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Phytotoxicity of alkaloids, coumarins and flavonoids isolated from 11 species belonging to the Rutaceae and Meliaceae families

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ABSTRACT

Meliaceae and Rutaceae families are known for the high diversity of their secondary metabolites, which include many groups that represent a rich source of structural diversity, and are good candidates as sources of allelochemicals that could be useful in agriculture. In the work described here the bioactivity profiles were evaluated for 3 alkaloids (1–3), 12 coumarins (4–15), 2 phenylpropanoic acid derivatives (16 and 17) and 14 flavonoids (18–31) from 11 species belonging to the Meliaceae and Rutaceae families. All compounds were tested in the wheat coleoptile bioassay and those that showed the highest activities were tested on the STS (Standard Target Species) *Lepidium sativum* (cress), *Lactuca sativa* (lettuce), *Lycopersicon esculentum* (tomato), and *Allium cepa* (onion).

Most of the isolated compounds showed phytotoxic activity and graveoline (3), psoralen (8), and flavone (18) were the most active, with bioactivity levels similar to that of the commercial herbicide Logran®. The results indicate that these compounds could be involved as semiochemicals in the allelopathic interactions of these plant species.

1. Introduction

Meliaceae and Rutaceae families are known for their high diversity of secondary metabolites, including many groups that represent a rich source of structural diversity. Alkaloids, coumarins, flavonoids, terpenes and limonoids are the largest groups of secondary plant metabolites and they show remarkable biological activities and have potential medicinal value (Tan and Luo, 2011; Champagne et al., 1992; Da Silva et al., 1984; Da Silva and Gottlieb, 1987).

The biological activity of the alkaloids has been actively explored and has been found to span a variety of properties (Michael, 2007, 2005). These include anticancer (Duarte et al., 2010; Zhai et al., 2012), antiparasitic (Silva et al., 2012; Mbeunkui et al., 2012; Mishra et al., 2009; Santos et al., 2009), anti-inflammatory (Souto et al., 2011), antimicrobial (Joosten and Veen, 2011), and insecticidal (Bermúdez-Torres et al., 2009; Li et al., 2009) properties amongst others.

Coumarins are a large group of compounds that are widely distributed in many plant species and they have a wide spectrum of biological activities, including insecticidal (Pavela and Vrchotová, 2013), anticancer (Harada et al., 2010), Alzheimer's disease (Anand et al., 2012) and antimicrobial (Al-Amiery et al., 2012; Guan et al., 2011; Souza et al., 2005; Godoy et al., 2005; Sardari et al., 1999).

Flavonoids are also an important group of compounds and are widely found in the plant kingdom. They have a diverse range of significant bioactivities, including antioxidant (Wolfe and Liu, 2008), allelopathic (Treutter, 2006), gastroprotective (Mota et al., 2009), anticancer (Pick et al., 2011; Benavente-García and Castillo, 2008; Marchand, 2002), anti-inflammatory (García-Lafuente et al., 2009; Kim et al., 2004), and antimicrobial (Cushnie and Lamb, 2011; Salas et al., 2011) along with other effects.

The increased interest in crop protection, along with the extensive use of agrochemicals and the problems associated with their use, has led to the search for new biologically active natural products (Dayan et al., 2009). The discovery of new allelochemicals is an attractive alternative to current conventional herbicides used

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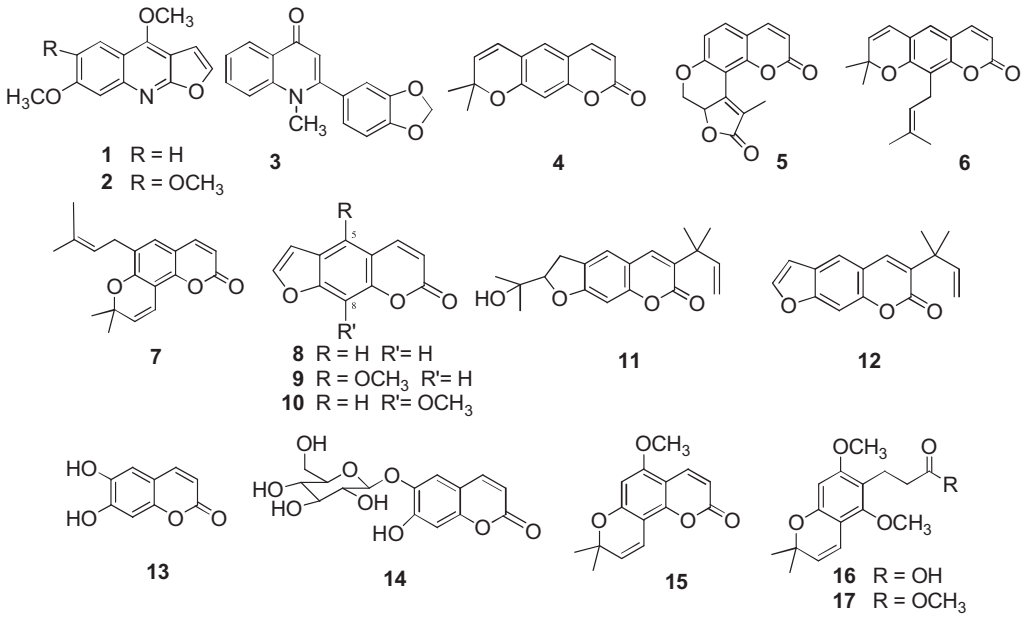


Fig. 1. Structures of the alkaloids (**1**–**3**) and coumarins (**4–17**) isolated from Meliaceae and Rutaceae families.

for weed control. Commercial herbicides have caused changes in populations of invasive species and environmental pollution and they have also enhanced the resistance phenomenon (Macías et al., 2007). In this context, Meliaceae and Rutaceae are good candidates to provide a source of allelochemicals for future use in agriculture.

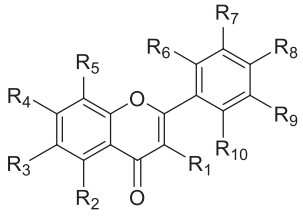
The aim of the work described here was to evaluate the bioactivity profiles of three alkaloids (**1**–**3**), twelve coumarins (**4**–**15**), two phenylpropanoic acid derivatives (**16** and **17**) (Fig. 1) and fourteen flavonoids (**18**–**31**) (Fig. 2) from eleven species belonging to the Meliaceae and Rutaceae families.

2. Results and discussion

2.1. Isolation of compounds

The extraction, isolation and identification (NMR, MS, IR, UV data) of the following compounds has been described previously: **2** and **9** from roots and aerial parts of *Ruta graveolens* (Paulini et al., 1989; Masuda et al., 1998); **4** from roots of *Citrus sinensis* grafted on *Citrus limonia* (Cazal et al., 2009); **5** from stems and leaves of *Raunia resinosa* (Velozo et al., 1997); **7** from the fruit of *Swinglea glutinosa* (Santos, 2005); **15**, **16** and **17** from stem and taproots of *Hortia oreadica* (Braga et al., 2012); **18**, **23** and **24** from the fruit, branches and leaves of *C. fruticosa* Bl. (Leite et al., 2009); **21** from the fruit of *Neoraputia magnifica* (Tomazela et al., 2000; Passador et al., 1997); **22** from leaves of *Neoraputia alba* (Arruda et al., 1993); **25** and **29** from stems and leaves of *Neoraputia paraensis* (Moraes et al., 2003); **26** and **27** from peel; and **31** from the fruit of *Murraya paniculata* (Ferracin et al., 1998). Metabolites **13**, **14**, **19**, **20**, **28** and **30** were obtained from commercial sources in order to carry out bioassays.

Compounds **1**, **3**, **8**, **11** and **12** were isolated from the roots and aerial parts of *R. graveolens*, and **10** and **15** were obtained from taproots of *H. oreadica* as described in Section 3.



18 $R_1,R_2,R_3,R_4,R_5,R_6,R_7,R_8,R_9,R_{10}$ = H
19 $R_1,R_2,R_3,R_6,R_7,R_8,R_9,R_{10}$ = H; R_4,R_5 = OH
20 $R_1,R_3,R_5,R_6,R_7,R_8,R_9,R_{10}$ = H; R_2,R_4 = OH
21 $R_1,R_3,R_5,R_6,R_7,R_{10}$ = H; R_2,R_4 = OCH_3; R_8,R_9 = OCH_2O
22 R_1,R_3,R_6,R_7,R_{10} = H; R_2,R_4,R_5 = OCH_3; R_8,R_9 = OCH_2O
23 R_1,R_3,R_5,R_6,R_{10} = H; R_2,R_4,R_7,R_8,R_9 = OCH_3
24 R_1,R_3,R_6,R_7,R_{10} = H; R_2,R_4,R_5,R_8,R_9 = OCH_3
25 R_1,R_3,R_6,R_{10} = H; R_2,R_4,R_5,R_7,R_8,R_9 = OCH_3
26 R_5,R_6,R_{10} = H; $R_1,R_2,R_3,R_4,R_7,R_8,R_9$ = OCH_3
27 R_3,R_6,R_{10} = H; $R_1,R_2,R_4,R_5,R_7,R_8,R_9$ = OCH_3
28 R_3,R_5,R_6,R_9,R_{10} = H; R_1,R_2,R_4,R_7,R_8, = OH

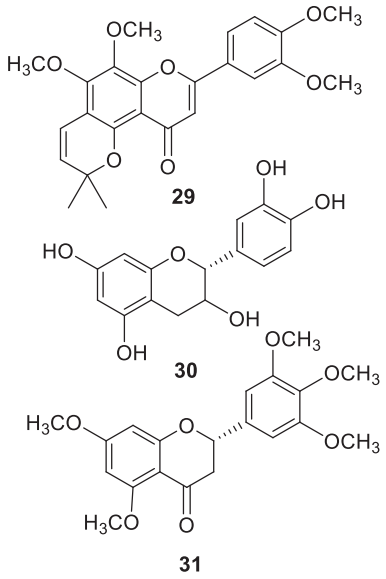


Fig. 2. Structures of the flavonoids (**18**–**31**) isolated from Meliaceae and Rutaceae families.

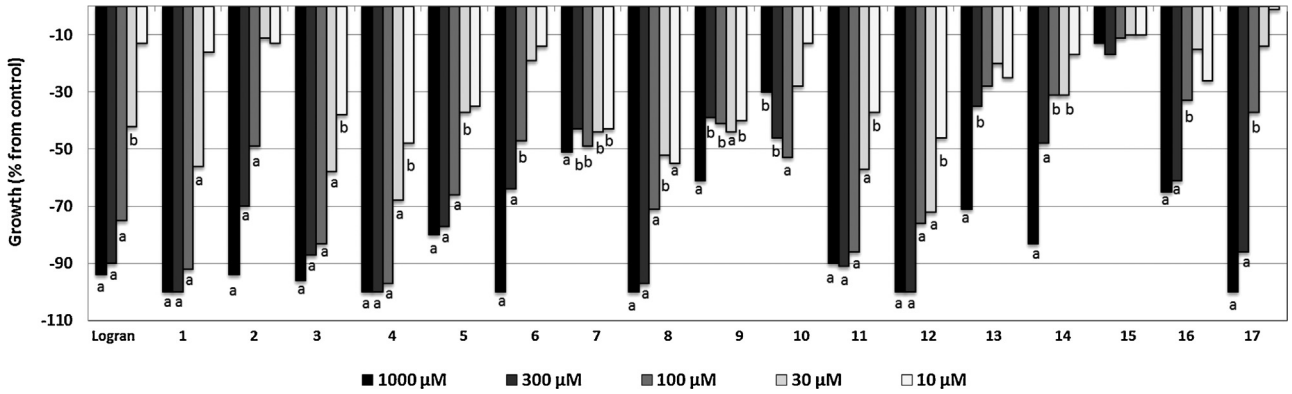


Fig. 3. Bioactivities obtained in the etiolated wheat coleoptile bioassay for compounds **1**–**17**. Values are expressed as percentage differences from the control and are not significantly different with $P > 0.05$ for the Mann–Whitney test. [a]Values significantly different with $P < 0.01$. [b]Values significantly different with $0.01 < P < 0.05$.

2.2. Coleoptile bioassay results

The etiolated wheat coleoptiles bioassay was used as an initial approach to evaluate the phytotoxicity of these compounds since it is a rapid test (24 h) that is sensitive to a wide range of bioactive substances (Cutler et al., 2000), including plant growth regulators, herbicides (Cutler, 1984), antimicrobials, mycotoxins and assorted pharmaceuticals (Jacyno and Cutler, 1993). The results are shown in Figs. 3 and 4, where negative values signify inhibition, positive values denote activation and zero represents control.

All alkaloids assayed were active. Evolitrine (**1**) and graveoline (**3**) presented the most consistent profiles, with levels of activity higher than – 85% at the first three concentrations tested (1 mM, 300 μM and 100 μM) (Fig. 3). Kokusagine (**2**) differs from **1** in the presence of an additional methoxyl group at C-6 but it only showed relevant activity levels at 1 mM and 300 μM (–94% and –70%).

Regarding coumarins, xanthyletin (**4**) was the most active of the pyranocoumarins (**4**–**7**, **15**). This compound completely inhibited coleoptile elongation at 1 mM, 300 μM and 100 μM and the bioactivity was retained upon dilution (–68%, 30 μM; 10 μM). Of the furanocoumarins, psoralen (**8**), chalepin (**11**) and chalepensin (**12**) showed good levels of activity. The most active compound was **12**, which completely inhibited the coleoptile elongation at 1 mM and 300 μM with the bioactivity maintained upon dilution (−76%, 100 μM; −72%, 30 μM and −46%, 10 μM). These results allow some structural correlations to be made. For example, linear pyranocoumarins and furanocoumarins are preferred to angular compounds (**4**, **8**, **11** and **12** vs. **5**, **7** and **15**). Furthermore, the absence of alkyl groups at C-8 (**4** vs.**6**) and methoxyl groups (**8** vs. **9**, **10** and **15**) leads to better results. However, the presence of prenyl groups bonded to C-3 does not lead to a decrease in the activity (**8** vs. **11** and **12**). Simple coumarins (**13** and **14**) and phenylpropanoic acid derivatives (**16** and **17**) showed inhibition effects of between −70% and −100% at the highest concentration, although the activities of these compounds decrease rapidly with dilution.

Regarding flavonoids, the most active compounds were flavone (**18**) and 3′,4′-methylenedioxy-5,7-dimethoxyflavone (**21**), which gave values of around −98% and −76% at 1 mM, respectively. 3′,4′,5,5′,7-Pentamethoxyflavone (**23**) did not show the highest levels of inhibition at 1 mM and 300 μM (−47% and −57%, respectively), but the bioactivity was maintained upon dilution (−51%, 100 μM; −44%, 30 μM and −44%, 10 μM) (Fig. 4). There is no clear substitution pattern that would indicate whether compounds are active or not. The introduction of hydroxyl and methoxyl groups seems to influence the bioactivity and small structural differences can change the activity markedly, as can be observed for **21** and **22** (Macías et al., 1997).

2.3. Phytotoxicity bioassay

The most active compounds, alkaloids **1** and **3**, pyranocoumarins **4** and **8**, furanocoumarins **11** and **12** and the flavones **18**, **21** and **23** were selected for phytotoxicity evaluation on the standard target species (STS) *Lepidium sativum* (cress), *Lactuca sativa* (lettuce), *Lycopersicon esculentum* (tomato), and *Allium cepa* (onion). The commercial herbicide Logran® was used as internal standard (Macías et al., 2000). The results of the bioassay are presented in Figs. 5 and 6, where data are presented as percentage differences from the control. The concentrations tested were

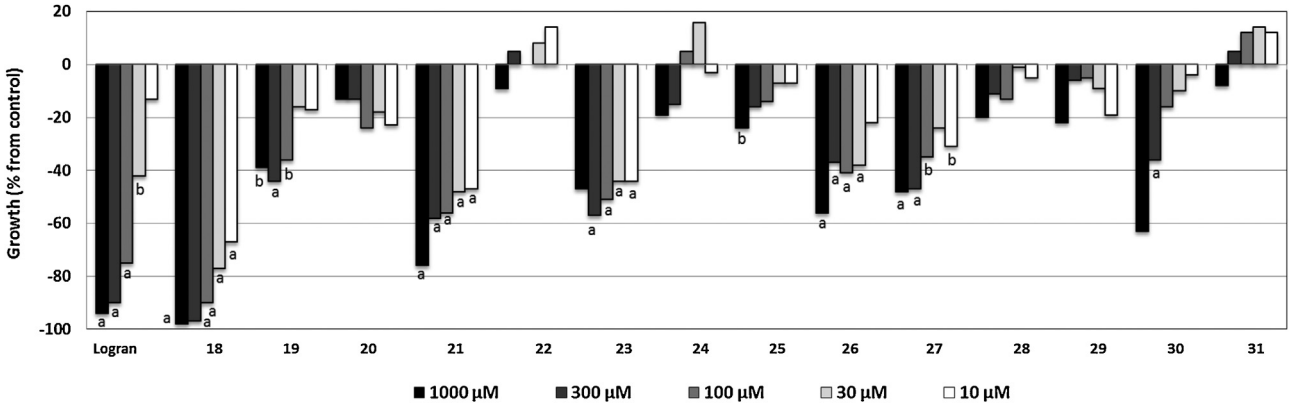


Fig. 4. Bioactivities obtained in the etiolated wheat coleoptile bioassay for compounds **18**–**31**. Values are expressed as percentage differences from the control and are not significantly different with $P > 0.05$ for the Mann–Whitney test. [a]Values significantly different with $P < 0.01$. [b]Values significantly different with $0.01 < P < 0.05$.

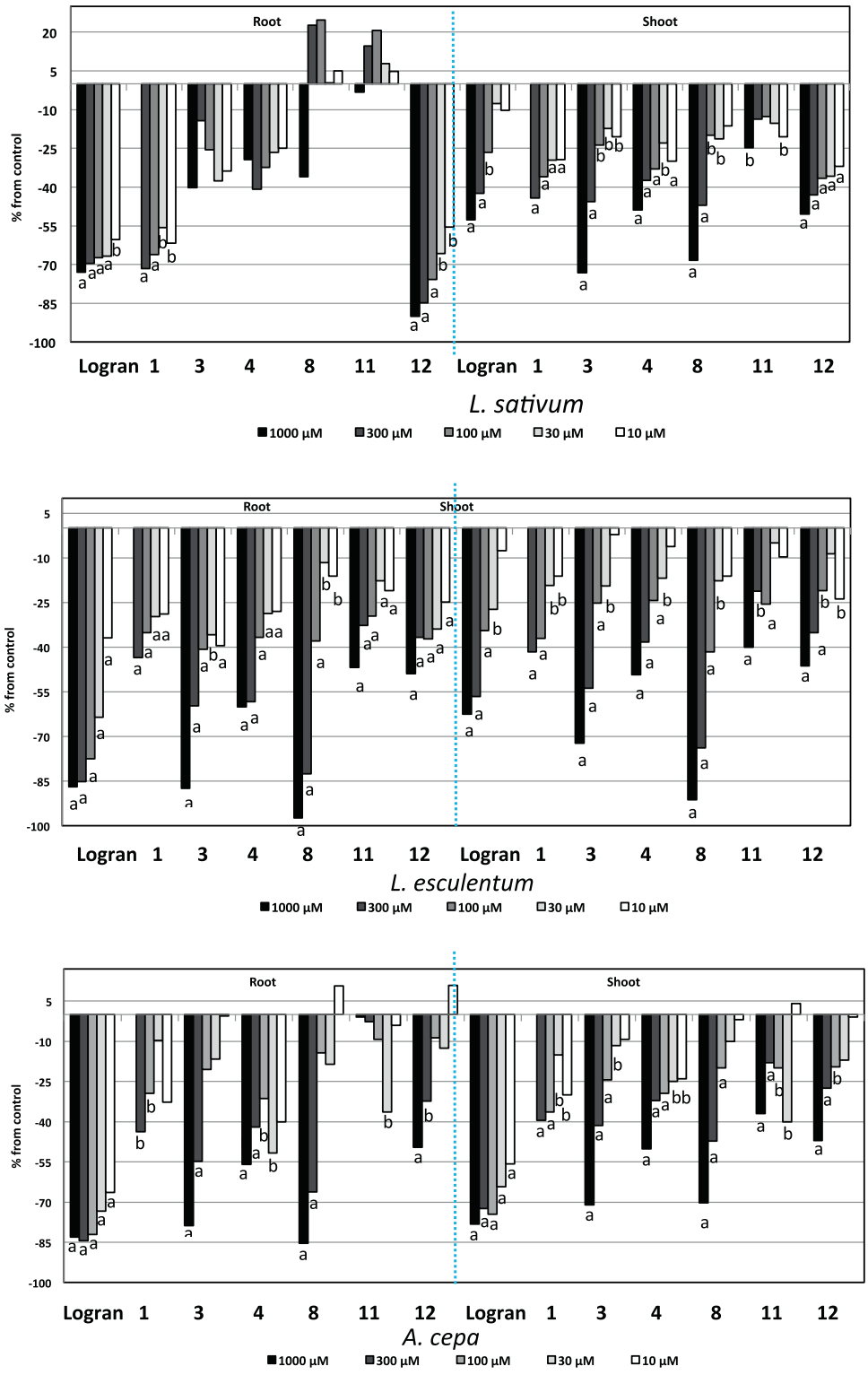


Fig. 5. Effects of the commercial herbicide Logran® and **1**, **3**, **4**, **8**, **11** and **12** on growth of standard target species. Values are expressed as percentage differences from the control and are not significantly different with $P > 0.05$ for the Mann–Whitney test. [a]Values significantly different with $P < 0.01$. [b]Values significantly different with $0.01 < P < 0.05$.

identical to those in the coleoptile bioassay, with the exception of **1**, for which the highest concentration was 300 µM.

The least affected parameter was germination, apart from compounds **3** and **8**, which inhibited germination of *L. sativum*, *L. esculentum* and *A. cepa*.

Regarding the dicotyledonous species, *L. sativum*, the compounds evolitrine (**1**) and chalepensin (**12**) were the most active and they inhibited root growth at all concentrations with similar levels to the herbicide Logran® (positive control). Graveoline (**3**) and psoralen (**8**) showed the best results on shoot growth, with values of −75% and −69%, respectively, at the highest concentration (10^{-3} M) (Fig. 5). Compounds **3** and **8** also affected the germination (−62% and −83%, respectively, at 1 mM).

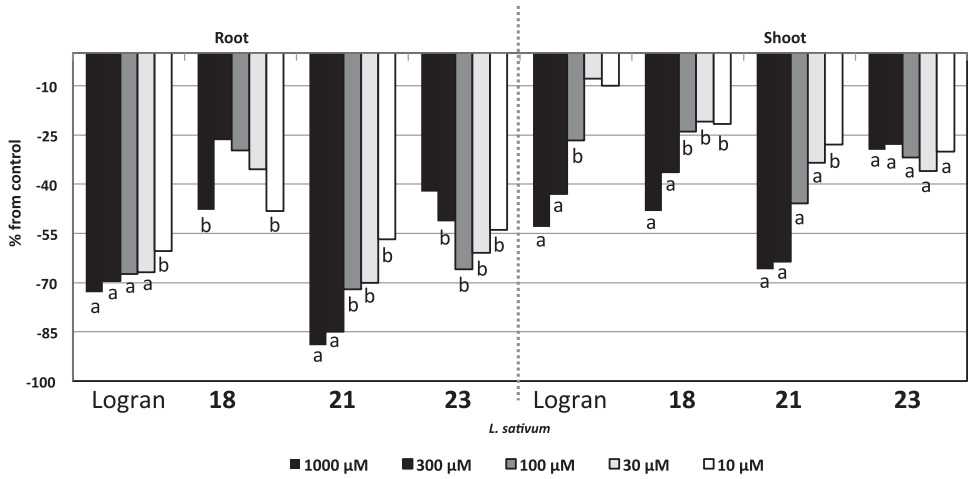


Fig. 6. Effects of the commercial herbicide Logran® and **18**, **21** and **23** on growth of *L. sativum*. Values are expressed as percentage differences from the control and are not significantly different with $P > 0.05$ for the Mann–Whitney test. [a]Values significantly different with $P < 0.01$. [b]Values significantly different with $0.01 < P < 0.05$.

Compound **21** was the most active flavone on germination and growth of *L. sativum* and it showed similar levels of phytotoxicity to the herbicide Logran® (Fig. 6). The effect on other species was not significant. The behavior of **18** and **23** was not significant on STS species.

Regarding *L. sativa*, the most active compounds were **8** and **12** and these affected the root growth at the highest concentration (−65% and −56%, respectively, at 1 mM). The growth of *L. esculentum* and *A. cepa* was also affected by the tested compounds – especially in the cases of **3** and **8**, which inhibited both parameters in both species.

Hale et al., 2004 demonstrated that **3** affected the growth of the aquatic plant *Lemma paucicostata* at 100 μM and caused tissue degradation at 250 μM and above. On the other hand, compounds **4**, **9**, and **10** have been previously tested on *L. sativa* and it was proposed that these compounds are responsible for the allelopathic activity of *Pilocarpus goudotianus* (Macías et al., 1993).

Although xanthyletin (**5**) and chalepin (**11**) have shown only moderate phytotoxicity levels on STS species, they inhibited the root growth of *Amaranthus hypochondriacus* (Anaya et al., 2005). Sampaio et al., 2012 evaluated the photosynthesis inhibition potential of furanocoumarins and highlighted chalepin (**11**) as a photosynthesis inhibitor.

We propose Meliaceae and Rutaceae to be good candidates to provide sources of allelochemicals that may be useful in agriculture. The isolated compounds showed phytotoxic activity and graveoline (**3**), psoralen (**8**) and flavone (**18**) were the most active, with bioactivity levels similar or even better than the commercial herbicide Logran®. The bioactivities of these compounds indicate that these products could also be involved as semiochemicals in the allelopathic interactions of these plant species.

3. Experimental

3.1. Plant material

R. graveolens was collected in Akai Ranch, located in Atibaia, São Paulo State, Brazil; *H. oreadica* Groppo was collected in Forest Reserve Adolpho Ducke, Itacoatiara, Amazonas State, Brazil; *C. sinensis* grafted on *C. limonia* was collected in Estação Experimental de Citricultura do Instituto Agronômico de Campinas, Cordeirópolis, SP, Brazil; *R. resinosa* (Nees et. Mart.) was collected in Cachoeiro do Itapemirim, Espírito Santo State, Brazil; *M. paniculata* was collected in São Carlos, SP, Brazil; *Cipadessa fruticosa* Bl and *N. magnifica* were collected in Viçosa, MG, Brazil; *N. alba* and *R. resinosa* (Nees et. Mart.) were collected in Cachoeiro do Itapemirim, Espírito Santo State, Brazil. The plants were identified by Dr. José R. Pirani from the Department of Botany, University of São Paulo and vouchers were deposited at the Herbarium at the same Department.

S. glutinosa (Bl.) Merr. was collected in Campinas, SP, Brazil, and identified by Prof. Dr. Maria Inês Salgado. A voucher specimen is deposited at the Herbarium of the Botany Department, Federal University of São Carlos (UFSCar) as number 7110.

3.2. Extraction and isolation

3.2.1. Isolation of *1*, *3*, *8*, *11* and *12* from Ruta graveolens

Dried roots (1.6 kg) and aerial parts (3.4 kg) were extracted with cold ethanol using a homogenizer for 3 days. The ethanolic solution was concentrated under reduced pressure and the crude extract of aerial parts was chromatographed using silica gel (CC) to afford the following fractions: hexane (13.8 g), CH_2Cl_2 (25.0 g) and MeOH (95.0 g). The liquid/liquid partition technique was used to fractionate the root extract into the following fractions: hexane (1.53 g), CH_2Cl_2 (1.55 g), EtOAc (1.87 g) and water (1.99 g). The CH_2Cl_2 fraction from the aerial parts was chromatographed on SiO_2 (70–230 mesh; 5.3 cm × 21.0 cm) in vacuum and eluted with hexane (A), CH_2Cl_2 (B) and MeOH (C) to yield three fractions. The CH_2Cl_2 fraction (B) from the aerial parts was fractionated on SiO_2, eluted with hexane, CH_2Cl_2, Me_2CO and MeOH to afford 20 fractions from which **8** (110.0 mg) and **12** (230.0 mg) were isolated. These new fractions were further purified by HPLC [Phenyl-Hexyl (0.7 × 30 cm) and particle size of 10 μm], with elution in the isocratic mode: MeOH:CH_2Cl_2, 1:1 (v/v), flow rate = 3.0 mL min^{-1}. Detection (Shimadzu SCL-10A) was carried out at λ = 254 and 365 nm and compound **11** (52.2 mg) was isolated. The MeOH fraction (C) from the aerial parts was purified on Sephadex LH-20 in isocratic mode (MeOH:CH_2Cl_2, 1:1 (v/v)) and compound **3** (35.2 mg) was isolated. The CH_2Cl_2 fraction from the roots was fractionated on SiO_2, eluted with hexane, CH_2Cl_2, Me_2CO and MeOH. A total of 30 fractions were collected and **1** (13.2 mg) was isolated.

The structures of the compounds were established by comparison of their spectroscopic and physical data with those reported in the literature (Hongwei et al., 2010; Masuda et al., 1998; Ngadjui et al., 1998; Oliveira et al., 1996; Kumar et al., 1995).

*3.2.2. Isolation of **10** and **15** from* H. oreadica

The taproots (3.3 kg) of *H. oreadica* were dried carefully by forced air at 40 °C and the sample was powdered. The sample was extracted to give the crude extracts as follows: hexane (86.0 g), CH_2Cl_2 (68.0 g) and MeOH (244.0 g). The CH_2Cl_2 extract was chromatographed on SiO_2 under vacuum, eluted with hexane (1.0 L), CH_2Cl_2 (1.0 L), EtOAc (3.0 L) and MeOH (1.0 L) to yield 4 fractions. The EtOAc fraction was chromatographed on SiO_2 (230–400 mesh; 4.0 cm × 24.0 cm), eluted with a hexane/MeOH gradient to give 20 new fractions. Fraction 8 was purified on SiO_2 (230–400 mesh; 5.0 cm × 30.0 cm) eluted with hexane/EtOAc with increasing polarity to afford compound **5** (128.0 mg). Fraction 13 was chromatographed on SiO_2 (230–400 mesh; 4.0 cm × 30.0 cm) using the same methodology described above to yield compound **10** (4.0 mg).

Structures of compounds were established by comparison of their spectroscopic and physical data with those reported in the literature (Melliou et al., 2005; Masuda et al., 1998).

The purities of all compounds were evaluated by HPLC prior the bioassay and they all had a purity higher than 98%.

3.3. Coleoptiles bioassay

Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in 15 cm diameter Petri dishes moistened with water and grown in the dark at 22 ± 1 °C for 3 days (Hancock et al., 1964). 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 a green safelight (Nitsch and Nitsch, 1956). Compounds were predissolved in DMSO and diluted to the final bioassay concentration with a maximum of 0.1% DMSO. Parallel controls were also run. The compounds to be assayed for biological activity were added to test tubes. Phosphate-citrate buffer (2 mL) containing 2% sucrose (Nitsch and Nitsch, 1956) at pH 5.6 was added to each test tube. 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 22 °C in the dark. The coleoptiles were measured by digitalization of their images. Data were statistically analyzed using Welch's test (Martín Andrés and Luna del Castillo, 1990). Data are presented as percentage differences from control. Thus, zero represents the control, positive values represent stimulation of the studied parameter, and negative values represent inhibition.

3.4. Phytotoxicity bioassay

The selection of target plants was based on an optimization process developed by us in our search for a standard phytotoxicity bioassay (Macías et al., 2000). Several Standard Target Species (STS) were proposed, including monocots *T. aestivum* L. (wheat) and *A. cepa* L. (onion) and dicots *L. esculentum* Will. (tomato), *L. sativum* L. (cress) and *L. sativa* L. (lettuce), which were assayed for this study. Bioassays were conducted using Petri dishes (50 mm diameter), with one sheet of Whatman No. 1 filter paper as support. Germination and growth were conducted in aqueous solutions at controlled pH using 10^{-2} M 2-[*N*-morpholino]ethanesulfonic acid (MES) and 1 M NaOH (pH 6.0).

Compounds to be assayed were dissolved in DMSO (0.1, 0.02, 0.01 and 0.002 M) and these solutions were diluted with buffer (5 μl DMSO solution/ml buffer) so that test concentrations for each compound (3×10^{-4}, 10^{-4}, 3×10^{-5} and 10^{-5} M) were achieved. This procedure facilitated the solubility of the assayed compounds. The number of seeds in each Petri dish depended on the seed size. 20 seeds were used for tomato, lettuce, cress and onion. Treatment, control or internal reference solution (1 ml) was added to each Petri dish. Four replicates were used for tomato, cress, onion and lettuce (80 seeds). 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 in the dark. Bioassays took 4 days for cress, 5 days for lettuce and tomato and 7 days for onion.

After growth, plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process. The commercial herbicide Logran®, a combination of 2-*tert*-butylamino-4-ethylamino-6-methylthio-1,3,5-triazine (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 according to a comparison study reported previously (Macías et al., 2000).

The herbicide was used at the same concentrations (1 mM, 300, 100, 30, 10 μM) and under the same conditions as those reported. Control samples (buffered aqueous solutions with DMSO and without any test compound) were used for all of the plant species assayed.

Evaluated parameters (germination rate, root length and shoot length) were recorded using a Fitomed© system (Castellano et al., 2001), which allowed automatic data acquisition and statistical analysis using its associated software. 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.

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