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Guaianolides from *Viguiera gardneri* inhibit the transcription factor NF-κB

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Abstract

Five guaianolides and a germacrolide were isolated from the leaf rinse extract of *Viguiera gardneri* (Asteraceae), together with known compounds. All compounds were detected in glandular trichomes collected from the leaves and were analyzed by HPLC. Structure elucidation was based on the analysis of spectroscopic data. Low energy conformations were obtained by quantum mechanical calculations. Three closely related guaianolides which were isolated as the main compounds were studied for their anti-inflammatory activity using the transcription factor NF-κB as molecular target. NF-κB DNA binding was inhibited at sesquiterpene lactones concentrations of 10 or 50 μM. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Viguiera gardneri*; Asteraceae; Heliantheae; Anti-inflammatory activity; Sesquiterpene lactones; NF-κB; Low energy conformation

1. Introduction

Viguiera (Asteraceae; Heliantheae) is a paraphyletic genus from the Helianthinae subtribe with approximately 200 species distributed in America (Blake, 1918). Its main metabolites are sesquiterpene lactones (STLs) of the germacranolide type as well as diterpenoids. The STL group includes mainly heliangolides and other minor groups such as germacrolides. Guaianolides and eudesmanolides were found only in few species. From the 72 *Viguiera* species recognized from South America, about 35 are restricted to "cerrado" areas in Brazil (Schilling et al., 2000). In contrast to the taxa from Central America with which exhaustive phytochemical studies have been carried out (Bohlmann, 1990; Romo De Vivar and Delgado, 1985), similar studies have not been reported so far for the Brazilian species of *Viguiera* (Da Costa et al., 1996, 2001; Spring et al., 2001).

Viguiera gardneri Baker is a perennial sunflower-like herb with alternate and oblong leaves belonging to the series Bracteatae of the South American section Paradosa whose members are spread in South America (Blake, 1918). This species shows capitate glandular trichomes in the leaf surface and in the anther appendages that are typical places where STLs accumulate (Spring, 1991).

In this work the leaf rinse extract of *V. gardneri* was chemically investigated and five STLs of the guaianolide type (**1–5**), one of the germacrolide type (**6**) (Fig. 1), as well as two flavonoids and a coumarin were isolated and identified. We investigated whether the three isolated guaianolides inhibit the transcription factor NF-κB, which serves as a central regulator of the human immune and inflammatory response. NF-κB regulates the transcription of inflammatory mediators such as cytokines, cyclooxygenase-II, nitric oxide synthase, immunoreceptors, cell adhesion molecules and hematopoetic growth factors.

Pharmacological inhibition of NF-κB in vivo may thus substantially modulate inflammatory processes. We have very recently demonstrated that several STLs inhibit activation of NF-κB (Castro et al., 2000a,b; Lyss et al., 1997; Rüngeler at al., 1998, 1999) and that selective

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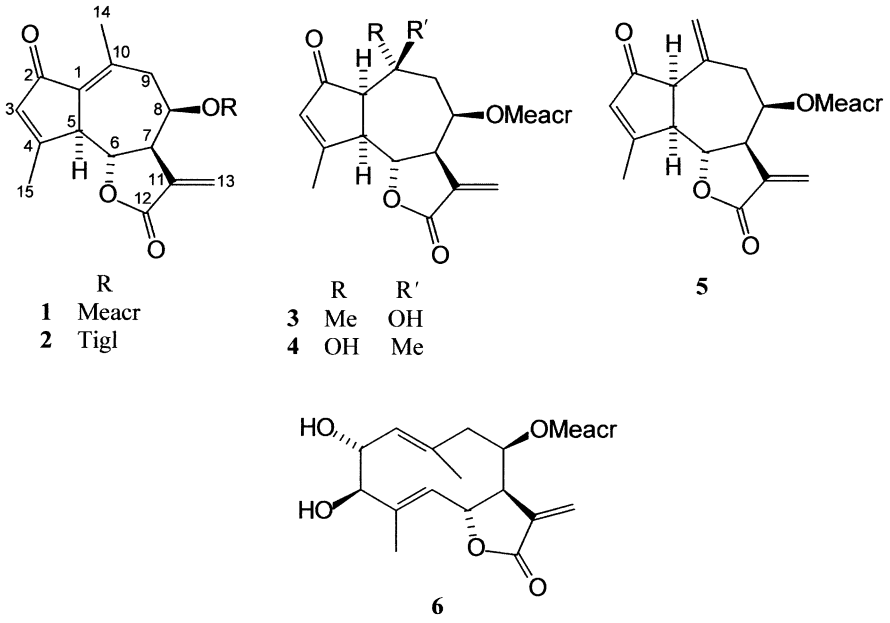

Fig. 1. Sesquiterpene lactones isolated from *V. gardneri*. The absolute configuration of the discussed compounds was not determined and the chemical formulae are given in analogy to known compounds.

alkylation of cysteine 38 in its p65 subunit is the crucial step for its inhibition (Garcia-Piñeres et al., 2001).

2. Results and discussion

The CH_2Cl_2 leaf rinse extract from *V. gardneri* was investigated by HPLC-UV-Vis-DAD analysis showing ca. 15 peaks in the chromatogram. Five main peaks and a minor peak whose UV spectra and chromatographic properties were characteristic of STLs (Spring et al., 2001) were separated. UV data of three other minor peaks indicated aromatic compounds, two of them being typical of flavonoid compounds and the third of a 6,7-dioxygenated coumarin. Intensive preparative HPLC analyses resulted in the isolation of 9 compounds which were analyzed by means of spectroscopic methods for structure elucidation.

^1H and ^{13}C spectroscopic NMR data of the guaianolides **1–4** (Fig. 1) are similar to each other and differ only in the presence of a hydroxyl group at C-10 in the epimers **3** and **4** and a 1(10) double bond in compounds **1** and **2**. The ^1H and ^{13}C NMR spectral data of these

Table 1
^1H NMR spectral data of STLs **1–6** from *V. gardneri* (300 MHz, [a]400 MHz; $CDCl_3$; TMS as int. std.; *J* in Hz)

H	**1**	**2**[a]	**3**	**4**	**5**	**6**[a]
1	–	–	2.68 *d* (6.5)	2.91 *d* (7.3)	3.33 *br d*	5.13 *br d* (9.7)
2	–	–	–	–	–	4.44 *dd* (9.7, 7.9)
3	6.23 *m*	6.23 *m*	6.07 *m*	6.16 *br s*	6.17 *m*	4.11 *d* (7.9)
4	–	–	–	–	–	
5	3.54 *d* (10.4)	3.53 *br d* (10.4)	3.23 *br dd* (10.2, 6.5)	3. 35 *br dd* (11, 7.3)	3.21 *br d* (7.2)	5.11 *br d* (9.1)
6	4.09 *dd* (10.4, 10)	4.10 *dd* (10.4, 10.1)	5.20 *dd* (10.2, 9.7)	4.76 *dd* (11, 9.6)	4.54 *dd* (8.9, 7.2)	5.06 *dd t* (9.1, 8)
7	3.15 *dddd* (10, 3.2, 2.9, 2)	3.13 *m*	3.14 *dddd* (9.7, 3.4, 3.1, 2.5)	3.19 *dddd* (9.6, 3.3, 3.2, 2)	3.21 *dddd* (8.9, 3.3, 3, 2)	2.96 *m*
8	5.75 *dd* (6.1, 2)	5.74 *br d* (6.3)	5.72 *ddd* (4.3, 3.4, 2.5)	5.79 *ddd* (3.8, 3.5, 2)	5.62 *m*	5.79 *m*
9α	2.86 *dd* (15, 6.1)	2.85 *dd* (14.7, 6.3)	2.31 *dd* (15.9, 3.4)	2.32 *dd* (15.7, 3.8)	2.56 *dd* (12, 4.4)	2.82 *dd* (14.6, 4.8)
9ß	2.74 *br d* (15)	2.71 *br d* (14.7)	2.00 *dd* (15.9, 4.3)	2.08 *dd* (15.7, 3.5)	2.49 *d* (12)	2.34 *dd* (14.6, 2.4)
13a	5.55 *d* (2.9)	5.53 *d* (2.9)	5.59 *d* (3.1)	5.66 *d* (3.2)	5.63 *d* (3)	5.65 *d* (3)
13b	6.24 *d* (3.2)	6.23 *d* (2.9)	6.32 *d* (3.4)	6.35 *d* (3.3)	6.36 *d* (3.3)	6.32 *d* (3)
14	2.35 *s*	2.35 *br s*	1.52 *s*	1.21 *s*	4.97 *br s* 5.07 *br s*	1.56 *s*
15	2.35 *s*	2.34 *br s*	2.35 *br s*	2.39 *br s*	2.35 *br s*	1.82 *s*
2′	–		–	–	–	–
3′a	5.97 *dq* (1.1, 1.1)	6.71 *q* (6.7)	6.04 *dq* (1.1, 1.1)	6.00 *dq* (1.1, 1.1)	6.00 *dq* (1.1, 1.1)	5.65 *m*
3′b	5.57 *dq* (1.7, 1.7)	–	5.56 *dq* (1.7, 1.7)	5.59 *dq* (1.7, 1.7)	5.56 *dq* (1.7, 1.7)	6.05 *br s*
4′	1.86 *dd* (1.1, 1.7)	1.75 *br s*	1.88 *dd* (1.1, 1.7)	1.89 *br s*	1.87 *dd* (1.1, 1.7)	1.93 *br s*
5′	–	1.77 *m*	–	–	–	–
-OH	–	–	3.47 *s*	–	–	–

Table 2
^{13}C NMR spectral data of STLs **1–6** from *V. gardneri*

C	1	2[b]	3[a]	4	5	6
1	134.3	132.3	58.9	57.0	55.8	125.8
2	195.3	n.o.	206.5	210.3	205.9	74.4
3	136.0	136.0	132.6	132.5	133.5	83.1
4	169.3	169.1	179.0	181.1	177.5	143.2
5	55.5	52.9	53.9	52.5	53.3	131.1
6	79.1	79.4	77.1	76.1	77.4	74.2
7	53.1	55.7	49.6	50.1	49.5	52.2
8	64.8	n.o.	67.0	65.3	66.7	71.0
9	41.0	41.0	46.6	46.2	41.5	43.5
10	146.5	146.6	74.4	73.9	138.2	135.9
11	132.6	n.o.	134.6	133.5	132.7	135.5
12	168.3	168.2	169.3	168.4	172.2	169.1
13	120.7	n.o.	122.9	123.0	123.1	121.5
14	23.0	n.o.	31.8	27.2	120.6	13.0
15	18.1	n.o.	20.1	20.1	22.6	17.9
1′	166.4	166.9	166.7	165.9	166.1	165.1
2′	135.7	138.9	136.0	135.4	135.5	135.0
3′	127.0	127.3	127.2	126.7	126.6	126.3
4′	19.8	n.o.	18.6	18.3	18.2	19.7
5′	–	n.o.	–	–	–	–

[a] 75 MHz, 100 MHz, $CDCl_3$; TMS as int. std.
[b] Data from HMBC; n.o. = signal not observed.

compounds are summarized in Tables 1 and 2, which will be discussed together according to two similar data sets. Low energy conformations of the STLs **1**, **3–6** were obtained by quantum mechanical calculations (Fig. 2). The theoretical values of the coupling constants were deduced from these molecular models. They agreed well with those obtained from the experiments.

HREIMS data of **1** indicated the molecular formula $C_{19}H_{20}O_5$. The IR spectrum has characteristic bands of carbonyl groups of a γ-lactone, a conjugated ester and a conjugated ketone at 1774, 1717 and 1686 cm^{-1}, respectively. The ^{1}H NMR spectroscopic data were similar to the 2-oxo-8-(2-methyl-2,3-epoxybutanoyl)-guaia-1(10),3,11(13)-trien-6,12-olide previously described in *Helianthus* (Gao et al., 1987) except for those from the acyl moiety. They agreed well with those from methacrylic acid. Data from COSY and the observed coupling constants led us to propose the assignment of the ester moiety at C-8. In addition clear NOEs were observed between H-8, H-7 (10%) and H-9α (10%) of **1** thus confirming the β-orientation of the ester side chain at C-8. ^{13}C NMR, DEPT 135 and HMBC experiments led to the assignment of most carbon atoms of this compound (Table 2).

Compound **2**, for which high resolution MS indicated a molecular formula of $C_{20}H_{22}O_5$, differed from **1** only in

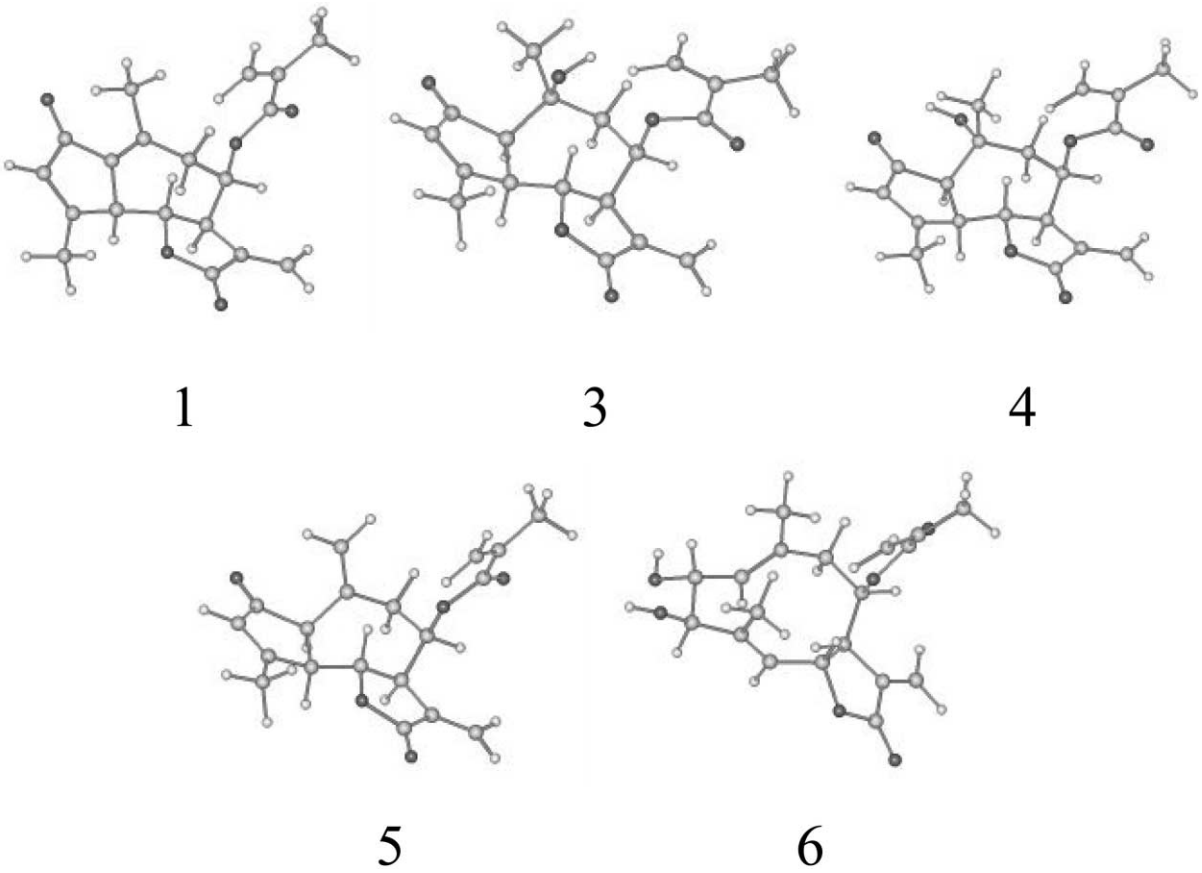

Fig. 2. Low energy conformations of the isolated STLs **1**, **3–6** as found by quantum mechanical calculations and by considering the NMR spectroscopic data (acyl side chains are not included in the conformational search).

the acyl moiety. NMR spectral data (^{1}H, COSY, ^{13}C and HMBC, Tables 1 and 2) along with the coupling constants confirmed the presence of the tigloyloxy moiety attached to C-8. Thus, compound **2** is a tiglate analogue of **1**.

MS data of **3** indicated the molecular formula $C_{19}H_{22}O_6$ which was established by HREIMS. IR data of **3** are very similar to those of **1**. They clearly show the presence of a hydroxyl group at 3471 and 3434 cm^{-1}, respectively. Careful inspection of the ^{1}H NMR spectral data showed that **3** had a hydroxyl group which has to be placed at C-10 as the singlet of the H-14 methyl group was shifted from δ 2.35 (**1**) to 1.52. In addition, the H-15 signal of **3** remained in the same position as **1** (δ 2.35) and showed a small coupling with H-3 (data from COSY), which appeared as a multiplet at δ 6.07 (Table 1). This proposal is also supported by the fact that the H-1 signal of **3** appears as a doublet at δ 2.68, and the H-5 signal as a broadened double doublet at δ 3.23, instead of a doublet as in **1** (δ 3.54). The H-6 signal appears as a double doublet at δ 5.20 (δ 4.09 in **1**), thus showing a deshielding effect of the hydroxyl group at C-10 in H-6, which must have a β-orientation. NOEs were observed between H-14, H-1 and H-9α. These data are in agreement with those of a known epoxyangelate analogue previously isolated from *Helianthus* (Gao et al., 1987).

The HREIMS data of **4** (Fig. 1) indicated the molecular formula $C_{19}H_{22}O_6$ while the IR spectrum is almost identical with that of **3**. Due to the α-orientation of the C-10 hydroxyl group the ^{1}H NMR spectral data of **4** (Table 1) differ from those of **3** only in the chemical shifts of three hydrogen signals: H-6, H-1 and H-14. The H-6 double doublet appears at δ 4.76 (instead of δ 5.20), the H-1 doublet at δ 2.91 (instead of δ 2.68) and the H-14 methyl group singlet at δ 1.21 (instead of δ 1.52). Correlation between these hydrogen atoms were observed in the COSY spectrum. The α-orientation of the hydroxyl group at C-10 is still supported by the absence of the deshielding effect of this same group in H-6. This structure could be assigned with certainty by careful analysis of NOESY data where interactions between H-6, H-9β and H-14 were observed.

Compound **5** (Fig. 1) has a molecular formula of $C_{19}H_{20}O_5$ determined by HREIMS. ^{1}H NMR (Table 1) and COSY spectrospic data were in accordance with 2-oxo-guaia-3,10(14),11(13)-trien-6,12-olide as basic guaianolide ring (Gutierrez and Herz, 1988) which was substituted with a methacryloyloxy residue at C-8. The H-15 methyl group signal at δ 2.35 is coupled with the H-3 signal at δ 6.17. The two broadened singlets at δ 4.97 and 5.07 indicate an exocyclic methylene at C-10. H-5 appears as a broadened doublet at δ 3.21 and H-1 appears as a broadened doublet at δ 3.33. All the carbon atoms were assigned based on ^{13}C NMR (Table 2) along with HMBC data.

Compound **6** has a molecular formula of $C_{19}H_{24}O_6$ as established by HREIMS. The analysis of the ^{1}H NMR and COSY spectroscopic data indicated a STL of the germacranolide type (*trans*, *trans*-germacrolide) as can be observed by the chemical shifts and coupling constants of H-1, H-5 and H-6 at δ 5.13, 5.11 and 5.06, respectively. The 2,3-*trans*-diol could be proposed by the presence of a double doublet at δ 4.44 ($J=9.7$ and 7.9 Hz) of H-2 and the doublet at δ 4.11 ($J=7.9$ Hz) of H-3. NOE between H-2 and H-14 was observed and a large coupling constant between H-2 and H-3 ($J=7.9$ Hz) confirmed their *trans* configuration. These data together led us to propose that the ten membered ring is in the $^{15}D_{5,1}D^{14}$ conformation (Watson and Kashyap, 1986). Calculation of the low energy conformation and the theoretical values of the coupling constants confirmed an up-up arrangement of the methyl groups of C-14 and C-15 (Fig. 2). The 8β-methacrylate side chain was easily assigned by the analysis of ^{1}H NMR data and COSY. The NMR spectroscopic data of **6** were almost identical with those reported in the 8β-5′-acetyl-angeloyloxy analogue previously isolated from species of *Eupatorium* (Herz et al., 1981). The chemical shifts of the ^{13}C were supported by HMBC data.

The flavonol isokaempferide, the flavone eupafolin and the coumarin prenyletin were identified on the basis of their spectroscopic data (UV, ^{1}H, and ^{13}C NMR). They were identical to those observed in the literature (Barberá et al., 1986; Da Costa et al., 1996).

V. gardneri is placed in the series Bracteatae (section Paradosa) and biosynthesizes STLs of a non-typical subgroup in the genus. The isolated compounds are not only found for the first time in nature, but this is also the first report of guaianolides in a Brazilian *Viguiera* species. So far only *V. sylvatica* of the section Diplostichis from Costa Rica (Central America; Tamayo-Castillo et al., 1989) and *V. tucumanensis* of the series *Bracteatae* from Argentina (South America; Meragelman et al., 1996) have shown guaianolides, also in co-occurrence with germacranolides. Other chemically investigated members from section Paradosa afforded heliangolides as the main compounds (Da Costa et al., 1996; Romo de Vivar and Delgado, 1985; Spring et al., 2001). This fact can be a useful feature when considering further chemotaxonomic considerations.

STLs are not only interesting compounds for chemotaxonomic studies, but they also possess a wide range of biological activities (Picman, 1986), among which the anti-inflammatory activity has been intensively studied (e.g. Hall et al., 1979, 1980). Very recently, it was shown that they partly exert this effect by inhibiting the transcription factor NF-κB (Castro et al., 2000a, b; Lyss et al., 1997, 1998; Rüngeler et al., 1998, 1999). In order to investigate whether the STLs **1**, **3** and **4** also interfere with this molecular target, Jurkat T cells were incubated with the respective STLs at various concentrations for 1 h and subsequently stimulated with TNF-α for 1 h. Total protein extracts were prepared and analyzed for

NF-κB DNA binding activity in an electrophoretic mobility shift assay (EMSA) (Fig. 3). Stimulation with TNF-α induced one novel DNA binding activity in Jurkat T cells (Fig. 3, lane 2). Antibody reactivity and competition assays identified this complex as a NF-κB p50/p65 heterodimer (data not shown; see Pahl and Baeuerle, 1995; Brown et al., 1993).

All compounds completely inhibited NF-κB DNA binding at concentrations between 10 and 50 μM without showing any cytotoxic effects (Fig. 3). STL **1** was the most active. Complete inhibition was observed at a 10 μM concentration (Fig. 3, lane 4). A very slight band for the NF-κB DNA complex could be observed at a 20 μM concentration of compound **4** (Fig. 3, lane 10), which differs from **1** by an α-hydroxy group at C-10 (Fig. 1). However, if the hydroxyl group is β-configured as in compound **3**, the concentration which causes complete inhibition increased to 50 μM (Fig. 3, lane 8). Interestingly, these guaianolides act as strong NF-κB inhibitors. However, they possess an α-methylene-γ-lactone, but not a further α,ß-unsaturated carbonyl structure, like e.g. in helenalin, which can easily react with nucleophiles in a Michael type addition. These structure elements were previously proposed as a prerequisite for a strong inhibition of NF-κB. STLs which possess only one of these reactive groups were less active (Rüngeler et al., 1999). A similar activity as observed for **1**, **3** and **4** was already proven for some monofunctional guaianolides.

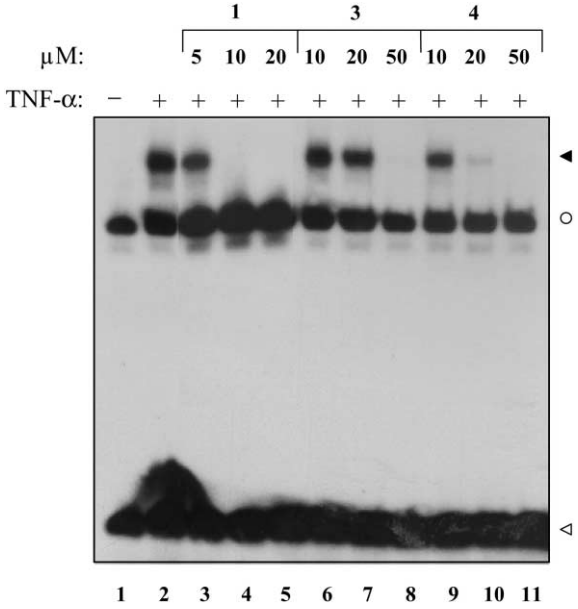

Fig. 3. The effect of the STLs **1**, **3** and **4** on NF-κB DNA binding. Lane 1 shows unstimulated control cells, in lane 2 cells were treated with 200 U/ml TNF-α alone. In lanes 3–8 the cells were pretreated for 1 h with various concentrations of studied STLs and subsequently stimulated with TNF-α for 1 h. A filled arrowhead indicates the position of NF-κB DNA complexes. The open circles denote a non-specific activity binding to the probe. The open arrowhead shows unbound oligonucleotide.

Further studies are in progress to explain the NF-κB inhibitory activity of these guaianolides.

Moreover, the guaianolides **1**, **3** and **4** inhibit the transcription factor NF-κB at micromolar concentrations. Thus, it is highly likely that preparations from *V. gardneri* may also exhibit anti-inflammatory activities in vivo, which has to be proven in further studies.

3. Experimental

3.1. General

IR spectra were recorded in $CHCl_3$ in a Nicolet-Protègè 460 and a Perkin Elmer 1600. ^1H and ^{13}C NMR spectra were run on a Bruker Advance DPX 300 (^1H, 300 MHz; ^{13}C, 75 MHz) and a Brücker Advance DPX 400 (^1H, 400 MHz; ^{13}C, 100 MHz), the chemical shifts in ppm and using TMS as int. standard. The NOE experiments were run at 300 MHz in $CDCl_3$. EIMS and HREIMS were measured on a Finnigan MAT8200; ESIMS on a Finnigan TSQ7000. HPLC analyses used a Shimadzu SCL-10Avp liquid chromatograph with a Shimadzu SPD-M10Avp UV-Vis-DAD detector operating with the Class-VP software, v. 5.02.

3.2. Plant material

Aerial parts of *V. gardneri* were collected in the surrounding area of Goiânia, in the S.A. Descoberto-Cidade Eclética highway (S 15°53′, W 48°19′, altitude 3388±316 ft), State of Goiás, Brazil, in April 1998. Plant material was identified by E. E. Schilling (Department of Botany, University of Tennessee, Knoxville, TN, USA) and by J. N. Nakajima (Instituto de Biologia, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil). A voucher specimen (FBC # 81) is deposited at the Herbarium of the Departamento de Biologia, FFCLRP, Universidade de São Paulo, Ribeirão Preto, SP, Brazil, with the code SPFR 4443.

3.3. Cell culture

Jurkat T cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 100 IU/ml penicillin and 100 μg/ml streptomycin (all Gibco-BRL).

3.4. Electrophoretic mobility shift assays

Total protein extracts from Jurkat T cells were prepared using a high-salt detergent buffer (Totex: 20 mM Hepes, pH 7.9, 350 mM NaCl, 20% (v/v) glycerol, 1% (w/v) NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.1% PMSF, 1% aprotinin). Cells were harvested by centrifugation, washed once in ice cold PBS (Sigma) and resuspended in four cell

volumes of Totex buffer. The cell lysate was incubated on ice for 30 min, then centrifuged for 5 min at 13,000 rpm at 4 °C. The protein content of the supernatant was determined and equal amounts of protein (10–20 μg) added to a reaction mixture containing 20 μg BSA (Sigma), 2 μg poly (dl-dC) (Boehringer), 2 μl buffer D+ (20 mM Hepes, pH 7.9; 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1% PMSF), 4 μl buffer F (20% Ficoll 400, 100 mM Hepes, 300 mM KCl, 10 mM DTT, 0.1% PMSF) and 10^5 c.p.m. (Cerenkov) of a ^{32}P-labeled oligonucleotide, made up to a final volume of 20 μl with distilled H_2O. Samples were incubated at room temp. for 25 min. NF-κB oligonucleotide (Promega) was labeled using γ-[^{32}P]-ATP (3,000 Ci/mM; Amersham) and a T4 polynucleotide kinase (Promega).

3.5. Calculation of the conformations for STLs *1*, *3–6*

We generated low-energy conformations of the STLs using the conformational search option of ChemPlus® (v. 2.0), which is operated under the Molecular Modeling package Hyperchem® (v. 5.1 Professional). Energy minimizations were performed with Hyperchem's semi-empirical quantum mechanical method AM1 using the Polak-Ribiere minimization algorithm. Starting structures were created with Hyperchem and initially minimized to an RMS gradient <0.01 kcal mol^{-1} Å^{-1}. All rotatable cyclic bonds were included as variable torsions and allowed to be changed simultaneously. The search was performed applying a usage directed search method and standard settings for duplication tests. A search run was terminated after energy minimization of 2500 unique starting geometries. Acyl side chains of the respective STLs were not included in the conformational search. The resulting structures were energy minimized to a RMS gradient as above. Coupling constants were calculated with PCMODEL™ using the MOPAC form of the conformations created with HyperChem™.

3.6. Extraction and isolation

Screening for STLs was performed by microsampling of glandular trichomes according to Spring (1991). The material for the HPLC runs (glands and leaf rinse extract) was first analyzed in the liquid chromatograph (Shimadzu ODS column, 4.6×250 mm, 5 μm, MeOH–H_2O 55:45, 1.0 ml min^{-1}, MeCN–H_2O 35:65, 1.3 ml min^{-1}, 2,5-dimethylphenol as int. standard, UV detection simultaneously at 225 and 265 nm and DAD). The compounds were isolated by means of prep. HPLC (Shimadzu ODS column, 20×250 mm, 5 μm, MeOH-H_2O 55:45, 6.0 ml min^{-1}, UV at 225 nm) from an extract of intact dried leaves (35.0 g) rinsed with CH_2Cl_2 for 10 min at room temp. in an incubator shaker. The extract was filtered through common filter paper and concentrated in vacuum to yield a dry residue (2.0 g), which was later re-suspended in MeOH. This material was partitioned twice with *n*-hexane. The MeOH-soluble phase was concentrated in vacuum and, in order to eliminate non-polar compounds, the residue was re-suspended in MeOH–H_2O 3:1. The supernatant was concentrated in vacuum to yield a STL-rich (IR analysis) residue (1.3 g). Several aliquots of this residue were finally chromatographed on the preparative column to afford compounds **1** (60 mg), **2** (1 mg), **3** (27 mg), **4** (42 mg), **5** (4 mg), **6** (25 mg), isokaempferide (20 mg), eupafolin (20 mg) and prenyletin (3 mg). All these compounds were observed in the analytical HPLC traces (conditions as given above) of the manually collected glands from the leaves.

3.6.1. 2-One-8β-methacryloyloxy-guaia-1(10),3,11(13)-trien-6α,12-olide (*1*)

White solid. UV-DAD λ_{max} 250 nm; IR ν_{max}^{CHCl3} cm^{-1}: 1145, 1619 (C=C), 1686 (C=O, ketone), 1717 (C=O, ester), 1774 (C=O, γ-lactone), 3071; for ^{1}H and ^{13}C NMR spectra, see Tables 1 and 2; EIMS 70 eV, *m/z* (rel. int.): 328 $[M]^+$ (38), 242 $[M–C_4H_6O_2]^+$ (17), 227 (10), 214 (8), 199 (10), 186 (11), 171 (9), 146 (8), 91 (16), 69 $[Meacr]^+$ (100); ESIMS, *m/z* (rel. int.): 329 $[M+H]^+$ (67), 361 $[M+MeOH+H]^+$ (100); HREIMS *m/z* $[M]^+$ 328.1310 (calc. for $C_{19}H_{20}O_5$).

3.6.2. 2-One-8β-tigloyloxy-guaia-1(10),3,11(13)-trien-6α,12-olide (*2*)

Colorless gum. UV-DAD λ_{max} 213 nm; for ^{1}H and ^{13}C NMR spectra, see Tables 1 and 2; EIMS 70 eV, *m/z* (rel. int.): 342 $[M]^+$ (97), 242 $[M–C_5H_8O_2]^+$ (49), 227 (19), 149 (40), 146 (13), 91 (18), 83 $[Tigl]^+$ (97), 55 (100); ESIMS, *m/z* (rel. int.): 343 $[M+H]^+$ (72), 764 $[2x (M+K+H^+)]^+$ (100), 375 $[M+MeOH+H]^+$ (39); CIMS (Isopropyl) 240 eV, *m/z* (rel. int.): 343 $[M+H]^+$ (12), 243 $[M–Tigl]^+$ (17), 101 $[OTigl+2H]^+$ (100), 83 $[Tigl]^+$ (10); CIMS (NH_3) 240 eV, *m/z* (rel. int.): 343 $[M+H]^+$ (100), 260 $[M–Tigl+H]^+$ (28), 243 $[M–C_5H_8O_2+H]^+$ (23), 83 $[Tigl]^+$ (8); HREIMS *m/z* $[M]^+$ 342.146725 (calc. for $C_{20}H_{22}O_5$).

3.6.3. 2-One-8β-methacryloyloxy-10β-hydroxy-guaia-3,11(13)-dien-6α,12-olide (*3*)

White solid. UV-DAD λ_{max} 220 nm; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1156, 1625 (C=C), 1693 (C=O, ketone), 1712 (C=O, ester), 1761 (C=O, γ-lactone), 3103, 3471 (OH); for ^{1}H and ^{13}C NMR spectra, see Tables 1 and 2; EIMS 70 eV, *m/z* (rel. int.): 346 $[M]^+$ (1), 328 $[M–H_2O]^+$ (1), 260 (11), 242 $[M–C_4H_6O_2]^+$ (35), 165 (22), 138 (30), 123 (16), 96 (34), 69 $[Meacr]^+$ (100); ESIMS, *m/z* (rel. int.): 369 $[M+Na]^+$ (19), 385 $[M+K]^+$ (45), 401 $[M+Na+MeOH]^+$ (100), 715 $[2x (M+Na)]^+$ (13); HREIMS *m/z* $[M]^+$ 346.1416 (calc. for $C_{19}H_{22}O_6$).

*3.6.4. 2-One-8β-methacryloyloxy-10α-hydroxy-guaia-3,11(13)-dien-6α,12-olide (**4**)*

White solid. UV-DAD λ_{max} 211 nm; IR $\nu_{max}^{CCCl_3}$ cm^{-1}: 1151, 1619 (C=C), 1678 (C=O, ketone), 1716 (C=O, ester), 1771 (C=O, γ-lactone), 3104, 3434 (OH); for ^1H ^{13}C NMR spectra, see Tables 1 and 2; EIMS 70 eV, *m/z* (rel. int.): 346 $[M]^+$ (1), 328 $[M–H_2O]^+$ (9), 260 (16), 242 $[M–C_4H_6O_2]^+$ (31), 196 (11), 165 (19), 96 (14), 69 [Meacr]$^+$ (100); ESIMS, *m/z* (rel. int.): 347 $[M+H]^+$ (21), 369 $[M+Na]^+$ (31), 385 $[M+K]^+$ (14), 401 $[M+Na+MeOH]^+$ (53), 715 $[(M\times2)+Na]^+$(100); HREIMS *m/z* $[M]^+$ 346.1416 (calc. for $C_{19}H_{22}O_6$).

*3.6.5. 2-One-8β-methacryloyloxy-guaia-3,10(14),11(13)-trien-6α,12-olide (**5**)*

Colorless gum. UV-DAD λ_{max} 215 nm; for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; EIMS 70 eV, *m/z* (rel. int.): 328 $[M]^+$ (14), 268 (7), 242 $[M- C_4H_6O_2]^+$ (29), 213 (6), 185 (6), 149 (15), 91 (8), 69 [Meacr]$^+$ (100); HREIMS *m/z* $[M]^+$ 328.1310 (calc. for $C_{19}H_{20}O_5$).

*3.6.6. 2α,3β-Dihydroxy-8β-methacryloyloxy-germacra-1(10),4,11(13)-trien-6α,12-olide (**6**)*

Colorless gum. UV-DAD λ_{max} 210 nm; for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; EIMS 70 eV, *m/z* (rel. int.): 348 $[M]^+$ (1), 276 (1), 262 (7), 218 (7), 192 (8), 149 (7), 121 (8), 95 (13), 69 [Meacr]$^+$ (100); ESIMS, *m/z* (rel. int.): 371 $[M+Na]^+$ (16), 387 $[M+K]^+$ (27), 403 $[M+Na+MeOH]^+$ (100), 419 $[M+K+MeOH]^+$ (7), 719 [2x $(M+Na)]^+$ (30), 735 [2x $(M+K)]^+$ (8); HREIMS *m/z* $[M]^+$ 348.1572 (calc. for $C_{19}H_{24}O_6$).

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