FULL PAPER

Evaluation of the Allelopathic Potential of Leaf, Stem, and Root Extracts of *Ocotea pulchella* Nees et Mart

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Despite the increase in recent decades in herbicide research on the potential of native plants, current knowledge is considered to be low. Very few studies have been carried out on the chemical profile or the biological activity of the Brazilian savanna (Cerrado) species. In the study reported here, the allelopathic activity of AcOEt and MeOH extracts of leaves, stems, and roots from *Ocotea pulchella* Nees was evaluated. The extracts were assayed on etiolated wheat coleoptiles. The AcOEt leaf extract was the most active and this was tested on standard target species (STS). *Lycopersicon esculentum* and *Lactuca sativa* were the most sensitive species in this test. A total of eleven compounds have been isolated and characterized. Compounds **1**, **2**, **4**, and **6** have not been identified previously from *O. pulchella* and ocoteol (**9**) is reported for the first time in the literature. Eight compounds were tested on wheat coleoptile growth, and spathulenol, benzyl salicylate, and benzyl benzoate showed the highest activities. These compounds showed inhibitory activity on *L. esculentum*. The values obtained correspond to the activity exhibited by the extract and these compounds may therefore be responsible for the allelopathic activity shown by *O. pulchella*.

Keywords: *Ocotea pulchella*, Ocoteol, Allelopathic activity, Cerrado, Allelochemicals.

Introduction

The Cerrado (the Brazilian savanna) is one of the most biodiverse vegetation formations in Brazil and it has experienced significant deforestation and fragmentation. The Cerrado contains at least 12,000 higher plant species but only a small fraction of these has been studied from the phytochemical viewpoint [1]. Allelochemicals arise from the secondary metabolism that operates in plant–plant interactions [2]. Furthermore, the distribution of these chemical compounds in plants is not uniform, either qualitatively or quantitatively, in space or time. Allelochemicals can be present in many parts of the plant, namely leaves, stems, roots, flowers, seeds, and bark. Under appropriate environmental conditions, these allelochemicals are released into the environment by volatilization, exudation from the roots, leaching from the aerial parts, and decomposition of plant remains. When these compounds are released into the environment they, or their degradation products [3], can have an effect on the growth and development of natural or implanted biological communities, either negatively or positively. Thus, these compounds can affect the development of neighboring plants, mainly in the germination and the growth of shoots and roots [4].

A knowledge of the main interactions between cultivated plants and secondary compounds extracted from native plants can be relevant in the management of weeds in agricultural ecosystems [5].

Weeds can be controlled using a combination of cultivation practices, such as sowing rates, mechanical weeding, crop rotation. and the use of competitive crops [6]. The use of synthetic herbicides is, however, the main method of weed control due to its high efficiency and practicality. Nevertheless, the intensive application of herbicides causes severe environmental damage and the evolution of herbicide-resistant weed populations [7]. Several factors may cause or accelerate the development of weed resistance to synthetic herbicides and these include the biological features of the plants and the chemical properties of the herbicide. The continued use of the same herbicide, or one with the same mode of action, is the main factor in the selection of resistant varieties. New regulations have reduced the number of synthetic pesticides available in agriculture due to the potential impact that they have on human health and the environment [8]. Knowledge of the role of chemical

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compounds extracted from plants, and their negative effects on the development of weeds, is important in the search for natural allelochemicals and synthetic derivatives for use as natural herbicides, since they have specific modes of action and are less damaging to the environment [9].

Lauraceae is a family with 55 genera and about 2500 – 3000 species of mostly tropical trees. Worldwide, this family is of considerable economic importance because it is used as a source of wood for construction (*Nectandra*, *Ocotea*, *Persea* spp.), as a crop (*Persea americana*) and to obtain flavors for the food industry, perfumery, and medicines (*Cinnamomum zeylanicum*, *C. cassia*) [10][11]. The genus *Ocotea* (Lauraceae) is widely represented in the American tropics with 300 – 400 species and it is also present in Brazil with about 150 species [12]. The large number of species in the genus *Ocotea* has attracted the interest of Brazilian phytochemical researchers [13]. Some species of this genus are used in popular medicine due to their antirheumatic, purgative, and tonic properties, and for the treatment of stomach disorders and abscesses [14], among other uses. The Lauraceae family is known to contain high levels of essential oils, which are mainly present in the leaves and have different reported biological activities [12]. Other species are considered to be aromatic and they are therefore used in perfumery and flavorings [15]. These oils have also shown herbicidal activity with inhibitory effects observed on germination, growth, and chlorophyll concentration in the plant species studied as targets [16].

Chemical studies carried out with species of the genus *Ocotea* have often shown the presence of aporphine alkaloids [17], lignans and neolignans [18], tannins, steroids, triterpenes, and phenylpropanoids [19]. In a phytochemical study on leaves of *O. pulchella*, it was found that leucoanthocyanidins, glycosides, flavonoids, flavonols, steroids, triterpenes, and anthocyanin were present in addition to the potential larvicidal, cytotoxic, and antioxidant properties [20]. To date, several compounds have been isolated from this plant and various biological activities have been identified. *O. pulchella* is widely distributed in the Cerrado (Brazilian savanna) and different extracts of this plant have also shown allelopathic activity, but the natural products responsible for the observed activity have not been identified [21].

The aim of the study described here was to determine the compounds responsible for the allelopathic activity of *O. pulchella*. In order to achieve this aim, a bioassay-guided isolation was carried out to identify the plant part and the extract that were the most allelopathic. The major components from this active extract were isolated, characterized, and tested in appropriate bioassays to identify the metabolites responsible for the observed activity.

Results and Discussion

Dried leaves, stems, and roots of *O. pulchella*, were extracted with AcOEt and MeOH, respectively. These extracts, at concentrations of 0.8, 0.4, and 0.2 mg/ml, were subjected to an etiolated wheat coleoptile bioassay. This bioassay showed a higher inhibition by the AcOEt extract from leaves, with a value of nearly 60% at 0.8 mg/ml, whereas the effects of stem and root extracts was of little significance when compared with that of the control. The MeOH extracts from leaves, stems, and roots showed lower levels of inhibition than the AcOEt extracts, *i.e.*, 20 – 40% at the highest concentration (0.8 mg/ml) (*Fig. 1*).

The differences in the activity between the leaf, stem, and root extracts are consistent with literature data in terms of the concentrations of allelochemicals in different plant parts [22].

The differences in the activity profiles between the MeOH and AcOEt extracts suggest that the most active metabolites have a medium polarity. Moreover, the activity profile of the AcOEt extract of leaves had a dose–response relationship in which an increase in the concentration from 0.2 to 0.8 mg/ml resulted in an increase of about 40% in inhibition (*Fig. 1*). Allelochemicals that typically have medium polarity belong to the groups of alkaloids, flavonoids, phenols, and terpenoids [23]. As in the present study, *Miranda et al.* [24] found greater inhibition in wheat coleoptiles exposed to AcOEt extracts. Therefore, the AcOEt extract of leaves was evaluated in a subsequent phytotoxicity bioassay.

A phytotoxicity test on standard target species (cress, onion, lettuce, and tomato) was employed to assess the bioactivity of the AcOEt extract of leaves from *O. pulchella* on the development of root, shoot, and seed germination. The parameter that was most affected by the AcOEt extract of leaves was root length, with inhibition values for *Lycopersicon esculentum* of around 65%. In addition, a dose-dependent inhibition was observed on the root tomato seedlings tested (*Fig. 2*). The sensitivity of roots to allelochemicals can be explained by the fact that roots are the first parts of the plant to emerge and they are in direct contact with the extracts, which can be absorbed directly [25].

In *Lactuca sativa*, the parameter that was affected the most was the root growth, with an inhibition of 45% at the highest concentration (0.8 mg/ml). *L. esculentum* was the only species that was significantly affected in relation to the germination and development of root and stem upon exposure to the AcOEt extract (*Fig. 2*). The observed differences in susceptibility between the target species are consistent with the literature data. Tolerance or resistance to allelochemicals may be specific and certain species may have a greater sensitivity than others; for example, lettuce (*L. sativa* L.) and tomato (*L. esculentum* L.) are bioindicators of allelopathic activity.

The herbicidal potential of the genus *Ocotea* has already been identified by other researchers. For example, *Borges et al.* [16] found that aqueous extracts of *Ocotea odoriferous* significantly reduced the germination, root growth, and chlorophyll content of *Sorghum bicolor* L. species.

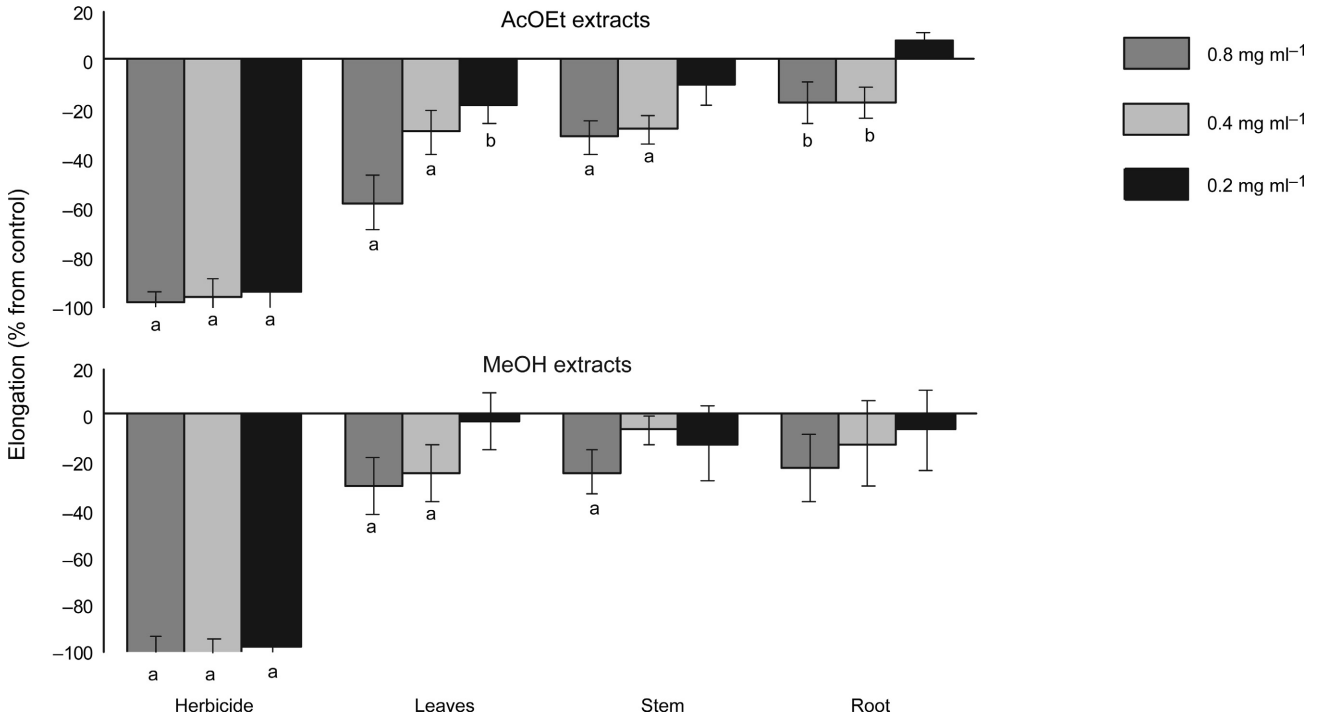

Fig. 1. Effects of leaf, stem, and root extracts from *Ocotea pulchella* and the herbicide *Logran*® on the elongation of etiolated wheat coleoptiles. Values are expressed as percentage difference from control. Levels of significance at $P < 0.01$ (a) and $0.01 < P < 0.05$ (b), according to the *Welch*'s test compared to control.

The AcOEt extract of leaves was selected for further study. This extract was purified by chromatography and the following allelochemicals were characterized: five sesquiterpenes (**1** – **5**), one fatty acid (**6**), two flavonoids (**7** and **8**), one phenylethanoid glucoside (**9**), two aromatic esters (**10** and **11**), and two sugars (**12** and **13**). The isolated compounds were identified by comparison of their spectroscopic data (^{1}H-NMR, ^{13}C-NMR, IR, and MS) with those reported in the literature for 1*β*,6*α*-dihydroxyeudesm-4(5)-ene (**1**) [26]; 1*β*-hydroxyeudesma-5,11-diene (**2**) [27]; T-cadinol (**3**) [28]; loliolide (**4**) [29]; spathulenol (**5**) [30]; dimorphecolic acid (**6**) [31]; afzelin (**7**) [32]; kaempferol-4′-*O*-rhamnopyranoside (**8**) [33]; benzyl salicylate (**10**); benzyl benzoate (**11**) [34]; both anomers of D-xylopyranose [35] (*Fig. 3*).

The chemical profile of the extracts revealed a high proportion of sesquiterpenes. Several biological activities [36] have been reported for sesquiterpenes **1** – **4** and these include anti-inflammatory [36a], antiallergic [36b], local anesthetic [36c], nematicidal on *Bursaphelenchus xylophilus* [36d], cytotoxic, antifungal, and bactericidal [36e], in addition to herbicidal activity on weeds [24][36f].

Compounds **1**, **2**, **4**, and **6** have not been isolated before from *O. pulchella*. Compound **6** is a polyunsaturated long-chain fatty acid and this class of compound has shown several biological activities, including antimicrobial, cytotoxic, antioxidant, and signaling [37][38].

Compound **9** (3.1 mg) was isolated from subfraction *A4.4* (54.5 mg) as a yellow amorphous solid. The HR-EI-TOF-MS data for **9** showed a molecular ion at *m*/*z* 433.1497 ([*M* – H]$^-$), which is consistent with the molecular formula $C_{22}H_{26}O_9$. The ^{1}H-NMR spectrum of **9** (*Table 1*) is consistent with the presence of an anomeric H-atom that resonates at *δ*(H) 4.24 and aromatic H-atoms between *δ*(H) 6.71 and 7.08. The ^{13}C-NMR spectrum is consistent with the presence of a sugar and aromatic rings. The sugar was identified as glucose by comparison of the chemical shifts and splitting patterns of the sugar H-atoms [39]. The anomeric C(1)-atom (*δ*(C) 104.4) was correlated to H–C(8′a) (*δ*(H) 3.91) and H–C(8′b) (*δ*(H) 3.66) in the HMBC spectrum. The H–C(8′a) and H–C (8′b) signals were correlated in the COSY spectrum and further correlated to the signal for H–C(7′) (*δ*(H) 2.81). The signal for H–C(7′)was correlated in the HMBC to three aromatic C-atom signals at *δ*(H) 130.0 (C(1′)), *δ*(C) 131.3 (C(2′), C(6′)), and C(8′) (*δ*(C) 71.7). These findings suggest that **9** has a phenylethanoid glycoside as a partial structure. The resonances at *δ*(H) 4.42 and 4.20 were assigned to H–C(6a) and H–C(6b), respectively, of the sugar moiety due to the correlation with H–C(5) (*δ*(H) 3.42) in the COSY spectrum. The phenylethanoyl substitution at C(6) was revealed by the HMBC experiment, in which H–C(6a) and H–C(6b) were correlated to the ester C=O C-atom (*δ*(C) 173.8 (C(8′′))) (*Table 1*). C(8′′) was correlated to H–C(7′′) at *δ*(H) 3.53, and H–C(7′′) was correlated to the aromatic C-atom resonances at *δ*(C) 126.3 (C(1′′)) and *δ*(C) 130.9 (C(2′′), C(6′′)) in the HMBC spectrum of **9**. The positions of the OH groups in the aromatic rings were determined by comparison of the chemical shifts with those of compounds with the same

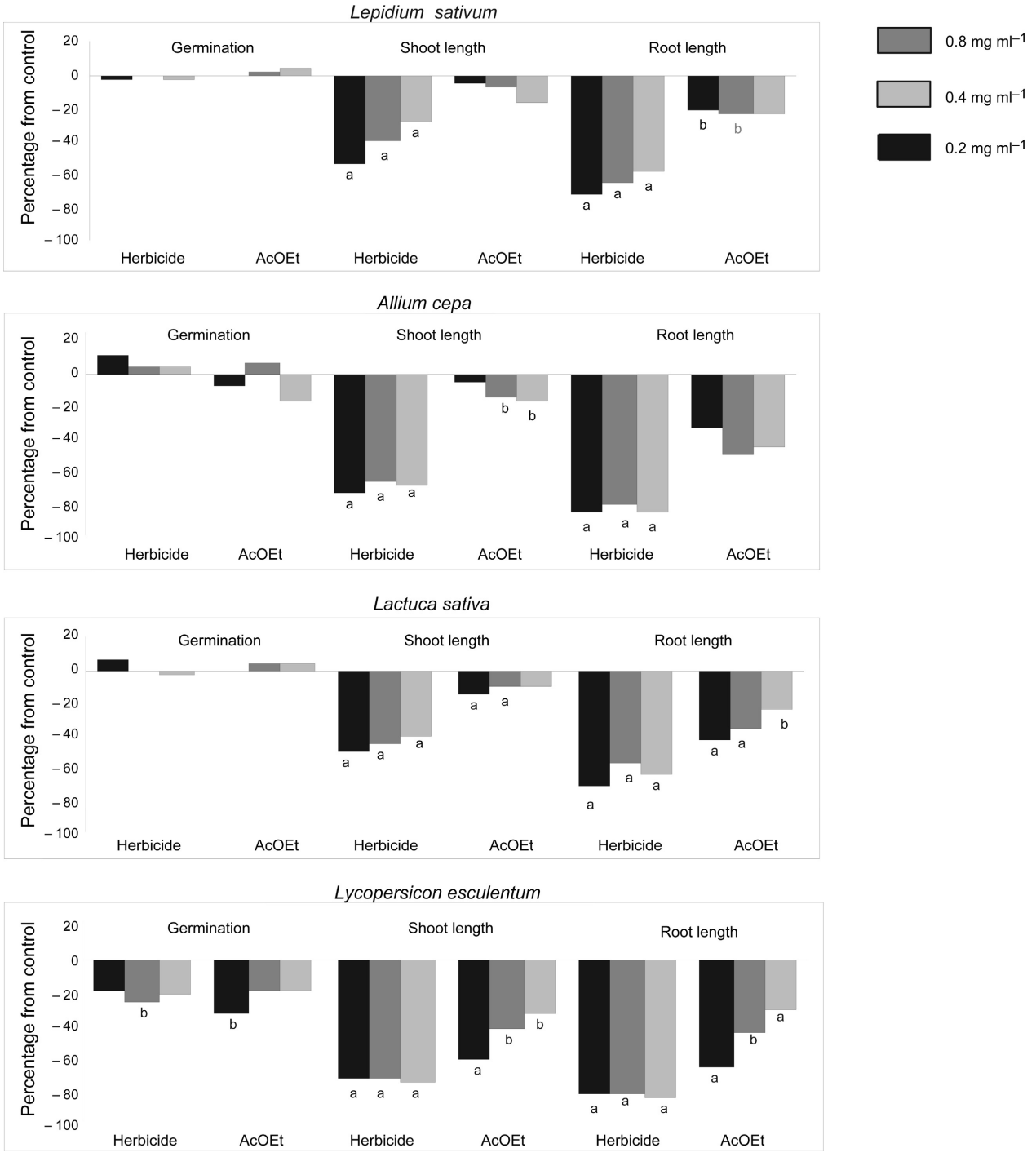

Fig. 2. Effects of the herbicide *Logran*® and the AcOEt extract of leaves from *Ocotea pulchella* on the growth of standard target species. Values are expressed as percentage difference from control. Levels of significance at $0.01 < P < 0.05$ (b) or $P < 0.01$ (a) according to *Welch*'s test compared to control.

hydroxylation pattern [4]. The corresponding aromatic C-atoms were assigned from the HSQC data for **9**. Therefore, the structure of **9** was assigned as shown in *Fig. 3*. This compound is described here for the first time and it has been named ocoteol.

The bioactivities of five sesquiterpenes (**1** – **5**), one flavonoid (**7**), and two aromatic esters (**10**, **11**) were assessed in the wheat coleoptile bioassay in a concentration range from 10^{-3} to 10^{-5}M. Compounds **8** and **9** were not tested due to the low amounts obtained. The results are summarized in *Fig. 4*.

Sesquiterpenes **1** – **5** showed significant inhibitory activity on coleoptile elongation at the two highest concentrations (10^{-3} and 3×10^{-4}M), with values between

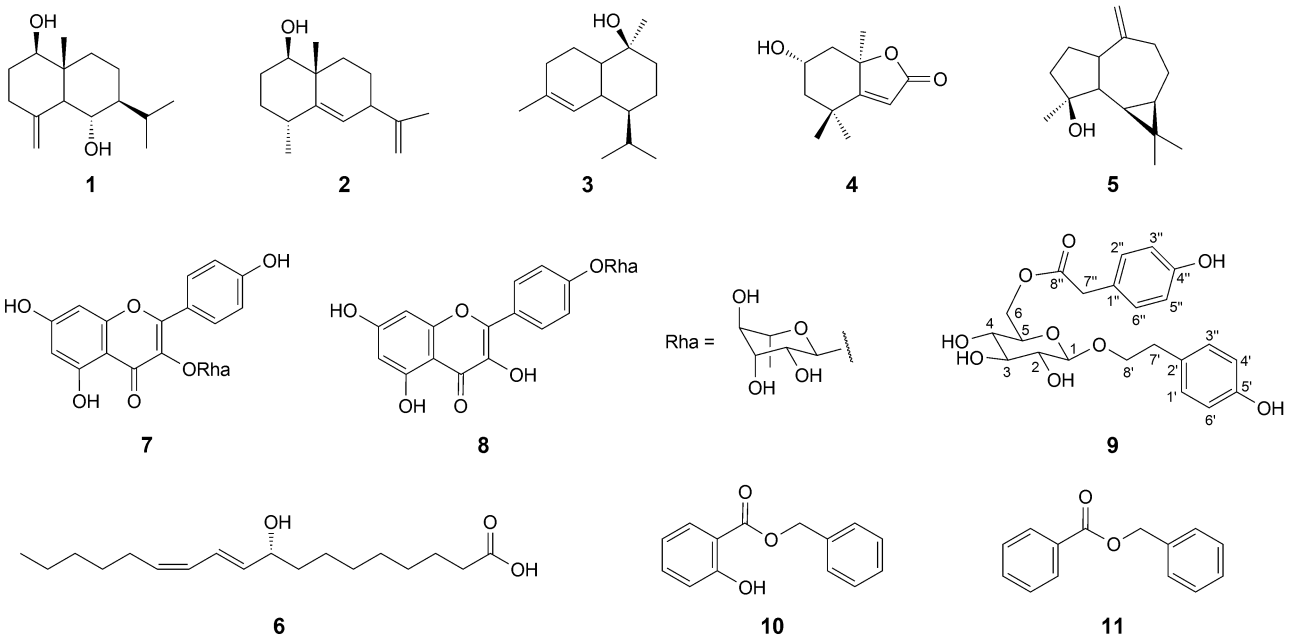

Fig. 3. Compounds isolated from leaves of canelinha (*Ocotea pulchella*).

Table 1. ^{1}H-NMR (600 MHz) and ^{13}C (125 MHz) spectroscopic data for compound **9** in $CDCl_3$

Position	δ(H)	δ(C)	gHMBC
1	4.24 (*d*, *J* = 7.5)	104.4	2
2	3.16 (*dd*, *J* = 8.5, 7.5)	75.3	
3	3.33 – 3.30 (*m*)	77.9	
4	3.28 – 3.20 (*m*)	72.1	
5	3.43 – 3.39 (*m*)	75.0	6a
6a	4.42 (*dd*, *J* = 12.0, 2.0)	64.9	
6b	4.20 (*dd*, *J* = 12.0, 6.0)		
1′		130.0	3′, 5′, 7′
2′	7.08 (*d*, *J* = 8.5)	131.3	6′
3′	6.69 (*d*, *J* = 8.5)	116.3	5′
4′		156.8	2′, 3′, 5′, 6′
5′	6.69 (*d*, *J* = 8.5)	116.3	3′
6′	7.08 (*d*, *J* = 8.5)	131.3	2′, 3′, 5′
7′	2.81 (*t*, *J* = 7.2)	36.4	2′, 6′
8′a	3.91 (*dt*, *J* = 9.5, 7.2)	71.7	7′
8′b	3.49 – 3.40 (*m*)		
1″		126.3	
2″	7.08 (*d*, *J* = 8.5)	130.9	7″
3″	6.68 (*d*, *J* = 8.5)	116.1	
4″		157.5	2″
5″	6.68 (*d*, *J* = 8.5)	116.1	
6″	7.08 (*d*, *J* = 8.5)	130.9	2″, 7″
7″	3.56 – 3.49 (*m*)	41.1	2″
8″		173.8	6a, 7″

δ in ppm, *J* in Hz.

60 and 100%, and compound **5** (spathulenol) was the most active of all the compounds tested, with 100% inhibition at 10^{-3} and 3×10^{-4}M. These values are higher than those of the commercial herbicide *Logran*® at these concentrations. The antibacterial activity and inhibitory effect on the growth of the phytopathogens *Fusarium oxysporum* f. sp. *dianthi* and *Botrytis cinerea* have also been also reported for compound **5** [40]. The flavonoid **7** (afzelin) showed low inhibitory activity, with a value of 50% at 10^{-3}M, and it showed only small effects at higher dilutions. Flavonoids represent an important class of polyphenols and their presence in plants seems to be related to defense functions, control of plant hormones, enzymes, and the inhibition of allelopathic agents. The two aromatic esters **10** and **11** (benzyl salicylate and benzyl benzoate) had the best activity profiles and the highest inhibitory activity on coleoptile elongation. These compounds showed greater than 90% inhibition at the highest concentrations (10^{-3} and 3×10^{-4}M) and even at the third concentration tested (10^{-4}M), at which it exceeded 80% inhibition. These values are higher than those of the commercial herbicide at the same concentrations. These compounds isolated from *O. pulchella* have shown cytotoxic activity on tumor cell lines (CCF-STTG1, Hep3B, HepG2, H-460, AGS, N-87, SW-620, MCF-7, and VERO), of which astrocytoma cells were the most resistant [12]. If therapeutic concentrations have been achieved in target tissues, it has been demonstrated that these components may be useful in the treatment of age-related inflammatory conditions.

The results obtained in this bioassay show that the most active compounds were **5** (spathulenol) and the aromatic esters **10** (benzyl salicylate) and **11** (benzyl benzoate). These activity values were corroborated by calculating the IC_{50} values for all of the compounds. It is noteworthy that compounds **10** and **11** showed similar or lower IC_{50} values

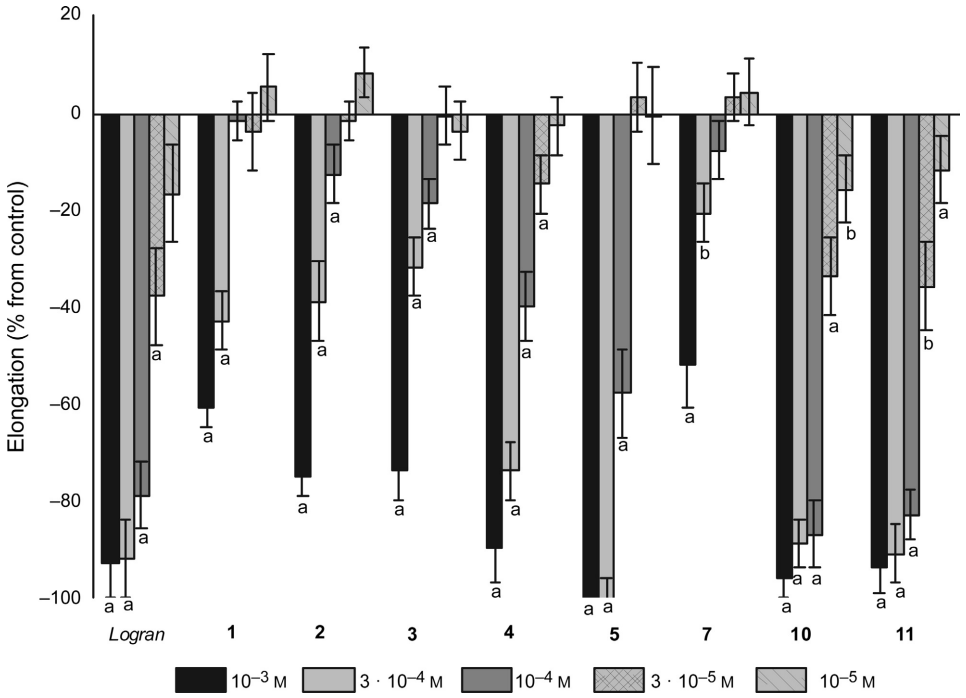

Fig. 4. Effects of compounds **1** – **5**, **7**, **10**, **11**, and the herbicide *Logran*® isolated from *Ocotea pulchella* on the elongation of etiolated wheat coleoptiles. Values are expressed as percentage difference from control. Levels of significance at $0.01 < P < 0.05$ (b) or $P < 0.01$ (a) according to *Welch*'s test compared to control.

Table 2. IC_{50} Values calculated from compounds **1** – **5**, **7**, **10**, **11**, and *Logran*® in the wheat coleoptile bioassay, using a sigmoidal dose–response variable slope model

Compound	IC_{50} [μg/ml]	r^2
1	512.4	0.9753
2	399.3	0.9918
3	487.2	0.9885
4	131.8	0.9963
5	89.3	0.9307
7	900.1	0.9969
10	38.5	0.9712
11	39.3	0.9794
Logran®	38.7	0.9895

compared to the herbicide *Logran*® (38.5, 39.3, and 38.7 μg/ml, for **10**, **11**, and *Logran*®, resp.) (*Table 2*).

Compounds **5**, **10** and **11** were selected for evaluation on *L. sativa* and *L. esculentum* (*Fig. 5*). *L. esculentum* was the species that was affected the most by the AcOEt extract of leaves. The compounds tested showed inhibitory activity on all three parameters assessed, especially at the first concentration (10^{-3}M), with values between 60% and 80% on root and stem growth and 40–60% on germination (*Fig. 5*). These three compounds also showed inhibitory activity on *L. sativa*, albeit with lower values than the initial AcOEt extract. It is worth highlighting the inhibition of root growth, with values close to 40% obtained with compound **11** at the highest concentration tested (10^{-3}M).

Conclusions

The AcOEt leaf extract from *O. pulchella* was the most promising in the study of allelopathic activity, with the species *L. esculentum* affected the most (inhibition values higher than 60% on root and stem growth).

Eleven compounds have been isolated from this extract and these were characterized. Compounds **1**, **2**, **4**, and **6** have not been isolated previously from *O. pulchella* and ocoteol (**9**) is described for the first time. Sesquiterpene **5,** and the aromatic esters **10** and **11** were the most active in the coleoptile bioassay, with inhibition values higher than 90%. The IC_{50} values for compounds **10** and **11** were similar to those of the herbicide *Logran*®. These three compounds showed inhibitory activity on *L. esculentum* – especially at the highest concentration tested, with values around 60% and 80% on root and stem growth, respectively. These activities are very similar to that exhibited by the AcOEt extract. Therefore, it can be concluded that spathuleol (**5**), benzyl salicylate (**10**), and benzyl benzoate (**11**) are responsible for the allelopathic activity shown by *O. pulchella*.

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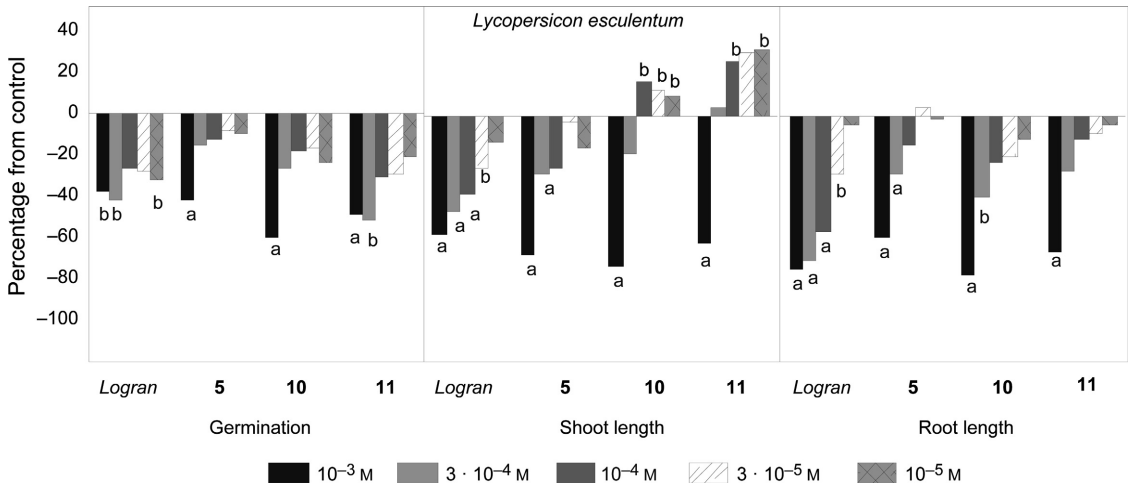

Fig. 5. Effects of the compounds spathulenol (**5**), benzyl salicylate (**10**), and benzyl benzoate (**11**) from *Ocotea pulchella* on *L. esculentum* growth. Values are expressed as percentage difference from control. Levels of significance at $0.01 < P < 0.05$ (b) or $P < 0.01$ (a) according to *Welch*'s test compared to control.

Experimental Part

General

Ultrasound extractions were performed in an ultrasonic bath (360 W; *J.P. Selecta*, Barcelona, Spain) in two series of 15 min. TLC: SiO_2 *60 F254* Al sheets and SiO_2 *60 RP-18 F254S* Al sheets (*Merck*, Darmstadt, Germany). Column chromatography (CC): SiO_2 *60A* (0.060 – 0.200) from *Acros Organics* (Geel, Belgium) and *Lichroprep RP 18* (40 – 63 μm) from *Merck*. HPLC: HPLC chromatograph (*Merck-Hitachi*, Tokyo, Japan) with RI detection. The columns used for HPLC were a semiprep. column (250 mm × 10 mm i.d., 10 μm *Lichrospher 100 RP-18*; *Merck*) with a guard column (*LiChrospher RP-18*; *Merck*) and an anal. column (250 mm × 4.5 mm i.d., 5 μm *Gemini 110A RP-18*; *Phenomenex*, Torrance, CA, USA) with a guard column (Security Guard Cartridges *Gemini RP-18*; *Phenomenex*). Optical rotation: *241 polarimeter* (*Perkin–Elmer*, Waltham, MA, USA; on the sodium D line) at r.t. IR Spectra (KBr): *Fourier* transform infrared (FT-IR) *Spectrum 1000* spectrophotometer (*Perkin–Elmer*); $\tilde{\nu}$ in cm^{-1}. NMR Spectra: *Agilent* 600, 500, and 400 MHz spectrometers (*Agilent*, Palo Alto, CA, USA); δ in ppm rel. to residual ^{1}H-signals of $CDCl_3$ (δ(H) 7.25) and the solvent signal (δ(C) 77.00), *J* in Hz. HR-MS: *Synapt G2 UPLC-QTOF ESI* mass spectrometer (*Waters*, Milford, MA, USA); in *m*/*z*.

Chemicals

$CHCl_3$, hexane, MeOH, CH_2Cl_2, AcOEt, and acetone (all *Hipersolv Chromanorm* for HPLC) were obtained from *VWR International* (Radnor, PA, USA). *MagniSolv* $CDCl_3$ (deuteration degree min. 99.8%) for NMR spectroscopy was obtained from *Merck*.

Preparation of Extracts and Isolation of Compounds

Ocotea pulchella (Lauraceae) leaves, stems, and roots were collected in September 2013 (dry season) from the Brazilian (Cerrado) area on the campus of Universidade Federal de São Carlos (UFSCar) in São Carlos-SP, Brazil (21°58′ to 22°00′ S and 47°51′ to 47°52′ W). A voucher specimen was filed in the herbarium of the Botany Department of the Federal University of São Carlos, with the number HUFSCar 8799. After collection, the leaves, stems, and roots were dried at 40 °C for 72 h and ground in an industrial mill.

Dried material (60 g) was defatted with hexane by applying an ultrasound-assisted extraction. The following quantities of hexane extracts were obtained: 1.7 g (leaves), 194 mg (stems), and 581 mg (roots). The material was subsequently dried (48 h at 40 °C in an oven) and subdivided into small portions of leaf, stem, and root powder. AcOEt and MeOH were used to extract defatted material and these extractions yielded the following amounts after removal of the solvent: leaves 3.39 g (AcOEt) and 3.16 g (MeOH); stems 1.1 g (AcOEt) and 2.1 g (MeOH); and roots 1.1 g (AcOEt) and 1.08 g (MeOH). Chlorophyll was removed from leaf extracts using mixtures of H_2O/MeOH and this treatment yielded four fractions: 20% MeOH (*Fr A*), 40 + 60% MeOH (*Fr.* B), 80% MeOH (*Fr.* C), and 100% MeOH (*Fr.* D), ending with CH_2Cl_2 as eluent on an *RP-18* chromatography column. These extracts (AcOEt and MeOH) from leaves (without chlorophyll), stems, and roots were bioassayed on etiolated wheat coleoptiles. The AcOEt extract from the leaves was the most active, and this was therefore studied in a phytotoxicity bioassay on target species.

Bearing in mind that the AcOEt extract of leaves was the most active, the remaining leaves (1.25 kg), previously defatted, were extracted with 10 l of AcOEt in portions of 15 g of plant material using an ultrasonic bath

(2 × 500 ml). This process yielded 33.9 g of material. Chlorophyll was removed from the extract using the same H_2O/MeOH mixtures as described above, and four (chlorophyll-free) fractions were obtained in decreasing order of polarity: *A* (5.7 g), *B* (1.2 g), *C* (1.0 g), and *D* (3.0 g).

Fr. A was chromatographed on *C-18* SiO_2 using H_2O/MeCN/MeOH mixtures of increasing polarity from 0% to 100%, with an increase each time of 10% of MeOH and finishing with 1:1 MeCN/MeOH (500 ml). This process afforded three fractions: *A1 – A3*. *Fr. A2* (2.1 g) was pooled and subjected to column chromatography with $CHCl_3$/MeOH/H_2O from 10% to 100% in MeOH and 1% water, with a 10% increase each time and finishing with 100% MeOH (500 ml of each polarity) to give six subfractions (*Frs. A2.1 – A2.6*). *Fr. A2.1* (365 mg) was subjected to column chromatography under the same conditions as *Fr. A2* to yield eight subfractions. The second subfraction (*Fr. A2.2*) yielded compound **7** (4.9 mg). *Fr. A2.6* was purified by HPLC (semiprep. column) eluting with H_2O/acetone (50:50 *v*/*v*, flow 3 ml/min) to give compound **8** (1.2 mg).

Fr. A3 (2.3 g) was subjected to column chromatography with $CHCl_3$/MeOH/H_2O from 10% to 100% in MeOH, with a 10% increase each mixture and finishing with 100% MeOH (500 ml), with 1% water at all polarities, to afford seven subfractions (*Frs. A3.1 – A3.7*). *Fr. A3.4* (54.5 mg) was purified by HPLC (semiprep. column) eluting with H_2O/acetone (50:50, flow 3 ml/min) to give compounds **9** (3.1 mg), **7** (10 mg), and **8** (1.8 mg). *Fr. A3.6* (1.6 g) was subjected to column chromatography with $CHCl_3$/MeOH/H_2O from 10% to 100% in MeOH and 1% water, with a 10% increase each time and finishing with 100% MeOH (500 ml of each polarity) to give four subfractions (*Frs. A3.6.1 – A3.6.4*). The spectroscopic data for subfraction *A3.6.4* were consistent with a mixture of two anomeric sugars of D-xylopyranose (500 mg).

Frs. B and *C* were chromatographed with a hexane/AcOEt gradient from 0 to 100% AcOEt, with a 10% increase each time, and finishing with 100% MeOH (500 ml of each polarity) to afford various subfractions: *Frs. B1 – B7* and *Frs. C1 – C6*. *Frs. B3* (15.5 mg) and *B5* (32.1 mg) yielded compounds **1** (10.1 mg) and **4** (22.1 mg). *Fr. C1* (278 mg) was subjected to column chromatography with a hexane/acetone gradient from 0% to 100% in acetone, with a 10% increase each time and finishing with 100% MeOH (100 ml of each polarity), to afford five subfractions (*Frs. C1.1 – C1.5*). *Fr. C1.1* (237 mg) was purified by HPLC (semiprep. column) eluting with hexane/AcOEt (85:15, flow 3 ml/min) to obtain compounds **2** (2.7 mg), **3** (3.1 mg), **5** (16 mg), **10** (3.6 mg), and **11** (52 mg). *Fr. C5* (85.5 mg) yielded compound **6** (2.8 mg).

Ocoteol (= **2-(4-Hydroxyphenyl)ethyl 6-*O*-[(4-Hydroxyphenyl)acetyl]-*β*-D-glucopyranoside**; **9**). Yellow amorphous solid. $[\alpha]_D^{25} = -22.4$ (*c* = 1.0, MeOH). UV (MeOH): 205 (2.53), 225 (2.41). IR (KBr): 3375, 2945, 1736, 1614, 1516, 1450, 813, 681. ^{1}H-NMR ($CDCl_3$, 600 MHz): see *Table 1*. ^{13}C-NMR ($CDCl_3$ 125 MHz): see *Table 1*. HR-EI-TOF-MS (neg.): 433.1497 ($[M - H]^-$; calc. 433.1499).

Coleoptile Bioassay

The extracts (AcOEt and MeOH) at concentrations of 0.8, 0.4, and 0.2 mg/ml were subjected to an etiolated wheat coleoptile bioassay. This test is widely used to evaluate the sensitivity of wheat to a wide range of bioactive substances [41].

Triticum aestivum *L. cv*

Hard seeds were pregerminated in water for 3 days in the dark at 22 ± 1 °C, stored in *Petri* dishes (15 cm diameter) and covered with *Whatman No. 1* filter paper with around 100 seeds in a volume of 15 ml of deionized H_2O. The roots and caryopses were removed from seedlings and the coleoptiles were removed. The latter were placed in a *Siles* guillotine and the apical 2 mm were cutoff and discarded [41]. The next 4 mm of the coleoptiles were removed and used for bioassays [42]. All manipulations were performed under a green safelight [43].

Crude extracts were dissolved in DMSO (0.1%) and diluted in phosphate-citrate buffer containing 2% sucrose at pH 5.6. Three control samples were used: buffer with DMSO, buffer alone, and an internal reference with *Logran*® herbicide (59% terbutryn and 0.6% triasulfuron). The commercial herbicide was used as an internal reference to allow comparison with a study reported previously [44].

The concentrations were 0.8, 0.4, and 0.2 mg/ml for extracts and 10^{-3}, 3×10^{-4}, 10^{-4}, 3×10^{-5}, and 10^{-5}M for compounds. Three replicates were performed for each dilution. Each assay was carried out on five coleoptiles and 2 ml of extract, buffer or *Logran*®. The tubes were rotated at 6 rpm in a roller tube apparatus (*Stuart Scientific*) for 24 h at 25 °C in the dark. After 24 h, the coleoptiles were removed and measured using Photomed software after digitalization of the images [44]. Data were statistically analyzed using a *Welch*'s test [45] and are presented as percentage difference from the control.

Germination and Growth Bioassay

The bioassay was conducted in a *Petri* dish (50 mm diameter) on *Whatman No. 1* paper as support. Germination and growth were conducted in aq. solns. with pH controlled using 10^{-2}M 2-[*N*-morpholino]ethanesulfonic acid and 1M NaOH (pH 6.0). The extracts were dissolved in DMSO and the resulting solns. were diluted with buffer (5 µl DMSO soln./ml buffer). Parallel controls were also run as described above for the coleoptile bioassay. The concentrations used were 0.8, 0.4, and 0.2 mg/ml for extracts, and 10^{-3}, 3×10^{-4}, 10^{-4}, 3×10^{-5}, and 10^{-5}M for compounds. Samples were prepared and added to the

seeds in a *Petri* dish, which was then covered with parafilm and incubated in the dark. Each treatment consisted of four replicates of 20 seeds per replicate (total of 80 seeds) and 1 ml of soln. in each *Petri* dish.

The selected targets species were based on the results of a previous phytotoxicity study [44]. The standard target species (STS) proposed included the dicotyledons tomato (*Lycopersicon esculentum* Will.), cress (*Lepidium sativum* L.), and lettuce (*Lactuca sativa* L.), and the monocotyledon onion (*Allium cepa* L.).

The *Petri* dishes were further incubated at 25 °C in a *Memmert ICE 700* controlled environment growth chamber. The photoperiod was 24 h of dark for onion, tomato, cress, and lettuce. Bioassays took 4 days for cress, 5 days for tomato, 6 days for lettuce, and 7 days for onion. After the specified incubation period, the seeds from each *Petri* dish were stored at −10 °C for 24 h to stop seedling growth before measurement. A commercial herbicide of known activity was used as a control, namely *Logran®* marketed by *Syngenta* [44].

Statistical Analysis

The data for germination rate, root length, and shoot length were recorded using a *Fitomed* system [46]. Data were analyzed statistically using the *Welch*'s test, with significance fixed at 0.01 and 0.05. The results are presented as percentage differences from the control. Zero represents control, positive values represent stimulation, and negative values represent inhibition. In order to achieve a more accurate analysis of the results, it was necessary to calculate a series of statistical parameters. As a consequence, IC_{50} values were calculated and the cluster analysis was carried out. The IC_{50} values were calculated using sigmoidal dose–response or dose–response variable slope models.

REFERENCES

[1] P. Novaes, J. M. G. Molinillo, R. M. Varela, F. A. Macías, *Phytochem. Rev.* **2003**, *12*, 839.

[2] R. K. Bethke, W. T. Molin, C. Sprague, D. Penner, *Weed Sci.* **2013**, *61*, 41.

[3] F. A. Macias, A. Oliveros-Bastidas, D. Marin, N. Chinchilla, D. Castellano, J. M. G. Molinillo, *J. Agric. Food Chem.* **2014**, *62*, 9450.

[4] H. Shimomura, Y. Sashida, T. Adachi, *Phytochemistry* **1987**, *26*, 2363.

[5] M. Khan, F. Hussain, S. Musharaf, *Afr. J. Agric. Res.* **2011**, *6*, 6391.

[6] D. A. Mortensen, L. Bastians, M. Sattin, *Weed Res.* **2000**, *40*, 49.

[7] J. J. O. Pinto, L. Galon, T. Dal Magro, S. O. Procópio, G. Concenço, C. F. Pinho, E. A. Ferreira. *Planta Daninha.* **2008**, *26*, 767.

[8] F. E. Dayan, C. L. Cantrell, S. O. Duke, *Bioorg. Med. Chem.* **2009**, *17*, 4022.

[9] C. L. Cantrell, E. Dayan, S. O. Duke, *J. Nat. Prod.* **2012**, *75*, 1231.

[10] P. J. Christoffoleti, R. F. Lopez-Ovejero, M. Nicolai, L. Vargas, S. P. C. Carvalho, A. C. Caetano, C. C. Carvalho, M. S. Moreira, 'Aspectos de resistência de plantas daninhas a herbicidas', 3ª edn., Atua. e rev., Piracicaba, Brazil, 2008.

[11] D. F. Coutinho, *J. Essent. Oil Res.* **2007**, *19*, 482.

[12] C. Chaverri, C. Diaz, J. F. Ciccio, *J. Braz. Chem. Soc.* **2011**, *22*, 741.

[13] H. Lorenzi, Árvores Brasileiras – Manual de Identificação e Cultivo de Plantas Arbóreas Nativas do Brasil, 4ª edn., Instituto Plantarum: São Paulo, 2002, 143.

[14] M. J. Camargo, M. L. D. Miranda, C. M. Kagamida, E. D. Rodrigues, F. R. Garcez, W. S. Garcez, *Quim. Nova.* **2013**, *36*, 1008.

[15] S. Takaku, W. A. Haber, W. N. Setzer, *Biochem. Syst. Ecology* **2007**, *35*, 525.

[16] E. E. L. Borges, E. S. Lopes, G. F. Silva, *Rev. Árvore* **1993**, *17*, 69.

[17] A. L. L. Lordello, M. Yoshida, *Phytochemistry* **1997**, *46*, 741.

[18] M. Ishige, M. Motidome, M. Yoshida, O. R. Gottlieb, *Phytochemistry* **1991**, *30*, 4121.

[19] C. C. Andrei, R. Braz-Filho, O. R. Gottlieb, *Phytochemistry* **1988**, *27*, 3992.

[20] D. P. Montrucchio, *Planta Med.* **2012**, *78*, 1543.

[21] A. C. R. Araújo, Ph. D. Thesis, Federal University of Paraná, Curitiba, **2012**.

[22] P. A. Monquero, 'Aspectos da biologia e manejo das plantas daninhas', Ed. RiMA, São Carlos, SP, 2014, p. 430.

[23] Y. O. Kim, J. D. Johnson, E. J. Lee, *J. Chem. Ecol.* **2005**, *31*, 1175.

[24] M. A. F. M. Miranda, R. M. Varela, A. Torres, J. M. G. Molinillo, S. C. J. Gualtieri, F. A. Macías, *J. Nat. Prod.* **2015**, *14*, 1.

[25] A. Tanveer, M. K. Jabbar, A. Kahliq, A. Matloob, R. N. Abbas, M. M. Javaid, *Chil. J. Agric. Res.* **2012**, *72*, 495.

[26] F. Nagashima, K. Kishi, Y. Hamada, S. Takaoka, Y. Asakaway, *Phytochemistry* **2005**, *66*, 1662.

[27] B. Blas, J. Zapp, H. Becker, *Phytochemistry* **2004**, *65*, 127.

[28] A. S. Feliciano, M. Medarde, M. Gordaliza, E. Del Olmo, J. M. M. Del Corral, *Phytochemistry* **1989**, *28*, 10, 2717.

[29] S. Isoe, S. Katsumura, B. H. Suong, T. Sakan, *Tetrahedron Lett.* **1971**, *16*, 1089.

[30] H. Iwabuchi, M. Yoshikura, W. Kamisako, *Chem. Pharm. Bull.* **1989**, *37*, 509.

[31] S. Tsuboi, S. Maeda, A. Takeda, *Bull. Chem. Soc. Jpn.* **1986**, *59*, 2050.

[32] W. S. Garcez, M. Yoshida, O. R. Gottlieb, *Phytochemistry* **1995**, *39*, 815.

[33] A. Ibrahim, S. I. Khalifa, I. Khafagi, D. T. Youssef, S. Khan, M. Mesbah, I. Khan, *Chem. Pharm. Bull.* **2008**, *56*, 1253.

[34] V. P. A. Talzi, *Russ. J. Appl. Chem.* **2006**, *79*, 107.

[35] P. A. J. Gorin, M. Mazuret, *Can. J. Chem.* **1975**, *53*, 1212.

[36] a) C. Ghelardini, N. Galeotti, L. D. C. Mannelli, G. Mazzanti, A. Bartolini, *II Farmaco* **2001**, *56*, 387; b) E. S. Fernandes, G. F. Passos, R. Medeiros, F. M. Da Cunha, J. Ferreira, M. M. Campos, L. F. Pianowski, J. B. Calixto, *Eur. J. Pharmacol.* **2007**, *569*, 228; c) T. R. Costa, O. F. L. Fernandes, S. C. Santos, C. M. A. Oliveira, L. M. Lião, P. H. Ferri, J. R. Paula, H. D. Ferreira, B. H. N. Sales, M. R. R. Silva, *J. Ethnopharmacol.* **2000**, *72*, 111; d) H. M. Zhang, G. L Wang, C. Q. Bai, P. Liu, Z. M. Liu, Q. Z. Liu, Y. Y. Wang, Z. L. Liu, S. S. Du, Z. W. Deng, *Molecules* **2011**, *16*, 9017; e) L. F. Da S. Machado, C. R. Kaiser, S. S. Costa, L. M. Gestinari, A. R. Soares, *Braz. J. Pharmacog.* **2010**, *20*, 441; f) O. A. Colom, S. Popich, A. Bardon, *Nat. Prod. Res.* **2007**, *21*, 254.

[37] A. P. Desbois, V. J. Smith, *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1629.

[38] F. Mezni, A. Maaroufi, M. Msallem, M. Boussaid, M. L. Khouja, A. Khaldi, *J. Med. Plant. Res.* **2012**, *6*, 5266.

[39] Y. Jo, M. Kim, M. H. Shin, H. Y. Chung, J. H. Jung, K. S. Im, *J. Nat. Prod.* **2006**, *69*, 1399.

[40] P. V. Farago, J. de F. Padilha-de-Paula, T. Nakashima, P. M. Döll-Boscardin, J. M. Budel, B. H. L. N. S. Maia, *Lat. Am. J. Pharm.* **2012**, *29*, 1242
[41] C. Hancock, H. Barlow, H. Lacey, *J. Exp. Bot.* **1964**, *15*, 166.
[42] F. A. Macías, R. Lacret, R. M. Varela, C. Nogueiras, J. M. G. Molinillo, *J. Chem. Ecol.* **2010**, *36*, 396.
[43] J. P. Nitsch, C. Nitsch, *Plant Physiol.* **1956**, *31*, 94.
[44] F. A. Macías, J. Galindo, D. Castellano, R. Velasco, *J. Agric. Food Chem.* **2000**, *48*, 5288.
[45] A. Martín, J. D. Luna, Bioestadistica para las ciencias de la salud, Ediciones Norma: Madrid, Spain, 1990.
[46] D. Castellano, Ph. D. Thesis, Universidad de Cádiz, Spain, **2002**.

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