Effect of Accessions and Environment Conditions on Coumarin, *O*-Coumaric and Kaurenoic Acids Levels of *Mikania laevigata*

Authors

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Abstract
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Coumarin, *o*-coumaric, and kaurenoic acid are bioactive compounds usually found in the leaves of *Mikania laevigata*. Genetic and environmental variations in the secondary metabolites of plants may have implications for their biological effects. Three different accessions of *M. laevigata* cultivated in four sites between the Equator and the Tropic of Capricorn in Brazil were evaluated aiming to present potential raw materials and discuss relationships among these three bioactive compounds. The results revealed effects of plant accessions and environmental factors and suggested two contrasting chemical phenotypes of *M. laevigata*. The first phenotype presented the highest levels of kaurenoic acid (2283 ± 316 mg/100 g) besides lower levels of coumarin (716 ± 61 mg/100 g), which was also stimulated by the environment and mild climate at the site nearest to the Tropic of Capricorn. The other phenotype presented the lowest levels of kaurenoic acid (137 ± 17 mg/100 g) besides higher levels of coumarin (1362 ± 108 mg/100 g), which was also stimulated by the environment and tropical climate at the site nearest to the Equatorial beach.

Supporting information available online at http://www.thieme-connect.de/products

Introduction
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The *Mikania* genus (Asteraceae) occurs mainly in South America, with extensions in Asia, North America, and Southern Africa [1]. *Mikania laevigata* Sch. Bip. ex Baker is a native and perennial liana and an endemic Brazilian species, commonly named as guaco, and is used in traditional medicine [2]. The chemical composition of the genus comprises derivatives of cinnamic acid and kaurene-type diterpenes [3]. The major bioactive compounds in the leaves of *M. laevigata* have been identified as derivatives of cinnamic acids [4,5] like coumarin and *o*-coumaric acid, along with kaurene-type diterpenes [5] (i.e., kaurenoic acid, benzoylgrandifloric acid, and cinnamoylgrandifloric acid).

Coumarin is the chemical marker described [6] and its presence in the leaves of *M. laevigata* produces the characteristic aromatic smell resembling vanilla [7]. Coumarin is a natural flavoring found in many foods [8,9], although its Tolerable Daily Intake has been limited (0.1 mg/kg of body weight) for consumption in foods [10]. The conversion of cinnamic acid to coumarin occurs by way of *o*-coumaric acid [11]. Both coumarin and *o*-coumaric acid showed important anti-inflammatory activity in allergic inflammation, and the whole extract of *M. laevigata* blocked the harmful effect of these isolated markers [4]. Coumarin also showed bronchodilator properties [12,13]. Another bioactive compound, kaurenoic acid, has been reported for its vasorelaxant and relaxing smooth muscle [14,15], anticonvulsant [16], analgesic [17], and antimicrobial [18,19] properties. Kaurenoic acid is biosynthesized from geranylgeranyldiphosphate (GGDP), a common C_{20} precursor for diterpenoids, and this bioactive compound may also be subsequently converted to other metabolites [5] or to bioactive gibberellins (GAs) in flowering plants [20].

In Brazil, the species *M. laevigata* is widely used due to antiallergic, antispasmodic, anti-inflammatory, antiulcer, antimicrobial, bronchodilator and relaxing smooth muscle properties [1,14,16,17,21–23]. The Brazilian Pharmacopoeia [24] recommends the use of *M. laevigata* leaves due to their balsamic effects to alleviate coughing and as an expectorant. The influence of genetic [25] and environmental [5,26] variations on the secondary
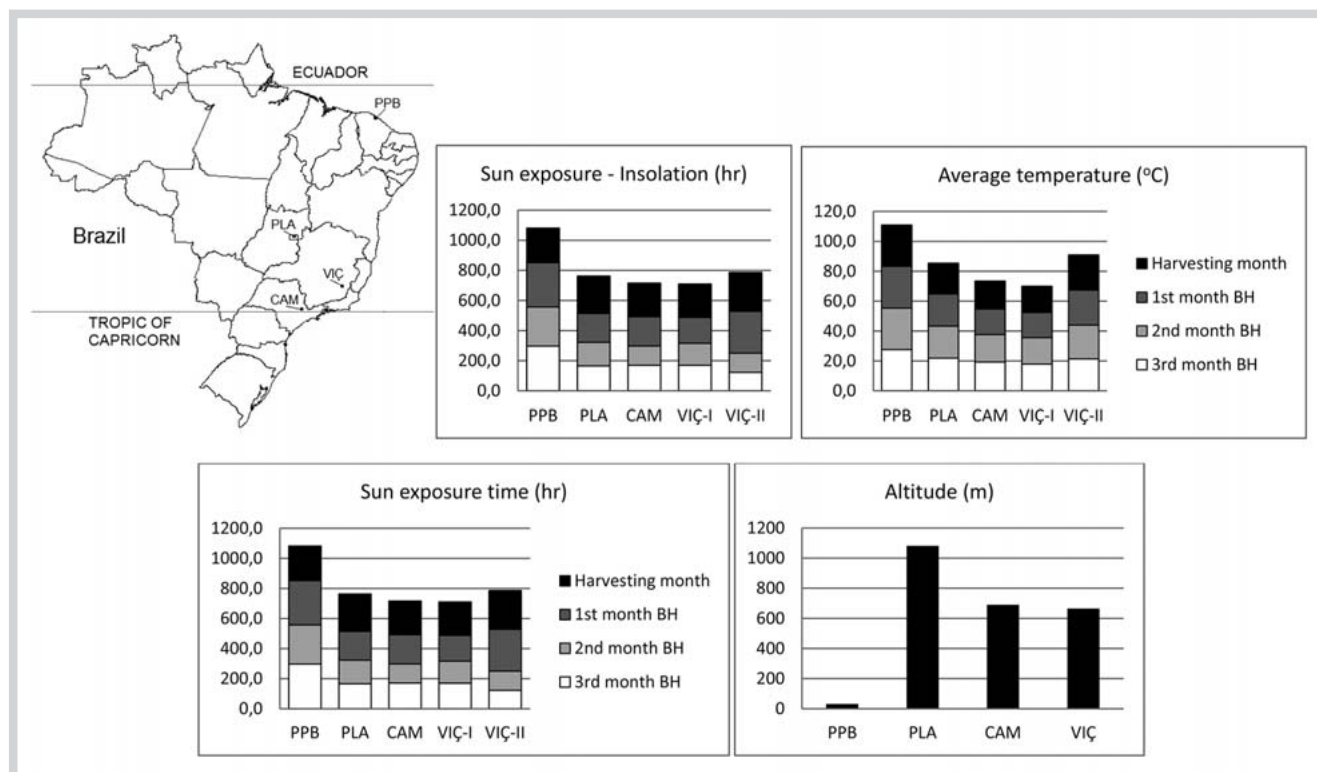
Fig. 1 Geographical sites (PPB: Paraipaba; PLA: Planaltina; CAM: Campinas; VIÇ-I: Viçosa winter; VIÇ-II: Viçosa summer) of cultivation of *M. laevigata* in Brazil and climatic conditions. Sun exposure or insolation, average tempera-ture, and total rainfall were evaluated at harvesting month, 1st month, 2nd month, and 3rd month before harvest (BH) [31].

metabolites of plants may have implications for their biological effects. Seasonal variation in the bioactive compounds of *M. laevigata* and *Mikania glomerata* Spreng. leaves [5] showed the highest coumarin in *M. laevigata* cultivated in the summer and under 80% shading, while kaurane-type diterpenes were higher in the winter, but favored by full sunlight. However, no reference was found about different chemotypes of *M. laevigata* or about the influence of geographic sites.

The increase in the use of herbal therapies raises concern about the quality of such products, regarding authenticity, purity, and the chemical composition of plant raw materials [27]. This study first discriminates three previously selected accessions of *M. laevigata* grown in four sites between the Equator and the Tropic of Capricorn in Brazil, aiming to present raw materials with the potential to produce safe and effective products. Then this study aims to discuss relationships among the accumulation of three bioactive compounds due to both plant accessions and environmental conditions, suggesting two chemical phenotypes of *M. laevigata*.

Results and Discussion
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Although *M. laevigata* is a wild species from Brazilian flora, it has been collected from the wild and under cultivation in backyards for a long period of time. Nowadays, most of the plant material used in the market or by local growers is cultivated. Plant accessions "Unaerp" and "Cpqba" are mostly used in the Southeast and South of Brazil, while "Cenargen" is usually used in the Mid-West. These accessions were selected from three Brazilian Germplasm

Collections based on their uses in Local Phytotherapy Programs, and the experiment was conducted cultivating them in four sites (○ **Fig. 1**). Leaf yield was higher for the accessions harvested in Paraipaba (○ **Fig. 2A**), mainly for the Unaerp accession; the leaf/stem ratio was higher for Paraipaba (○ **Fig. 2B**) and also for the Unaerp accession harvested in Campinas. Paraipaba is the most tropical site and this influence is discussed below.

Three bioactive compounds were clearly separated and quantified using HPLC-PDA (○ **Fig. 3**). Their contents ranged from 716 ± 61 to 1384 ± 105 mg/100 g for coumarin, from 136 ± 18 to 411 ± 87 mg/100 g for *o*-coumaric acid, and from 137 ± 17 to 2283 ± 316 mg/100 g for kaurenoic acid, depending on the accession and the place of cultivation (○ **Fig. 4**). Other reports found only traces of *o*-coumaric acid [3] and lower coumarin levels (ranging from not detected to 520 mg/100 g) [3, 6, 27] than the values found in this study. The difference could be due to the extractor solvent (ethanol) used by those authors, which, when evaluated in our study, extracted only 60% of the coumarin (peak area 1333 ± 55 mAU/s) that had been extracted by 70% ethanol (2185 ± 21 mAU/s). In contrast, the extraction of kaurenoic acid was similar for ethanol (961 ± 40 mAU/s) and 70% ethanol (1034 ± 73 mAU/s). Rocha et al. [28] also found a higher extraction of coumarin using 70% ethanol (0.47 mg/mL) than 94% ethanol (0.05 mg/mL) in leaves of *M. glomerata*. Although Bertolucci et al. [3] achieved a very good recovery of bioactive compounds using ethanol, the recovery expressed only the free compounds in the extracts (not those compounds stuck to the fibers and plant structure, whose hydrogen bonds could be hydrolyzed by water). However, additional differences observed could also be due to the accessions (different plant materials).
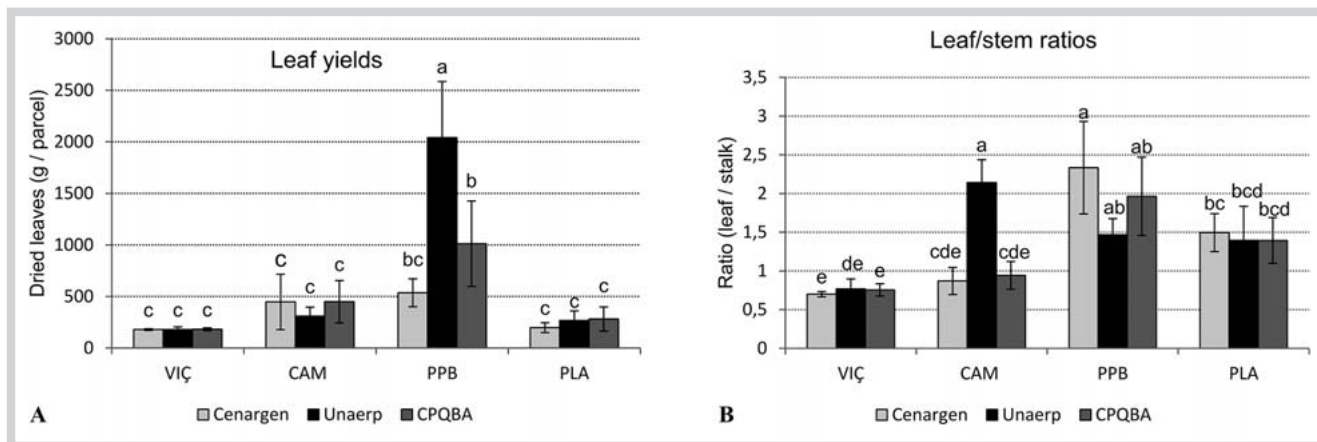
Fig. 2 Leaf yields (dry matter) (**A**) and leaf/stem ratios (**B**) ± standard deviation of three *Mikania* accessions (Cenargen, Unaerp, and Cpqba) harvested in Viçosa (VIÇ), Campinas (CAM), Paraipaba (PPB), and Planaltina (PLA), Brazil. Each bar is the average of seven repetitions; means with the same letters are not significantly different at the level of 0.05 of probability according to orthogonal contrasts.
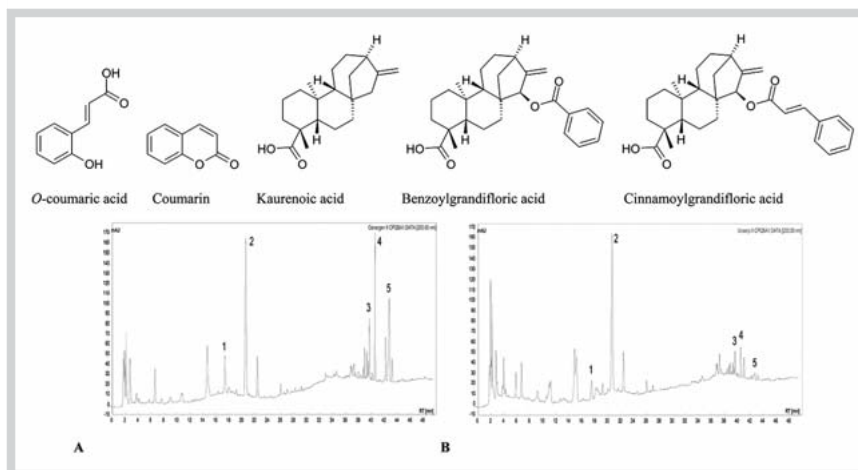
Fig. 3 Structures and chromatograms of bioactive compounds determined in the leaves of the Cenargen (**A**) and Unaerp (**B**) accessions of *M. laevigata* harvested in Campinas, SP, Brazil.

Fig. 4 Bioactive compounds ± standard deviation in the leaves (dry matter) of three *Mikania* accessions (Cenargen, Unaerp, and Cpqba) harvested in Viçosa (VIÇ), Campinas (CAM), Paraipaba (PPB), and Planaltina (PLA), Brazil. Each bar is the average of seven repetitions; means with the same letters are not significantly different (orthogonal contrasts applied in the ANOVA of ○ **Table 1**).
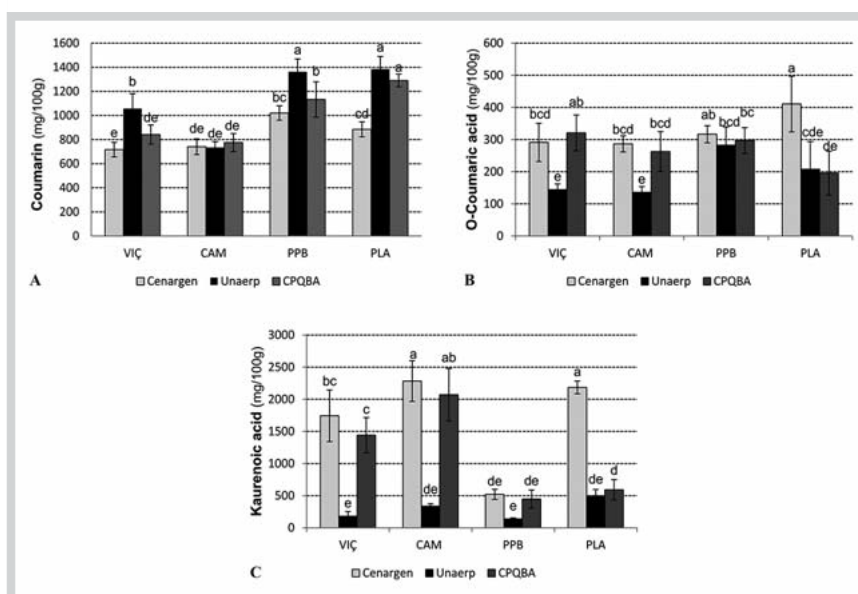
Table 1 Two-way ANOVA using site and *Mikania* accession factors.

	Coumarin				Kaurenoic acid				o-Coumaric			
	Sum Sq	Df	F value	Pr (> F)	Sum Sq	Df	F value	Pr (> F)	Sum Sq	Df	F value	Pr (> F)
Site	2 984 485	3	65,876	< 2.2E-16	15 386 994	3	105,672	< 2.2e-16	55 963	3	6,055	9,80E-04
Accession	1 179 600	2	39,055	2,15E-12	27 734 274	2	285,703	< 2.2E-16	251 846	2	40,8735	1,38E-12
Site : accession	–	–	–	–	11 043 836	6	37,922	< 2.2e-16	173 532	6	9,3878	1,34E-07
Residuals	1 147 722				3 494 659	72			221 817	72		
	AIC = 485.52				AIC = 434.13				AIC = 586.72			

When performing the comparisons of means through the contrasts (**Table 1**), the Cenargen accession showed the lowest coumarin levels (**Fig. 4A** and **Table 2**), while the Cpqba and Unaerp accessions did not differ among themselves. Considering the geographical regions, leaves from Campinas presented significantly lower coumarin levels than leaves from Planaltina, Paraipaba, and Viçosa (**Table 3**). Campinas is in the Southeast of Brazil nearest to the Tropic of Capricorn, showing characteristics of a milder climate (**Fig. 1**). Otherwise, the samples from Paraipaba and Planaltina presented the highest coumarin (**Fig. 1 S**, Supporting Information) and their levels did not differ in themselves. Paraipaba and Planaltina are more tropical sites, and Paraipaba was distinguished from the other sites of cultivation by its longer sun exposure time and the highest daily average temperature. Planaltina presented the highest altitude and highest total rainfall. Another phenylpropanoid [26], chlorogenic acid, also presented higher levels in tobacco leaves from the site that had the highest altitude and the longest sun exposure time.

Light and temperature are known for their importance to plant metabolism and development, and they certainly influenced these results. However, comparing both seasonal harvests (**Table 4** and **Fig. 2 S**, Supporting Information), coumarin contents of Viçosa II (sampling in the summer) and Viçosa I (sampling in the late winter) did not differ. Although Bertolucci et al. [5] suggest that rainfall could be important to coumarin accumulation, coumarin profiles of the three accessions were also similar in both seasons, even with the highest rainfall in the summer (**Fig. 1**). Unlike what was observed in this study, Bertolucci et al. [5] found an increase in coumarin cultivated in the summer (Lavras, MG, Brazil) with similar environmental conditions to those of Viçosa (MG, Brazil), but showing lower insolation in the summer. So the stability of coumarin levels found in both seasons of the Viçosa study could be due to the greater insolation found in the Viçosa summer (**Fig. 1**).

Unlike coumarin, the levels of *o*-coumaric acid were higher in the Cenargen than in the Unaerp accession (**Fig. 4B** and **Table 2**). These results suggest that even though *o*-coumaric acid is biosynthesized, it is not always converted or not yet converted into coumarin at the same rate as it is accumulated, mainly in the Cenargen accession. However, for *o*-coumaric acid, an interaction was also observed between accession and site (**Table 1**). The tropical climate of Paraipaba at sea level probably induced the increase in the *o*-coumaric accumulation in the Unaerp accession (**Fig. 4B**), even as the high altitude of Planaltina (Central Plateau) probably inhibited the *o*-coumaric acid accumulation in the Cpqba accession. Planaltina would have favored the biosynthesis and accumulation of *o*-coumaric acid in the Cenargen accession (**Fig. 4B**) as well as stimulating the oxidative biosynthesis of coumarin from *o*-coumaric acid in the Unaerp and Cpqba accessions (**Fig. 4A**).

Table 2 Global mean comparison among three *Mikania* accessions according to orthogonal contrasts.

Bioactive compound	Accession comparison	Orthogonal contrast
Coumarin	Cenargen X Cpqba	t = 3.39; p value = 0.001
	Cenargen X Unaerp	t = 4.79; p value < 0.001
	Cpqba X Unaerp	t = 1.67; p value = 0.101
o-Coumaric acid	Cenargen X Cpqba	t = 2.94; p value = 0.005
	Cenargen X Unaerp	t = 6.61; p value < 0.001
	Cpqba X Unaerp	t = 3.80; p value < 0.001
Kaurenoic acid	Cenargen X Cpqba	t = 2.77; p value = 0.008
	Cenargen X Unaerp	t = 9.58; p value < 0.001
	Cpqba X Unaerp	t = 6.15; p value < 0.001

Table 3 Global mean comparison among four sites of cultivation according to orthogonal contrasts.

Bioactive compound	Site comparison	Orthogonal contrast
Coumarin	Campinas X Paraipaba	t = 10.15; p value < 0.001
	Campinas X Planaltina	t = 8.28; p value < 0.001
	Campinas X Viçosa I	t = 2.80; p value = 0.010
	Paraipaba X Planaltina	t = 0.23; p value = 0.818
	Paraipaba X Viçosa I	t = 5.60; p value < 0.001
	Planaltina X Viçosa I	t = 5.05; p value < 0.001
o-Coumaric acid	Campinas X Paraipaba	t = 3.62; p value = 0.001
	Campinas X Planaltina	t = 1.32; p value = 0.195
	Campinas X Viçosa I	t = 0.91; p value = 0.370
	Paraipaba X Planaltina	t = 0.94; p value = 0.358
	Paraipaba X Viçosa I	t = 2.11; p value = 0.044
	Planaltina X Viçosa I	t = 0.56; p value = 0.577
Kaurenoic acid	Campinas X Paraipaba	t = 5.72; p value < 0.001
	Campinas X Planaltina	t = 1.76; p value = 0.087
	Campinas X Viçosa I	t = 1.69; p value = 0.100
	Paraipaba X Planaltina	t = 4.00; p value < 0.001
	Paraipaba X Viçosa I	t = 4.49; p value < 0.001
	Planaltina X Viçosa I	t = 0.13; p value = 0.895

Comparing both seasonal harvests (**Table 4** and **Fig. S2**, Supporting Information), *o*-coumaric acid levels found in the leaves from Viçosa II (sampling in the summer) were significantly higher than the leaves from Viçosa I (sampling in the late winter) for all evaluated accessions. In contrast, Bertolucci et al. [5] did not find quantifiable levels of *o*-coumaric acid in the leaves of *M. laevigata*. However, considering that *o*-coumaric acid is the precursor of coumarin [11], this result supports the possibility of coumarin increasing in summer, as reported by Bertolucci et al. [5], but not observed for Viçosa in this work, probably due to the higher insolation.

Table 4 Bioactive compounds of the three *Mikania* accessions of *M. laevigata* harvested from Viçosa in the late winter (Viçosa I) and in the summer (Viçosa II).

Accession	Winter	Summer
Coumarin (mg/100 g)		
Cenargen	716.2 ± 60.5 Aa	757.9 ± 79.1 Aa
Cpqba	842.0 ± 79.0 Ba	805.5 ± 69.0 Ba
Unaerp	1056.9 ± 123.4 Ca	1007.1 ± 137.2 Ca
***O*-coumaric (mg/100 g)**		
Cenargen	291.1 ± 59.4 Ab	512.8 ± 30.5 Aa
Cpqba	321.0 ± 55.6 Ab	474.5 ± 20.7 Aa
Unaerp	144.3 ± 17.8 Bb	359.0 ± 27.5 Ba
Kaurenoic acid (mg/100 g)		
Cenargen	1743.5 ± 401.4 Aa	1720.3 ± 110.5 Aa
Cpqba	1441.7 ± 274.3 Aa	1647.0 ± 178.0 Aa
Unaerp	180.8 ± 73.5 Bb	527.4 ± 238.8 Ba

Each result is mean ± standard deviation (dry matter) of seven repetitions; same small letters indicate that bioactive compounds did not differ between seasons and same capital letters indicate that the bioactive compounds did not differ among *Mikania* accessions (p < 0.05)

For kaurenoic acid, the Unaerp accession from all four cultivation sites and all three accessions cultivated in Paraipaba presented the lowest levels (○ **Fig. 4 C**). In contrast, the other two evaluated accessions from both Campinas and Viçosa presented the highest levels of kaurenoic acid, together with the Cenargen accession from Planaltina. Paraipaba presented a tropical (low latitude and high insolation, which disperses less if compared with higher latitudes) and coastal influence (low altitude, high temperature, and a smaller temperature range than inland areas besides soil salinity) in the metabolism of this diterpene. Another metabolite derived from kaurenoic and cinnamic acids (cinnamoylgrandifloric acid) in the leaves of *M. laevigata* also followed kaurenoic acid levels. Cinnamoylgrandifloric acid was reduced in samples from the Unaerp accession (○ **Fig. 2**) and from Paraipaba. The content of another diterpene (duvatriene 1,3-diol) found in tobacco [26] was also decreased in a region of China with a lower altitude and higher average temperature. Kaurenoic acid is also used by plants for GA biosynthesis [29]. Usually, higher GAs are produced when the plant is exposed to cold temperatures, and Bertolucci et al. [5] also found higher levels of kaurenoic acid in the winter, when temperatures dropped lower than in the summer. Indeed, accessions harvested in Paraipaba presented higher leaf/stem ratios (○ **Fig. 2 B**), suggesting that plants lack GAs in Paraipaba. Also, the Unaerp accession harvested in Campinas showed higher leaf/stem ratios, suggesting lower levels of GAs in this accession. Differences depending on the seasonal harvests (**Fig. S2**, Supporting Information) showed that profiles of accessions cultivated in Viçosa were similar in both seasons, but that kaurenoic acid was higher only for the Unaerp accession in the summer (○ **Table 4**). Viçosa in the summer presented higher temperatures and higher insolation than in the late winter (○ **Fig. 1**). Although Bertolucci's seasonal study [5] reported variations in temperatures similar to Viçosa, that study noted higher kaurenoic acid in the winter, probably due higher insolation. Planaltina presented a higher altitude and latitude, but its weather was somewhat similar to that of Viçosa II (summer) and it also showed levels of kaurenoic acid (493 ± 103 mg/100 g) for the Unaerp accession similar to those found in Viçosa II (527 ± 239 mg/100 g). However, higher levels for the Cenargen and lower levels for the Cpqba accession were observed in Planaltina, showing the interaction between the accession site for kaurenoic acid accumulation.

The relationships among these bioactive compounds also imply the coumarin biosynthesis from *o*-coumaric acid [11]. The ratio of coumarin and *o*-coumaric acid (CO/OC ratio) ranged from 2.3 to 3.5 for Cenargen and Cpqba accessions grown in Viçosa I and Campinas (both sites presented similar sun exposure, temperature, and altitude). For the Unaerp accession, this ratio ranged from 5.1 to 7.8, suggesting a faster and more efficient process of coumarin biosynthesis in this accession. For Paraipaba, the three *Mikania* accessions presented nearer CO/OC ratios ranging from 3.0 to 5.9, suggesting that, in this coastal region in the summer, coumarin biosynthesis seems to have been induced in the Cenargen and Cpqba accessions. For Viçosa II (summer), where higher levels of *o*-coumaric acid were accumulated in all three accessions, these CO/OC ratios were lower, ranging between 1.2 and 1.9 for Cenargen and Cpqba and between 2.2 and 3.4 for the Unaerp accession. In Planaltina (where the weather was somewhat similar to Viçosa II), the Cenargen accession also maintained the lowest CO/OC ratio (1.8 to 2.3), sustained by higher *o*-coumaric accumulation. However, Unaerp and Cpqba accessions, which accumulated lower *o*-coumaric levels (○ **Fig. 4 B**), provided the most elevated CO/OC ratios, ranging from 5.5 to 11.7. These results suggest that higher insolation could mainly increase *o*-coumaric acid levels, while higher temperatures and altitudes could increase coumarin accumulation. Furthermore, coumarin accumulation in the Cenargen accession seems to happen more slowly. However, the extent to which genetic or environmental factors alone or in combination contribute to this biosynthetic pathway is not clear. Besides, not all factors that could influence these results were controlled (such as soil) and, therefore, further influences cannot be excluded.

O-coumaric acid presented a positive correlation with kaurenoic acid, especially in samples from Planaltina (Pearson correlation coefficient R = 0.93), Viçosa (R = 0.90 and 0.82), and Campinas (R = 0.80), while a negative correlation was observed between kaurenoic acid and coumarin.

So these results suggest two contrasting phenotypes of *M. laevigata* with different profiles of coumarin and kaurenoic acid. The high coumarin accumulation found in the Unaerp accession could be associated with its ability to respond to the environmental conditions, stimulated by tropical climate, as occurred in Paraipaba. The high kaurenoic acid found in the Cenargen accession could be associated with the GAs and other metabolites, such as cinnamoylgrandifloric acid, which are especially stimulated by a mild climate such as was found at Campinas and Viçosa. Paraipaba, the most tropical site near the sea with the highest sun exposure and temperatures, produced the lowest levels of kaurenoic acid (diterpene), while Campinas, which is positioned closer to the Tropic of Capricorn, accumulated the lowest coumarin (phenypropanoid) levels. The chemical profile of *M. laevigata* accessions suggests that the Cenargen accession could be a promising material for herbal therapies due to its potential for giving a lower variability of coumarin and the highest accumulation of kaurenoic acid, especially in a "mild" climate.

Material and Methods
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General
Coumarin (> 99%; Sigma), *o*-coumaric acid (2-Hydroycinnamic acid 97%; Sigma-Aldrich), and kaurenoic acid (97% by HPLC, provided by CPQBA) were used as reference materials. Stock solutions of coumarin, *o*-coumaric acid (20 mg/100 mL), and kaurenoic acid (50 mg/100 mL) in 70% ethanol were diluted to a series of working solutions (from 0.1 to 200 µg/mL). HPLC analysis was carried out in a ProStar Varian system equipped with a ternary pump, autosampler, PDA detector, and Galaxie PS-335/Software 1.9. The column used was C18 Zorbax XDB (250 × 4.6 mm, 5 mm). Acetonitrile (grade HPLC) and phosphoric acid (86%) were used for HPLC elution.

Plant materials
The *Mikania* accessions were originally obtained from the three major germplasm collections in Brazil, i.e, Embrapa Genetic Resources and Biotechnology (Cenargen), the University of Campinas (CPQBA), and the University of Ribeirão Preto (Unaerp). Voucher specimens of the three accessions were deposited at the Embrapa Genetic Resources and Biotechnology Herbarium (CEN 84 536, CEN 84 537, CEN 84 538) and identified by an expert taxonomist in the *Mikania* genus (Dr. Mara Ritter, Federal University of Rio Grande do Sul). Cuttings of three plant accessions were sent, rooted, and planted at four sites in Brazil (Paraipaba, CE, latitude 03.26°S, longitude 39.08°W; Planaltina, DF, latitude 15.62°S, longitude 47.65°W; Viçosa, MG, latitude 20.76°S, longitude 42.86°W; Campinas, SP, latitude 22.54°S, longitude 47.03°W) using a spacing of 1.0 × 2.5 m with espaliers, according to field recommendations and preestablished protocol [30]; the experiments were in full sun and irrigated. Each *Mikania* accession was represented by 7 repetitions (5 plants/parcel), making 21 samples per site and making 84 samples in all four evaluated harvest sites. The branches (70%) from each parcel were collected at the beginning of flowering (except for Paraipaba and Viçosa II), which occurred over several months, depending on the geographical site. In Viçosa (MG), where plants flowered first, branches were collected twice, first on September 3, 2013 (late winter) and on then February 27, 2014 (summer). In Campinas (SP), branches were collected on November 9, 2013 (spring); Paraipaba (CE) on January 18, 2014 (summer); and Planaltina (DF) on May 27, 2014 (autumn). Climatic conditions according to the National Institute of Meteorology [31] are represented in ○ **Fig. 1**. The leaves were separated from their branches. At least 10% of representative samples were dried in a ventilated oven (55 °C) according to drying temperatures evaluated by Radünz et al. [32]. Dried samples (about 100 g) of each repetition were packed and shipped to the phytochemical laboratory at Embrapa. The dried leaves were powdered (40 mesh) and stored under refrigeration (4 °C) until assays were carried out. Moistures were determined for constant weight (105 °C).

Extraction
Efficiencies of different polarity solvent systems [3, 5, 8, 9, 28, 33] for coumarin, *o*-coumaric, and kaurenoic acid extraction were tested using ethanol, ethanol-water (50 : 50 v/v), ethanol-water (70 : 30 v/v), methanol, methanol-water (50 : 50 v/v), and methanol-water (80 : 30 v/v). Ethanol-water (70 : 30 v/v) presented the best efficiencies and was used as the extraction solvent. Exhaustive and single extractions using 50 or 100 mL of ethanol-water (70 : 30 v/v) by sonication [5, 8, 33] (30 min) or shaker [9, 28] (120 min) were not different from each other.

Powdered dry leaves (0.5 g) were extracted with 50 mL of ethanol-water (70 : 30 v/v), staying in an ultrasonic bath for 30 min at 30 °C. After centrifugation at 4800 rpm for 10 min, the supernatant was retained and stored at − 20 °C. Before the HPLC injection, the extract was newly centrifuged at 12 000 rpm, and 10 µL of the centrifuged fraction was injected into the chromatographic system.

HPLC analysis
Chromatographic separation (○ **Fig. 3**) was performed using a gradient of acetonitrile and aqueous phosphoric acid 0.1% as the mobile phase: acetonitrile 0 min, 16%; 12 min, 20%; 22 min, 30%; 35 min, 90%; 36–48 min, 100%; 49–50 min, 16%; flow 1.0 ml/min. The UV absorption was monitored at the length of maximum absorption (PDA) and, for quantitative analysis, the wavelength representing the highest intensity for each compound was chosen. Coumarin, *o*-coumaric, and kaurenoic acid peaks were identified by comparison of the retention time with the standards, by the profile of the spectrum provided by PDA, and co-eluting with the standards.

Each bioactive compound was quantified using an external calibration curve, their contents were corrected by moisture (%) of the dried leaves, and the results were expressed in mg/100 g of leaf (dry matter). The linearity (between 0.2 and 200 µg/mL; r = 0.99999), the limit of detection (0.1 µg/mL), the limit of quantification (0.2 µg/mL), and the inter-day precision (2.6% at 200 µg/mL; 1.2% at 100 µg/mL; 3.4% at 10 µg/mL; 5.0% at 1.0 µg/mL and 7.3% at 0.1 µg/mL) were calculated by means of repeated analysis of 15 points (between 0.1 and 200 µg/mL) on 7 different days. Recovery of coumarin ranged from 99 to 103%. The intraday precision (0.8 to 2.5%) was determined by ten repeated analyses of two *M. laevigata* samples. For the *o*-coumaric and kaurenoic acid, the linearity was between 0.2 and 150 µg/mL (r = 0.9999), the limit of detection was 0.1 µg/mL, and the limit of quantification was 0.2 µg/mL.

Statistical analysis
Statistical analysis was performed using the free software R Core Team for Windows (2014). For variable expression of coumarin, *o*-coumaric, and kaurenoic acids, two-way ANOVA was done with site and plant accession factors (○ **Table 1**). The choice of the best model for each bioactive compound was performed by Akaike information criterion (AIC), selecting the model that had the lowest AIC. The best models were accession + site (AIC 485.52225) for coumarin, accession + site + accession × site (AIC 434.1309) for *o*-coumaric acid, and accession + site + accession × site (AIC 586.7275) for kaurenoic acid. With the selected model, comparisons between means were developed using orthogonal contrasts. Test F was used to compare the variability of accessions and sites related to each bioactive compound. The two samples from Viçosa were compared by test T at the 0.05 significance level.

Supporting information
A boxplot displaying the coherence between bioactive compounds and harvest sites, and a radar chart for the compounds in the leaves of *M. leavigata* harvested in Viçosa are available as Supporting Information.

Acknowledgments
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Conflict of Interest
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The authors declare no conflict of interest.

Affiliations

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