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Post-emergence herbicidal activity of *Eucalyptus globulus* Labill. essential oil

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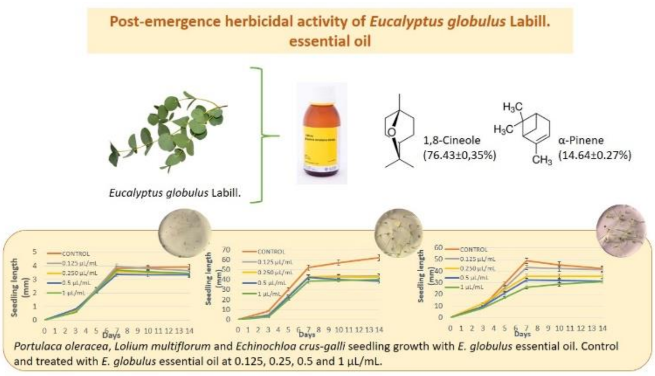
Graphical Abstract



Abstract.

Weed resistances to synthetic herbicides, as well as consequent health and environmental problems, are important items to find more eco-friendly natural alternatives to weed control. *Eucalyptus globulus* Labill. essential oil has been traditionally used against respiratory troubles as well as insect repellent due to 1,8-cineole content. Chemical composition of commercial *E. globulus* essential oil and its phytotoxic activity against three common annual weeds (*Portulaca oleracea* L., *Echinochloa crus-galli* (L) Beau. and *Lolium multiflorum* Lam.) has been studied. Twenty-eight compounds reaching 99.83% of the total essential oil were identified by gas chromatography-mass spectrometry analysis. The oxygenated monoterpene 1,8-cineole (76.43±0.35%), followed by the monoterpene hydrocarbon α-pinene (14.64±0.27%) were the main compounds. *E. globulus* essential oil lacks of phytotoxicity against the seed germination of the tested weed, showing significant effect on hypocotyl and radicle elongation of *E. crus-galli* at the highest dose (1 μL/mL) assayed and radicle inhibitory effects at all concentrations applied (0.125, 0.25, 0.50 and 1 μL/mL) against *L. multiflorum*. *E. globulus* essential oil could be used in the management of *E. crus-galli* due to its post-emergence herbicidal activity.

Introduction

Eucalyptus *(Eucalyptus globulus* Labill.) is a tree belonging to Myrtaceae family whose leaves are traditionally used in cough and flu disorders by its anticatarrhal, expectorant and antipneumonic properties [1,2]. Its essential oil has been included in a mixture with other volatile oils to relieve muscular aches, arthritis and respiratory troubles [3], being recently corroborated the mucolytic effect of the fluid extract obtained from the leaves of *E. globulus* together *Borago officinalis* L. and *Sambucus nigra* L. [4]. In addition, their essential oil is also a recognized insecticidal agent commonly used not only as an alternative pediculicide with a 100% of effectiveness in humans [5) but also as insect repellent against harmful creatures in agriculture, such as housefly (*Musca domestica*) and *Acanthoscelides obtectus* [6–8]. In this sense, *E. globulus* essential oil is being studied for pest control in food production, due to its wide-spectrum antimicrobial activity against storage foodstuff pathogens, like certain bacteria including *Escherichia coli* and *Pseudomonas aeruginosa* [9], fungal strains with a dose-dependent fungicidal effect against *Aspergillus flavus* and *A. parasiticus* and their aflatoxin production [10] and also against the normal development of *Fusarium verticillioides* by delaying spore germination causing a reduction in fumonisin production, too [11], as well as against other food spoilage microorganisms, such as yeasts strains (*Candida albicans* and *Sacchromyces cerevisiae*) [9]. Regarding this, there is an increased interest in the research of the industrial application of these properties, for instance *E. globulus* essential oil is incorporated as a natural antimicrobial ingredient in edible films exhibiting its antimicrobial and antioxidant properties and consequently enhancing microbial safety and shelf-life of food [12].

It is interesting to note the pesticide activity of natural compounds and their potential applications, particularly for a sustainable agriculture [13,14] due to the rising agrochemical problematic: the World Health Organization (WHO) warns about synthetic pesticides that have been seen to cause serious public health effects along years as consequence of the presence of considerable levels of pesticide residues in ground and surface water, as well as in food purchased in supermarkets, with their subsequently cause of human acute poisonings and even more cancer and other chronic illnesses [15,16]. Furthermore, it is of indispensable consideration the constant emergence of resistances by practically every type of organisms after the extensive use of pesticides making this fact one of the top four environmental problems in the world [15].

According to this, it is popularly known the case of glyphosate, the world's best known herbicide, whose resulting resistances have been described in many worldwide species, like common ragweed (*Ambrosia artemisiifolia* L.) in several row crops of the south-eastern USA following other still unknown mechanisms of action [17], annual ryegrass (*Lolium rigidum* L.) in Australia [18] or barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) in cotton fields of the midsouthern United States [19].

Regarding this, the phytotoxic effect of several essential oils continues to be studied against seed germination and seedling growth of some weeds [20]. For instance, *Citrus aurantiifolia* essential oil has demonstrated herbicidal effect against three agricultural weeds, *Avena fatua*, *Echinochloa crus-galli* and *Phalaris minor*, reducing their germination at ≥0.25-0.50 mg/mL as well as the coleoptile and root growth at ≥0.10-0.50 mg/mL [21]. Thyme (*Thymus vulgaris*), summer savory (*Satureja hortensis*), clove (*Syzgium aromaticum*) and cinnamon (*Cinnamomum zeylanicym*) essential oils have provided phytotoxic results causing electrolyte leakage and cell death of dandelion leaf (*Taraxacum officinale* Weber in Wiggers) [22], showing also *S. hortensis* essential oil nanoemulsion changes on germination, growth and morphophysiological features of *Amaranthus retroflexus* L. and *Chenopodium album* L. [23].

Regarding *E. globulus* essential oil, it is able to exert strong deleterious effects on the germination of *Amaranthus retroflexus* and *Portulaca oleracea* L. [24], seed germination and seedling growth of *Parthenium hysterophorus* L. [25] as well as on germination percentage and germination rate, radicle length, plumule length, primary root and pedicle length, and seedling height of *A. blitoides* and *Cynodos dactylon* (L.) Pers., at increasing concentrations [26].

Together the phytotoxic effects it is important to find selective herbicides that only disturb the seed germination and seedling development of weeds, without toxic effects on food crops.

So, the aims of this work are firstly to standardize through gas chromatography-mass spectrometry analysis the chemical composition of the commercial *E. globulus* essential oil in order to assure its main compounds and secondly, to determine their *in vitro* phytotoxic activity against seed germination and seedling growth of *P. oleracea*, a cosmopolitan annual weed of tropical and subtropical climates, *L. multiflorum*, a grass distributed along temperate climates affecting mostly cereals and *E. crus-galli*, an annual plant seriously influencing irrigation crops, especially rice, in order to obtain eco-friendly herbicides.

Materials and Methods

Essential Oil

Commercial sample of eucalyptus (*E. globulus* Labill.) (Batch 0065901) essential oil purchased from Guinama Lab. (Valencia, Spain), was stored at 4 ºC until chemical analysis and phytotoxic studies were carried out.

Seeds

Mature seeds of annual weeds of common purslane (*Portulaca oleracea* L.), Italian ryegrass (*Lolium multiflorum* Lam.) and barnyard grass (*Echinochloa crus-galli* (L.) Beauv.), were purchased from Herbiseed, UK (website: www.herbiseed.com).

Gas Chromatography-Mass Spectrometry Analysis

GC-MS analysis was carried out with a 5973N Agilent apparatus, equipped with a capillary column (95 dimethylpolysiloxane- 5 % diphenyl), HP-5MS UI (30 m long and 0.25 mm i.d. with 0.25 µm film thickness). The column temperature program was 60 ^{o}C during 5 min, with 3 ^{o}C/min increases to 180 ^{o}C, then 20 ^{o}C/min increases to 280 ^{o}C, which was maintained for 10 min. The carrier gas was helium at a flow-rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the m/z 30-500 range with an ionizing voltage of 70 eV.

Identification

The individual compounds were identified by MS and their identity was confirmed by comparison of their Kovat's retention index calculated using standard hydrocarbons relative to C_8-C_{32} *n*-alkanes, and mass spectra with reference samples or with data already available in the NIST 2005 Mass Spectral library and in the literature [27].

Herbicidal Activity

Sets of 20 seeds each with five replicates per treatment were homogenously distributed in Petri dishes (9 cm diameter) between two layers of filter paper (Whatman No.1) moistened with 4 mL of distilled water and with 0 (control), 0.125, 0.250, 0.5, and 1µL/mL of *E. globulus* essential oil. Petri dishes were sealed with parafilm and incubated in a germination chamber Equitec EGCS 301 3SHR model, according to previous assays [28] alternating 30.0 ± 0.1ºC 16 h in light and 20.0 ± 0.1ºC 8 h in dark and with (*E. crus-galli*) and without (*P. oleracea, L. multiflorum*) humidity. To evaluate the herbicidal activity of the essential oil, the number of germinated seeds was counted and compared with those of untreated seedlings. Emergence of the radicle (≥1 mm) was used as an index of germination and seedling length (hypocotyl and/or radicle) data was recorder after 3, 5, 7, 10 and 14 days in each replicate.

Statistical Analysis

Experiments were made with five replicates. Resulting data were subjected to one-way analysis of variance with IBM SPSS statistics 22 software. Tukey's *post hoc* test was used when variances remained homogeneous (Levene's test) and T3 Dunnett's *post hoc* one was employed if not, assuming equal variances. Differences were considered to be significant at $p \leq 0.05$.

Results and Discussion

Chemical Composition of *E. globulus* Essential Oil

Twenty-eight compounds reaching 99.83% of the total commercial *E. globulus* essential oil were identified by gas chromatography-mass spectrometry analysis. Compounds are clustered (Table 1) in homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes and listed according to Kovat's retention index calculated in GC on apolar HP-5MS column.

Highest quantities of monoterpene compounds (98.78%) were found in *E. globulus* essential oil. Twelve monoterpene hydrocarbons (21.93±0.08%) and seven oxygenated monoterpenes (76.85±0.13%) constituted the monoterpene fraction. The oxygenated monoterpene 1,8-cineole (76.43±0.35%) followed by the monoterpene hydrocarbon α-pinene (14.64±0.27%) were the main compounds. Among the monoterpene hydrocarbons, also large quantities of β-pinene (3.26±0.03%) and γ-terpinene (2.00±0.02%) were found.

β-Caryophyllene and longifolene with 0.51±0.01 and 0.30±0.00%, respectively, were the principal components among the sesquiterpene hydrocarbons. No higher percentages than 0.1% were found among the others sesquiterpene hydrocarbons identified.

Finally, caryophyllene oxide (0.04±0.00%) was the only oxygenated sesquiterpene found in *E. globulus* essential oil here analyzed.

The results obtained were similar to recent research [29] with 1,8-cineole (76.6%) and α-pinene (12.9%) as the main compounds of *E. globulus* essential oil and different from those obtained from samples of Pakistan [30] in which 1,8-cineole is the main compounds (56.5%) followed by limonene (28%) and α-pinene (4.2%).

Eucalyptus spp. are characterized by a great variability of the main compounds in their essential oils. Thus, in samples collected in Pakistan, large amount of citronellal (22.3%) and citronellol (20.0%) have been found in *E. citriodora* essential oil. Limonene (14.3%) and terpinen-4-ol (10.2%) were the main compounds in *E. crebra* essential oil; 1,8-cineole (15.2%) and α-pinene (12.1%) in *E. tereticornis* essential oil, while linalool (17.0%) followed by 1,8-cineole (16.1%) were the principal components in *E. camaldulensis* [31]. However *E. camaldulensis* is also characterized by a high content of spathulenol (41.46%) and *p*-cymene (21.92%) in samples collected in Valencia (Spain) [32]. The different chemical composition may be attributed to geographical, environmental and climatic differences [31].

Table 1. Chemical composition of commercial *E. globulus* essential oil

RT	RI	Compound	*E. globulus* Peak area (%)
		Monoterpene hydrocarbons	***21.93±0.08***
6.58	925	α-Thujene	0.01±0.00
6.88	933	α-Pinene	14.64±0.27
7.36	936	Camphene	0.18±0.00
7.58	952	Thuja-2,4(10)-diene	0.01±0.00
8.48	973	β-Pinene	3.26±0.03
9.11	987	Myrcene	0.77±0.01

9.65	998	α-Phellandrene	0.58±0.01
10.20	1012	α-Terpinene	0.14±0.01
11.25	1037	(*Z*)-β-Ocimene	0.11±0.01
11.68	1047	(*E*)- β-Ocimene	0.03±0.00
12.13	1056	γ-Terpinene	2.00±0.02
13.46	1083	Terpinolene	0.22±0.01
		Oxygenated monoterpenes	***76.85±0.13***
11.10	1033	1,8-Cineole	76.43±0.35
13.87	1091	Linalool	0.02±0.01
14.04	1094	α-Pinene oxide	0.01±0.00
17.59	1171	Terpinen-4-ol	0.04±0.01
18.01	1479	*p*-Cymen-8-ol	0.01±0.01
18.24	1184	α-Terpineol	0.31±0.02
22.62	1278	Bornyl acetate	0.03±0.00
		Sesquiterpene hydrocarbons	***1.01±0.00***
25.38	1341	α-Cubebene	0.05±0.00
26.21	1360	Longicyclene	0.01±0.00
27.71	1393	Longifolene	0.30±0.00
28.34	1408	β-Caryophyllene	0.51±0.01
29.74	1443	α-Humulene	0.06±0.00
30.72	1467	γ-Muurolene	0.01±0.01
31.68	1490	α-Muurolene	0.01±0.00
32.60	1513	δ-Cadinene	0.06±0.00
		Oxygenated sesquiterpenes	***0.04±0.00***
34.85	1571	Caryophyllene oxide	0.04±0.00
		TOTAL	**99.83±0.21**

RI, retention index relative to C_8-C_{32} *n*-alkane on HP-5MS column; values are mean ± standard deviation of three samples.

1,8-Cineole, the main constituent of the essential oil from *E. globulus* and other species, is a well-known wide-spectrum antibacterial agent [7]. Synergic effects against antibiotic-resistant pathogens have been observed with its combination with the sesquiterpene hydrocarbon aromadendrene, the main compound of essential oil from the fruits of *E. globulus* [33], and also with chlorhexidine digluconate, against methicillin-resistant *Staphyloccocus aureus* in planktonic and biofilm cultures [34,35].

On the other hand, its combination with α-pinene, the second main component of *E. globulus* essential oil here analysed provide beneficial effects against cellular oxidative stress, preventing reactive oxygen species-induced damage involved in the pathogenesis of several neurodegenerative diseases such as Alzheimer's disease [36].

Seed Germination and Seedling Growth Inhibition of *P. oleracea*, *L. multiflorum* and *E. crus-galli*, by *E. globulus* Essential Oil

The phytotoxic effect of *E. globulus* essential oil against seed germination and seedling growth of three well-known weeds, *P. oleracea*, *E. crus-galli* and *L. multiflorum*, is shown in Table 2 and Figures 1-3.

E. globulus essential oil had no effect against *P. oleracea*, *E. crus-galli* and *L. multiflorum* seed germination. No significant differences were found between control and all dose (0.125, 0.25, 0.5 and 1 µL/mL) assayed (Table 2).

Table 2. *In vitro* effects of *E. globulus* essential oil against *P. oleracea*, *L. multiflorum* and *E. crus-galli* seed germination and seedling growth.

Concentration (µL/mL)	***P. oleracea***		
	Germination	Hypocotyl growth	Radicle growth
Control	67.00±4.06 a	2.40±0.34 a	1.50±0.28 a
0.125	70.00±6.12 a	2.33±0.43 a	1.29±0.16 a
0.25	64.00±2.45 a	2.08±0.13 a	1.32±0.19 a
0.5	66.00±4.85 a	1.83±0.35 a	1.50±0.33 a
1	70.00±1.58 a	1.83±0.19 a	1.81±0.22 a
Concentration (µL/mL)	***E. crus-galli***		
	Germination	Hypocotyl growth	Radicle growth
Control	74.00±3.32 a	22.97±1.75 a	18.92±1.26 a
0.125	87.00±2.55 a	22.95±1.20 a	18.34±1.09 a,b
0.25	73.00±2.55 a	17.76±1.09 a,b	17.70±0.73 a,b
0.5	74.00±3.32 a	17.92±1.83 a,b	13.45±0.82 a,b
1	78.00±5.39 a	15.81±2.14 b	15.38±1.78 b
Concentration (µL/mL)	***L. multiflorum***		
	Germination	Hypocotyl growth	Radicle growth
Control	67.00±2.00 a	29.18±1.26 a	32.92±2.63 a
0.125	55.00±2.74 a	24.46±0.55 a	19.35±1.22 b
0.25	63.00±3.39 a	25.08±1.55 a	18.01±0.78 b
0.5	63.00±5.39 a	24.77±1.97 a	13.95±1.68 b
1	63.00±3.00 a	24.60±0.61 a	15.49±1.19 b

[a] Values are mean of five replications ± error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at p>0.05 according to T3 Dunnet and Tukey tests.

According to seedling growth, no significant differences in the seedling development (hypocotyl and radicle) of *P. oleracea* were observed after the application of *E. globulus* essential oil at all the concentrations assayed in comparison to control (Table 2, Figure 1).

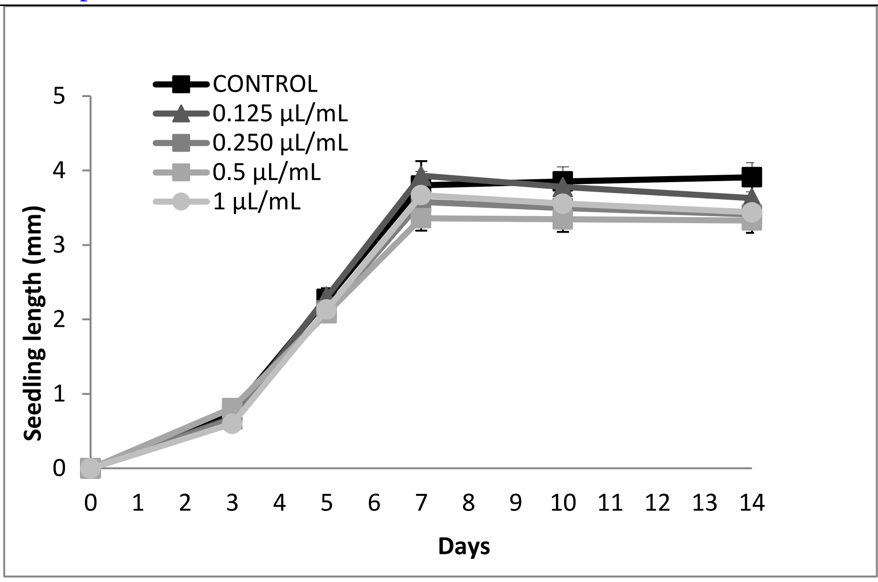


Figure 1. *P. oleracea* seedling growth with *E. globulus* essential oil. Control and treated with *E. globulus* essential oil at 0.125, 0.25, 0.5 and 1 µL/mL.

However, both hypocotyl and radicle of *E. crus-galli* were significantly inhibited at the highest dose tried (1 µL/mL) reaching 31.17 and 18.71% of growth reduction with respect to control (Table 2, Figure 2). Regarding *L. multiflorum* evolution, although no significant differences were observed in its hypocotyl growth (Table 2), the radicle development was considerably reduced between 41.22-52.95% without differences between all concentrations applied (0.125, 0.25, 0.50 and 1 µL/mL) (Table 2, Figure 3).

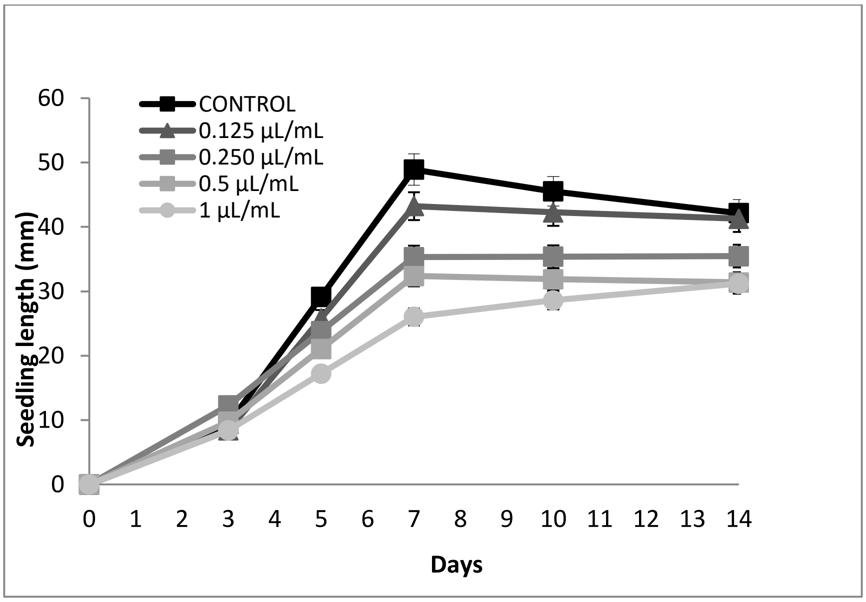


Figure 2. *E. crus-galli* growth with *E. globulus* essential oil. Control and treated with *E. globulus* essential oil at 0.125, 0.25, 0.5 and 1 µL/mL.

Regarding *L. multiflorum* evolution, although no significant differences were observed in its hypocotyl growth (Table 2), the radicle development was considerably reduced between 41.22-52.95%

without differences between all concentrations applied (0.125, 0.25, 0.50 and 1 µL/mL) (Table 2, Figure 3).

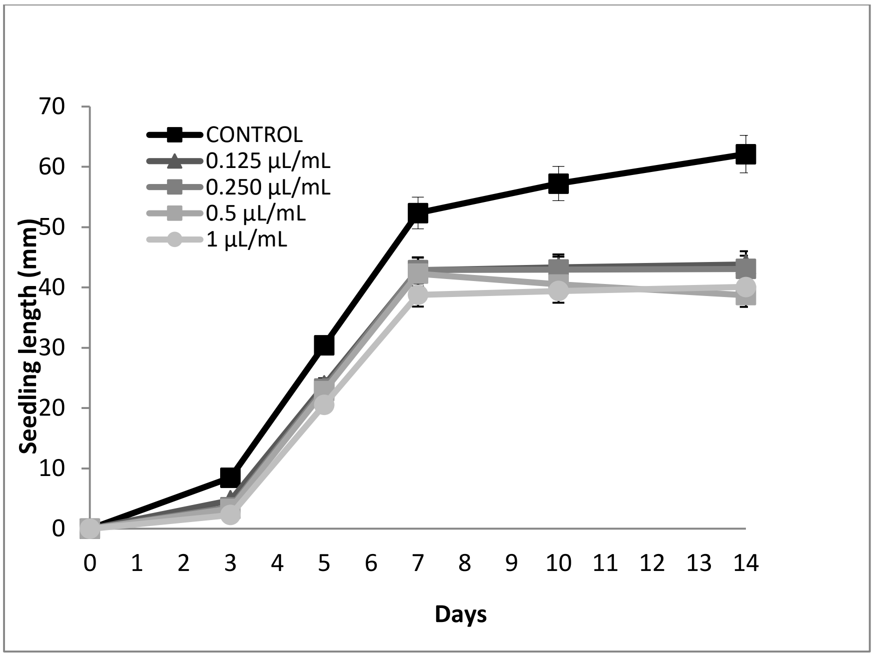


Figure 3. *L. multiflorum* seedling growth with *E. globulus* essential oil. Control and treated with *E. globulus* essential oil at 0.125, 0.25, 0.5 and 1 µL/mL.

The herbicidal potential is closely related with the essential oil composition, weeds and doses applied. In this ways, at the same doses and weed, *E. globulus* essential oil with 1,8-cineole (76.43%) has no phytotoxic effect against *P. oleracea* seed germination and seedling growth whereas *E. camaldulensis* essential oil with spathulenol (41.46%) as main compound was able to completely inhibit the seed germination of this cosmopolitan weed [32].

Regarding the weed, *E. globulus* essential oil showed at the doses employed significant effect on *E. crus-galli* seedling growth without phytotoxic effects against *E. crus-galli* seed germination while a higher doses (100 and 250 µg/mL*)* *E. tereticornis* essential oil with α-pinene, 1,8-cineole and β-pinene as the main compounds significantly affected both seed germination and seedling growth of *E. crus-galli* at [37]. Finally lower doses of *E. globulus* essential oil showed significant effects on seed germination and seedling development of others weed such as *Amaranthus blitoides* and *Cynodon dactylon* [26].

Conclusions

E. globulus essential oil with 1,8-cineole (76.43%) and α-pinene (14.64%) as the main compounds represents a potentially effective bioherbicide in the management of *E. crus-galli*. Further studies are needed with higher doses of *E. globulus* essential oil in order to corroborate a potential use also as a pre-emergent herbicide as well as its phytotoxic effects against food crops mainly rice and other cereals.

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