Allelopathic Potential of *Rapanea umbellata* Leaf Extracts

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The stressful conditions associated with the Brazilian savanna (Cerrado) environment were supposed to favor higher levels of allelochemicals in *Rapanea umbellata* from this ecosystem. The allelopathic potential of *R. umbellata* leaf extracts was studied using the etiolated wheat coleoptile and standard phytotoxicity bioassays. The most active extract was selected to perform a bioassay-guided isolation, which allowed identifying lutein (**1**) and (−)-catechin (**2**) as potential allelochemicals. Finally, the general bioactivity of the two compounds was studied, which indicated that the presence of **1** might be part of the defense mechanisms of this plant.

Introduction. – Allelopathic interactions arise due to the production of secondary metabolites that exert beneficial or prejudicial effects on the growth and development of plants and neighboring microorganisms [1][2]. Natural phytotoxins produced by plants might be used as natural herbicides with more specific and less harmful effects to the environment than man-made compounds [3]. The advantages of allelochemical herbicides include their H_2O solubility, the absence of halogenated molecules, alternative modes of action, more specific interactions with the target plants, activity at lower concentrations, and lower environmental damage compared to conventional herbicides [4][5]. For these reasons, research on allelopathy plays a fundamental role in agroecosystem interactions.

In recent years, our studies on allelopathy have been focused on the search for natural herbicide models and/or bioactive compounds from species of economic interest [6–8]. More recently, the interest has also been focused on the prospecting of allelochemicals from species that grow in less widely studied ecological communities and experience high biotic and abiotic stress, because bioactive compounds are produced in large amounts by plants under these conditions [9]. The neotropical Brazilian savanna is of great interest from the chemical ecology (allelopathy) point of view, because this ecosystem has a marked seasonal climate [10], and the species grow in nutritionally poor soils [11]. Furthermore, very few studies have been carried out on the isolation, identification, and potential of secondary metabolites from plants of the Cerrado [12][13].

Cytotoxic and phytotoxic bioassays are important tools for the isolation of allelochemicals. The cytotoxic bioassay used here was adapted by *Cutler* [14] from the

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assay published by *Hancock et al.* [15] to evaluate auxin effects on wheat etiolated coleoptiles. This general activity assay is based on the inhibition or stimulation of the cell elongation in undifferentiated tissues and is used to evaluate the phytotoxic effect of allelochemicals on the germination and initial growth of target plants.

Rapanea umbellata (MART.) MEZ (Myrsinaceae) is an arboreal, evergreen species that grows in the Cerrado [16]. Recent preliminary studies on extracts from leaves, stems, and roots indicated that only *R. umbellata* leaves showed phytotoxic effects on infesting species [13]. This finding suggests an allelopathic interaction. Moreover, subsequent studies proved that the extracts and compounds from *Rapanea* species have anthelmintic, antifungal, molluscicidal, and trypanocidal activities [17–19]. Prenylated benzoic acids, triterpenoid saponins, and flavonoids, as well as their glycosides, have been isolated in various studies of *Rapanea* species [17][19–22].

The present study reports the allelopathic potential of *R. umbellata* leaf extracts. The aim was to bioprospect the extracts by the isolation and purification of allelopathic secondary metabolites that could be used in the future as herbicides of natural origin. First, the most phytotoxic *R. umbellata* leaf extract was determined by using the etiolated wheat coleoptile and standard phytotoxicity bioassays. Then, a bioassay-guided fractionation of this extract was carried out, to isolate and identify the most active chemical constituents. The structures of these compounds were characterized by ^{1}H- and ^{13}C-NMR spectroscopy, and, finally, the bioactivity profiles of the isolated compounds were studied.

Results and Discussion. – *Selection of the Extract with the Highest Bioactivity.* The dried leaves of *R. umbellata* (0.5 kg) were defatted with hexane (6.50% yield). Then, the plant residue was extracted with CH_2Cl_2 (0.90% yield), AcOEt (1.05% yield), acetone (4.70% yield), MeOH (2.96% yield), and dist. H_2O (H_2O; 6.72% yield). The extracts were subjected to the etiolated wheat coleoptile bioassay [23], a rapid test that is sensitive to a wide range of bioactive substances [14][24][25], including plant growth regulators, herbicides, antimicrobials, mycotoxins, and assorted pharmaceuticals [14]. Three dilutions (0.8, 0.4, and 0.2 mg/ml), prepared from the dried extracts, were tested.

The effect of the extracts and of the herbicide *Logran®* (used as internal standard) on the elongation of etiolated wheat coleoptiles is shown in *Fig. 1*. Besides the hexane fraction, the extracts that showed the highest inhibition activities were the AcOEt, acetone, and CH_2Cl_2 extracts, which showed the highest effect at the highest concentration tested (0.8 mg/ml), with inhibition values of −74, −74, and −58%, respectively. At the same concentration, the MeOH and H_2O extracts showed lower inhibitory activities (−43 and −16%, resp.). The AcOEt and acetone extracts retained good activity levels at lower concentrations. Indeed, the inhibition values of these extracts at 0.4 and 0.2 mg/ml were −52 and −37%, respectively, for the AcOEt extract and −43 and −38%, respectively, for the acetone extract.

To further compare the activities of the extracts, IC_{50} values were calculated using a sigmoidal dose–response model. The results allow the extracts to be ranked in decreasing order of activity as follows: AcOEt ($IC_{50}=0.36$ mg/ml, $r^2=0.9979$) > acetone ($IC_{50}=0.42$ mg/ml, $r^2=0.9806$) > CH_2Cl_2 ($IC_{50}=0.68$ mg/ml, $r^2=0.9922$) > MeOH ($IC_{50}=0.93$ mg/ml, $r^2=0.9745$) > H_2O ($IC_{50}=3.74$ mg/ml, $r^2=0.9851$). As well

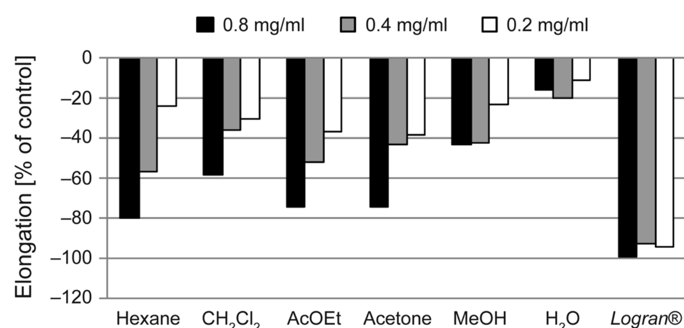

Fig. 1. *Effect of the hexane, CH_2Cl_2, AcOEt, acetone, MeOH, and H_2O leaf extracts of* Rapanea umbellata *and the herbicide* Logran® (used as positive control) *on the elongation of etiolated wheat coleoptiles.* Inhibition values are expressed as percentage difference from the control.

when considering the IC_{50} values, the AcOEt, acetone, and CH_2Cl_2 extracts were the most active ones. Hence, the MeOH and H_2O extracts were discarded, due to their lower activity. The differences in the activity profiles between the extracts suggest that the most active metabolites might be of intermediate polarity.

The most active extracts (AcOEt, acetone, and CH_2Cl_2) were selected for further phytotoxicity evaluation. The concentrations tested in the standard phytotoxicity assay were identical to those used for the etiolated wheat coleoptile bioassay. The standard target species (STS) tested were *Lactuca sativa* L. (lettuce), *Lycopersicum esculentum* Mill. (tomato), *Lepidium sativum* L. (cress), and *Allium cepa* L. (onion) [18].

The behavior of the evaluated extracts on the germination and growth of *L. esculentum* is inhibitory in all cases. As shown in *Fig. 2*, the highest inhibitory effects on the germination were caused by the acetone extract, which showed higher inhibition percentages than the herbicide *Logran*® at 0.8 and 0.4 mg/ml (−81 *vs.* −50% and −61 *vs.* −39%, resp.). The acetone extract showed also inhibitory activity on the root growth of this species (0.8 mg/ml, −63%; 0.4 mg/ml, −71%; 0.2 mg/ml, −21%). On the other hand, the activities of the AcOEt extract on the root and shoot growth was less persistent with dilution than that of the acetone extract. With regard to the second dicotyledonous species, *L. sativum*, the effects of the extracts were of medium significance. The acetone extract was the most active on the shoot growth of cress, with activity values of *ca.* −41% at the highest concentration. Neither extract showed a significant effect on the germination and shoot growth of the third dicotyledonous species, *L. sativa* (*Fig. 2*), but the acetone extract significantly inhibited its root growth (*ca.* −35% at 0.8 mg/ml).

In the monocotyledonous species *A. cepa*, the most affected parameter was the root growth, and the most active extract was the acetone extract. Indeed, this extract showed inhibitory effects on the root lengths of −63, −48, and −36% at 0.8, 0.4, and 0.2 mg/ml, respectively (*Fig. 2*).

In summary, the extracts that showed the highest standard phytotoxic activities were the acetone and AcOEt extracts. In contrast, the CH_2Cl_2 extract did not show relevant activity in this bioassay. Finally, the general activity in the coleoptile and the standard phytotoxic bioassays and the higher extraction yield (4.70 *vs.* 1.05%) justified

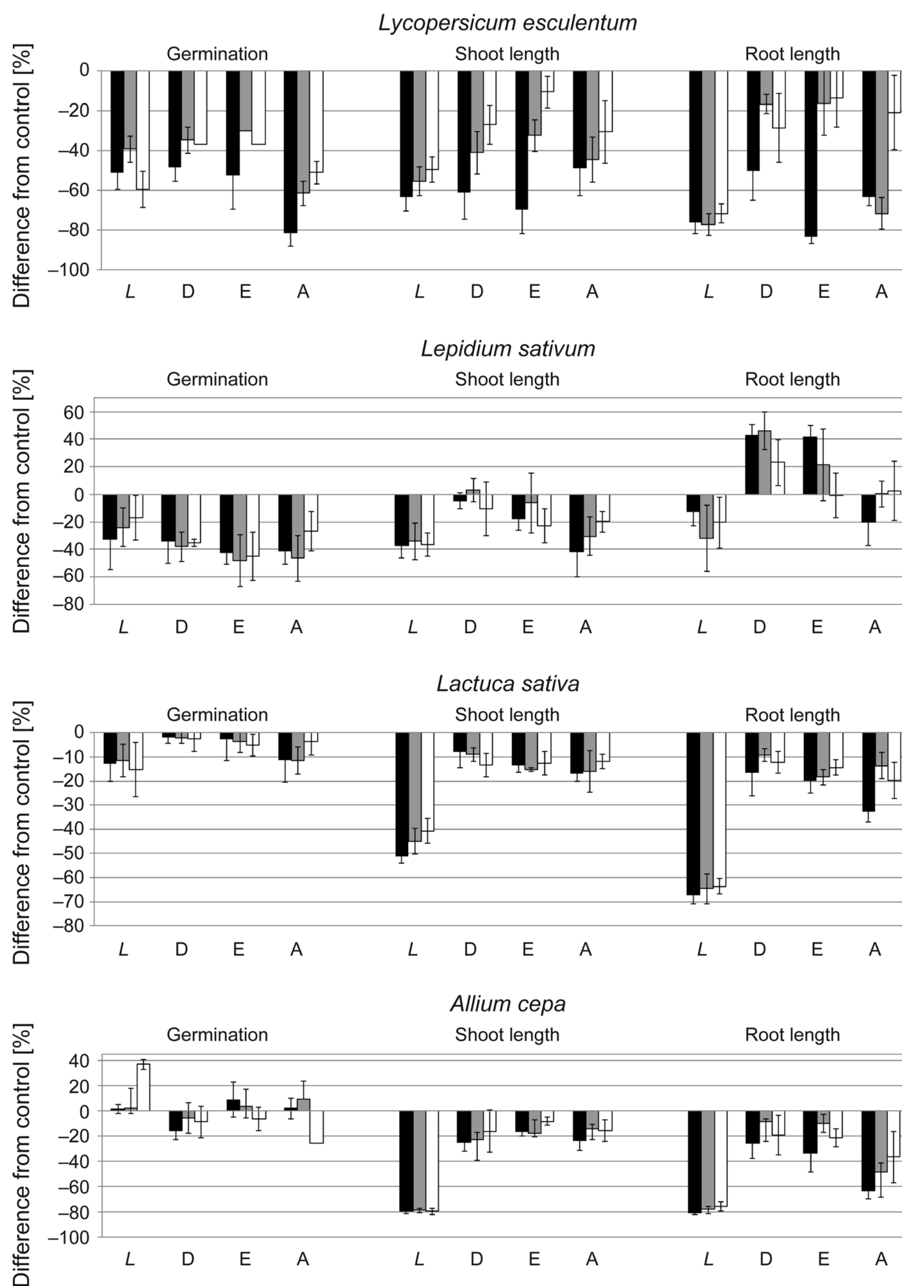

Fig. 2. *Effect of different concentrations* (■ 0.8 mg/ml, ■ 0.4 mg/ml, □ 0.2 mg/ml) *of the herbicide* Logran® (*L*; used as positive control) *and the* CH_2Cl_2 (D), *AcOEt* (E), *and acetone* (A) *leaf extracts of* Rapanea umbellata *on the growth of standard target species.* Values are expressed as percentage difference from the control.

the selection of the acetone extract for the bioassay-guided fractionation, with the aim of isolating and characterizing the bioactive constituents.

Bioassay-Guided Fractionation. The most active extract (acetone) was chromatographed on silica gel using hexane/acetone mixtures of increasing polarity as mobile phase. Due to their chromatographic profiles, fractions *Frs. E* (0.162 g, 0.81% yield), *G* (0.409 g, 1.74% yield), and *H* (0.900 g, 4.50% yield) were selected for the bioassay with etiolated wheat coleoptiles (*Fig. 3*). Three dilutions, prepared from the dried fractions, were used in this assay (0.8, 0.4, and 0.2 mg/ml).

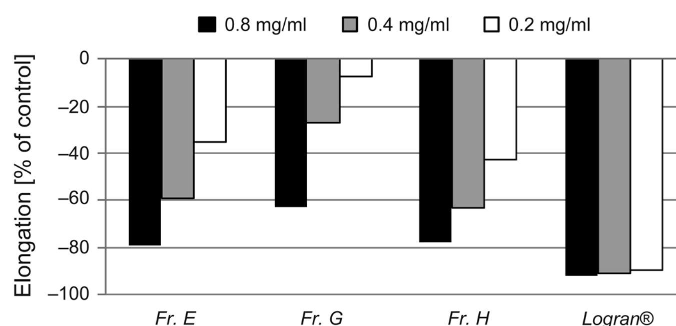

Fig. 3. *Effects of* Fractions E, G, *and* H *of the acetone extract of* Rapanea umbellata *leaves and of the herbicide* Logran® (used as positive control) *on the elongation of etiolated wheat coleoptiles.* Values are expressed as percentage difference from the control.

The results show that *Frs. E* and *H* inhibited the coleoptile elongation by more than −70% at 0.8 mg/ml. In particular, *Fr. E* presented inhibition values close to −80% at this concentration. On the other hand, the activity of *Fr. G* was −63% at 0.8 mg/ml and it decreased rapidly upon dilution. Considering also the IC_{50} values of *Frs. E* (IC_{50} = 0.31 mg/ml, r^2 = 0.9925), *G* (IC_{50} = 0.82 mg/ml, r^2 = 0.9582), and *H* (IC_{50} = 0.26 mg/ml, r^2 = 0.9968), *Frs. E* and *H* were selected for further fractionation by chromatography.

The chromatographic separation of the bioactive *Frs. E* and *H* allowed the isolation of one carotenoid, lutein (**1**), and one flavonoid, (−)-catechin (**2**), respectively (*Fig. 4*). The spectroscopic data and physical constants obtained for compounds **1** and **2** were found to be identical to those previously reported for lutein [26] and (−)-catechin [27], respectively. These compounds were isolated here for the first time from *R. umbellata*.

Bioassay Results. Finally, the bioactivities of the compounds isolated from *R. umbellata* were evaluated. The results presented in *Fig. 5* show that only compound **1** had a good level of activity, with an inhibition percentage of *ca.* −74% at the highest concentration tested (10^{-3} M). The bioactivity observed for **1** in the etiolated wheat coleoptiles bioassay was consistent with those previously reported for this compound [28]. Indeed, **1** was the most active of the twelve compounds isolated from *Withania aristata* leaves. Moreover, this compound showed activity on the germination of *L. sativa* and *L. esculentum* as well as on the root and shoot lengths of *L. esculentum* [28]. Additionally, *Oliveira* [29] observed that the fraction containing **1** in *Solanum lycocarpum* CH_2Cl_2 leaf extracts showed inhibitory activity in the coleoptile bioassay. This compound has also shown antimicrobial and antifungal activity in tests with

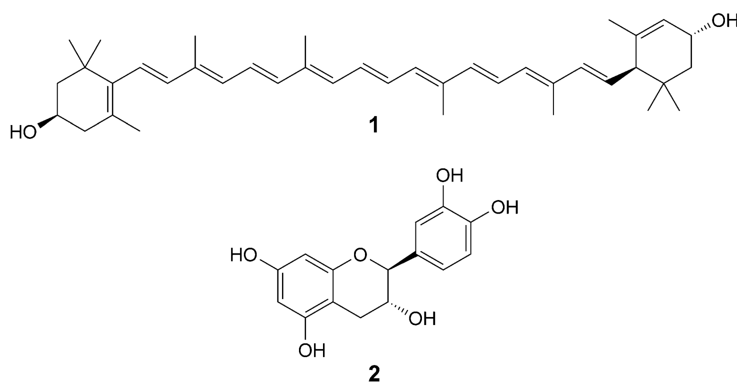

Fig. 4. *Compounds isolated from the acetone extract of* Rapanea umbellata *leaves: lutein* (**1**) *and (−)-catechin* (**2**)

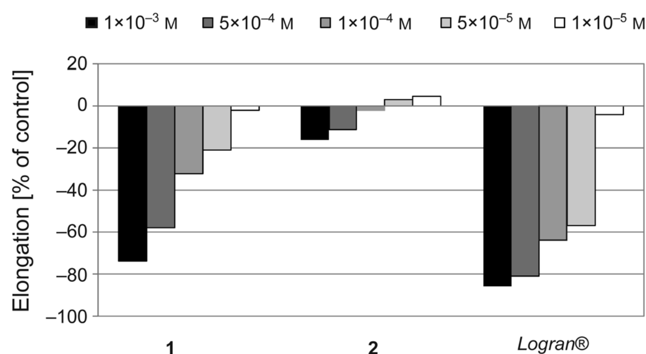

Fig. 5. *Effects of compounds* **1** *and* **2**, *isolated from* Rapanea umbellata *leaves, and of the herbicide* Logran® (used as positive control) *on the elongation of etiolated wheat coleoptiles.* Values are expressed as percentage difference from the control.

Tageles erecta pigment [30]. Therefore, compound **1** might be of interest for the production of herbicides of natural origin against infesting plants. Furthermore, the results might indicate a better establishment of *R. umbellata* in the Brazilian Cerrado by allelopathy, as the presence of **1** might provide this species with more resistance to weeds or invasive species.

(−)-Catechin (**2**) is one of the four isomers of catechin [31], and this compound did not show notable inhibitory effects in the coleoptile bioassay in the present study. In contrast, for (+)-catechin, *Lôbo et al.* [27] have shown growth inhibition and for epicatechin, they have demonstrated germination reduction in *Mimosa pudica* and *Senna obtusifolia* [27]. Compound **2** has been highlighted as the new weapon of *Centauria maculosa*, a native of Eurasia, to invade and establish itself in North American environments [32][33]. Hence, since the publication of these results, there has been some controversy concerning **2**, as to whether it should be classed as phytotoxic or not.

A number of authors considered **2** as phytotoxic, due to the observed inhibition of germination and growth of aerial and root parts of target plants in bioassays under illumination for a photoperiod of 16 h [34]. However, recent studies also indicated that different levels of susceptibility to compound **2** might depend on plant populations, type of substrate, formation of (±)-catechin–metal complexes in the soil, or interaction of **2** with bivalent ions [35–38].

The results reported here are consistent with those obtained by *Chobot et al.* [31] and *Duke et al.* [39][40], who described **2** as a non-phytotoxic compound. These authors had also performed growth bioassays on different target plants with a photoperiod of 12 h at 25°. *Duke et al.* [39][40] affirmed that the phytotoxity levels of (±)-catechin were much lower than those found for other natural substances that are considered toxic, beyond being a common substance in superior plants [39]. *Duke et al.* [40] refuted the idea that **2** could cause oxidative stress, because, according to these authors, **2** is an extremely strong antioxidant. *Chobot et al.* [31] rejected the hypothesis that **2** might be a new weapon, because it inhibits growth in concentrations far higher than other known phytotoxic substances.

Conclusions. – The acetone and AcOEt extracts obtained from *R. umbellata* leaves might be an interesting source of a natural herbicide model or of bioactive compounds, as exemplified by the activity levels shown in the wheat coleoptile and phytotoxicity bioassays. Compounds **1** and **2**, isolated for the first time from *R. umbellata*, were obtained from the acetone extract, one of the most bioactive extracts tested here. The promising inhibitory activity of **1** in the coleoptile bioassay indicated that **1** might be of interest for the production of herbicides of natural origin against weeds. It also indicated that the presence of **1** might be part of the defense mechanism of this plant and contribute to the success of this species in a variety of agroecosystems. On the other hand, **2** did not show bioactivity, a finding that supports the studies that described this compound as non-phytotoxic.

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; grade *15111*, *Merck*). Prep. TLC: SiO_2 plates (grade *5554*, *Merck*). Semiprep. TLC: SiO_2 *G 1500/LS 254* plates (200 × 200 × 0.25 mm; *Schleicher & Schuell*, ref. 391132). HPLC: *Merck LiChrospher SI 60* (250 × 10 mm; 10 μm) column, *Merck–Hitachi* instrument with *RI* detection. Optical rotations: *Perkin-Elmer 241* digital polarimeter; r.t. IR Spectra: *Perkin-Elmer FT-IR Spectrum 1000* or *Mattson 5020* spectrometer; $\tilde{\nu}$ in cm^{-1}. ^{1}H- and ^{13}C-NMR Spectra: *Varian INOVA-400* NMR spectrometer, in $CDCl_3$ or (D_4)MeOH; δ in ppm rel. to solvent signals ($CDCl_3$, δ(H) 7.25, δ(C) 77.0; (D_4)MeOH, δ(H) 3.30, δ(C) 49.0). HR-MS: *VG AUTOESPEC* mass spectrometer (70 eV); in *m/z* (rel. %).

Plant Material. Leaves of *Rapanea umbellata* Mart. Mez were unsystematically collected in July 2008 in the area of the Cerrado (Brazilian neotropical savanna) near the Universidade Federal de São Carlos (UFSCar), Campus São Carlos, São Carlos, SP, Brazil (21° 58′–22° 00′ S and 47° 51′–47° 52′ W).

Voucher specimens (No. 7276) have been deposited with the Herbarium of the Department of Botany of the UFSCar, Brazil.

Extraction, Bioassay-Guided Fractionation, and Isolation. The plant material was dried in an oven at 40° for 72 h and powdered in an industrial mill. Dried material (0.5 kg) was extracted with hexane at r.t. to obtain 32.54 g of defatted plant material (hexane extract). The plant residue was re-extracted with CH_2Cl_2, AcOEt, acetone, MeOH, and dist. H_2O, to yield, after removal of the solvent, 4.51, 5.19, 23.56, 14.78, and 33.60 g of extract, respectively. The effect of these extracts on the elongation of etiolated wheat coleoptiles was investigated and the most bioactive extracts were assayed for phytotoxicity towards standard target species (STS). The acetone extract, one of the most active extracts tested, was chromatographed (SiO_2; hexane/acetone mixtures of increasing polarity) to afford nine fractions, *i.e.*, *Frs. A–I*.

Frs. E, *G*, and *H* showed the best chromatographic profiles and were bioassayed with etiolated wheat coleoptiles. *Frs. E* and *H* showed higher bioactivity in the wheat coleoptile bioassay than *Fr. G* and were re-chromatographed. *Fr. E* (hexane/acetone 80:20 *v*/*v*; 0.162 g) was subjected to CC (SiO_2; hexane/AcOEt mixtures of increasing polarity) to afford five fractions, *Frs. E1–E5*. *Fr. E3* (0.0774 g) was purified by HPLC (SiO_2; hexane/AcOEt 75:25 *v*/*v*) to yield lutein (**1**; 12.9 mg). *Fr. H* (hexane/acetone 40:60; 0.900 g) was separated by CC (SiO_2; $CHCl_3$/MeOH mixtures of increasing polarity) to afford nine fractions, *Frs. H1–H9*. *Fr. H5* (0.145 g) was subjected to CC (*Sephadex LH-20*; hexane/$CHCl_3$/MeOH 2:1:1) to yield (−)-catechin (**2**; 41.9 mg).

Coleoptile Bioassay. Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in *Petri* dishes (diameter, 15 cm), moistened with H_2O, and grown in the dark at $25 \pm 1°$ for 4 d [15]. The roots and caryopses were removed from the shoots. The latter were placed in a *Van der Weij* guillotine, and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for the bioassays. All manipulations were performed under green safelight [41].

The crude extracts, fractions, and compounds were dissolved in DMSO and diluted with phosphate-citrate buffer (pH 5.6, containing 2% sucrose) [41] to the final bioassay concentration (final DMSO concentrations $\leq 0.1\%$). The following concentrations were assayed: 0.8, 0.4, and 0.2 mg/ml for both crude extracts and fractions, and 10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5}, and 10^{-5} M for pure compounds. The diluted extracts, fractions, or pure compounds were added to test tubes together with phosphate-citrate buffer (2 ml). Five coleoptiles were placed in each test tube (three tubes per dilution) and the tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at 25° in the dark. The coleoptiles were measured by digitalization of their images. Each assay was carried out in duplicate, and parallel controls were run. Data were presented as percentage differences from the control. Thus, zero represents the control; positive values represent stimulation of the studied parameter, and negative values represent inhibition. Data were statistically analyzed using *Welch*'s test [42].

Phytotoxicity Bioassays. The selection of the target plants was based on an optimization process carried out to set up a standard phytotoxicity bioassay [19]. The standard target species (STS) assayed in this study comprised the monocot *Allium cepa* L. (onion) and the dicots *Lycopersicum esculentum* MILL. (tomato), *Lepidium sativum* L. (cress), and *Lactuca sativa* L. (lettuce).

The bioassays were conducted in *Petri* dishes (diameter, 50 mm) with one sheet of *Whatman No.1* filter paper as a support. Germination and growth were conducted in aq. solns. at controlled pH (10^{-2} M 2-[*N*-morpholino]ethanesulfonic acid (MES) and 1M NaOH; pH 6.0). The extracts to be assayed were dissolved in DMSO, and these solns. were diluted with buffer (5 μl DMSO soln./ml buffer) so that for each extract test concentrations of 0.2, 0.4, and 0.8 mg/ml were achieved. This procedure facilitated the solubility of the assayed extracts. Four replicates were used for tomato, cress, onion, and lettuce, each containing 20 seeds. Treatment, control, or internal reference soln. (1 ml) was added to each *Petri* dish.

After adding seeds and aq. solns., the *Petri* dishes were sealed with *Parafilm*, to ensure closed-system models, and incubated at 25° in a *Memmert ICE 700* controlled-environment growth chamber in the dark. The bioassays took 4 d for cress, 5 d for lettuce and tomato, and 7 d for onion. After growth, the plants were frozen at $-10°$ for 24 h to avoid subsequent growth during the measurement process.

The commercial herbicide *Logran*® (combination of *N*-(1,1-dimethylethyl)-*N*′-ethyl-6-(methylsulfanyl)-1,3,5-triazine-2,4-diamine (Terbutryn, 59.4%) and 2-(2-chloroethoxy)-*N*-{[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl}benzenesulfonamide (Triasulfuron, 0.6%); *Syngenta*) was used as an

internal reference, according to a comparison study reported previously [23]. This reference was used at the same concentrations as the extracts (0.2, 0.4, and 0.8 mg/ml) and under the same conditions as reported previously [23]. Control samples (buffered aq. solns. with DMSO and without any test compound) were used for all of the plant species assayed.

The evaluated parameters (germination rate, shoot length, and root length) were recorded with a *Fitomed*© system [43], which allowed automatic data acquisition and statistical analysis by using the associated software. Data were analyzed statistically using *Welch*'s test, with significance levels fixed at 0.01 and 0.05. Results were presented as percentage differences from the control. Zero represented the parameter of the control, positive values stimulation, and negative values inhibition.

Determination of IC_{50} *Values.* After adjusting the activity data to a logarithmic concentration scale, IC_{50} values were obtained using a sigmoidal dose – response model according to *Eqn. 1:*

$$Y = Y_{\min} + (Y_{\max} - Y_{\min})/(1 + 10^{\log(IC_{50} - X)}) \quad (1)$$

where X indicates the logarithm of the concentration, Y indicates the response (phytotoxicity), and $Y_{\max}$ and $Y_{\min}$ are the maximum and minimum values of the response, respectively. Goodness of fit is described by the determination coefficient (r^2). The IC_{50} and r^2 values were obtained using *GraphPad Prism*® software v. 4.00.

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