol)	1031	50.01 ± 2.35	52.26 ± 4.16	21.64 ± 0.17	55.47 ± 9.17
Linalool	1098	0.11 ± 0.01	0.10 ± 0.01	0.09 ± 0.00	0.11 ± 0.03
α-Fenchol	1118	0.09 ± 0.01	0.07 ± 0.00	0.04 ± 0.01	0.06 ± 0.00
Terpinen-4-*ol*	1179	0.57 ± 0.09	0.38 ± 0.03	3.04 ± 0.01	0.49 ± 0.14
α-Terpineol	1194	0.85 ± 0.02	0.78 ± 0.08	1.70 ± 0.01	1.02 ± 0.15
trans-Carveol	1217	0.23 ± 0.01	0.22 ± 0.06	0.08 ± 0.01	0.09 ± 0.03
cis-p-Mentha-1(7),8-dien-2-ol	1228	0.74 ± 0.09	0.67 ± 0.08	0.15 ± 0.01	0.24 ± 0.10
Piperitone	1152	0.07 ± 0.01	0.06 ± 0.01	--	0.39 ± 0.01
Thymol	1288	0.11 ± 0.02	0.10 ± 0.05	0.36 ± 0.01	--
Carvacrol	1295	0.33 ± 0.08	0.12 ± 0.03	1.36 ± 0.03	--
Trans-Pinocarveol	1139	3.05 ± 0 .27	2.16 ± 0.43	0.58 ± 0.01	1.59 ± 0.65
Pinocarvone	1161	0.73 ± 0.08	0.48 ± 0.07	0.16 ± 0.01	0.35 ± 0.13
Borneol	1172	0.14 ± 0.03	0.10 ± 0.01	0.08 ± 0.02	0.09 ± 0.02
Carvotanacetone	1247	0.10 ± 0.01	--	0.38 ± 0.02	--
cis-p-Mentha-2,8-dien-1-ol	1135	0.05 ± 0.01	0.07 ± 0.02	--	0.01 ± 0.00
p-Cymen-8-ol	1186	0.87 ± 0.02	0.76 ± 0.14	0.42 ± 0.00	0.43 ± 0.07
Sesquiterpene hydrocarbons		1.45	2.10	1.37	1.51
β-Gurjenene	1437	1.11 ± 0.29	1.60 ± 0.20	1.12 ± 0.00	1.22 ± 0.86
allo-Aromadendrene	1459	0.34 ± 0.10	0.50 ± 0.27	0.25 ± 0.00	0.29 ± 0.00
Oxygenated sesquiterpenes		9.94	13.81	15.08	7.29
Spathulenol	1575	0.81 ± 0.14	1.64 ± 0.40	1.04 ± 0.01	0.20 ± 0.03
Viridiflorol	1584	1.10 ± 0.19	1.38 ± 0.15	1.43 ± 0.07	0.96 ± 0.51
Globulol	1584	6.46 ± 1.53	7.46 ± 0.59	10.37 ± 0.09	5.28 ± 2.83
10-*epi*-*Y*-Eudesmol	1622	0.71 ± 0.12	0.87 ± 0.07	1.04 ± 0.01	0.61 ± 0.36
Y-Eudesmol	1654	0.08 ± 0.02	0.24 ± 0.04	0.17 ± 0.02	0.08 ± 0.03
β-Eudesmol	1654	0.24 ± 0.07	0.42 ± 0.17	0.77 ± 0.01	0.16 ± 0.10
Isobicyclogermacrenal	1729	0.54 ± 0.18	1.80 ± 1.78	--	--
tau-Muurolol	1641	--	--	0.26 ± 0.01	Tr
Others		0.87	0.41	0.13	0.57
Isotorquatone	1801	0.30 ± 0.08	--	--	--
Isopentyl isovalerate	1105	0.57 ± 0.03	0.41 ± 0.06	0.13 ± 0.01	0.57 ± 0.02
Total identified		96.01	94.11	91.83	96.42

Compounds listed in order of elution in the HP-5 MS column, t= trace (< 0.01%). RI, retention index relative to C_8-C_{26} n-alkanes on the HP-5MS. Peak area percentages are calculated in GC on apolar HP-5 MS column. Values are means ± standard error

Germination tests

EO2 showed greater activity against weeds seed germination than the other EOs tested being the most effective completely inhibiting both *A. hybridus* and *L. perenne* seed germination at the highest concentration applied, 8 µl ml^{-1}, (Table 5) and also at this dose showed a good activity against *E. crus-galli*, reducing its germination 94.7%. In *C. album* the doses of 4 and 8 µl ml^{-1} reduced its germination 75% (Table 5). EO1 also inhibited completely *L. perenne* germination at the highest concentration applied, but against the other species showed less activity than EO2, and similar to EO3 and EO4 (Tables 3, 7 and 9). *L. perenne* and *A. hybridus* were the most sensitive species to this EOs, followed by *E. crus-galli* and *C. album*, which was the most resistant. EO1 and EO4 did not controlled *C. album* germination instead EO3 reduced it significantly 83.3% (Tables 3, 7 and 9).

Seedling growth

All the EOs tested reduced the seedling length of the species against they were assayed (Tables 4, 6, 8 and 10). EO 1 was active in *A. hybridus*, and *E. crus-galli* at concentrations higher than 0.5 µl ml^{-1} (Table 4). Against *L. perenne* in the first test EO1 showed activity also at 0.5 and 1 µl ml^{-1} but in the second test at concentrations greater than 2 µl ml^{-1}. *C. album* was the most resistant species, reducing its seedling length only when EO1 was applied at 8 µl ml^{-1} (Table 4). EO2 reduced *E. crus-galli* and *L. perenne* seedling length at concentrations higher than 1 µl ml^{-1} and *C. album* again was the most resistant species, being controlled at concentrations higher than 4 µl ml^{-1} (Table 6).

EO3 showed great activity against all the species tested (Table 8): in *A. hybridus*, it caused a significant decrease in seedlings length, at concentrations greater than 0.25 µl ml^{-1}. In *C. album* (test 1), *L. perenne* and *E. crus-galli* it was

effective at doses higher than 0.5 µl ml^{-1}. However, in *C. album* (test 2) was effective at concentrations higher than 1 µl ml^{-1} (Table 8). EO4 was very active in *A. hybridus*, reducing its growth when applied at 0.125 µl ml^{-1} or higher concentrations (Table 10). In *E. crus-galli*, was effective at concentrations greater than 1 µl ml^{-1}. Against *C. album* showed inhibitory effect at concentrations above 0.5 (test 1) or 1 (test 2) and reduced *L. perenne* seedling growth at concentrations greater than 2 µl ml^{-1}.

Table 3. Effects of essential oil from leaves of *Eucalyptus camaldulensis* plants cultivated in pots irrigated with normal water (EO1) on *Amaranthus hybridus*, *Chenopodium album*, *Echinochloa crus-galli* and *Lolium perenne* seeds germination

Treatment (µl ml^{-1})		*A. hybridus*	*C. album*	*E. crus-galli*	*L. perenne*
Test 1	0 (Control 1)	72.00 ± 5.39 a	51.11 ± 8.11 a	26.00 ± 5.81 b	70.00 ± 7.60 a
	0.125	78.00 ± 3.39 a	32.00 ± 8.31 b		
	0.25	56.00 ± 10.42 a	22.00 ± 7.68 b		
	0.5	19.00 ± 5.79 b	23.00 ± 6.04 b	49.00 ± 5.67 a	45.00 ± 4.77 b
	1	6.00 ± 4.85 b	27.00 ± 3.39 b	45.00 ± 4.53 a	36.00 ± 5.21 b
Test 2	0 (Control 2)		24.00 ± 6.60 a	76.00 ± 3.40 a	56.00 ± 3.67 a
	1		23.00 ± 9.30 a	54.00 ± 6.53 b	42.00 ± 3.39 b
	2		9.00 ± 4.85 a	38.00 ± 3.89 bc	34.00 ± 6.00 b
	4		14.00 ± 6.96 a	33.00 ± 6.33 cd	4.00 ± 1.87 c
	8		20.00 ± 5.70 a	22.00 ± 5.12 d	0.00 ± 0.00 d

Values are means ± standard error of five replicates of 10/20 seeds each after 14 days of incubation. Within each species, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test

Table 4. Effects of essential oil from leaves of *Eucalyptus camaldulensis* plants cultivated in pots irrigated with normal water (EO1) on seedling length (mm) of *Amaranthus hybridus*, *Chenopodium album*, *Echinochloa crus-galli* and *Lolium perenne*

Treatment (µl ml^{-1})		*A. hybridus*	*C. album*	*E. crus-galli*	*L. perenne*
Test 1	0 (Control 1)	23.44 ± 1.10 a	13.92 ± 0.64 a	67.41 ± 2.64 a	51.99 ± 3.52 a
	0.125	23.11 ± 0.70 a	11.40 ± 1.14 ab		
	0.25	19.51 ± 2.36 a			
	0.5	13.49 ± 1.17 b	10.13 ± 1.88 ab	35.85 ± 2.62 b	26.46 ± 1.67 b
	1	11.39 ± 5.90 b	12.32 ± 1.96 a	39.13 ± 1.78 b	22.84 ± 4.07 b
Test 2	0 (Control 2)		12.06 ± 1.73 a	59.99 ± 3.26 a	57.92 ± 1.81 a
	1		12.33 ± 1.96 a	40.55 ± 1.60 b	48.72 ± 3.76 a
	2		11.62 ± 2.68 a	31.51 ± 2.13 c	30.30 ± 4.38 b
	4		9.41 ± 0.54 ab	21.81 ± 1.91 d	18.51 ± 1.85 c
	8		4.66 ± 0.96 b	7.98 ± 0.97 e	

Values are means ± standard error of five replicates of 10/20 seedlings each after 14 days of incubation. Within each species, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test

Table 5. Effects of essential oil from leaves of *Eucalyptus camaldulensis* plants cultivated in pots irrigated with treated waste water (EO2) on *Amaranthus hybridus*, *Chenopodium album*, *Echinochloa crus-galli* and *Lolium perenne* seeds germination

Treatment (µl ml^{-1})		*A. hybridus*	*C. album*	*E. crus-galli*	*L. perenne*
Test 1	0 (Control 1)	60.00 ± 3.16 a	69.00 ± 6.00 a	26.00 ± 5.81 a	70.00 ± 7.60 a
	0.125	9.00 ± 4.30 b	58.00 ± 6.82 ab		
	0.25	1.00 ± 1.00 c	45.00 ± 5.24 b		
	0.5	0.00 ± 0.00		27.00 ± 6.51 a	40.00 ± 3.94 b
	1	0.00 ± 0.00	42.00 ± 9.03 b	25.00 ± 6.19 a	27.00 ± 4.23 b
Test 2	0 (Control 2)		24.00 ± 6.60 a	76.00 ± 3.40 a	56.00 ± 3.67 a
	1		19.00 ± 5.10 ab	46.00 ± 4.00 b	28.00 ± 5.39 b
	2		9.00 ± 1.87 bc	46.00 ± 10.30 b	23.00 ± 6.04 b
	4		6.00 ± 1.87 c	40.00 ± 11.40 b	9.00 ± 3.32 c
	8		6.00 ± 2.45 c	4.00 ± 2.45 c	0.00 ± 0.00 d

Values are means ± standard error of five replicates of 10/ 20 seeds each after 14 days of incubation. Within each species, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test

Table 6. Effects of essential oil from *Eucalyptus camaldulensis* plants cultivated in pots irrigated with treated waste water (EO2) on seedling length (mm) of *Amaranthus hybridus*, *Chenopodium album*, *Echinochloa crus-galli* and *Lolium perenne*

Treatment (µl ml^{-1})		*A. hybridus*	*C. album*	*E. crus-galli*	*L. perenne*
Test 1	0 (Control 1)	12.88 ± 1.45 a	13.92 ± 0.64 a	67.41 ± 2.64 a	51.99 ± 3.39 a
	0.125	15.97 ± 2.64 a	12.99 ± 0.74 a		
	0.25	8.26 a	10.11 ± 0.59 b		
	0.5				26.46 ± 1.67 b
	1		8.72 ± 0.52 b	42.11 ± 0.95 b	22.84 ± 4.07 b
Test 2	0 (Control 2)		12.06 ± 1.73 a	59.99 ± 3.26 a	57.92 ± 1.81 a
	1		8.64 ± 0.94 ab	44.98 ± 1.46 b	22.08 ± 3.23 bc
	2		6.46 ± 2.09 bc	27.50 ± 2.09 c	30.30 ± 4.38 b
	4		4.35 ± 0.68 bc	17.67 ± 2.03 d	18.51 ± 1.85 c
	8		3.37 ± 1.05 c	9.57 ± 5.36 d	

Values are means ± standard error of five replicates of 10/20 seedlings each after 14 days of incubation. Within each species, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test

Table 7. Effects of essential oil from leaves of *Eucalyptus camaldulensis* trees irrigated with normal water (EO3) on *Amaranthus hybridus*, *Chenopodium album*, *Echinochloa crus-galli* and *Lolium perenne* seeds germination

Treatment (µl ml^{-1})		*A. hybridus*	*C. album*	*E. crus-galli*	*L. perenne*
Test 1	0 (Control 1)	89.00 ± 4.85 a	69.00 ± 6.00 a	26.00 ± 5.81 a	69.00 ± 7.37 a
	0.125	3.00 ± 7.48 b	54.00 ± 7.48 a		
	0.25	5.00 ± 3.16 b	44.00 ± 10.17 a		
	0.5	3.00 ± 7.52 b	33.00 ± 7.52 a	15.00 ± 2.69 a	36.00 ± 4.76 b
	1	2.00 ± 8.72 b	49.00 ± 8.72 a	37.00 ± 6.84 a	17.00 ± 5.59 c
Test 2	0 (Control 2)		24.00 ± 6.60 a	76.00 ± 3.40 a	56.00 ± 3.67 a
	1		22.00 ± 5.15 a	51.00 ± 5.86 b	22.00 ± 6.82 b
	2		16.00 ± 3.67 a	38.00 ± 3.89 bc	15.00 ± 7.42 b
	4		11.00 ± 4.30 ab	33.00 ± 6.33 cd	1.00 ± 1.00 c
	8		4.00 ± 2.92 b	22.00 ± 5.12 d	1.00 ± 1.00 c

Values are means ± standard error of five replicates of 10/ 20 seeds each after 14 days of incubation. Within each species, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test

Table 8. Effects of essential oil from leaves of *Eucalyptus camaldulensis* trees irrigated with normal water (EO3) on seedling length (mm) of *Amaranthus hybridus*, *Chenopodium album*, *Echinochloa crus-galli* and *Lolium perenne*

Treatment (µl ml^{-1})		*A. hybridus*	*C. album*	*E. crus-galli*	*L. perenne*
Test 1	0 (Control 1)	24.26 ± 1.43 a	13.92 ± 0.64 a	63.23 ± 3.20 a	51.99 ± 3.39 a
	0.125	18.83 a	13.43 ± 0.63 a		
	0.25	9.53 ± 1.41 b	12.66 ± 1.41 ab		
	0.5	9.93 ± 0.20 b	10.34 ± 0.78 bc		28.27 ± 3.99 b
	1	9.74 ± 0.44 b	10.05 ± 0.59 c	38.09 ± 1.66 b	20.49 ± 4.45 b
Test 2	0 (Control 2)		12.06 ± 1.73 a	59.99 ± 3.26 a	57.92 ± 1.81 a
	1		6.59 ± 0.59 b	36.79 ± 2.68 b	22.53 ± 3.89 b
	2		3.94 ± 0.25 b	31.34 ± 3.19 bc	20.49 ± 4.45 b
	4		3.97 ± 0.81 b	25.07 ± 2.12 c	
	8		3.45 ± 0.36 b	12.82 ± 0.95 d	10.69 b

Values are means ± standard error of five replicates of 10/20 seedlings each after 14 days of incubation. Within each species, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test

Table 9. Effects of essential oil from leaves of *Eucalyptus camaldulensis* trees irrigated with treated waste water (EO4) on *Amaranthus hybridus*, *Chenopodium album*, *Echinochloa crus-galli* and *Lolium perenne* seeds germination

Treatment (µl ml^{-1})		*A. hybridus*	*C. album*	*E. crus-galli*	*L. perenne*
Test 1	0 (Control 1)	73.00 ± 4.06 a	26.00 ± 5.81 a	69.00 ± 7.37 a	69.00 ± 6.00 ab
	0.125	17.00 ± 7.35 b			77.00 ± 8.31 a
	0.25	16.00 ± 7.31 b			79.00 ± 6.40 a
	0.5	5.00 ± 0.00 bc	28.00 ± 5.33 a	36.00 ± 4.76 b	53.00 ± 9.30 b
	1	2.00 ± 1.22 c	41.00 ± 7.52 a	23.00 ± 3.67 b	46.00 ± 9.41b
Test 2	0 (Control 2)		76.00 ± 3.40 a	56.00 ± 3.67 a	24.00 ± 6.60 a
	1		51.00 ± 5.86 b	22.00 ± 6.82 b	32.00 ± 3.00 a
	2		38.00 ± 3.89 bc	15.00 ± 7.42 b	15.00 ± 2.74 a
	4		33.00 ± 6.33 cd	1.00 ± 1.00 c	37.00 ± 8.89 a
	8		22.00 ± 5.12 d	1.00 ± 1.00 c	21.00 ± 8.57 a

Values are means ± standard error of five replicates of 10/ 20 seeds each after 14 days of incubation. Within each species, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test

Table 10. Effects of essential oil from *Eucalyptus camaldulensis* trees irrigated with treated waste water (EO4) on seedling length (mm) of *Amaranthus hybridus, Chenopodium album, Echinochloa crus-galli* and *Lolium perenne*

Treatment (µl ml^{-1})		*A. hybridus*	*C. album*	*E. crus-galli*	*L. perenne*
Test 1	0 (Control 1)	24.26 ± 1.43 a	13.92 ± 0.64 a	67.41 ± 2.64 a	51.99 ± 3.39 a
	0.125	14.55 ± 2.97 b	15.28 ± 0.78 a		
	0.25	12.26 ± 2.54 b	14.04 ± 0.42 a		
	0.5	11.51 ± 2.18 b	11.77 ± 0.96 b		40.10 ± 3.52 a
	1	7.28 ± 2.03 b	10.05 ± 0.59 b	33.66 ± 2.4 b	47.18 ± 5.16 a
Test 2	0 (Control 2)		12.06 ± 1.73 a	59.99 ± 3.26 a	57.92 ± 1.81 a
	1		10.36 ± 0.66 ab	41.13 ± 2.79 b	45.87 ± 6.34 ab
	2		7.50 ± 1.08 bc	36.5 ± 1.66 b	44.46 ± 5.39 ab
	4		5.80 ± 0.52 c	28.44 ± 3.04 c	29.55 ± 2.27 b
	8		5.17 ± 1.83 c	14.09 ± 2.99 d	22.95 ± 11.53 b

Values are means ± standard error of five replicates of 10/20 seedlings each after 14 days of incubation. Within each species, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test

Discussion

The irrigation with treated wastewater caused differences in the chemical composition of the *E. camaldulensis* EOs studied. Mahumane *et al.* (2016) reported that mature leaves produced higher EO yields in comparison to the younger leaves. Many studies showed that instability of EO composition and yield of *Eucalyptus globulus* Labill. was influenced by the season and leaf age (Silvestre *et al*., 1997). Batish *et al.* (2008) showed that the amount of EO produced varies with season, climate change, species and the age of the plant.

EOs secondary metabolites vary also with the physiological and developmental stage of the plant (Einhellig, 1996). Kong *et al.* (1999) showed that the quantity of volatile oils increased under stress conditions. The application of treated wastewater (TWW) at a reasonable rate improved growth and productivity of some herbaceous species. However, the main problem that can arise from excessive and continuous application of TWW is phytotoxicity due to high content of salts and heavy metals (Bedbabis *et al*., 2010).

The important amount of EOs present in old leaves can be explained by their physiological stage, as with age, growth stimulating hormones like gibberellins and cytokinins decrease. Then, the biosynthesis of these hormones and terpenoid-rich EOs occurs via the mevalonic acid pathway and this may result in a shift of precursors towards the synthesis of the latter (Batish *et al*., 2006)

The chemical composition of *E. camaldulensis* EO from leaves was dominated by major volatile compounds *1,8*-cineole, *p*-cymene and *α*-pinene. Previous studies reported that *E. camaldulensis* EOs yielded from 0.26% to 3.48% being the highest amount found for plants cultivated in Taiwan (Su *et al*., 2006). Also, 1,8-cineole was the major compound detected in many *E. camaldulensis* EOs, the quantities were approximately 50% in EOs from plants cultivated in Iran, the Democratic Republic of the Congo, Brazil, Egypt and Nigeria (Barbosa, 2016). The EO from leaves of *E. camaldulensis* trees growing in Spain wild as ornamentals presented spathulenol and p-cymene as the major compounds (Verdeguer *et al*., 2009). In the EO of *E. camaldulensis* from Taiwan *α*-pinene, *p*-cymene and *α*-phellandrene were identificated as the principal constituents (Cheng *et al*., 2009).

Chemical analysis showed that the profile of monoterpenes changed according to the *Eucalyptus* ages and treatments. All the *E. camaldulensis* Eos obtained contained important monoterpenes percentages (75.25-87.05%). Oxygenated monoterpenes was the main phytochemical class on juvenile leaf EOs irrigated with WW and TWW. The changes in the EO composition were rather quantitative than qualitative. However, the EOs obtained from old plants, presented qualitative differences in their composition. A highest percentage of monoterpene hydrocarbons were found on the EO from trees irrigated with WW, while the EO from trees irrigated with TWW presented a chemical profile more similar to the EOs from young plants, also with higher content in oxygenated monoterpenes.

Ben Jemâa *et al.* (2012) reported that terpene emissions are influenced by many biotic and abiotic elements (light and temperature) and species-specific in particular. A qualitative and quantitative variation in chemical composition of *Eucalyptus* EOs was found in terpene levels according to the season.

Mahumane *et al.* (2016) showed that changes in chemical composition due to leaf age were noted at different stages of maturity. Higher percentage of limonene and *α*-terpineol were reported in young leaves, while higher levels of *α*-pinene and *1,8*-cineole were noted in mature leaves. EOs from different aromatic plants belonging to Lamiaceae, Compositae, Myrtaceae and Verbenaceae families have been reported to have allelopathic effects (Verdeguer *et al*., 2009). The allelopathic effect of the EOs is related to the EO composition and the species on which they are applied. In this sense, our results corroborate that the great differences in the EOs composition of *E. camaldulensis* produced a strong or more selective phytotoxic effect on the weed species tested. According to other authors (Scrivanti *et al*., 2003; López *et al*., 2009), the EOs rich in oxygenated compounds, were more active than EOs with high percentages of hydrocarbon compounds.

Conclusions

To summarize, this work studied the effect of TWW on chemical composition and herbicidal activities of *E. camaldulensis* EOs. The chemical composition of EOs obtained from *E. camaldulensis* plants and trees was largely

influenced by the water type used for irrigation. Irrigation with TWW was beneficial to increase the quality of the chemical composition of *E. camaldulensis* EOs. All the EOs studied showed herbicidal effects, inhibiting the germination and seedling growth of the species tested. They could be an alternative to synthetic herbicides more respectful with human health and the environment.

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Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

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