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Phytotoxins from *Tithonia diversifolia*

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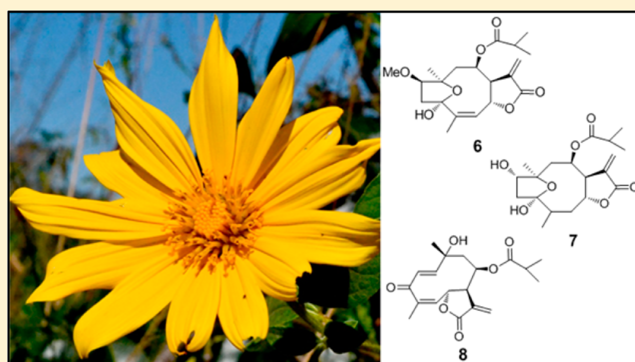
Supporting Information

ABSTRACT: *Tithonia diversifolia* (Mexican sunflower) is a dominant plant of the Asteraceae family, which suggests it produces allelochemicals that interfere with the development of surrounding plants. The study described herein was conducted to identify the compounds that have phytotoxic activity in *T. diversifolia* extracts. Ethyl acetate extracts of the leaves, stems, and roots showed significant inhibition of wheat coleoptile growth, and the leaf extract had similar inhibitory effects to a commercial herbicide. Fourteen compounds, 12 of which were sesquiterpene lactones, have been isolated. Two sesquiterpene lactones are reported for the first time and were isolated as an inseparable mixture of 8*β*-*O*-(2-methylbutyroyl)-tirotundin (**4**) and 8*β*-*O*-(isovaleroyl)tirotundin (**5**). Their structures were determined by spectroscopic analysis, including NMR techniques and mass spectrometry. The sesquiterpene lactones 1*β*-methoxydiversifolin (**6**), tagitinin A (**7**), and tagitinin C (**8**) were the major products identified. These compounds were active on etiolated wheat coleoptiles, seed germination, and the growth of STS and weeds. The phytotoxic activity shown by these sesquiterpene lactones indicates that they are the compounds responsible for the activity exhibited by the initial extracts.



Invasive species are the second leading cause of global diversity loss after habitat fragmentation.[1] Invasive plants can affect ecological systems by altering many communities and ecosystems,[2] outcompeting native flora, and establishing monocultures.[3] Some of these species were introduced initially as crops but were considered subsequently as weeds.[4] Attempts have been made to explain invasive plant mechanisms through numerous ecological studies.

It is worth considering the possibility that some invasive species may gain an advantage by virtue of having secondary compounds that present novel challenges to native species.[5,6] These biochemical weapons function as unusually powerful allelopathic agents that may be highly inhibitory to other plants in invaded communities.[7]

Allelopathy can be defined as the positive or negative effects on the growth of other plants caused by secondary metabolites produced by plants and released into the environment.[8] These compounds can be released from plants by volatilization, root exudation, leaching, and decomposition of plant material.[9] It has been demonstrated that the phenomenon of allelopathy is an important ecological mechanism that influences the dominance and succession of plants, climax formation, communities, agriculture management, and productivity.[10,11]

The identification of allelochemicals from invasive plants is an important step in gaining a complete understanding of the allelopathic phenomenon. Such information may provide an insight into the establishment of invasive plants in a given environment and may also guide the development of new tools for natural herbicide models[12] that are more environmentally benign than synthetic pesticides.[13]

Tithonia diversifolia (Hemsl.) A. Gray (Mexican sunflower) is a perennial shrub of the family Asteraceae.[14] It originated in Mexico and Central America, but it was introduced into a wide range of other countries for ornamental proposes or for its pharmacological action,[15] its use as green manure, and the control of soil erosion.[16] However, this plant has become a problem due its invasive behavior in several countries.[14,17]

Many classes of secondary metabolites have been isolated from *Tithonia* species, including sesquiterpenoids, diterpenoids, and flavonoids.[17] More than 150 compounds have been isolated from *T. diversifolia*,[18] and these include the sesquiterpene lactones tirotundin, tagitinin A, and tagitinin C.[19]

Sesquiterpene lactones are present in large quantities in the family Asteraceae, and they are an important class of secondary metabolites responsible for pharmacological and phytotoxic activities.[20−25] The sesquiterpene lactones tagitinin A and tagitinin C were reported to reduce seed germination and seedling growth of certain plants.[26] This phytotoxicity suggests

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the possibility of allelopathic activity on the growth of other plants under natural conditions.[27] The incorporation of dry *T. diversifolia* leaves into the soil was reported to inhibit the growth of rice seedlings.[28] In other studies it was found that *T. diversifolia* aqueous extracts show phytotoxic activity on the germination and growth of *Amaranthus* species.[29,30]

The aim of the study described herein was to bioprospect *T. diversifolia* extracts through the isolation and purification of secondary metabolites with phytotoxic activity. It was envisaged that the results could explain the invasive behavior of the plant and that the compounds identified could be used as natural herbicide models in the future. In this study, plant material (roots, leaves, and stems) was extracted using ethyl acetate and methanol as solvents. Extracts and purified compounds were tested in etiolated wheat coleoptile and phytotoxicity bioassays. The most phytotoxic extract was fractionated in order to isolate and identify its chemical components. The structures of the compounds were elucidated by spectroscopic studies. The bioactivity profiles of the isolated compounds were assayed on the standard target species (STS)[31] (lettuce, watercress, tomato, and onion) and on the weeds barnyardgrass and brachiaria. The Poaceae species barnyardgrass (*Echinochloa crus-galli* L.) and brachiaria (*Urochloa decumbens* (Stapf) R.D. Webster) are important weed species throughout the world. Barnyardgrass is a weed that is native to Asia, but it invades rice plantations around the world.[32] Brachiaria is especially invasive in South America.[33] Our hypothesis is that allelochemicals from *T. diversifolia* have phytotoxic potential, and they can inhibit the development of other invasive weeds such as those tested in this study.

■ RESULTS AND DISCUSSION

Dried leaves (60 g), stems (60 g), and roots (60 g) of *Tithonia diversifolia*, defatted with hexane, were extracted with ethyl acetate (EtOAc, 1.04%, 0.14%, and 0.18% yield, respectively) and methanol (MeOH, 3.28%, 2.15%, and 1.05% yield, respectively). Chlorophyll was removed from the leaf extracts. The extracts, at concentrations of 0.8, 0.4, and 0.2 mg mL^{-1}, were subjected to an etiolated wheat coleoptile bioassay.[34] This test is used widely to evaluate the sensitivity of wheat to a wide range of bioactive substances.[35]

The leaf, stem, and root EtOAc extracts generally showed higher inhibition than the methanol (MeOH) extracts. The leaf EtOAc extract was the most active, and this also showed the best activity profile, as activity was retained upon dilution, even at 0.2 mg mL^{-1} (−74%) (Figure 1).

In order to compare the activities of the extracts, IC$_{50}$ values were calculated using a sigmoidal dose−response model. The results allowed the extracts to be ranked in decreasing order of activity as follows: EtOAc leaf extract (IC$_{50}$ = 73 μg mL^{-1}, r^2 = 0.9955) > MeOH leaf extract (IC$_{50}$ = 190 μg mL^{-1}, r^2 = 0.9988) > EtOAc stem extract (IC$_{50}$ = 240 μg mL^{-1}, r^2 = 0.9994) > EtOAc root extract (IC$_{50}$ = 720 μg mL^{-1}, r^2 = 0.9983) > MeOH root extract (IC$_{50}$ = 1520 μg mL^{-1}, r^2 = 0.9627) > MeOH stem extract (IC$_{50}$ = 3670 μg mL^{-1}, r^2 = 0.9654). It can be seen from the IC$_{50}$ values that the leaf EtOAc, leaf MeOH, and stem EtOAc extracts were the most active ones. The differences in the activity profiles and the similarity between the leaf MeOH and stem EtOAc extracts suggested that the most active metabolites are of intermediate polarity. The leaf EtOAc extract showed a more consistent activity profile with dilution when compared with the leaf MeOH extract. Therefore, the leaf and stem EtOAc extracts were evaluated in a phytotoxicity bioassay using the concentrations described above.

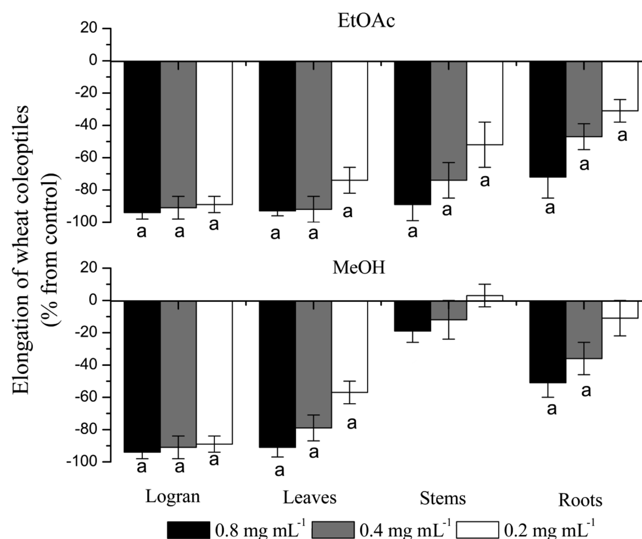

Figure 1. Effects of the herbicide Logran, ethyl acetate (EtOAc), and methanol (MeOH) leaf, stem, and root extracts of *Tithonia diversifolia* on the elongation of etiolated wheat coleoptiles. Values are expressed as percentage difference from control. Levels of significance at $p < 0.01$ (a) according to Welch's test.

The standard target species were lettuce, tomato, cress, and onion (Figure 2), and the weed species were barnyardgrass and brachiaria (Figure 3). The parameter that was affected the most by the extracts was root length. The root sensitivity 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 extracts, which can be absorbed directly. As a consequence, the roots are exposed to peak periods and concentrations of phytotoxins.[36]

The inhibition by the leaf extract at the highest concentrations was equal to that of the herbicide Logran at 0.8 and 0.4 mg mL^{-1} on STS (Figure 2) and barnyardgrass (Figure 3) roots, with values above 80% inhibition for the highest concentration in most species. Moreover, the leaf extract at 0.8 mg mL^{-1} was more active than the herbicide on the germination of tomato and cress (Figure 2). The inhibition of leaf extracts at the two highest concentrations on the shoot lengths of tomato, onion, and cress was similar to that obtained with the herbicide (Figure 2). On the other hand, the stem extract showed similar inhibition potencies to the herbicide on the shoot length of tomato, onion, and cress and on the root length of tomato and onion (Figure 2). The germination of weeds was not affected by the extracts or the herbicide, and a similar lack of activity was observed for the shoot and root lengths of brachiaria (Figure 3).

These results show that the extracts of *T. diversifolia* have phytotoxic activity on STS and weed species. The identification of the individual components and the evaluation of their bioactivity could provide a better understanding of the ability of invasive plants to become dominant[37] and thus facilitate the discovery of new tools for crop protection.

The EtOAc leaf extract was selected to continue the study, as it was the most active extract in both bioassays. The remaining defatted material (1.8 kg) was extracted with EtOAc, and the chlorophyll was removed to give 24.3 g of material as four chlorophyll-free fractions: A (3.831 g), B (10.766 g), C (3.831

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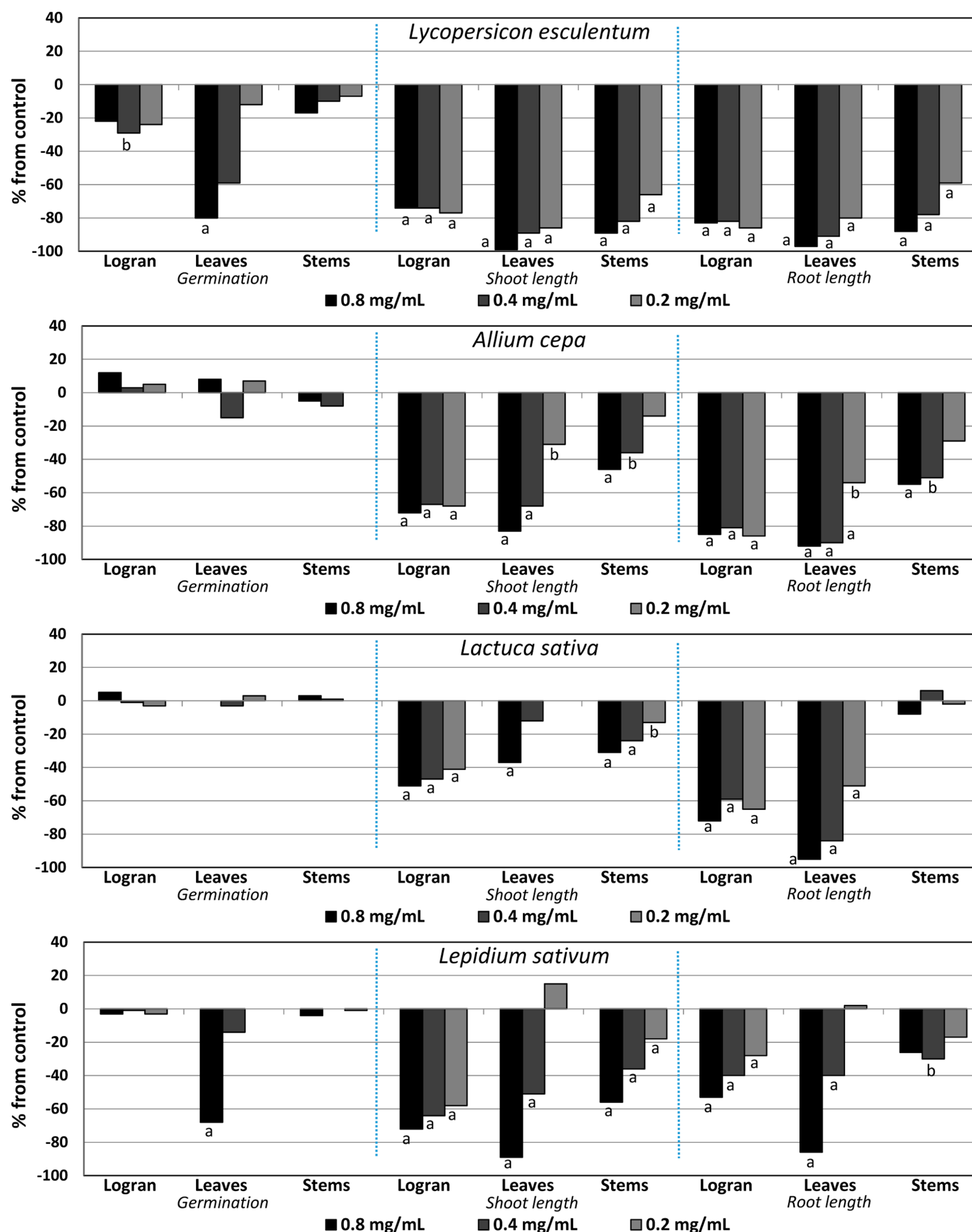

Figure 2. Effects of the herbicide Logran and ethyl acetate (EtOAc) leaf or stem extracts of *Tithonia diversifolia* on standard target species growth. Values are expressed as percentage difference from control. Levels of significance at $0.01 < p < 0.05$ (b) or $p < 0.01$ (a) according to Welch's test.

g), and D (5.875 g). These fractions were chromatographed on silica gel using hexane/acetone mixtures of increasing polarity. This process afforded subfractions (A) A1, A2, and A4; (B) B1, B2, B3, and B4; and (C) C3, C4, C5, C6, and C8. These subfractions were purified, and the allelochemicals were identified.

The chromatographic separation of subfractions led to the isolation of 14 compounds: 12 sesquiterpene lactones (**1**−**12**), one secosesquiterpene (**13**), and one diterpene (**14**). The isolated compounds **1**−**3** and **6**−**14** were identified by comparison of their spectroscopic data (^{1}H NMR, ^{13}C NMR, IR, and MS) to those reported in the literature for tagitinin F-3-*O*-methyl ether (**1**),[38] 3-methoxytirotundin (**2**),[39] tirotundin (**3**),[40] 1*β*-methoxydiversifolin (**6**),[38] tagitinin A (**7**),[41] tagitinin C (**8**),[41] 3*β*-acetoxy-8*β*-isobutyryloxyreynosin (**9**),[39] 3*β*-acetoxytithifolin (**10**),[39] 3*α*-acetoxycostunolide (**11**),[42] 8*β*-

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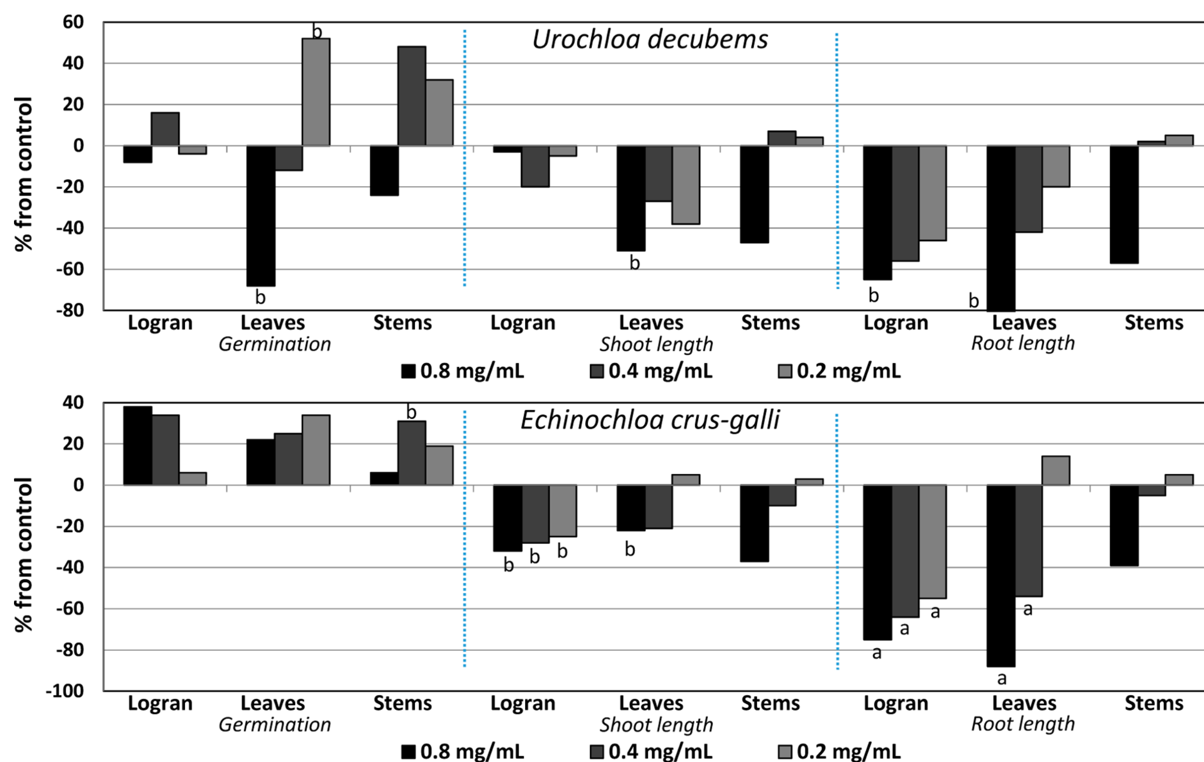
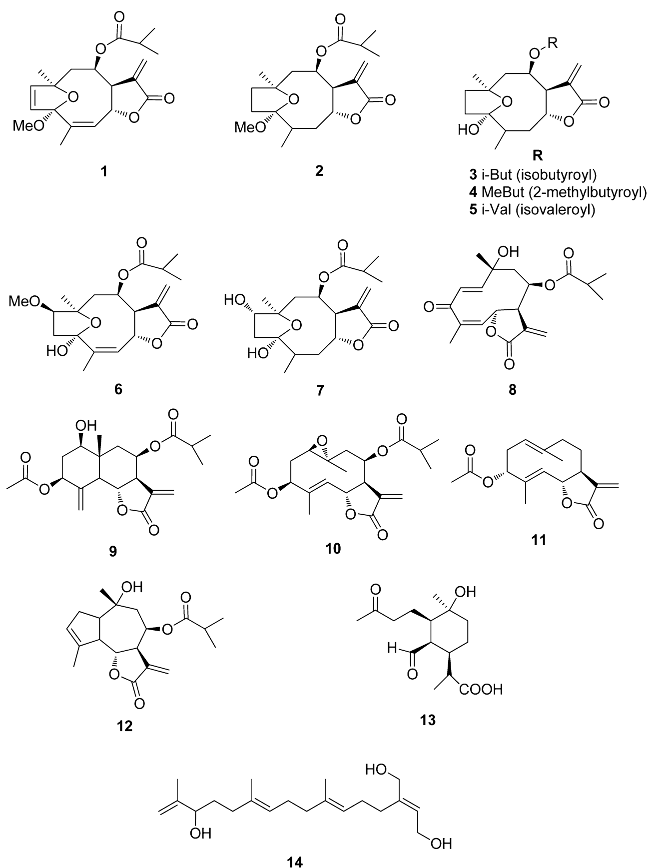


Figure 3. Effects of the herbicide Logran and ethyl acetate (EtOAc) leaf or stem extract of *Tithonia diversifolia* on weed growth. Values are expressed as percentage difference from control. Levels of significance at $0.01 < p < 0.05$ (b) or $p < 0.01$ (a) according to Welch's test.



isobutyryloxycumambranolide (**12**),[43] 2-formyl-4-hydroxy-4*α*-methyl-3-(3-oxobutyl)cyclohexaneacetic acid (**13**),[44] and (2*E*,6*E*10*E*)-3-(hydroxymethyl)-7,11,15-trimethylhexadeca-2,6,10,15-tetraene-1,14-diol (**14**).[45] Compound **11** is reported for the first time from *T. diversifolia*.

Compounds **4** and **5** were isolated from fraction C3.3 as an apparent single component after successive purifications using HPLC techniques. However, the ^{1}H and ^{13}C NMR spectra showed that this was a mixture of two sesquiterpene lactones in a 3:2 ratio. The (+)-HRESITOFMS showed two signals at m/z 367.2116 and 389.1938 according to the adducts $[M + H]^+$ and $[M + H + Na]^+$ and is consistent with the presence of a single compound, and the (−)-HRESITOFMS data showed other signals at m/z 365.1976 and 389.1914 corresponding to $[M - H]^-$ and $[M - H + Na]^-$. These peaks are consistent with a molecular formula of $C_{20}H_{30}O_6$, for both isomeric compounds. The ^{1}H and ^{13}C NMR spectra contained signals similar to those of tirotundin (**3**)[40] apart from the absence of the signals corresponding to the isobutyroyl ester side chain at C-8. The spectra contained signals from two different chains, and these were inferred as corresponding to two different compounds. It was not possible to identify which signal corresponded with which side chain from the ^{1}H−^{1}H COSY spectrum, since the signals were very close together. However, the 1D TOCSY (total correlation spectroscopy) spectrum proved to be more informative and allowed the determination of the exact chemical shifts of the signals from each ester side chain. Signals corresponding to the methyl groups of both chains were identified in the TOCSY spectrum. These signals were selected since they were more shielded than the others.

The procedure known as "array-TOCSY" with different values of mix (mixing time) between 0.010 and 0.15 s provided different subspectra (Figure 4). The rise in the mixing time value allowed greater distance correlations in spin systems to be visualized whenever the magnitude of the coupling constant of the neighboring proton allows this effect. Each side-chain proton of the sesquiterpene lactones was assigned (Figure 4).

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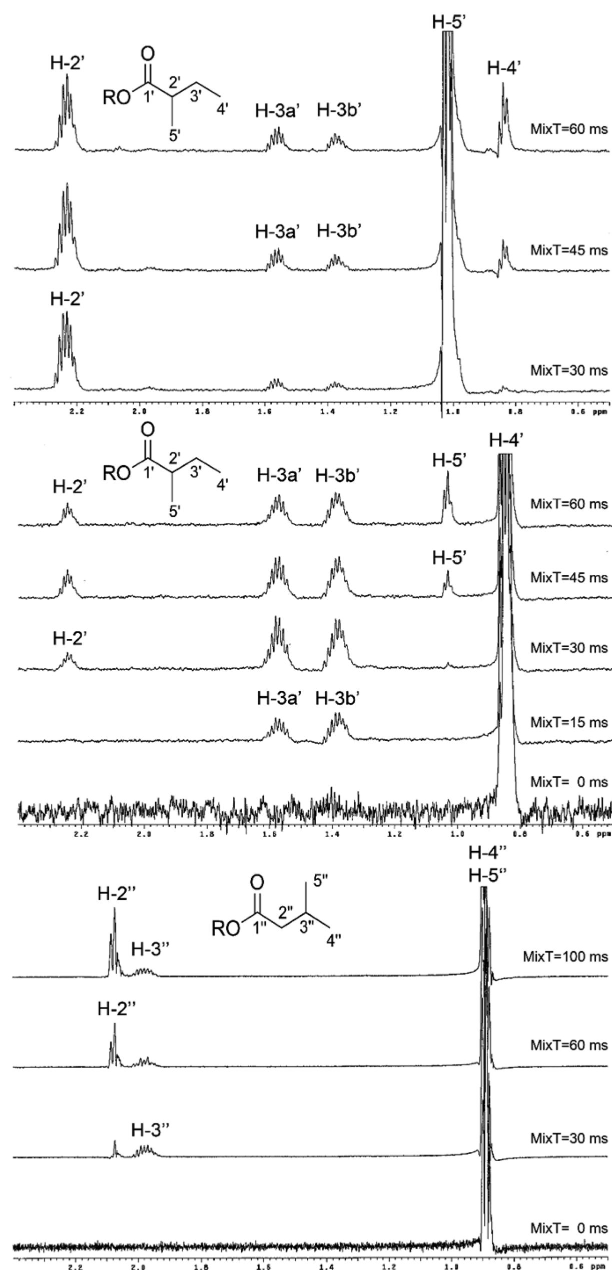

Figure 4. Selected 1D TOCSY NMR spectra of the signals corresponding to ester side chains of the fraction containing compounds **4** and **5** (600 MHz, $CDCl_3$).

Starting with the methyl proton at δ 0.83 (H-4′), correlations for δ 1.38 (H-3′a), 1.58 (H-3′b), 1.02 (H-5′), and 2.22 (H-2′) in the 1D TOCSY spectrum were observed, and these results are consistent with a 2-methylbutyroyl ester[38] (Table 1). In the same way, 1D TOCSY subspectra correlations were obtained by selective excitation of the methyl protons at δ 0.86 and 0.87 (H-4″, H-5″) from the other compound, and the connectivity established with signals at δ 1.95 (H-3″) and 2.06 (H-2″) corresponded to an isovaleroyl chain (Table 1).[39]

The zTOCSY technique is a two-dimensional total *J*-correlation spectroscopy for scalar coupled spin systems with a zero-quantum filter for artifact suppression. Thus, the zTOCSY experiment contains a zero-quantum filter and usually results in cleaner spectra than the TOCSY experiment. The

Table 1. ^{1}H **NMR (600 MHz) and** ^{13}C **(125 MHz) Spectroscopy Data for Compounds 4 and 5 in** $CDCl_3$

position	δ_C type	δ_H (J in Hz)	HMBC
1	38.74, CH_2	2.22, m; 1.97, m	C-2, C-3, C-9, C-10
2	38.17;[a] 38.15,[a] CH_2	1.85 m	C-1, C-3
3	108.77;[a] 108.76,[a] C		
4	43.23;[a] 43.22,[a] CH	1.98, d (7)	C-2, C-3, C-5, C-6
5	38.05;[a] 38.03,[a] CH_2	2.11, ddd (1.5, 7, 9.3); 1.84, ddd (1.5, 7, 9.3)	C-3, C-4, C-6, C-7, C-15
6	81.18;[a] 81.14,[a] CH	4.53, ddd (1.5, 6.5, 9.3)	C-4, C-7, C-8
7	47.72;[a] 47.59,[a] CH	4.06, dddd (2.9, 3.4, 4.1, 6.5)	
8	69.80;[a] 69.55,[a] CH	5.54, ddd (4.1, 4.8, 11.1)	C-6, C-7
9	42.11;[a] 42.08,[a] CH_2	1.85, dd (4.8, 14.7); 1.74, dd (11.1, 14.7)	C-1, C-7, C-8, C-10, C14
10	80.19;[a] 80.18,[a] C		
11	136.93;[a] 137.00,[a] C		
12	169.39, C		
13	121.58;[a] 121.55,[a] CH_2	6.25, d (2.9); 5.52, d (3.4)	C-7, C-11, C-12
14	26.86;[a] 26.81,[a] CH_3	1.44, s	C-1, C-9, C-10
15	18.56;[a] 18.54,[a] CH_3	1.10, d (7)	C-3, C-4, C-5
2-methylbutyroyl			
1′	175.80, C		
2′	41.16, CH	2.22, tq (7)	C-1′, C-3′, C-4′, C-5′
3′	26.56, CH_2	1.38, dq (7); 1.58, dq (7)	C-1′, C-2′, C-4′, C-5′
4′	11.60, CH_3	0.83, t (7)	C-2′, C-3′
5′	16.86, CH_3	1.02, d (7)	C-2′, C-3′
isovaleroyl			
1″	172.19, C		
2″	43.34, CH_2	2.06, dd (15, 7)	C-1″, C-3″, C-4″, C-5″
3″	25.63, CH	1.95, m	C-1″, C-2″, C-4″, C-5″
4″	22.22, CH_3	0.86, d (7)	C-2″, C-3″, C-5″
5″	22.34, CH_3	0.87, d (7)	C-2″, C-3″, C-4″

[a] ^{13}C assignments of compounds **4** and **5** may be interchanged.

zTOCSY spectrum allowed the structural sequences proposed above to be performed (Figure S10, Supporting Information).

A full assignment of the ^{1}H and ^{13}C NMR spectroscopic data for these new sesquiterpene lactones is reported with $^{1}H-^{1}H$ COSY, gDQCOSY, HSQC, HMBC, and NOESY data. As a result, the structure of **4** was established as 8*β*-*O*-(2-methylbutyroyl)tirotundin, and the structure of **5** as 8*β*-*O*-(isovaleroyl)tirotundin. Neither of these two compounds has been reported previously in the literature.

The bioactivities of compounds **1**−**3**, **6**−**12**, and **14** were tested in a wheat coleoptile bioassay in the concentration range 10^{-3} to 10^{-5} M (except for compound **2**, which had a concentration limit of 3×10^{-4} M). Compound **13** was not tested due to the low amount obtained, and compounds **4** and **5** were not evaluated because they were obtained as a mixture.

All of the compounds tested showed high inhibitory activities against coleoptile elongation at the highest concentration used.

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Compounds **1** (94%), **3** (94%), **6** (98%), **7** (91%), **8** (96%), and **12** (93%) showed similar or higher activity in comparison to the commercial herbicide Logran at 10^{-3} M (Figure 5).

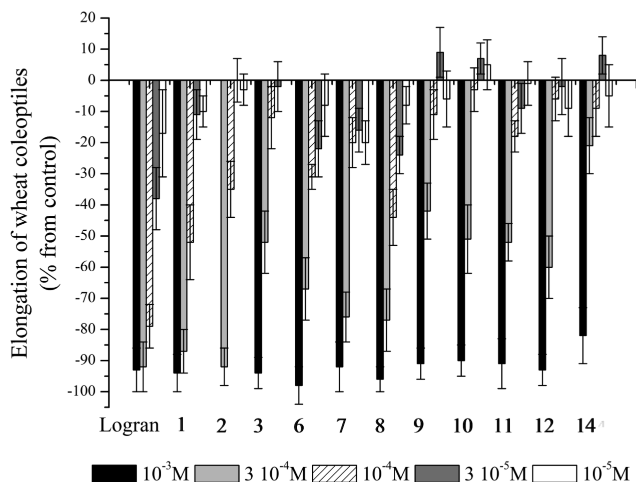

Figure 5. Effects of compounds tagitinin F-3-*O*-methyl ether (**1**), 3-methoxytirotundin (**2**), tirotundin (**3**), 1*β*-methoxydiversifolin (**6**), tagitinin A (**7**), tagitinin C (**8**), 3*β*-acetoxy-8*β*-isobutyryloxyreynosin (**9**), 3*β*-acetoxytithifolin (**10**), 3*α*-acetoxycostunolide (**11**), 8*β*-isobutyryloxycumambranolide (**12**), and (2*E*,6*E*10*E*)-3-(hydroxymethyl)-7,11,15-trimethylhexadeca-2,6,10,15-tetraene-1,14-diol (**14**) from *Tithonia diversifolia* on the elongation of etiolated wheat coleoptiles.

Compounds **1**−**3**, **6**−**8**, and **10**−**12** were also active at a concentration of 3×10^{-4} M, with more than 50% inhibition obtained. Compound **2** had similar activity to the commercial herbicide at 3×10^{-4} M, but its activity decreased from a concentration of 10^{-4} M.

The compounds with the best activity profiles in the wheat coleoptile bioassay were **1**, **2**, **6**, **7**, and **8**, as these had inhibition values higher than 65% at the second concentration tested (3×10^{-4} M). These activity values were corroborated by calculating the IC_{50} values for all compounds. The IC_{50} values were calculated using a sigmoidal dose−response or dose−response variable slope models (Table 2).

Compounds **6** (4.82 g), **7** (4.17 g), and **8** (3.62 g) were the major components and accounted for 20%, 17%, and 15%, respectively, of the EtOAc extract (24.3 g) studied. Phytotoxicity bioassays on the germination and development of STS seeds (Figure 6) and weeds (Figure 7) were carried out on **6** (1*β*-methoxydiversifolin), **7** (tagitinin A), and **8** (tagitinin C). In contrast, very small amounts of compounds **1** and **2** were obtained, and these were therefore not evaluated on the seeds.

Table 2. IC_{50} Values Calculated from Compounds 1−3, 6−12, and 14 in the Wheat Coleoptile Bioassay, Using a Sigmoidal Dose−Response Variable Slope Models

compound	IC_{50} (μg mL^{-1})	r^2
1	98.15	0.9953
2	125.3	0.9993
3	288.1	0.9993
6	177.5	0.9814
7	203.6	0.9395
8	113.0	0.9951
9	357.0	0.9530
10	283.7	0.9967
11	287.4	0.9971
12	265.0	0.9947
14	528.3	0.9877
Logran	38.69	0.9895

The parameter that was affected least was germination. Significant differences were not observed between the compounds and the herbicide for the STS (Figure 6). On the other hand, compounds **6** and **8** showed significant inhibition (almost 80%) of the seed germination for brachiaria, and this was higher than that of the standard herbicide used (Figure 7). These results suggest possible selectivity and the potential for use for this compound in the biological control of brachiaria when applied in pre-emergence.

Shoot length was affected only by compounds at the highest concentration assayed, and in most cases inhibition values were equal to those of the commercial herbicide. The values were close to 95% for compounds **6** and **8** on tomato, 90% for **6** on onion, and 65% for **6** on cress (Figure 6). Root length was the variable that was most affected by the allelochemicals, mainly at a concentration of 10^{-3} M. The isolated compounds were more phytotoxic than the standard herbicide in most cases. Compound **6** was more active at 10^{-3} M than the herbicide on root length for brachiaria (Figure 7). Compounds **6**, **7**, and **8** were active on the root length of tomato (96%, 85%, and 97%, respectively) and onion (90%, 77%, and 86%, respectively). Compounds **6** and **8** also showed activity on lettuce and cress (Figure 6). These compounds are also active on the root length of weeds with inhibition values on root growth of brachiaria and barnyardgrass above 70%, higher than the value obtained for the herbicide (Figure 7).

The extracts (Figures 2 and 3) and compounds (Figures 6 and 7) tested were active on STS plants and weeds. The wide spectrum of biological activity shown by the sesquiterpene lactones means that they have potential uses in medicine and agriculture. Indeed, such compounds have been described as having antitumor,[46] cytotoxic,[47,48] anti-inflammatory,[49] antimicrobial,[50] antifungal,[51,52] antifeedant,[53] and allelopathic[22,23] properties.

In addition to their phytotoxic activity, the sesquiterpene lactone tagitinin A was reported to have insecticidal properties,[54] and tagitinin C was reported to have antiplasmodial activity, as it was active against a chloroquine-sensitive strain.[55]

The results obtained in this study show that the extracts and pure compounds have similar activity profiles in coleoptile and seedling growth bioassays. The amounts in which natural compounds are biosynthesized and their biological activities, including phytotoxicity, suggest that they may be involved in the defense mechanism of this plant,[56] and this also supports our hypothesis that *T. diversifolia* has phytotoxic effects and could inhibit the development of other plants. These compounds would be the allelochemicals responsible for the activity shown by this plant. The great potential of *T. diversifolia* could be exploited in the biological control of weeds and as natural herbicide models for crop protection.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined at room temperature on a model 241 polarimeter (PerkinElmer, Waltham, MA, USA) (on the sodium D line). Infrared (IR) spectra (KBr) were recorded on a Fourier transform infrared (FT-IR) Spectrum 1000 spectrophotometer (PerkinElmer). Nuclear magnetic resonance (NMR) spectra were run on 600, 500 and 400 MHz spectrometers (Agilent, Palo Alto, CA, USA). Chemical shifts are

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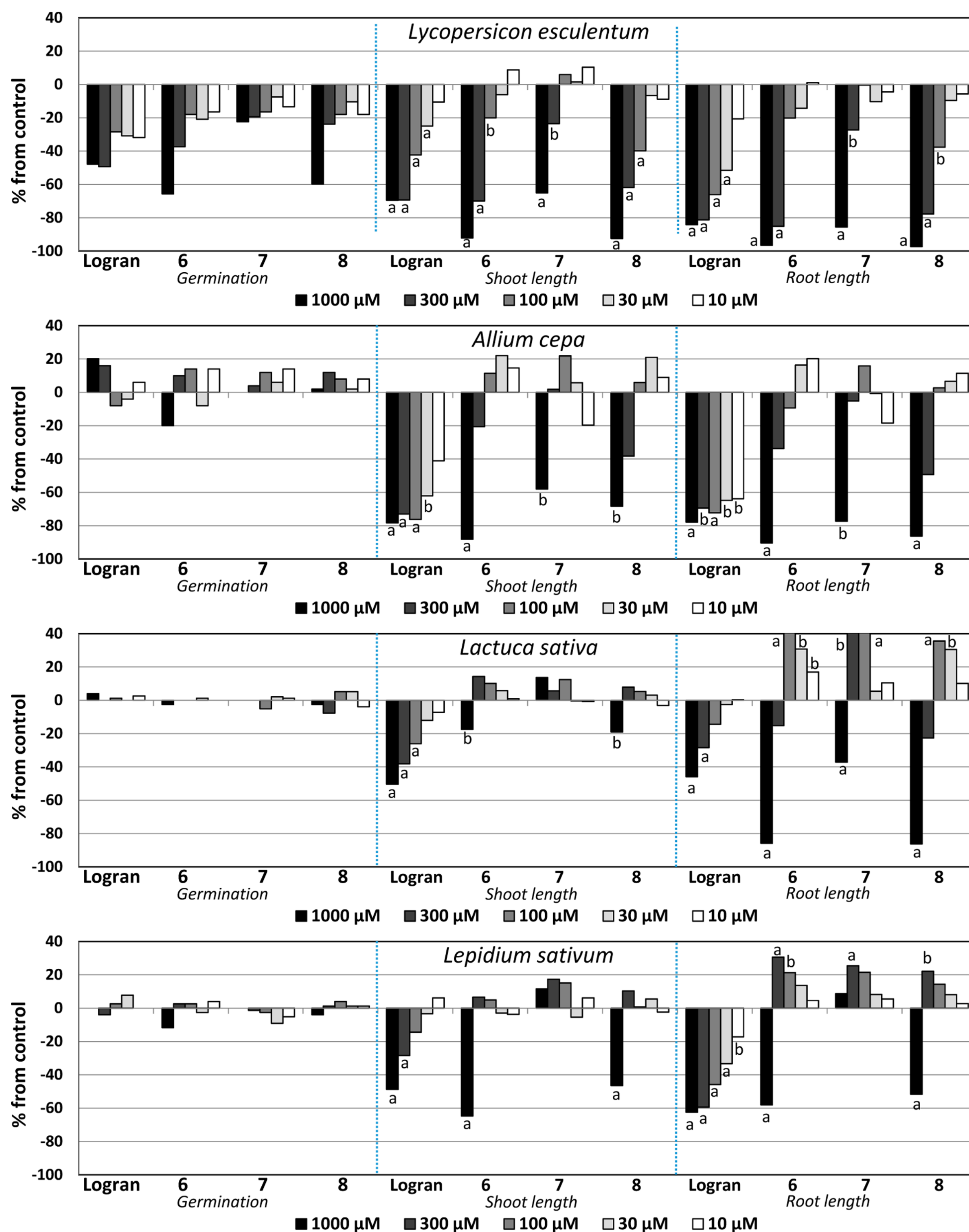

Figure 6. Effects of compounds 1*β*-methoxydiversifolin (**6**), tagitinin A (**7**), and tagitinin C (**8**) from *Tithonia diversifolia* on standard target species growth. Values are expressed as percentage difference from control. Levels of significance at $0.01 < p < 0.05$ (b) or $p < 0.01$ (a) according to Welch's test.

given in ppm with respect to residual ^{1}H signals of $CHCl_3$-d_1 (δ 7.25), and ^{13}C signals are referenced to the solvent signal (δ 77.00). HRMS were obtained on a Synapt G2 UPLC-QTOF ESI mass spectrometer (Waters, Milford, MA, USA). HPLC was carried out on a HPLC chromatograph (Merck-Hitachi, Tokyo, Japan), with RI detection. Silica gel 0.060−0.200, 60A from Acros Organics (Geel, Belgium), and Lichroprep RP 18 (40−63 μm) from Merck (Darmstadt, Germany) were used for column chromatography. TLC was carried out on silica gel 60 F254 aluminum sheets and TLC silica gel 60 RP-18 F254S aluminum sheets from Merck. Compounds were visualized under $UV_{254/366}$ light and by spraying with H_2SO_4/H_2O/HOAc (4:16:80). HPLC columns used were a semipreparative column 250 mm × 10 mm i.d., a 10 μm Lichrospher 250-10 Si60 (Merck) with a LiChrospher Si60 guard column (Merck), and an analytical column

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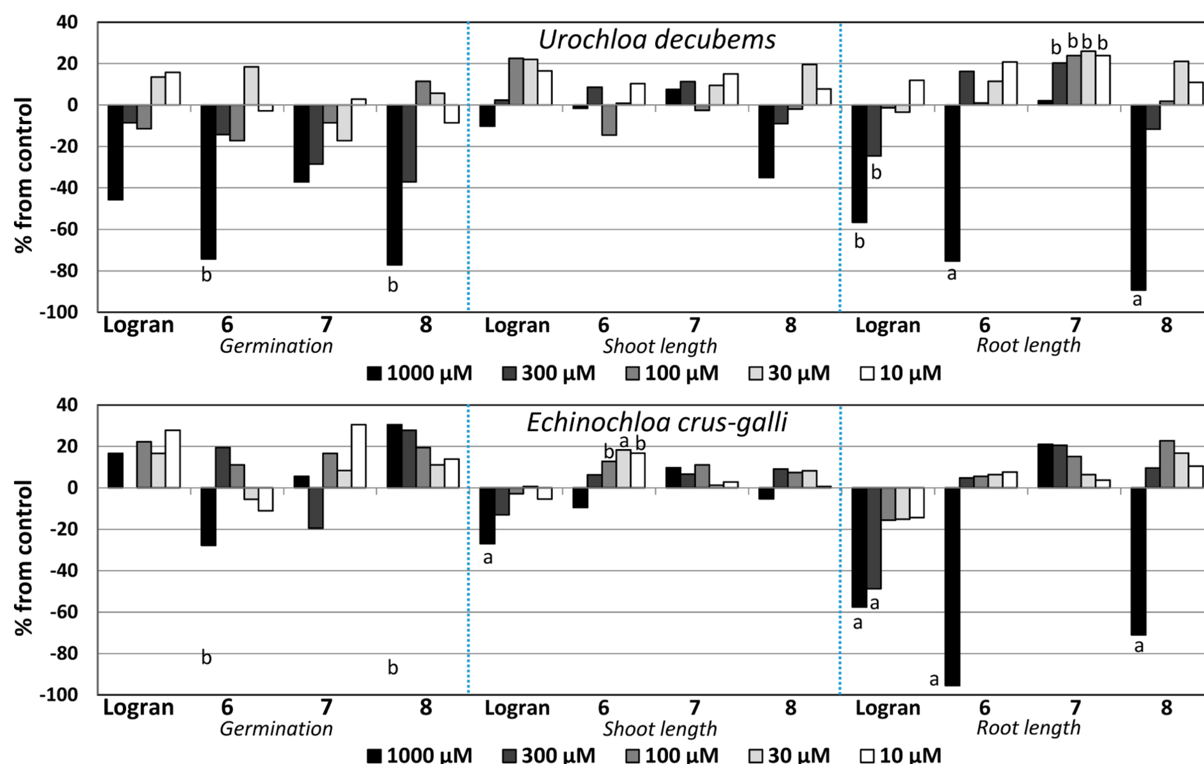

Figure 7. Effects of compounds 1β-methoxydiversifolin (**6**), tagitinin A (**7**), and tagitinin C (**8**) from *Tithonia diversifolia* on weed growth. Values are expressed as percentage difference from control. Levels of significance at $0.01 < p < 0.05$ (b) or $p < 0.01$ (a) according to Welch's test.

250 mm × 4.60 mm × 10 μm Luna 10 μm Silica (2) 100 Å (Phenomenex). Ultrasound-assisted extractions were performed in an ultrasonic bath (360 W, J.P. Selecta, Barcelona, Spain).

Chemicals. Chloroform, *n*-hexane, methanol, dichloromethane, ethyl acetate, and acetone Hipersolv Chromanorm for HPLC were obtained from VWR International (Radnor, PA, USA). MagniSolv chloroform-D1 deuteration degree min. 99.8% for NMR spectroscopy was obtained from Merck.

Plant Material. Leaves, stems, and roots were randomly gathered from plants in the area of Cerrado (Brazilian savanna) from Universidade Federal de São Carlos (UFSCar) São Carlos campus, São Carlos, SP, Brazil (21°58′ to 22°00′ S and 47°51′ to 47°52′ W), during the dry season (August 2013). Plant species was identified by one of the authors (S.C.J.G.). The *T. diversifolia* exsiccates were deposited in the Herbarium of the Department of Botany of UFSCar, Brazil (voucher 8728).

Extraction and Isolation. The plant material was dried in an oven (for 72 h at 40 °C) and powdered using an industrial mill. The dried material from each part of the plant (60 g) was extracted with hexane at room temperature using an ultrasonic bath in order to defat the material. The following quantities were obtained: roots 92.8 mg (hexane), leaves 401.5 mg (hexane), and stems 52.7 mg (hexane). The defatted material was extracted with ethyl acetate (EtOAc) and methanol (MeOH). These extractions yielded, after removal of the solvent, the following quantities: roots 105 mg (EtOAc) and 628.6 mg (MeOH); leaves 625 mg (EtOAc) and 1966 mg (MeOH); stems 84 mg (EtOAc) and 1291 mg (MeOH). Chlorophyll was removed from leaf extracts using mixtures of H_2O/MeOH, 20% (fraction A), 40% + 60% (fraction B), 80% (fraction C), and 100% (fraction D), and finally with chloroform as eluent on an RP-18 chromatography column. The EtOAc and MeOH extracts were tested in bioassays with etiolated wheat coleoptiles, and the most active were bioassayed for phytotoxicity on standard target species.

The EtOAc leaf extract was the most active in both bioassays, and the remaining leaves (1.8 kg), previously defatted, were therefore extracted using 10 L of EtOAc in portions of 180 g of plant with 2 × 500 mL each using an ultrasonic bath. This process yielded 35 g of material. The chlorophyll was removed from this extract, and four chlorophyll-free fractions were obtained in decreasing order of polarity, A (3.8306 g), B (10.7661 g), C (3.8310 g), and D (5.8751 g), which were chromatographed with a hexane/EtOAc gradient from 0 to 100% in EtOAc, with a 10% increase each time, and finishing with 100% MeOH (500 mL of each polarity), to afford various subfractions: (A) A1, A2, and A4; (B) B1, B2, B3, and B4; (C) C3, C4, C5, C6, and C8. Subfraction B1 (22.1 mg) was purified by HPLC (semipreparative column) eluting with hexane/EtOAc (80:20 v/v, flow 3 mL/min) to obtain compounds **1** (5 mg) and **2** (2.2 mg). Subfractions B2 (3.56 g), B3 (2.32 g), and B4 (3.32 g) yielded compounds **6** (3.56 g), **8** (2.32 g), and **7** (3.32 g), respectively. Subfraction C3 (505.5 mg) was subjected to column chromatography with a hexane/acetone gradient from 20% to 100% in acetone, with a 10% increase each time and finishing with 100% MeOH (250 mL of each polarity) to afford nine subfractions (C3.1 to C3.9). Subfraction C3.2 (98 mg) was purified by HPLC (semipreparative column) eluting with hexane/EtOAc (70:30 v/v, flow 3 mL/min) to afford compounds **11** (3.5 mg), **12** (10 mg), and **13** (1.3 mg). Subfraction C3.3 (300 mg) was purified by HPLC (semipreparative column) eluting with hexane/EtOAc (50:50, flow 3 mL/min) to yield compounds **3** (11.3 mg), **10** (7.8 mg), and an isomeric mixture of **4+5** (21.3 mg). This mixture **4+5** was obtained after successive purifications using analytical columns eluting with hexane/EtOAc (60:40, flow 1 mL/min) and hexane/acetone (50:50, flow 1 mL/min) as a single peak. Subfraction C4 (768 mg) afforded compound **8** (768 mg). Subfraction C5 (295.5 mg) was purified by HPLC (semipreparative column) eluting with hexane/EtOAc (60:40, flow 3 mL/min) to yield compounds **8** (68.4 mg) and **9** (14.2 mg). Subfraction C6 (756 mg) yielded compound **7** (756 mg). Subfraction C8 (120.7 mg) yielded compound **14** (120.7 mg). Subfractions A1 (1.26g), A2 (467 mg), and A4 (854 mg) yielded compounds **6** (1.26 g), **8** (467 mg), and **7** (854 mg), respectively.

*8β-O-(2-Methylbutyroyl)tirotundin (**4**) and 8β-O-(isovaleroyl)-tirotundin (**5**) (mixture of isomers):* white, amorphous power; IR (KBr) ν_{max} 3400, 1770, 1735 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data see Table1; positive-ion EISMS m/z 389 (100) $[M + Na]^+$; positive-ion HRESIMS m/z 389.1938 [M +

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Na]$^+$ (calcd for $C_{20}H_{30}O_6Na$ 389.1940); 367.2116 [M + H]$^+$; negative-ion EISMS m/z 365 (100) [M − H]$^-$; negative-ion HRESIMS m/z 365.1976 [M − H]$^-$ (calcd for $C_{20}H_{30}O_6$ 365.1964); MS−MS, ESI-positive [367 (M + H)], 349 (M − H_2O + H), 247 (M − H_2O − $C_5H_{10}O_2$ + H); ESI-negative [365 (M − H)], 263 (M − $C_5H_{10}O_2$ − H), 101 ($C_5H_9O_2$).

Compounds **1**−**3**, **6**−**12**, and **14** were bioassayed on etiolated wheat coleoptiles, and the active major compounds **6**, **7**, and **8** were bioassayed for phytotoxicity on standard target species and weeds.

Coleoptile Bioassay. Wheat (*Triticum aestivum* L. cv. Duro) seeds were sown in 15 cm diameter Petri dishes moistened with water and grown in the dark at 25 ± 1 °C for 3 days.[35] 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 bioassays. All manipulations were performed under a green safelight.[57] Crude extracts or pure compounds were dissolved in DMSO (0.1%) and diluted in phosphate-citrate buffer containing 2% sucrose[57] at pH 5.6 to the final bioassay concentrations (0.8, 0.4, and 0.2 mg mL^{-1} for extracts and 10^{-3}, 3×10^{-4}, 10^{-4}, 3×10^{-5}, and 10^{-5} M for compounds).

Parallel controls were also run. The commercial herbicide Logran, for which the original formulation is a combination of N^2-*tert*-butyl-N^4-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine (terbutryn, 59.4%) and 1-[2-(2-chloroethoxy)phenylsulfonyl]-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea (triasulfuron, 0.6%), was used as an internal reference, at the same concentrations and under the same conditions as reported previously.[34] Buffered aqueous solutions with DMSO and without any test compound were used as a control for all the plant samples assayed.

Five coleoptiles and 2 mL of solution were placed in each test tube (three tubes per dilution), and the tubes were rotated at 6 rpm in a roller tube apparatus for 24 h at 25 °C in the dark. The coleoptiles were measured by digitalization of their images. Data were statistically analyzed using Welch's test[58] and are presented as percentage difference from the control. Positive values represent stimulation, and negative values represent inhibition.

Phytotoxicity Bioassays. The selection of target plants was based on a study for a standard phytotoxicity bioassay reported previously.[34] Several standard target species were proposed, including the dicotyledons tomato (*Lycopersicon esculentum* Will.), cress (*Lepidium sativum* L.), and lettuce (*Lactuca sativa* L.) and the monocotyledon onion (*Allium cepa* L.), which were assayed for this study. Two weed species were added as target plants in this bioassay, the monocotyledons barnyardgrass (*Echinochloa crus-galli* L.) and brachiaria (*Urochloa decumbens* (Stapf) R.D. Webster).

Bioassays were conducted using Petri dishes (50 mm diameter) with one sheet of Whatman No.1 filter paper as a support. Germination and growth were conducted in aqueous solutions at controlled pH by using 10^{-2} M 2-[*N*-morpholino]ethanesulfonic acid and 1 M NaOH (pH 6.0). The extracts or compounds to be assayed were dissolved in DMSO, and these solutions were diluted with buffer (5 μL DMSO solution/mL buffer) so that test concentrations for each extract (0.8, 0.4, and 0.2 mg mL^{-1}) and compound (10^{-3}, 3×10^{-4}, 10^{-4}, 3×10^{-5}, and 10^{-5} M) were achieved. Parallel controls were also run as described before for the coleoptile bioassay.

Four replicates were used for tomato, cress, onion, and lettuce, each containing 20 seeds. Treatment, control, or internal reference solution (1 mL) was added to each Petri dish. After adding seeds and aqueous solutions, Petri dishes were sealed with Parafilm to ensure closed-system models. Seeds 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 and 16/8 h light/dark for barnyardgrass and brachiaria. Bioassays took 4 days for cress, 5 days for tomato, 6 days for lettuce, 7 days for onion, and 8 days for barnyardgrass and brachiaria. After growth, plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process.

The germination rate, root length, and shoot length were recorded using a Fitomed system.[59] Data were analyzed statistically using Welch's test, with significance fixed at 0.01 and 0.05. Results are presented as percentage differences from the control. Zero represents control, positive values represent stimulation, and negative values represent inhibition.

■ ASSOCIATED CONTENT

Supporting Information

1D and 2D NMR spectra for compounds **4** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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