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Constituents of glandular trichomes of *Tithonia diversifolia*: Relationships to herbivory and antifeedant activity

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ABSTRACT

The herbivory activity of the bordered patch larvae (*Chlosyne lacinia*, Lepidoptera) on leaves of a Brazilian population of *Tithonia diversifolia* and the antifeedant potential of its leaf rinse extract were investigated. The caterpillars fed only on the adaxial face, where the density of glandular trichomes is very low, and avoided the abaxial face, which contains high levels of trichomes. Deterrent activity against the larvae was observed in leaf discs treated with leaf rinse extract at concentrations of 1–5% of fresh leaf weight. High-performance liquid chromatography (HPLC) analysis indicated that sesquiterpene lactones are the main constituents of the glandular trichomes. Dichloromethane rinse extracts of the leaves and inflorescences were chemically investigated, and 16 compounds were isolated and identified: 14 sesquiterpene lactones, a flavonoid and a diterpenoid. In this study, five sesquiterpene lactones are described for the first time in the genus, including two lactones, one of which has an unusual seco-guaianolide skeleton. Our findings indicate that the caterpillars avoid the sesquiterpene-lactone-rich glandular trichomes, and provide evidence for the antifeedant activity of the dichloromethane leaf rinse extract. In addition, a study of the seasonal variation of the main constituents from the leaf surface throughout a year demonstrated that a very low qualitative but a very high quantitative variation occurs. The highest level of the main metabolite tagitinin C was observed between September and October and the lowest was from March to June, the later corresponding to the period of highest infestation by the larvae.

1. Introduction

The ecological interactions between herbivores and host plants can indicate which morphological, chemical or phenological feature is more important to the plant when interacting with fauna (Andrade et al., 1999). The glandular trichome density on leaves is one of the morphological characteristics that can determine the number of herbivores feasting on certain plant species (Woodman and Fernandes, 1991; Fernandes, 1994). Moreover, several classes of secondary metabolites have been identified in the glandular trichomes (Castro et al., 1997; Monteiro et al., 2001; Spring, 2000; Valkama et al., 2003). Thus, the trichomes can act as antifeedant and antimicrobial defenses in ecological interactions (Monteiro et al., 2001; Valkama et al., 2003). Capitate glandular trichomes are found in most of the taxa of the tribe Heliantheae (Asteraceae) and usually store sesquiterpene lactones (STLs) (Spring, 1989), which have been shown to present biological activities and toxic properties (Schmidt, 1999).

In some species of Lepidoptera, some STLs can reduce the growth rate, increase the number of pupation days, reduce the pupal weight, and reduce the survival rate (Picman, 1986). Passreiter and Isman (1997) showed that some germacranolides and furanoheliangolides from *Neurolaena lobata* (L.) R.Br. ex Cass. (Asteraceae: Heliantheae) are feeding deterrents to *Spodoptera litura* (F.) (Noctuidae). In two other previous studies, Dutta et al. (1986, 1993) reported that STLs isolated from *Tithonia diversifolia* show feeding deterrence against the grubs of *Epilachna vigintioctopunctata* (F.) (Coleoptera: Coccinellidae) and other four Lepidoptera. However, in these studies, no relationship was established among herbivory rate, feeding deterrence and the content of glandular trichomes.

Tithonia diversifolia (Hemsley) A. Gray (Asteraceae: Heliantheae), or Mexican sunflower, is a shrub that is native to Mexico and Central America was introduced in Africa, Australia, Asia and

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South America (Goffin et al., 2002). Previous phytochemical studies of this genus have shown that the major constituents include three subtypes of STLs: heliangolides, furanoheliangolides and eudesmanolides (Pérez et al., 1988, 1992).

We observed that the leaves of Brazilian *T. diversifolia* have many glandular trichomes on the abaxial surface. The chemical profile of these structures from HPLC-UV/Vis-DAD analysis followed by comparison with data from our in-house pure compound library indicated that STLs were the major constituents. As already mentioned, some STLs are feeding deterrents. Nevertheless, field observations indicated high levels of infestation of the Brazilian *T. diversifolia* with the bordered patch larvae *Chlosyne lacinia* (Geyer) (Lepidoptera: Nymphalidae) during a certain period of the year. These observations led us to investigate this relationship and identify the putative secondary metabolites involved.

Therefore, the main aim of this work was to evaluate the herbivory activity of *C. lacinia* larvae and the feeding deterrent potential of the leaf rinse extract of a Brazilian population of *T. diversifolia*. The chemical profiles of the foliar glandular trichomes and the leaf and inflorescence rinse extracts were also obtained. In addition, the three main constituents of the leaf rinse extract were analyzed by comparing HPLC peak areas, indicating that there is a relative variation of such compounds over the year. Moreover, the quantitative variation of the main metabolite (tagitinin C) over one year was determined. Concerning the feeding deterrent potential, our aims were to determine: (1) if *C. lacinia* larvae present behavioral strategies to avoid foliar glandular trichomes; and (2) if the leaf rinse extract, in which STLs are the main constituents, presents antifeedant effects on the larvae.

2. Results and discussion

The CH_2Cl_2 leaf rinse extracts of the aerial parts of *T. diversifolia* contained 16 compounds: 14 STLs (**1**–**14**), a diterpenoid (**15**) and a flavonoid (**16**) (Fig. 1). Ten compounds (**1**–**3**, **5**–**8**, **11**, **12** and **14**) were isolated from the extract of the inflorescences (phase A), and eight compounds (**1**, **4**, **9**, **10**, **12**, **13**, **15** and **16**) were isolated from the extract of the leaves (phase B). Large quantities of **6** and **8** were also detected in phase B, but they have not been purified. In addition to **1**, HPLC analysis showed that these two compounds are the main constituents of the foliar glandular trichomes. Compounds **2**, **7** and **13**, which have previously been isolated from *Greenmaniella resinosa* W.M. Sharp (Zdero et al., 1987), are being described for the first time in the genus *Tithonia*. Compounds **11** and **14** are new natural products and were identified by NMR spectroscopic analyses (^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY, HMQC, HMBC, NOE) and by high-resolution electrospray ionization mass spectrometry (HR-ESIMS). Compound **14** was found to be a novel STL with an unusual seco-guaianolide skeleton (Fig. 1). The other structures were identified by comparison of their NMR spectroscopic data with those reported in the literature, as well as by comparison with authentic samples from our library. The already known compounds are the STLs tagitinin A (**8**) (Baruah et al., 1979), tagitinin C (**1**) (Baruah et al., 1979), tagitinin E (**4**) (Schuster et al., 1992), tagitinin F (**6**) (Zdero et al., 1987), tagitinin C methylbutyrate (**2**) (Zdero et al., 1987), 1β,2α-epoxy tagitinin C (**3**) (Zdero et al., 1987), tirotundin (**10**) (Herz and Sharma, 1975), the 1,10-epoxy heliangolides **5** (Zdero et al., 1987), **7** (Zdero et al., 1987) and **9** (Schuster et al., 1992), the guaianolides 4β,10α-dihydroxy-3-oxo-8β-isobutyryloxyguai- 11(13)-en-6α,12-olide (**12**) (Zdero et al., 1987) and **13** (Zdero et al., 1987) as well as the diterpene *ent*-kaurenoic acid (**15**) (Da Costa et al., 1996) and the flavonoid hispidulin (**16**) (Pereira et al., 1997).

Compound **11** was obtained as a colorless and amorphous solid. Its molecular formula was $C_{20}H_{28}O_8$, as determined by HR-ESIMS ($[M+H]^+$, m/z = 397.1848 and $[M+Na]^+$, m/z = 419.1657). The ^{1}H and ^{13}C NMR spectroscopic data (Table 1) of **11** showed many signals similar to those of the already known semi-synthetic compound epoxywoodhousin (Herz and Bhat, 1972), the data from which data was used to determine the proposed structure. The isobutyrate ester side-chain at C-8 was confirmed by the septet at δ 2.41 (H-2′) and the two methyl doublets at δ 1.04 and 1.05 (J = 7.0 Hz) in the ^{1}H NMR spectrum, as well as the ^{13}C signals at δ 176.4, 34.5, 19.5 and 19.0 (C-1′ to C-4′, respectively). The two doublets (1H each) at δ 6.28 and δ 5.61 (J = 2.8 and 2.5 Hz) were assigned to H-13a and H-13b, respectively, and confirmed as the α-methylene-γ-lactone moiety. These signals were coupled to that of H-7 (δ 4.32 *dddd*, J = 2.5, 2.8, 4.1 and 4.8 Hz), which in turn was coupled to those of H-6 (δ 5.12 *d*, J = 4.8 Hz) and H-8 (δ 5.52 *ddd*, J = 4.1, 4.8 and 11.1 Hz), as shown by the ^{1}H–^{1}H COSY correlation data (Table 1). As anticipated, H-9a and H-9b (δ 1.74 and 1.66, respectively) appeared as double doublets owing to their geminal coupling (J = 14.7 Hz) and the couplings with H-8 (J = 11.1 and 4.8 Hz, respectively), thus indicating the β-orientation of the isobutyrate ester, common to the other known STLs. Two 3H singlets at δ 1.50 and 1.44 were assigned to H-14 and H-15, respectively, and this was also supported by data from HMQC and HMBC analyses. The signal of H-1 appeared as a doublet of doublets at δ 3.99 (J = 6.5 and 10.4 Hz) owing to its coupling with the double of doublets of H-2a (δ 2.56, J = 6.5 and 12.6 Hz) and H-2b (δ 1.91, J = 10.4 and 12.6 Hz). A 3H singlet at δ 3.38 was assigned to a methoxy group, which was attached to C-1, as observed in the HMBC correlation data. The β-orientation of this group was proposed based on the data from NOE (Fig. 2) since the signal of H-1 was strongly increased when H-14 was irradiated. The ^{1}H and ^{13}C NMR spectra of **11** did not show any signal that indicated a double bond or a dihydro system between C-4 and C-5. The signal of H-5 appeared as a singlet at δ 3.34 and no coupling was observed with H-6. This observation led to the assumption that both H-5 and H-6 are located in the same plane (β-oriented) as this is the only orientation that allows a dihedral angle between H-5 and H-6 of approximately 90°. This was also confirmed by the NOE enhancement observed in the signal of H-5 when H-6 was irradiated. The chemical shift of H-5 (δ 3.34) shows that it is close to an oxygen atom. An epoxy group was proposed to occur between C-4 and C-5 because C-4 is quaternary and H-15 occurs as a singlet. This was confirmed by the presence of the epoxy signals for C-4 (δ 67.2) and C-5 (δ 66.2) in the ^{13}C NMR spectroscopic data. The β-orientation of H-15 was proposed based on the NOE data (Fig. 2) since its irradiation strongly increased the signal of H-5. The remaining carbon atoms were confirmed at their respective positions based on analyses of the ^{13}C NMR, HMQC and HMBC data; all assignments are shown in Table 1.

Compound **14** was obtained as a colorless and amorphous solid. Its molecular formula of $C_{19}H_{24}O_7$ was determined by HR-ESIMS ($[M+H]^+$, m/z = 365.1614 and $[M+Na]^+$, m/z = 387.1440). ^{1}H NMR data (Table 2) showed signals with values similar to those of a known semi-synthetic compound (Chowdhury et al., 1983), the data from which were used in the assignment of the proposed structure. The α-methylene-γ-lactone moiety was confirmed by the presence of the two doublets of H-13a at δ 6.38 (J = 3.4 Hz) and H-13b at δ 5.67 (J = 3.0 Hz) coupled to H-7 at δ 3.22 (*dddd*, J = 1.8, 3.0, 3.4 and 10.0 Hz) in the ^{1}H NMR spectrum. The isobutyrate ester side-chain was readily assigned based on the two 3H doublets at δ 1.13 and 1.16 (J = 7.0 Hz) and the septet at δ 2.56 (J = 7.0 Hz). Starting from H-7 and based in the ^{1}H–^{1}H COSY data it was possible to assign H-8, H-9a, H-9b, H-6 and H-5, which appeared at δ 5.80 (*ddd*, J = 1.8, 2.8 and 4.0 Hz), 2.54 (*dd*, J = 2.8 and 15.2 Hz), 2.00 (*dd*, J = 4.0 and 15.2 Hz), 5.13 (*dd*, J = 10.0 and 10.3 Hz) and 3.37 (*dd*, J = 9.0 and 10.3 Hz), respectively. The signal of H-1 at δ 2.82 (*ddd*, J = 7.7, 9.0 and 13.9 Hz) was in turn coupled to

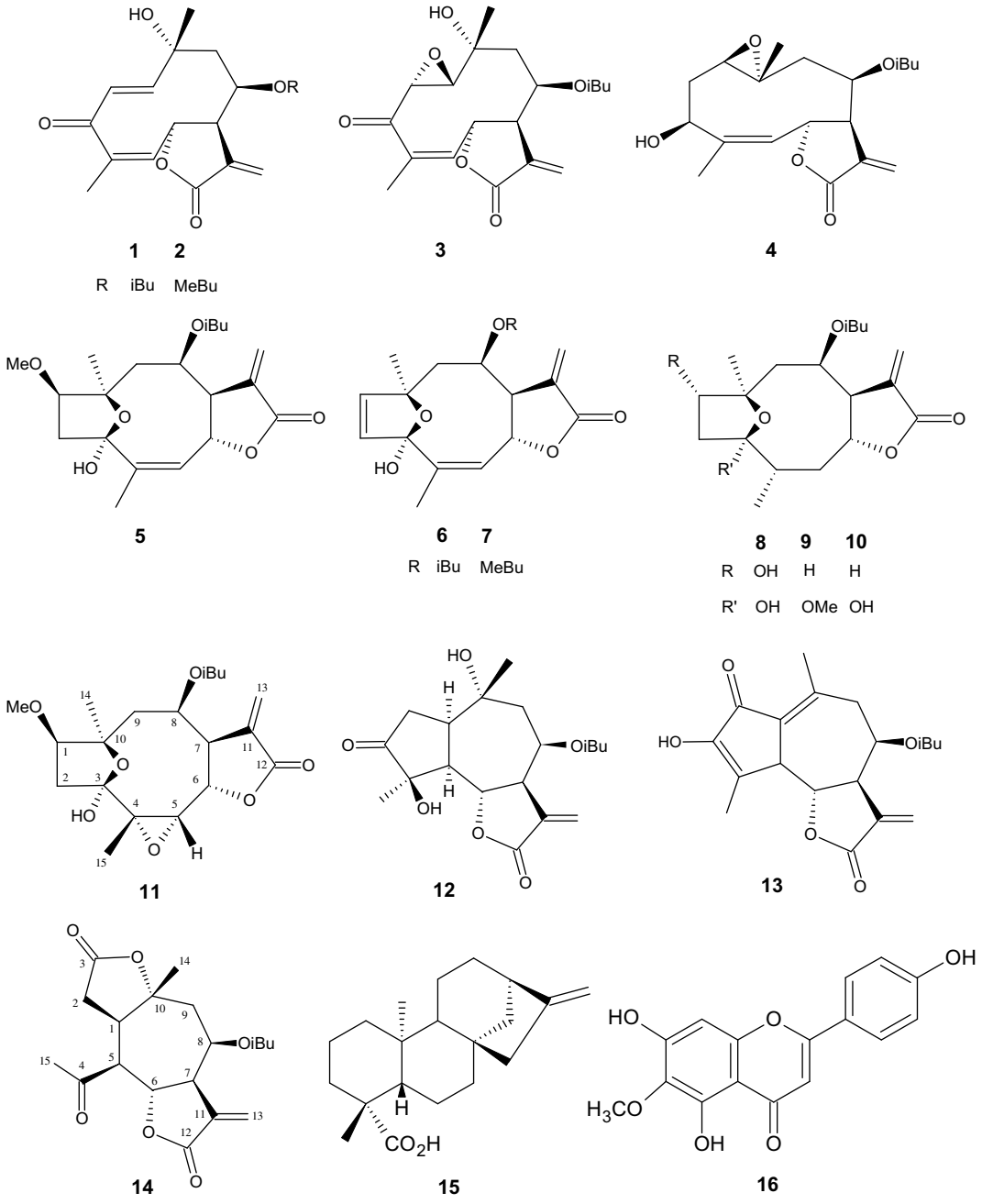

Fig. 1. Structures of the compounds isolated from *T. diversifolia*.

H-2a and H-2b, which appeared as two doublets of doublets at δ 2.66 (*dd*, $J = 7.7$ and 16.4 Hz) and 2.60 ($J = 13.9$ and 16.4 Hz), respectively; this was also confirmed by analyses of the ^{1}H–^{1}H COSY data (Table 2). Two 3H singlets at δ 1.51 and 2.37 were respectively assigned to H-14 and H-15 and confirmed by the HMQC and HMBC data (Table 2). Data from NOE experiments (Fig. 3) supported the relative stereochemistry of both methyl groups of H-14 and H-15, as well as H-8, H-1 and H-5. The ^{13}C NMR spectroscopic data (Table 2) confirmed the presence of the six quaternary carbons C-3, C-4, C-10, C-11, C-12 and C-1′ at δ 172.6, 206.7, 87.6, 133.6, 167.8 and 175.6, respectively. Together, the analyzed data led us to propose that the structure of compound **14** is that of a new natural seco-guaianolide with an unusual skeleton. Usually, the seco-guaianolides are cleaved between C-9 and C-10, but cleavage between C-3 and C-4 as in **14** is uncommon. The biosynthetic origin of **14** in this plant can be proposed starting from the guaianolide **12**. As already mentioned, this di-γ-lactone has only been obtained previously by semi-synthesis involving further oxidation and rearrangement of an enol lactone formed by Baeyer–Villiger oxidation of cyclotagitinin C (Chowdhury et al., 1983). In that study, compound **14** was the main component of a mixture consisting of three derivatives and only an incomplete assignment of the ^{1}H NMR spectroscopic data was presented. Moreover, the ^{13}C NMR and 2D NMR data were not reported in the previous study.

As reported in this work, the secondary metabolite chemistry of the leaf and inflorescence rinse extracts of this Brazilian population of *T. diversifolia* is very rich, comprising different skeletal subtypes of STLs as well as a kaurane diterpene and a flavone, being the highest chemodiversity observed so far for this species (Baruah

Table 1
NMR spectroscopic data for compound **11** ($CDCl_3$, 400 MHz for ^1H and 100 MHz for ^{13}C, δ in ppm)

Position	δ_H (*J* in Hz)	^1H–^1H	δ_C	$^1J_{CH}$	$^2J_{CH}$	$^3J_{CH}$
1	3.99 *dd* (6.5, 10.4)	H-2b, H-2a	86.7	H-1	H-2a, H-2b	H-14, –OCH_3
2a	2.56 *dd* (6.5, 12.6)	H-1, H-2b	40.5	H-2a, H-2b		–OCH_3
2b	1.91 *dd* (10.4, 12.6)	H-1, H-2a				
3			101.5		H-2a, H-2b	H-5, H-15
4			67.2		H-5, H-15	
5	3.34 *s*		66.2	H-5	H-6	H-15
6	5.12 *d* (4.8)	H-7	75.0	H-6	H-5	
7	4.32 *dddd* (2.5, 2.8, 4.1, 4.8)	H-6, H-8, H-13a, H-13b	42.0	H-7	H-6	H-13a, H-13b
8	5.52 *ddd* (4.1, 4.8, 11.1)	H-7, H-9a, H-9b	71.2	H-8	H-9a, H-9b	H-6
9a	1.74 *dd* (11.1, 14.7)	H-8, H-9b	35.0	H-9a, H-9b		H-1
9b	1.66 *dd* (4.8, 14.7)	H-8, H-9a				
10			82.8		H-9a, H-9b, H-14	H-2a
11			136.3		H-13a	
12			169.6			H-6, H-13a, H-13b
13a	6.28 *d* (2.8)	H-7	123.5	H-13a, H-13b		
13b	5.61 *d* (2.5)	H-7				
14	1.50 *s*[a]		26.7	H-14		H-1, H-9a, H-9b
15	1.44 *s*[a]		21.0	H-15		H-5
1′			176.4		H-2′	H-3′, H-4′
2′	2.41 *septet* (7.0)	H-3′, H-4′	34.5	H-2′	H-3′, H-4′	
3′	1.05 *d* (7.0)[a]	H-2′	19.5	H-3′	H-2′	H-4′
4′	1.04 *d* (7.0)[a]	H-2′	19.0	H-4′	H-2′	H-3′
–OCH_3	3.38 *s*[a]		59.1	–OCH_3	H-1	

[a] Integrated to 3H.

Table 2
NMR spectroscopic data for compound **14** ($CDCl_3$, 400 MHz for ^1H and 100 MHz for ^{13}C, δ in ppm)

Position	δ_H (J in Hz)	^1H–^1H	δ_C	$^1J_{CH}$	$^2J_{CH}$	$^3J_{CH}$
1	2.82 *ddd* (7.7, 9.0, 13.9)	H-2b, H-2a	43.0	H-1	H-2a, H-2b, H-5	H-9a, H-14
2a	2.66 *dd* (7.7, 16.4)	H-1, H-2b	32.0	H-2a, H-2b	H-1	
2b	2.60 *dd* (13.9, 16.4)	H-1, H-2a				
3			172.6		H-2a, H-2b	
4			206.7		H-5, H-15	H-1, H-6,
5	3.37 *dd* (9.0, 10.3)	H-1, H-6	51.9	H-5		H-15
6	5.13 *dd* (10.0, 10.3)	H-5, H-7	76.3	H-6	H-5	
7	3.22 *dddd* (1.8, 3.0, 3.4, 10.0)	H-6, H-8, H-13a, H-13b	46.5	H-7	H-6, H-8	H-9a, H-13a, H-13b
8	5.80 *ddd* (1.8, 2.8, 4.0)	H-7, H-9a, H-9b	64.6	H-8	H-9a	
9a	2.54 *dd* (2.8, 15.2)	H-8, H-9b	46.0	H-9a, H-9b		H-14
9b	2.00 *dd* (4.0,15.2)	H-8, H-9a				
10			87.6		H-1, H-9a, H-9b, H-14	H-2a, H-2b, H-5,
11			133.6			
12			167.8			H-13a, H-13b
13a	6.38 *d* (3.4)	H-7	123.3	H-13a, H-13b		
13b	5.67 *d* (3.0)	H-7				
14	1.51 *s*[a]		23.6	H-14		H-1, H-9b
15	2.37 *s*[a]		33.5	H-15		
1′			175.6		H-2′	H-3′, H-4′
2′	2.56 *septet* (7.0)	H-3′, H-4′	34.1	H-2′	H-3′, H-4′	
3′	1.16 *d* (7.0)[a]	H-2′	18.9	H-3′	H-2′	H-4′
4′	1.13 *d* (7.0)[a]	H-2′	19.0	H-4′	H-2′	H-3′

[a] Integrated to 3H.

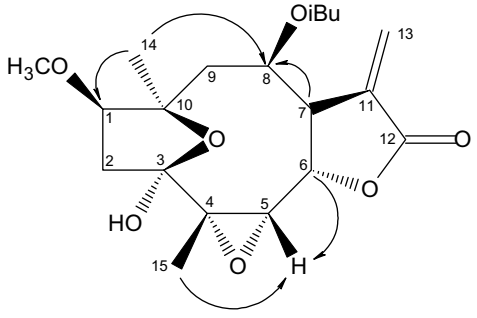

Fig. 2. NOE data of compound **11**.

et al., 1994; Gu et al., 2002; Kuo and Chen, 1998; Pereira et al., 1997). Nevertheless, the skeletal types and substitutional features of most of the isolated compounds are typical of the genus *Tithonia*, the only exception being the guaianolides **12** and **13** and the seco-guaianolide **14**. As already mentioned, the known compounds **2**, **7** (heliangolides) and **13** are also present in *Greenmaniella resinosa* (Heliantheae: Melampodiinae) (Zdero et al., 1987). The same is true for **3** and **12**, as previously reported by Pereira et al. (1997). Our results, therefore, indicate a new chemical relationship between *Tithonia* and *Greenmaniella* regarding heliangolides and guaianolides. Considering morphological characteristics, this relationship has been briefly discussed in a previous study (Sharp, 1935) and corroborates our finding.

Field observations have shown that the *C. lacinia* larvae at early stages of development (first or second instars) scrape the adaxial surface of leaves of *T. diversifolia* without passing over the abaxial face, thereby always resulting in a thin film that corresponds to the abaxial epidermis. Analysis of this epidermis revealed the presence of large amounts of intact glandular trichomes, which are the structures that contain STLs. Measurements of the herbivory rate on adaxial (76.1 ± 14.9%) and abaxial leaf surfaces of *T. diversifolia* by *C. lacinia* showed that these caterpillars feed only on the adaxial surface ($p < 0.0001$), indicating a total preference for this side of the leaf. Data from experiments in which only the abaxial face was analyzed showed that *C. lacinia* does not present herbivory during the three periods evaluated (8, 16 and 24 h). Electron microscopy analysis showed that the density of trichomes on the abaxial surface was much greater than on the adaxial surface. On the abaxial surface, the tector trichome (nonglandular hairs, uni or multicellular, simple or branched with short basil and pitted thick-walled cells) density was 22.1 ± 0.3 per mm^2 and the glandular trichome density was 17.8 ± 1.2 per mm^2. On the adaxial surface, the tector trichome density was 8.6 ± 0.3 per mm^2 and there were no glandular trichomes. Statistical analyses showed differences in the glandular and tector trichome contents between the two surfaces of the leaves ($p < 0.001$).

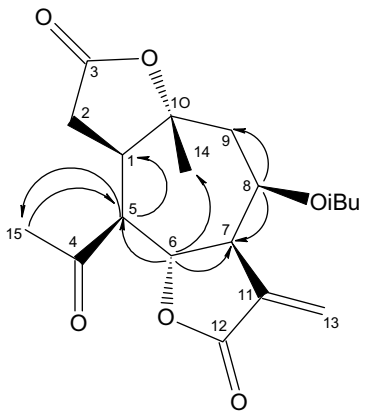

Fig. 3. NOE data of compound **14**.

Different concentrations (0.5, 1, 3 and 5% w/w of a fresh leaf disc) of phase B (leaf rinse extract) of *T. diversifolia* were applied to leaf discs and the herbivory rate was measured against a control leaf disc treated only with MeOH. Only the 0.5% concentration was not significantly different compared with the control ($p > 0.05$). As depicted in Fig. 4, the larvae showed a total preference for the control leaf discs when compared with the discs rinsed with 5, 3 and 1% of phase B ($p < 0.0001$). The experiments using 5% of phase B demonstrated an herbivory rate of 0.6 ± 1.1%, while its control showed 88.6 ± 12.4%. The experiments in which leaf discs were treated with 3% and 1% of phase B presented herbivory rates of 3.9 ± 4.0% and 3.8 ± 5.2% whereas control discs presented 89.1 ± 14.0% and 56.7 ± 14.4%, respectively. There were no significant differences in feeding deterrence among the concentrations of 5% (99.2 ± 1.3%), 3% (95.5 ± 4.4%) and 1% (93.7 ± 7.5%) ($p > 0.05$).

Chlosyne lacinia is found from Arizona to Texas in the USA, in Central America and in southern Argentina (Clark and Faet, 1997). Females oviposit primarily on sunflower (*Helianthus annuus* L.) and other Asteraceae species, for example, *Acanthospermum hispidum* DC, *Ambrosia polystachia* L. and *Vernonia* Schreb. spp. (Justus et al., 2003). This is the first time these larvae were shown to use *T. diversifolia* as a host plant.

In this work, we found that *C. lacinia* larvae fed on *T. diversifolia* leaves, gnawing exclusively on the adaxial surface. The herbivory activity of these larvae results in a thin film epidermis on the abaxial surface, rich in intact glandular trichomes containing STLs that are not consumed. This indicates an herbivory behavior that either avoids or reduces the intake of glandular trichomes and their constituents. The feeding preference for the adaxial leaf surface suggests a relationship with the lower percentage of tector trichomes and the absence of glandular trichomes on this side of the leaf.

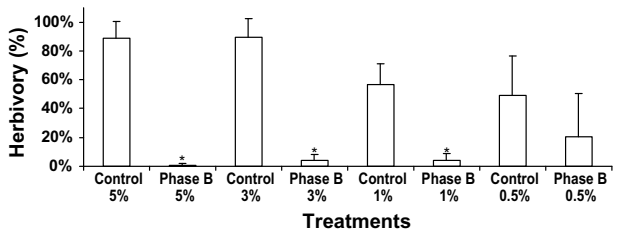

Fig. 4. Evaluation of the antifeedant activity of phase B (leaf rinse extract) of *T. diversifolia* on *C. lacinia* larvae at different concentrations ($n = 15$ for each concentration). *Means significant difference from the respective control; Paired t test, $p < 0.05$.

The STLs isolated from the leaves of *T. diversifolia* are characterized by the presence of an exocyclic methylene group conjugated with a carbonyl group from a γ-lactone, which are associated with the selective alkylation of nucleophilic groups present in enzymes, such as sulphydryl groups. This feature can explain the pharmacological and toxicological activities of these metabolites (Picman, 1986; Schmidt, 1999) and may be related to their ability in inhibiting the feeding of *C. lacinia* at concentrations from 1% of *T. diversifolia* phase B.

The qualitative analysis of phase B (leaf rinse extract) as well as the glandular trichome profile of *T. diversifolia* by HPLC using the isolated compounds as reference indicated that they are rich in STLs. Moreover, three of these STLs, the tagitinins A, C and F (structures **8**, **1** and **6**, respectively, Fig. 1), were found to be the main constituents in the analyzed material. To determine if these STLs are present in the trichomes throughout the year, and if they are responsible for the observed antifeedant activity, HPLC qualitative analysis of the leaf rinse extracts and quantitative analysis of tagitinin C, the main metabolite, were performed monthly for one year (see Section 4.4). Twenty individuals of a population of *T. diversifolia* were selected for the analyses. From January to December, leaves from these individuals were collected, air-dried, and the rinsed extracts were obtained with CH_2Cl_2 and further analyzed by HPLC. The qualitative analysis of leaf rinse extracts showed that tagitinins A, C and F (8, 1, 6) are the main compounds throughout the year, with tagitinin C (1) presenting highest concentrations followed by tagitinins A (8) and F (6). The highest concentrations of these three STLs were observed during September–November, a period that corresponds to springtime in Brazil (Fig. 5). The larvae of *C. lacinia* show herbivory on the leaves of *T. diversifolia* during April–June (autumn), which corresponds to the period of the year when the level of the main STLs is lower (Fig. 5). This suggests that the caterpillars avoid the feeding behavior when higher concentrations of these secondary metabolites are present.

The quantification of the main metabolite of the leaf rinse extract, tagitinin C (**1**) (Table 3), was performed using HPLC (see Section 4.4). It was found that the highest concentrations of tagitinin C (1) occur in September and October (0.33% and 0.22% in the dried leaves and 6.9% and 7.1% in the leaf rinse extract, respectively), and that the lowest occur in March–June (Table 3), which corresponds to the period that *C. lacinia* larvae are present in the leaves of *T. diversifolia*. In general, there is a high quantitative variation in the concentration of this metabolite in the leaves of *T. diversifolia* throughout the year. These results support the hypothesis that STLs do present an ecological function. Moreover, it is important to notice that after the high level of infestation by *C. lacinia* larvae

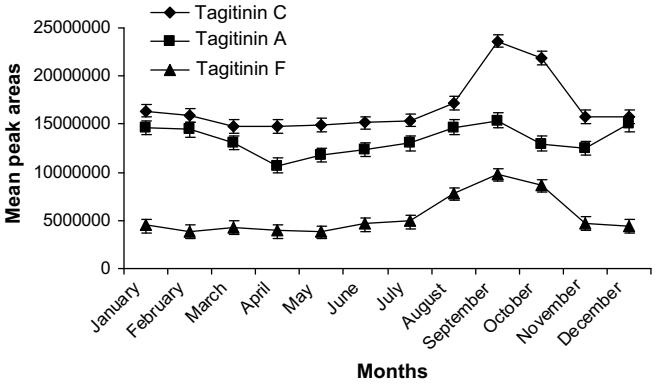

Fig. 5. Mean peak areas of the three main compounds detected in the leaf rinse extract of *T. diversifolia* from January to December 2004. Tagitinin C (**1**), tagitinin A (**8**) and tagitinin F (**6**).

Table 3
Weight of the leaf rinse extract and content of tagitinin C (**1**) in the extract and in dried leaves of *T. diversifolia*

Months	Weight of leaf rinse extract (mg)	Tagitinin C in the extract (mg)	% of Tagitinin C in the extract	% of Tagitinin C in dried leaves
January	282.0	17.5	6.2	0.18
February	265.0	13.5	5.1	0.14
March	177.0	8.8	5.0	0.09
April	193.0	7.4	3.8	0.07
May	186.0	8.0	4.3	0.08
June	255.0	9.7	3.8	0.10
July	290.0	11.9	4.1	0.12
August	373.0	15.2	4.1	0.15
September	481.0	33.3	6.9	0.33
October	315.0	22.4	7.1	0.22
November	289.0	16.8	5.8	0.17
December	271.0	9.8	3.6	0.10

on leaves of *T. diversifolia* takes place, the levels of STLs increase monthly, suggesting that the females oviposit when the level of the main STLs is lower during the year. Further studies should be performed in order to understand the relationships between these STLs, the herbivory as well as the antifeedant activity displayed by the CH_2Cl_2 leaf rinse extract of *T. diversifolia.*

3. Concluding remarks

Our results show that the glandular trichomes of the investigated population of *T. diversifolia* are rich in secondary metabolites, especially STLs. Moreover, the larvae of *C. lacinia* have a characteristic behavior while feeding on the leaves of *T. diversifolia*: they shave the adaxial surface without passing over the epidermis of the abaxial surface where the glandular trichomes are located. Together, the results in this study allow us to conclude that the caterpillars of *C. lacinia* recognize and avoid the secondary metabolites present in the glandular trichomes. We suggest that STLs stored in these glandular trichomes, which were isolated from the CH_2Cl_2 leaf rinse extract, may be responsible for this antifeedant behavior.

4. Experimental

4.1. General procedures

IR spectra were recorded in $CHCl_3$ using a Nicolet-Protégé 460 spectrometer. NMR spectra were run on a Bruker DPX 400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C). Samples were dissolved in $CDCl_3$, and the spectra were calibrated at the solvent signals at 7.26 (^1H) or 77.0 (^{13}C); the chemical shifts were in ppm. High-resolution ESI-MS spectra were recorded using an Ultro-TOF Bruker, fitted with an electrospray ion source operating in the positive ion mode. HPLC analyses were performed using a Shimadzu SLC-10A*vp* liquid chromatography controller, operating with the Class-VP software v. 5.02, equipped with Shimadzu UV-DAD detector SPD-M10A *vp* and a Shimadzu column ODS (4.6 × 250 mm, 5 μm). Optical rotation was measured in $CHCl_3$ in a Perkin–Elmer 241 polarimeter.

4.2. Plant material

Leaves and inflorescences from *T. diversifolia* were collected in May, 2002, by S.R. Ambrósio in Ribeirão Preto, SP, Brazil. The plant material was identified by F.B. Da Costa by comparison with a previous sample identified by E.E. Schilling (University of Tennessee, USA) and J.N. Nakajima (Universidade Federal de Uberlândia, MG, Brazil). A voucher specimen (FBC #126) is deposited at the herbarium SPFR of the Department of Biology, FFCLRP, Universidade de São Paulo, Ribeirão Preto, SP, Brazil.

For the qualitative and quantitative analysis, five leaves of 20 previously selected individuals of *T. diversifolia* (*ca.* 2 m high, containing more than 300 leaves), were collected monthly (between the 5th and 10th days) during 2004. The leaves collected from the selected individuals were mixed and air-dried. Ten grams of this material were rinsed with CH_2Cl_2 (400 mL) before analysis.

4.3. Extraction and isolation

Screening for STLs was performed by microsampling of glandular trichomes according to Schorr et al. (2002). The material (glandular trichomes and leaf rinse extract) was first analyzed by HPLC and compounds were further isolated by preparative techniques. Air-dried intact inflorescences (2.5 kg) and leaves (3.7 kg) were rinsed for 3 min at room temperature (about 26 °C) with CH_2Cl_2, (10 and 18 L) respectively, yielding a light-yellow extract for both plant materials. Each extract was filtered through filter paper to yield dry residue, (24 and 82 g) respectively, after solvent evaporation under reduced pressure. Each residue was re-suspended in MeOH, diluted with H_2O (7:3, v:v), and successively partitioned with *n*-hexane and CH_2Cl_2. After solvent evaporation under reduced pressure, the rinse extract of the inflorescences produced 12.0 and 9.8 g of residues, respectively, whereas the leaves yielded 32.3 and 44.7 g of residues, respectively. The residues were further analyzed by IR spectroscopy. The IR spectra of both CH_2Cl_2 residues of the inflorescences and leaves, known as phase A and B, respectively, showed strong bands of carbonyl stretching of γ-lactones at about 1760 cm^{-1}.

Phase A was separated over silica gel 60H (Merck, art. no. 7736) using vacuum liquid chromatography (VLC; Pelletier et al., 1986) with increasing amounts of EtOAc in *n*-hexane. Solvent was removed under reduced pressure in a rotary evaporator. Seven fractions (500 mL each) were obtained and IR analysis showed the presence of carbonyl groups of γ-lactones in fractions 4 (2.3 g), 5 (2.6 g) and 6 (1 g). Fraction 4 was fractionated by VLC as described above to give five other fractions (250 mL each, fractions 4.1–4.5). Compound **6** (5.0 mg) was isolated from fraction 4.4 (200 mg) after preparative TLC (silica gel PF_{254}, 1 mm thickness, Merck, art. no. 7730, *n*-hexane–EtOAc 4:1+1% HOAc, UV 254 nm) followed by medium pressure chromatography (flash chromatography; Still et al., 1978) using silica gel 60 (Merck, art. no. 9385, 0.040-0.063 mm) and isocratic *n*-hexane–EtOAc 4:1+1% HOAc as mobile phase. Fraction 4.5 (775 mg) was also fractionated by flash chromatography (*n*-hexane–EtOAc–$CHCl_3$ 5:2:3+1% HOAc) yielding 25 new fractions (10 mL each), which were grouped into eight fractions (from 4.5.1 to 4.5.8) after TLC analysis. Fractions 4.5.5 (120 mg) and 4.5.7 (260 mg) were purified using radial chromatography (Chromatotron, Harrison Research Inc., model 8924; *n*-hexane–Et_2O–EtOAc 6:3:1+2% HOAc, isocratic, flow rate 2 mL/min, UV 254 nm) and produced an additional amount (8 mg) of **6** as well as compounds **5** (14.7 mg) and **1** (12 mg). Fraction 4.5.8 (15 mg) was purified by HPLC using an analytical column, in portions of 0.5 mg (MeOH–H_2O 7:3, flow rate 1 mL/min, UV 210 nm) yielding an additional 5 mg of **6**. Fraction 4.6 (1.12 g) was fractionated using flash chromatography (*n*-hexane–Et_2O–EtOAc 4:3:3+2% HOAc), yielding 25 fractions (10 mL each), which were grouped into six fractions (4.6.1 to 4.6.6) after TLC analysis. Fraction 4.6.5 (20 mg) corresponded to compound **1**, whereas fraction 4.6.4 (733 mg) was further purified by flash chromatography (*n*-hexane–Et_2O–EtOAc 5:2:3+1% HOAc) as mentioned above. Fraction 4.6.4.4 (520 mg) also corresponded to compound **1**, whereas fraction 4.6.4.3 (20 mg) was purified using preparative TLC (*n*-hexane–*iso*-PrOH–$CHCl_3$ 5:0.5:4.5+1% HOAc, UV 254 nm) yielding compound

2 (2 mg). Compound **12** (5 mg) was isolated by HPLC (MeOH–H_2O 55:45, flow rate 0.9 mL/min, UV 210 nm) from fraction 4.6.6 (12.5 mg). Fraction 5 was separated by VLC, as described for the previous fraction, yielding six new fractions. Fraction 5.6 (220 mg) was fractionated by flash chromatography (*n*-hexane–*iso*-PrOH–$CHCl_3$ 5:0.5:4.5+2% HOAc) as previously described, and three new fractions were obtained. Fraction 5.6.1 (14 mg) was purified by HPLC (MeOH–H_2O 55:45) yielding compound **7** (1.5 mg), whereas fraction 5.6.3 (20 mg) also purified by HPLC (MeOH–H_2O–MeCN 50:45:5, flow rate 1 mL/min, UV 210 nm), contained compound **3** (5 mg). Fraction 6 (1 g) was further fractionated by VLC and yielded seven new fractions. Fraction 6.2 (130 mg) was fractionated by flash chromatography (*n*-hexane–Me_2CO–$CHCl_3$ 5:1:4+1% HOAc) yielding seven other fractions after TLC analysis. Fractions 6.2.2 (2 mg) and 6.2.6 (4 mg) corresponded to compounds **11** and **8**, respectively. Fraction 6.3 (370 mg) was fractionated again in the same way as described for fractions 6.2 and 6.3.6 (100 mg), and also separated by flash chromatography (*n*-hexane–*iso*-PrOH–$CHCl_3$ 6:0.25:3.75+2% HOAc) to give an additional amount of **8** (47 mg) and 2.5 mg of compound **14**. The authenticity of the new compounds **11** and **14** was confirmed by HPLC–DAD–MS analysis. Based on the results, it could be assured that both compounds were present in the leaf rinse extract, therefore demonstrating that **11** and **14** are natural products and not artifacts produced during the extraction procedure. Analysis and peak identification by HPLC–DAD–MS were performed on a Shimadzu LC-20A HPLC apparatus with a diode array detector (CBM-20A, Shimadzu) coupled to an UltrOTof (Bruker Daltonics) ESI-qTOF mass spectrometer. The following elution gradient was employed, with a flow rate of 3 mL/min: solvent A: H_2O; solvent B: MeCN; elution profile: 0–20 min, 20–50% B (linear gradient); 20–23 min, 50–100% B.

Phase B was initially fractionated over silica gel by VLC as described above, yielding eight fractions. Fraction 2 (150 mg, *n*-hexane–EtOAc 3:7) was fractionated by flash chromatography (*n*-hexane–EtOAc 9:1+1% HOAc) and five fractions were obtained after TLC analysis. A solid mass appeared in fraction 2.2 (70 mg), and compound **15** was obtained after washing the solid with MeOH. Fraction 3 (1 g) was fractionated by VLC yielding six fractions. Fraction 3.6 (430 mg) was shown to contain a significant amount of **1**, which was re-purified using flash chromatography (*n*-hexane–EtOAc 7:3 + 1% HOAc) and yielded compound **1** (40 mg). Fraction 3.5 (300 mg) was separated by flash chromatography (*n*-hexane–EtOAc–$CHCl_3$ 6:1:3+1% HOAc) and seven fractions were obtained after TLC analysis. Fraction 3.5.6 (80 mg) was initially fractionated by flash chromatography (*n*-hexane–EtOAc–$CHCl_3$ 6.5:0.5:3+1% HOAc) yielding four new fractions, and fraction 3.5.6.1 (12 mg) was shown to be compound **13**. Fraction 3.5.7 (100 mg) was also fractionated by flash chromatography (*n*-hexane–*iso*-PrOH–$CHCl_3$ 6.5:0.5:3+1% HOAc) yielding five other fractions. After several HPLC injections (MeOH–H_2O 55:45, flow rate 0.9 mL/min, UV 210 nm) of samples containing 0.5 mg each, fraction 3.5.7.5 (20 mg) gave compounds **9** (2 mg) and **10** (2.5 mg). Fraction 5 (500 mg) was fractionated using flash chromatography (*n*-hexane–EtOAc–$CHCl_3$ 6:2:2+ 1% HOAc) yielding six fractions after TLC analysis. Yellow crystals appeared in fraction 5.5 (50 mg) and 15 mg of compound **16** were obtained after washing the crystals with CH_2Cl_2. Fraction 5.6 (40 mg) was purified by HPLC (MeOH–H_2O 55:45) and compounds **4** (5 mg) and **12** (3 mg) were obtained. Compounds **6** and **8** were found at high concentrations in fractions 4 (3.1 g) and 6 (2.6 g), respectively, but they were not purified because they had previously been purified from phase A.

4.4. Qualitative and quantitative analysis using HPLC

In order to perform the seasonal qualitative analysis, several mobile phases were evaluated to develop an HPLC method for separating the main metabolites present in phase B (leaf rinse extracts) of the selected individuals of *T. diversifolia*. The system consisting of a Shimadzu ODS column eluted with MeCN–H_2O (45:55) at a flow rate of 1 mL/min and UV detection at 210 nm gave the best chromatogram resolution. The previously isolated STLs were used as standard compounds to characterize the metabolites present in the rinse extracts. The extracts were injected in triplicate and variations in the mean peak areas of the three main metabolites (tagitinins A, C and F; compounds **8**, **1** and **6**) over the year were calculated from January to December 2004.

The quantification of tagitinin C (**1**) was carried out using MeOH–H_2O 48:52, flow rate 0.6 mL/min, and UV detection at 254 nm, a wavelength at which this metabolite is easily detectable (Goffin et al., 2003). The method was validated to determine the precision, accuracy and reproducibility, among other parameters. Calibration curves were constructed using standard solutions of tagitinin C in the concentration range of 5–500 μg/mL. Each sample (20 μL) was measured in triplicate and the software Class-VP was used to generate a standard curve from average detector responses. The linear regression of the curve gave the following equation: $y = 0.00009x - 4.5702$, showing a correlation coefficient (R^2) of 0.9999. The precision and accuracy were calculated using three standard solutions of tagitinin C (10, 100 and 500 μg/mL), which were measured four times to determine the standard deviation of the method in the same day (intra-day variation) and in five different successive days (inter-day variation). The values of the coefficient of variation were less than 10% for precision and 70–130% for accuracy. The detection limit was 0.02 μg and the quantification limit was 0.07 μg, calculated by UV detection at 254 nm. Based on the regression curve, the concentrations of tagitinin C over the year in the leaf rinse extracts from the selected *T. diversifolia* were calculated.

4.5. Collection and rearing of larvae

The larvae of *C. lacinia* were collected from the leaves of *T. diversifolia* from April to June 2004, which corresponds to their most abundant period. The first instars were reared in the laboratory with 75% of relative humidity and environmental temperature (25 °C). Identification of the Lepidoptera species was carried out by Prof. I.R. Diniz, Department of Zoology, Universidade Federal de Brasília (UnB), Brasília, DF, Brazil.

The microscopic observations were performed with a stereoscopic loop (Leica MZ 75 with a Leica DFC 280 photographic camera) in the Laboratory of Ecology, FFCLRP, USP.

4.6. Evaluation of *C. lacinia* larvae preference by the abaxial and adaxial faces of *T. diversifolia* leaves

The first set of experiments tested the leaf surface preference. Two leaf discs (1.1 cm diameter each) were placed in a Petri dish with wet filter paper. One leaf disc was placed with the abaxial side upward and the other with the adaxial side upward. Three 12-h food-deprived larvae of *C. lacinia* in early stages of growth (second and third instars) were placed in the Petri dish ($n = 15$) and the herbivory rate was measured for 8 h. The percentage of consumed area in each disc was measured as the herbivory rate through digitalization of the disc images and analysis by the software SIARCS (Integrated System for Root and Ground Cover Analysis), version 3.0 (EMBRAPA, Brasília, DF, Brazil).

After evaluating the preference of *C. lacinia* larvae for either the abaxial or adaxial faces, a second set of experiments was performed to evaluate the herbivory on the abaxial surface. The method used in these experiments was the same as described above. However, only one leaf disc in which the abaxial surface was exposed was used. The leaf discs were offered to larvae for 8, 16

and 24 h, and 20 Petri dishes ($n = 20$) were used for each time period.

4.7. Electron microscopy of the leaf surface of T. diversifolia

Five leaves of *T. diversifolia* were analyzed by electron microscopy using a Jeol-JSM 5200 microscope, with a 20 kV acceleration tension, at the Laboratory of Electron Microscopy, Department of Cellular Biology and Pathogenic Bioagents, Faculdade de Medicina de Ribeirão Preto, USP.

Fragments of leaves (6 mm^2) were tagged, indicating the specimen, and the leaf faces were analyzed. The preparation of the material followed the recommendations of Bondada et al. (1996). As the material was already dried to the critical point, the infiltration phase with EtOH 30% and the dry phase were not necessary. The sample documentation was made with a MAMIYA machine and T-MAX 120 film, and the number of tector trichomes and glandular trichomes were highlighted in both leaf faces.

4.8. Evaluation of the deterrent activity of the leaf rinse extract on C. lacinia larvae

The antifeedant activity of phase B of *T. diversifolia* was evaluated using the same type of experiment as described above (Section 4.6), using two leaf discs (1.1 cm diameter each) in each Petri dish. On the adaxial side of one leaf disc, different amounts of phase B (0.5%, 1%, 3% and 5% w/w of a fresh leaf disc), re-suspended with MeOH (20 μL) were added. In the other leaf disc only MeOH (20 μL) was added (control). For each concentration, 15 tests ($n = 15$) were performed. Three 12-h food-deprived larvae of *C. lacinia* in early stages of growth (second and third instars) were used in each experiment. The herbivory rates of the two leaf discs (control and extract) were measured after 8 h of exposure and calculated by digitalization of the disc images and analysis by SIARCS.

The feeding deterrence was calculated according to the method of Passreiter and Isman (1997): percentage of deterrence = 100 − [(consumed area of the treated disc × 100)/(consumed area of control disc)]. This was calculated only for the concentrations at which the consumed area of the treated disc showed significant differences when compared with the control.

4.9. Statistical analysis

Results are expressed as means ± standard error of the mean (S.E.M.). Statistical analyses were performed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. The significance level considered in all tests was 0.05.

4.10. 1β-methoxy-3α-hydroxy-3,10β-4,5α-diepoxy-8β-isobutyroyloxy-germacra-11(13)-en-6α,12-olide (**11**)

Colorless and amorphous solid. HR-ESIMS *m*/*z* 397.1848 $[M+H]^+$ (calc. for $C_{20}H_{28}O_8$, 397.1862); $\alpha_{25}^{D} = -16.67$ ($CHCl_3$; *c* 0.21). For ^1H and ^{13}C NMR spectroscopic assignments, see Table 1.

4.11. 4-oxo-8β-isobutyroyloxy-3,4-secoguaia-11(13)-en-6α,12,10α,3-diolide (**14**)

Colorless and amorphous solid. HR-ESIMS *m*/*z* 365.1614 $[M+H]^+$ (calc. for $C_{19}H_{24}O_7$, 365.1600); $\alpha_{25}^{D} = -15.23$ ($CHCl_3$; *c* 0.17). For ^1H and ^{13}C NMR spectroscopic assignments, see Table 2.

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