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Biochemical Systematics and Ecology

Phytotoxic effect of bioactive compounds isolated from *Myrcia tomentosa* (Myrtaceae) leaves

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ARTICLE INFO

ABSTRACT

The aim of this study was to assess the phytotoxic potential of leaves of *Myrcia tomentosa*, as well as to isolate and identify the main bioactive compounds. The results for the coleoptile and phytotoxicity bioassays indicated the ethyl acetate extract for the phytochemistry study, owing to the high activity and the maintenance of the activity at lower concentrations. This extract was chromatographed and subjected to ^{1}H NMR and ^{13}C NMR. Two major active compounds were isolated from the ethyl acetate extract of leaves of *M. tomentosa*: avicularin and juglanin. The fractions where these compounds were isolated showed potent inhibition of coleoptile growth. This paper is the first report on the presence of the flavonoids avicularin and juglanin in species of Myrtaceae from Neotropical savanna and provides a basis for future studies on the bioprospecting of *M. tomentosa*.

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1. Introduction

Natural products with biological activity are the main sources for new chemical structures and are useful in the development of molecules with potential utilization in pharmacology, agronomy and other areas. Increasing interest is being focused on secondary metabolites with putative roles in chemical communication between organisms (Macías et al., 2008). In the Neotropical savanna (cerrado) plants grow in nutritionally poor soils that are acidic and have high aluminum levels (Haridasan, 2008), so the replacement of predated leaves represents a high cost (Fine et al., 2006) and competition for nutrients is intense. This ecosystem has markedly seasonal climate, suffering drought from April to September and fire episodes from December to March (Klink and Machado, 2005). Studies have shown that bioactive compounds are produced in large amounts by plants submitted to biotic or abiotic stress (Chaves and Escudero, 1999). It follows that the cerrado is a promising ecosystem in the search for bioactive compounds. Currently, there are few studies about the isolation, identification and potentiality of the bioactive compounds from plants of the cerrado.

Myrcia tomentosa Glaz. (Myrtaceae) is a deciduous, heliophytic and pioneer tree. It occurs in semi-deciduous upland forest and savanna in the Brazilian states of Goiás, Minas Gerais, Sao Paulo and Mato Grosso do Sul (Lorenzi, 2002). In the Neotropical savanna (cerrado *sensu stricto*), this species inhibits the growth of other plants around it, indicating the production of allelochemicals that alter the growth patterns of other species (pers. obs). Although others species of this botanic family have showed phytotoxic potential (Souza Filho et al., 2006) so far, no studies have proved the bioactivity or the chemical profile characterization of *Myrcia tomentosa*. Thus, we aimed to assess the phytotoxic potential of leaves of *Myrcia*

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tomentosa, as well as isolate and identify the main bioactive compounds. In this study, *M. tomentosa* compounds were isolated through a bioassay-guided isolation using an etiolated wheat coleoptile and phytotoxicity bioassays. Their structures were characterized by nuclear magnetic resonance of ^{1}H NMR and ^{13}C NMR.

2. Materials and methods

2.1. Plant material

Leaves of *Myrcia tomentosa* Glaz. (Myrtaceae) were collected between July and August (dry season) in Neotropical savanna (cerrado *sensu stricto*) in the campus of Universidade Federal de São Carlos, São Carlos city, São Paulo state, Brazil (21° 58′ to 22° 00′ S by 47° 51′ to 47° 52′ W). A voucher specimen (8318) was deposited at the Herbarium at Universidade Federal de São Carlos (HUFSCar). The leaves were collected, dried in an incubator at 40 °C for 48 h, powdered in an electric grinder and stored in a vacuum pack at room temperature (±25 °C) until use in the Laboratory of allelopathy, at the Universidad de Cádiz, Spain.

2.2. Extraction and isolation

Ground dried leaves of *Myrcia tomentosa* (0.5 kg) were first mixed with hexane (10 l) in a capped glass bottle, which was ultrasonicated for 30 min in order to defat the material. The plant material was filtered off and the process repeated three times. After the third filtration, the extracted plant material was subjected to the same procedure with a series of other organic solvents: dichloromethane (DCM), ethyl acetate (EtOAc), acetone (ACE), methanol (MeOH) and water. The various sets of extracts were pooled and evaporated under reduced pressure (Fig. 1).

These extracts were tested with etiolated wheat coleoptiles and in a phytotoxicity bioassay. The ethyl acetate extract (18 g) was the most active. This was chromatographed on a silica gel (SiO$_2$) column and eluted with hexane/acetone mixtures of increasing polarity. When eluted with hexane/acetone (9:1) the extract yielded fractions F1 (72.1 mg), F2 (790.2 mg), F3 (396.2 mg) and F4 (37.5 mg); elution with hexane/acetone (1:4) yielded fraction F5 (935.9 mg); elution with hexane/acetone (2:3) yielded fraction F6 (768.2 mg); elution with hexane/acetone (3:2) yielded fractions F7 (128.2 mg), F8 (159.7 mg), F9 (284.6 mg) and F10 (390.3 mg) and; that with pure acetone yielded fraction F11 (10,809.9 mg).

The fractions F8 and F9 (Fig. 1) showed the better chromatographic profile and showed bioactivity in the wheat coleoptile bioassay (Fig. 4). These fractions were re-chromatographed on a silica gel column with hexane/acetone mixtures of increasing polarity as eluent, followed by methanol. Fraction F8 eluted with hexane/acetone (3:2) yielded sub-fraction F8 A (9.5 mg); with hexane/acetone (1:1), it yielded sub-fractions F8 B (10 mg) and F8 C (80.9 mg); with hexane/acetone (2:3), it yielded sub-fraction F8 D (19.9 mg) and with pure methanol, sub-fraction F8 E (3.3 mg). Fraction F9 eluted with hexane/acetone (1:1) yielded sub-fractions F9 A (10.9 mg) and F9 B (8.1 mg); when eluted with hexane/acetone (3:2) it yielded sub-fractions F9 C (24.2 mg) and F9 D (95 mg); with hexane/acetone (7:3) it yielded sub-fraction F9 E (79.3 mg) and with pure methanol, sub-fraction F9 F (74.4 mg).
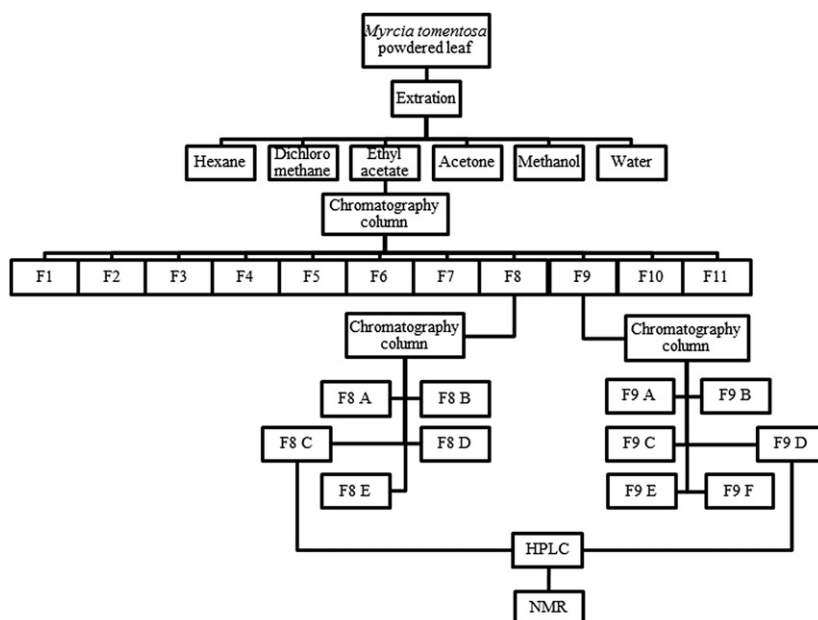

Fig. 1. Fluxogram of the extractions and fractionations carried out with the *Myrcia tomentosa* powdered leaf.
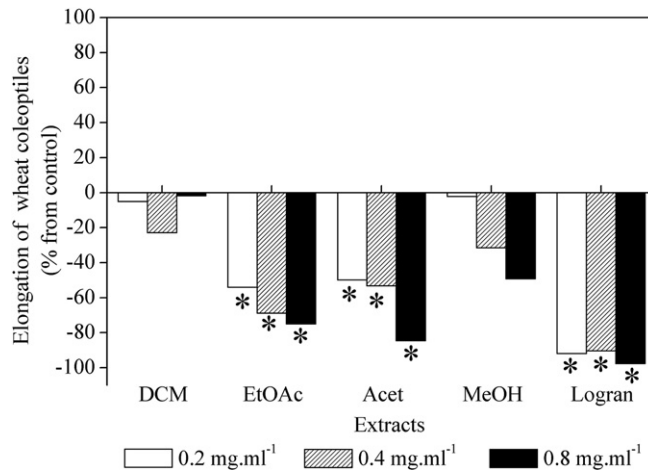
Fig. 2. Effect of *Myrcia tomentosa* leaves extracts on the elongation of etiolated wheat coleoptiles. Values are expressed as percentage difference from the control. Dichloromethane (DCM), ethyl acetate (EtOAc), acetone (Acet) and methanol (MeOH). (*) Significantly different from control, with $p < 0.05$ for the Wilcoxon test.

Sub-fractions F8 C and F9 E were subjected to HPLC with Merck LiChrospher RP-18 columns (10 μm, 250 × 10 mm) and methanol/water (3:2) as eluent, 3 ml min^{-1} flow rate and IR detector to purify these sub-fractions. This process afforded 2 products: F8 CI (13.6 mg) and F9 EI (28.9 mg). F8 CI and F9 EI were analyzed in Varian INOVA 600 NMR spectrometers (^{1}H and ^{13}C NMR) with deuterated methanol (CD$_3$OD), to identify the compounds.
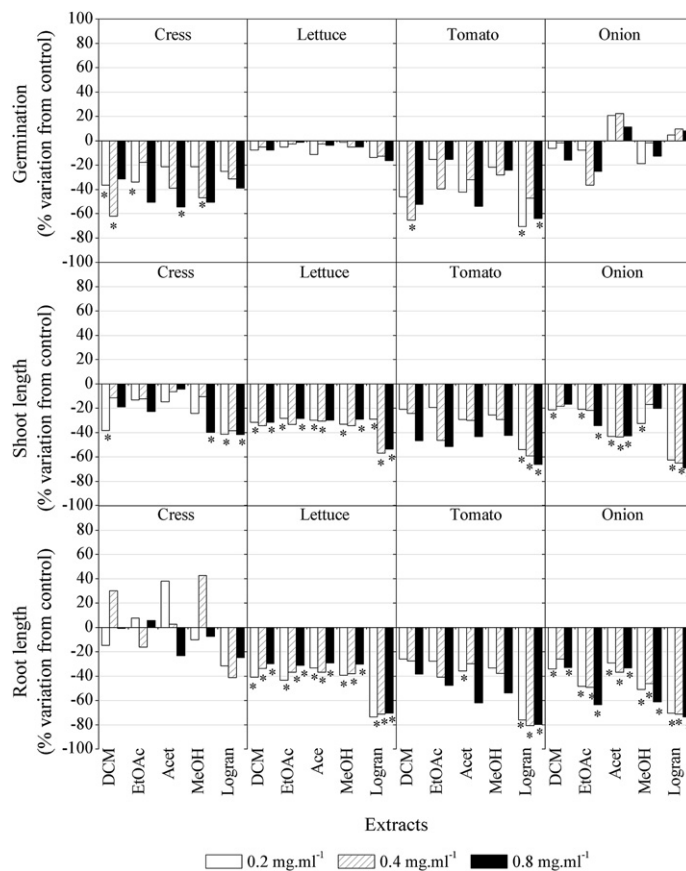

Fig. 3. Effect of *Myrcia tomentosa* leaves extracts on the growth of standard target species. Values are expressed as percentage difference from the control. Dichloromethane (DCM), ethyl acetate (EtOAc), acetone (Acet) and methanol (MeOH). (*) Significantly different from control, with $p < 0.05$ for the Wilcoxon test or the Student's *t*-test.
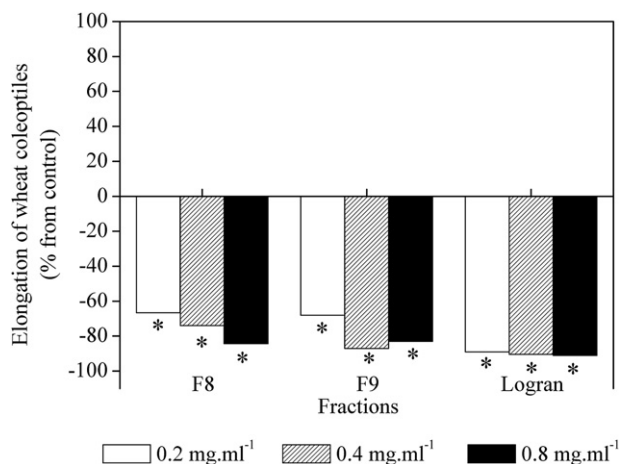
Fig. 4. Effect of the fractions F8 and F9 on the elongation of etiolated wheat coleoptiles. Values are expressed as percentage from the control. (*) Significantly different from control, with $p < 0.05$ for the Wilcoxon test.

2.3. Coleoptile bioassay

Wheat seeds (*Triticum aestivum* L. cv. Pizon) were sown in Petri dishes (15 cm diameter) holding one sheet of filter paper moistened with distilled water and grown in the dark at 22 ± 1 °C for 3 d. Roots and caryopses were removed from the shoots. Shoots were placed in a Van der Weij guillotine, the apical 2 mm were cut off and discarded and the next 4 mm of the coleoptiles were removed and used for the bioassay. All manipulations were performed under a green safelight (Macías et al., 2010).

Solutions used for this assay were prepared from 9.7 mg of each dry extract pre-dissolved in 60 μl of dimethyl sulfoxide (DMSO) and diluted with 12 ml of phosphate-citrate buffer containing 2% sucrose at pH 5.6 giving 0.8 mg ml^{-1}. Dilutions of 0.4 and 0.2 mg ml^{-1} were prepared by adding buffer. Three control samples were used: buffer with DMSO, buffer alone and an internal reference with Logran® herbicide (59% of terbutryn and 0.6% of triasulfuron). The commercial herbicide was used as an internal reference to allow comparison with a study reported previously (Macías et al., 2000). This herbicide was used at the same concentrations (0.2, 0.4 and 0.8 mg ml^{-1}) and under the same conditions as the compounds extracted from *M. tomentosa*.

Three test tubes (repetitions) with five coleoptiles and 2 ml of extracts, fractions, sub-fractions, buffer or Logran were used in the assay ($n = 15$). The tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at 22 °C in the dark. After 24 h the coleoptiles were removed and measured, after digitalization of their images (Macías et al., 2010).

2.4. Phytotoxicity bioassays

For each dry extract 26 mg was dissolved in 160 μl of DMSO and 32 ml of buffer (10 mM 2-(N-morpholino)-ethanesulfonic acid (MES) and 1 mM of NaOH, pH = 5.6) to obtain stock extract at 0.8 mg ml^{-1}. Dilutions of 0.4 and 0.2 mg ml^{-1} were prepared by adding buffer. Three control samples were used: buffer with DMSO, buffer alone and an internal reference with Logran® herbicide (as described above). This herbicide was used at the same concentrations (0.2, 0.4 and 0.8 mg ml^{-1}) and under the same conditions as the compounds extracted from *M. tomentosa*.

The selection of target plants was based on an optimization process described by Macías et al. (2000). Several standard target species (STS) were assayed, including the dicotyledons *Lepidium sativum* L. (cress), *Lactuca sativa* L. (lettuce), and *Lycopersicon esculentum* Will. (tomato) and monocotyledon *Allium cepa* L. (onion).

Bioassays were carried out in Petri dishes (5 cm diameter) holding one sheet of filter paper moistened with 1 ml of the extract or control. Twenty seeds were placed in each Petri dish, four replicates being used for each target species ($n = 80$). Petri dishes were sealed with Parafilm®, to ensure a closed system, and were kept in a germination chamber at 25 °C in the dark. Bioassays took 4 d for cress, 5 d for lettuce and tomato and 7 d for onion. After growing, plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process (Macías et al., 2010).

2.5. Mathematical and statistical data analysis

Coleoptile lengths, germination rates, root and shoot lengths were calculated as percentage difference from control. Zero represents control, positive values represent stimulation and negative values inhibition.

The design of the laboratory experiments was completely randomized. The data was subjected to the Shapiro–Wilk normality test. The statistical significance of the differences between treatments and control were tested by the Student *t*-test,

for normal data, or by the Wilcoxon test, for non-normal data, both at the 5% level. All analyzes were carried out in R (R Development Core Team, 2009).

3. Results and discussion

3.1. Coleoptile bioassay

Ethyl acetate and acetone extracts significantly inhibited coleoptile elongation at all concentrations tested in a dose-dependent manner (Fig. 2). The hexane extract was obtained for removing less polar compounds and was not further analyzed. The dichloromethane extract did not show a conventional dose-dependent effect, as the greatest inhibition was observed at 0.4 mg ml^{-1} and not 0.8 mg ml^{-1}, may be because of the poor solubility of the products in this extract (Fig. 2). Among the most active extracts, only the ethyl acetate extract maintained its activity at lower concentrations (Fig. 2).

3.2. Phytotoxicity bioassays

Germination rate of cress seeds was significantly inhibited by dichloromethane (0.2 and 0.4 mg ml^{-1}), ethyl acetate (0.2 mg ml^{-1}), acetone (0.8 mg ml^{-1}) and methanol (0.4 mg ml^{-1}) extracts, whereas lettuce seeds, subjected either to the extract or to the herbicide did not differ significantly from the control and the germination rate of tomato seeds was suppressed by dichloromethane (0.4 mg ml^{-1}) extract and herbicide (0.2 and 0.8 mg ml^{-1}) alone. Acetone extract and herbicide stimulated the germination rate of onion and the other extracts had no effect on that target plant (Fig. 3). Comparing the germination rate of the target species, lettuce and onion seeds were less sensitive to the extracts.

Inhibition of seed germination by chemical compounds can play an important role in the regulation of plant succession (Fenner, 2000). The varied responses of the different target species ensure the selectivity of the species during establishment under natural conditions, influencing the population dynamics. In addition, the co-evolution of species may be related to secondary metabolites via the ability of some plants to detoxify or metabolize them (Hofmann et al., 2006; Schulz and Wieland, 1999).

The shoot length of cress seedlings was significantly reduced by dichloromethane (0.2 mg ml^{-1}) and methanol extracts (0.8 mg ml^{-1}) and by the herbicide (0.2 and 0.8 mg ml^{-1}); lettuce seedling shoots were significantly shortened by all extracts at 0.2 and 0.8 mg ml^{-1} and by herbicide at all concentrations; tomato seedling shoots were significantly reduced by herbicide at all concentrations and onion seedlings were inhibited by all extracts, at least at one of the concentrations and by herbicide at all concentrations (Fig. 3). All extracts stimulated cress root growth at one concentration while the herbicide inhibited cress root growth at all concentrations. The shoot growth of lettuce and onion seedlings was inhibited by all extracts at the two tested concentrations and by herbicide at all concentrations. The tomato seedling roots had their growth inhibited only by acetone extract at 0.2 mg ml^{-1} and by herbicide (Fig. 3). In view of these results, the extract obtained with ethyl acetate was the one showing a more consistent activity. It was the most active on the root of lettuce, tomato and onion and shoot of lettuce and tomato.

The results of the phytotoxicity bioassays show that the target species respond differently to the extracts (Fig. 3), confirming that the susceptibility of the target species to phytotoxic substances under laboratory conditions depends on the physiological and biochemical characteristics of each species (Kobayashi, 2004). The influence of the extracts on germination depends on the size and permeability of the seed coat (Hanley and Whiting, 2005). Such species-dependent responses to allelochemicals can influence the plant species composition of natural ecosystems and can be used to design selective herbicides in agro ecosystems.

3.3. Chemical fractionation and purification

The results described above for the coleoptiles and phytotoxicity bioassay indicated the ethyl acetate extract for detailed phytochemical analysis, owing to the high activity and the maintenance of this activity at lower concentrations. Another characteristic favoring the choice of this extract was its chromatographic simplicity (observed by TLC analysis). The ethyl acetate extract was chromatographed on SiO$_2$, resulting in 11 fractions. The fractions F8 and F9 exhibited a major component each and had sufficient material for further study (more than 100 mg) and thus were tested by the coleoptile bioassay. The results made clear that fractions F8 and F9 significantly inhibited coleoptile growth (Fig. 4) (−84.3% at 0.8 mg ml^{-1}; −73.9% at 0.4 mg ml^{-1} and −66.6% at 0.2 mg ml^{-1} for F8 and −83.2% at 0.8 mg ml^{-1}, −86.8% at 0.4 mg ml^{-1} and −68.9% at 0.2 mg ml^{-1} for F9). Both fractions were re-chromatographed on the silica gel column. F8 yielded five sub-fractions and F9 six. Subfractions F8 C and F9 E were purified by HPLC, resulting in 2 products. The products F8 CI and F9 EI were pure substances and were subjected to ^{1}H NMR and ^{13}C NMR. The NMR data for F8 CI and F9 EI coincide with those described in the literature for the compounds juglanin (Fiorentino et al., 2009) and avicularin (Kim et al., 1994), respectively (Fig. 5). The aglycone of juglanin and avicularin are the flavonoids kaempferol and quercetin, respectively, and both flavonoids contain the same sugar, *O*-arabinofuranoside, coupled to the flavonol nucleus.

Flavonoids constitute the largest class of plant phenolics. The carbon skeleton of flavonoids contains 15 carbons arranged in two aromatic rings linked by a chain of three carbons. *Myrtacea* is characterized by flavonoids, flavones being rare. Some studies have reported the presence of flavonoids in species of the genus *Myrcia*: C-methylated flavonoids might be found in
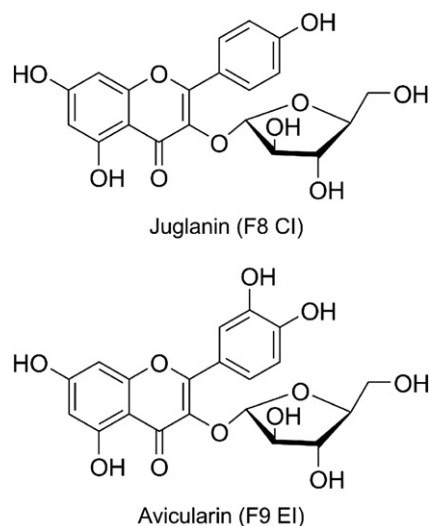
Fig. 5. Flavonoid glycosides isolated from *Myrcia tomentosa* leaves.

leaves of *Myrcia citrifolia* (Gottlieb et al., 1972); myrciacitrin I, II, III, IV and V (Matsuda et al., 2002), quercitrin, guaijaverin and desmanthin-1 have been isolated from leaves of *Myrcia multiflora* (Jung et al., 2006) and mearnsitrin and myricitrin were found in *Myrcia uniflora* (Ferreira et al., 2006). The isolation of glycosylated flavonols from *M. tomentosa* leavescould establish the first approach to the chemical relationship between this species and others belonging to the *Myrtacea* family.

Recent studies have reaffirmed the link between flavonoids and plant architecture by showing that flavonoid-defective mutants display a wide range of alterations to root and shoot development (Buer and Djordjevic, 2009). The affected traits include alterations to root growth, lateral root density, root hair development and length, shoot/flower organ number, overall architecture and stature, and seed organ density (Buer et al., 2010). The mechanisms by which the alterations at the level of flavonoids promote these physiological changes are not well-defined. According to recent research (Buer et al., 2010), three likely mechanistic possibilities are: direct effects of the flavonoids on unidentified molecular targets, indirect effects mediated by the ability of flavonoids to modulate the levels of auxin, or through ROS regulation. Flavonoids influence auxin transport (Peer and Murphy, 2007), the plant defense (Treutter, 2005), modulate the levels of reactive oxygen species (ROS) and promote the allelopathic effect (Bais et al., 2006), such as kaempferol, quercetin and naringenin (Macías et al., 2007).

According to the results of the coleoptile bioassay (Fig. 4), fraction F8 and F9 (Fig. 5) had similar inhibitory activity. Both flavonoids contain the same sugar, *O*-arabinofuranoside, coupled to the aromatic nucleus kaempferol in juglanin and quercetin in avicularin. What differentiates these nuclei is the presence of a hydroxyl attached to carbon number 3′ in the avicularin molecule (Fig. 5). The biological activity of flavonoids is related to chemical structure, particularly of the hydroxyl groups (Cunha et al., 2007).

The present study reported the phytotoxic activity of the fractions from which juglanin and avicularin were isolated, corroborating other studies that reporting this activity (Berrehal et al., 2010; Rodrigues et al., 2010; Fiorentino et al., 2009; Esposito et al., 2008; Melos et al., 2007). Therefore, the species *Myrcia tomentosa* should be of interest in studies on the development and production of natural herbicides. Furthermore, the presence of these flavonoids might clarify, in part, the high frequency and distribution in cluster of these species in the community. This is the first report of the presence of juglanin and avicularin in a species of Myrtaceae from the Neotropical savanna and provides a basis for future studies on bioprospecting *Myrcia tomentosa*.

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