Transformed Cell Suspension Culture of *Galphimia glauca* Producing Sedative Nor-friedelanes

Authors **Anabel Ortíz**[1], **Alexandre Cardoso-Taketa**[2], **Mario Rodríguez Monroy**[3], **Jesús Arellano**[3], **Georgina Hernández**[3], **María Luisa Villarreal**[1]

Affiliations
[1] Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, México
[2] Centro de Investigación en Productos Bióticos, Instituto Politécnico Nacional, Yautepec, Morelos, México
[3] Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México

Key words
- *Galphimia glauca*
- Malpighiaceae
- transformed suspension cell cultures
- triterpenes
- nor-friedelanes
- sedative

Abstract

The Mexican species *Galphimia glauca* (Cav.) Kuntze (Malphigiaceae) synthesises a family of sedative and anxiolytic nor-secofriedelanes, designated as galphimines. These active principles accumulate at low concentration in the aerial parts of plants from wild populations. Transformed calluses and cell suspension cultures of this species were established in order to induce a greater production of nor-friedelanes. The cell suspension line GgBa was selected and grown over a period of two years of continuous subculturing in MS nutrient medium in the absence of growth regulators. PCR and Southern blot analyses were employed in order to confirm that the *rol* A gene had been integrated into the plant genome. Batch cultures of the GgBa cell line were grown over a 32-day period and first-order growth kinetics was observed, reaching a specific growth rate (μ) of 0.13 d^{-1}. The production of glaucacetalin A (**10**), a triterpenoid related to the known galphimines, was quantified in the nutrient medium by HPLC. The transformed cell suspension culture GgBa also synthesised a novel nor-friedelane, given the name glaucacetalin D (**13**). High-resolution spectroscopic and spectrometric techniques were employed to elucidate the structure of **13**. This triterpene has never been observed in wild plant tissues or in other *in vitro* cultures. Maslinic acid (**14**) was identified in cell biomasses. The triterpene production of the cell line GgBa was as follows: glaucacetalin A, 2.7 mg/L; glaucacetalin D, 2.9 mg/L and maslinic acid, 2.4 mg/g dry weight. The sedative activity of compounds **10** and **13** was demonstrated in ICR mice by using the sodium pentobarbital-induced hypnosis model. No cytotoxicity of **10** and **13** was exhibited against KB, MCF-7 and HF6 human cancer cell lines.

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Correspondence
Dr. María Luisa Villarreal
Centro de Investigación en Biotecnología
Universidad Autónoma del Estado de Morelos
Av. Universidad 1001
Col. Chamilpa
Cuernavaca
62209 Morelos
Mexico
Phone: + 52 77 73 29 70 57
Fax: + 52 77 73 29 70 30
luisav@cib.uaem.mx

Introduction

A family of nine nor-secofriedelanes, designated as galphimines A–I (**1–9**) was isolated and elucidated from extracts manifesting sedative effects, obtained from the aerial parts of *Galphimia glauca* (Cav.) Kuntze. This member of the Malphigiaceae family is traditionally employed for the treatment of central nervous system disorders [1, 2]. Taking these compounds and using *in vitro* models [1], galphimine B (**1**) was shown to have sedative activity, whereas galphimines A (**3**), B (**1**) and E (**2**) displayed anxiolytic activity in mice [3]. The yield of galphimines, harvested from wild specimens is low, and their accumulation in leaves fluctuates depending on stationary as well as ontogenic variables. Moreover, in a recent investigation we demonstrated that these sedative compounds only accumulate in populations of *G. glauca,* growing in restricted locations within Mexico [4]. This situation makes it difficult to obtain an adequate supply of raw plant material from this species for conducting pharmacological and clinical assays and for increased commercialisation of this plant. There is a pressing need to characterise the triterpenoid profile of *G. glauca,* as a prerequisite for establishing the biosynthetic pathway that is involved in the production of active principles. It is also important to undertake biotechnological investigations in order to obtain plant genotypes with the capacity to accumulate greater and controlled quantities of these sedative compounds. In previous investigations, we established *in vitro* cell and organ culture systems for *G. glauca.* Initially, we developed callus and cell suspension cultures, where nutritional and hormonal factors,

as well as different process formats were evaluated and optimised, in order to induce cell growth and synthesis of compounds **1** and **2** [5,6]. More recently, we established transformed hairy root lines by infecting cotyledons of *G. glauca* with *Agrobacterium rhizogenes* ATCC 15834 [7]. These transformed cultures synthesised galphimine E (**5**), three new nor-friedelanes structurally related to galphimines, designated as glaucacetalins A–C (**10–12**), and maslinic acid (**14**) [8] (**Fig. 1**). Even though these results indicate that it is possible to use hairy root cultures to produce nor-friedelanes of *G. Glauca*, as with other plant species, the scale-up of growth in bioreactors of greater volume may prove difficult, thus making it difficult to use this technology on a commercial scale [9]. Transformed cell cultures represent a potentially valuable system, capable of synthesising an important number of secondary metabolites [10]. In comparison with non-transformed systems, these transformed cultures exhibit stable and homogeneous fast growing biomasses that can more easily be scaled-up into high capacity bioreactors, than is the case for roots [11].

This paper focuses on the establishment of transformed callus and cell suspension cultures of *G. glauca* that were observed emerging from the hairy roots of this species. Polymerase chain reaction (PCR) and Southern blot analysis were used to verify the transformed state of the cultures. The accumulation of compound **10** was evaluated using a chemical analysis of the culture medium, and a new nor-friedelane (**13**) was also isolated and elucidated, using high resolution spectroscopic and spectrometric techniques. The neuropharmacological effects of **10** and **13** were evaluated by using ICR mice in the elevated plus maze, as well as the sodium pentobarbital-induced hypnosis model. Cytotoxic activity of **10** and **13** against KB (nasopharyngeal carcinoma), MCF-7 (breast carcinoma) and HF6 (colon carcinoma), was also evaluated.

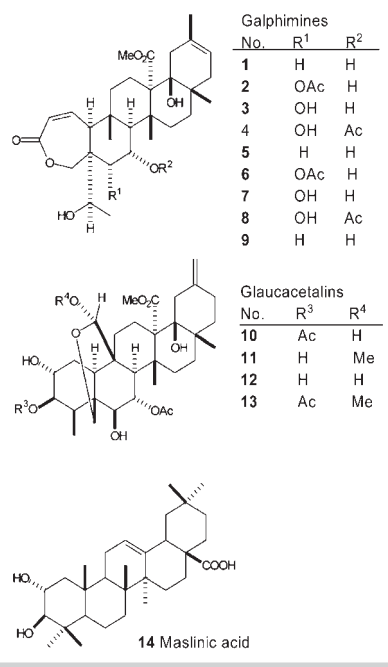

Fig. 1 Triterpenes synthesized by *G. glauca*: galphimines (wild plants), glaucacetalins and maslinic acid (transformed cell cultures).

Material and Methods

General experimental procedures

Data acquisition and processing were performed with MassLynx 4.0 software. High-resolution electrospray mass spectra (ESI-HR-MS) in the positive ion mode were obtained on a Q-TOF Ultima Global hybrid quadrupole time-of-flight instrument (Waters-Micromass), equipped with a pneumatically assisted electrospray (Z-spray) ionisation source and an additional sprayer (lock spray) for the reference compound. 300 MHz ^{1}H- and 75.4 MHz ^{13}C-NMR spectra were recorded on a Bruker spectrometer while HMBC measurements were determined on a Bruker 500 MHz spectrometer. The instrumentation for HPLC analysis consisted of a Waters (Millipore Corp.) 600E multisolvent delivery system, equipped with a Waters W996 diode array detector (216 nm) and an automatic sample delivery system. Control of equipment, data acquisition, processing, and management of chromatographic information was performed, using the Millenium 32 software programme (Waters).

Plant material

Undifferentiated calluses were obtained from tissue growing in small aggregates in the centre of the hairy root line VYT, which was previously established by infection of *G. glauca* explants with *Agrobacterium rhizogenes* ATCC 15834 [7]. Voucher specimens of *G. glauca* are on deposit at the Instituto Mexicano del Seguro Social Herbarium (IMSSM: 11061), Centro Médico Nacional SigloXXI, Mexico City.

Culture of transformed calluses

Small callus tissue was separated from hairy roots and transferred to solid B5 medium without phytohormones, and supplemented with 3% sucrose and 1.5 g/L polyvinylpyrrolidone (PVP), incubated at 25 °C, under continuous white light (illumination of 25 $\mu Mn^{-2} \cdot s^{-1}$) and subcultured every 15 days.

Cell suspension cultures

Cell suspension cultures were established by disgregation of friable calluses, using an inoculum of 1.1 g/L dry weight. Suspensions were initiated in 250-mL Erlenmeyer flasks, each containing 100 mL MS medium supplemented with 3% sucrose and 1.5 g/L of polyvinylpyrrolidone, in the absence of phytohormones. The cultures were agitated in a rotary shaker (110 rpm) in light conditions as described above, and subcultured every 15 days.

For kinetic studies, the cultures were grown in a batch mode for 32 days, and the subsequent parameters on growth and metabolite production were measured every three days (three replicates): dry weight, fresh weight, pH, cell viability, and triterpene production. Fresh weight was determined as filtered biomass, dry weight determined after lyophilisation, and cell viability was defined using the fluorescein diacetate method. HPLC analysis for the identification and quantification of triterpenes is described in the corresponding section.

PCR and Southern blot analysis

DNA from transformed calluses, cell suspensions cultures, untransformed plant controls, as well as from *A. rhizogenes* ATCC 15834, was isolated using the Puregene DNA purification kit (Gentra Systems), as described by the manufacturer.

The polymerase chain reaction (PCR) was employed in order to confirm the presence of *rol A* and *rol C* genes in calluses and cell cultures. PCR experiments were performed using the following reported specific primer pairs [12]: the nucleotide sequences for positions 21–42 (5′-CGTTGTCGGAATGGCCCAGACC-3′) and 268–246 (5′-CGTAGGTCTGAATATTCCGGTCC-3′) were used to amplify

a DNA fragment of 248 bp of *rol A*; and the sequences for positions 51–70 (5′-TGTGACAAGCAGCGATGAGC-3′) and 550–531 (5′-GATTGCAAACTTGCACTCGC-3′) were used to amplify a fragment of 487 bp of *rol C* gene. Each PCR reaction contained PCR SuperMix (Invitrogen), 200 nM of each primer and 300 ng of genomic DNA. PCR conditions were: 5 minutes at 95 °C, followed by 35 cycles, one minute at 95 °C, one minute at 50 °C, one minute at 72 °C. The amplified fragments were resolved in 1.0% (w/v) agarose-TAE gel and visualised by staining with ethidium bromide.

For Southern blot analysis, DNA (15 µg per reaction) was digested with *Eco*R1, electrophoresed in 1% agarose gel and subsequently blotted onto a nylon membrane (Hybond Plus Amersham). The membrane was pre-hybridised in Rapid-hyb buffer (Amersham Biosciences) for 2 h at 65 °C and hybridised overnight to a ^{32}P-labelled probe for *rol A* gene. The probe was obtained by PCR using DNA from *A. rhizogenes* ATCC 15834 as template and the gene-specific primers for *rol A* gene, as previously described. Labelling was carried out using the Amersham Megaprime DNA labelling system. Following hybridisation, the membrane was washed for 30 min in 2 × SSC, 0.1% SDS at 65 °C; 30 min in 0.5 × SSC, 0.1% SDS at 65 °C and 45 min in 0.1 × SSC, 0.1% SDS at 65 °C. It was then exposed to X-ray film, between intensifying screens for 24 h, at − 70 °C.

Isolation and purification of nor-friedelanes

The nutrient medium collected after several subcultures was subjected to three extractions, each using 30 mL of EtOAc. The extracts were combined and evaporated until dry. Subsequently the residue was fractionated over a silica gel column, using a gradient of $CHCl_3$ and MeOH (0–10% v/v) as eluent. Fractions were collected, examined by TLC and combined, in order to yield a crude mixture of nor-friedelanes (**10** and **13**). The final purification was achieved using silica gel 60 (15–40 µm) and an isocratic elution of $CHCl_3$ and MeOH (5% v/v), where an enriched fraction with compound **13** (12 mg) was chromatographed and 10 fractions (each of 0.8 mL) were collected. Fraction number 8 yielded 3.1 mg of the pure compound **13**.

Identification of glaucacetalin D (13)

Colourless oil, $C_{35}H_{54}O_{11}$; MS-MS (ESI-TOF, 60 V): m/z = 671.3 [M + Na]$^+$, 611, 511, 479, 451, 419, 391, 373, 267, 249, 151; HR-MS (ESI-TOF): m/z = 671.3406 [M + Na]$^+$ (calcd. for $C_{35}H_{52}O_{11}Na$: − 0.2 ppm error). ^{1}H- and ^{13}C-NMR: see ◉ **Table 1**.

Identification and quantification of triterpenes

Cell suspension samples were harvested every three days, providing biomasses and nutrient media. The media (100 mL) were filtered from the biomasses and extracted (3×), using 10 mL of EtOAc. The organic phases were dried, filtered and reduced to 500 µL, for analysis with HPLC following filtration through a Millipore filter (0.5 µm). The biomasses were lyophilised, ground, and extracted (3×) with EtOAc (10 mL) for 10 min, by sonication at room temperature. Each extract was reduced to 500 µL following filtration through a millipore filter (0.5 µm).

Preparation of standards: For preparation of standards, purification of compounds **10, 13** and **14** was carried out as described earlier [2, 7].

The identity of **14** was confirmed using ^{1}H- and ^{13}C-NMR spectroscopic analysis and by comparing with reported data [13].

Identification of compounds by HPLC: Compounds **10** and **13** were identified in cell cultures samples using an HPLC procedure previously described [7], comparing retention times of authentic samples, by HPLC co-elution experiments. This analysis was performed using a Symmetry C_{18} column (Waters; 5 µm, 4.6 × 250 mm), an isocratic elution of CH_3CN-H_2O (1 : 1), a flow rate of 0.7 mL/min, and 10 µL of the sample injection (1 mg/100 µL).

Quantitative determinations: Suitable calibration curves for **10, 13** and **14** were constructed by injecting a series of standard solutions, ranging between 0.02–2 mg/L. The standards reached a purity > 99% following HPLC analysis. Determinations were carried out in duplicate and the peak area was measured to obtain average results for each calibration curve. Correlation coefficients of the calibration curves (> 0.9907) demonstrated that the relationship between peak areas and concentrations of each standard was linear under these chromatographic conditions. Sample solutions (500 µL) were injected and the peak areas were compared with calibration curves.

Neuropharmacological assays

Sodium pentobarbital induced-sleeping time test: Experiments conducted on ICR male mice were performed according to the law and institutional guidelines and approved by the Ethics and Security Committee from the Centro de Investigación en Biotecnología, UAEM. Food and water were available *ad libitum* and the mice (35–40 g) were conditioned to a 12-h light/dark cycle, at a temperature of 22 ± 1 °C. Animals were arbitrarily assigned to one of the following categories: group 1: negative controls, where each animal was intraperitoneally (*i.p.*) injected with 0.75 mL/100 g of the vehicle (8% Tween 80, Sigma, in saline); group 2: positive controls that were injected with *i.p.* 0.1 mg/100 g of diazepam (Relazepam from Pisa Lab.); groups 3, 4 and 5: that were treated individuals, injected *i.p.* with 5 mg/100 g of compounds **1, 10** and **13**, respectively, dissolved in the same vehicle as used for the controls. Following their injection, all tested animals were evaluated according to two neuropharmacological models; the elevated plus maze [14] and the sodium pentobarbital-induced hypnosis [15].

Elevated plus maze test: The aluminium platforms of the elevated plus maze were painted white with impermeable epoxy resin. The model consisted of two flat opposing open arms (10 cm wide and 50 cm long) and two opposite enclosed arms, perpendicular to the first pair and of the same size, but with 20 cm high plexiglass enclosing walls. The junction area of the four arms (central platform, 10 × 10 cm) intersected to form the shape of a plus sign. The walls were easily removed for cleaning after each animal was tested. The whole apparatus was elevated to approximately 50 cm above the floor. The testing room was quiet and dimly lit. Mice were individually placed on the open arms, facing the centre of the maze. After administration of the test sample (25 min), the activity of the mouse was recorded in relation to the time spent in the enclosed or open platforms. The scores were evaluated by applying the formula [open/(open + enclosed)] × 100.

Sodium pentobarbital-induced sleeping time test: Sodium pentobarbital (40 mg/kg) was *i.p.* injected into each mouse, 30 min after the administration of vehicle, diazepam and the tested compounds (**1, 10** and **13**). Sleeping time was defined as the interval between onset, up until the loss of righting reflex through to recovery. Results were scored in relation to the number of observations per vehicle (6), diazepam (5), compound **1** (5), compound **10** (7), and compound **13** (5) in both pharmacological tests [14, 15].

The SAS (SAS Institute, Inc.) 9.1 programme was used for statistical analysis. The results are presented as mean ± S.D. Data were

Position	10		13	
	δ_H	δ_C	δ_H	δ_C
1a	1.71	25.5	1.67	25.9
1b	2.43		2.34	
2	3.85	66.5	3.85	66.9
3	4.61 t (2.9)	76.9	4.62 t (3.3)	77.5
4	1.98	37.5	1.98	37.7
5		42.7		43.0
6	3.49	85.4	3.52	85.9
7	5.54 dd (10.9, 7.0)	78.7	5.55 dd (11.0, 6.7)	79.3
8	2.34 d (10.9)	51.6	2.43 d (11.1)	52.3
9		40.6		40.8
10	1.42	40.9	1.40	41.6
11a	0.85	31.1	0.82	30.8
11b	2.20		2.00	
12a	1.95	23.3	1.93	23.8
12b	2.10		2.08	
13		57.4		57.8
14		43.4		43.8
15a	1.25	29.9	0.97	30.3
15b	2.95		2.93	
16a	0.90	34.7	1.28	35.1
16b	1.98		2.05	
17		37.5		37.9
18		78.1		78.2
19a	2.10	42.5	2.25	42.9
19b	2.98 d (15.2)		3.01 d (15.3)	
20		144.7		145.1
21a	1.98	28.6	2.03	29.0
21b	2.30		2.33	
22a	1.04	35.7	1.08	36.2
22b	2.04		2.07	
23	1.09 d (7.3)	13.5	1.10 d (7.3)	13.9
24a	3.78 d (11.5)	54.8	3.76 d (11.2)	55.1
24b	4.23 d (11.5)		3.97 d (11.2)	
25	5.93 d (3.1)	94.2	5.34	101.7
26	1.35 s	21.7	1.54 s	23.3
27		175.6		176.0
28	1.05 s	25.5	1.08 s	25.9
29a	4.47 q (2.4)	108.9	4.50 q (2.4)	109.3
29b	4.68 q (2.4)		4.70 q (2.4)	
25-OCH_3			3.36 s	55.7
27-OCH_3	3.47 s	51.4	3.49 s	51.8
3-Ac	2.07 s	21.1/170.5	2.08 s	21.5/170.7
7-Ac	2.08 s	22.1/173.9	2.10 s	22.5/174.3

Table 1 ^{1}H- and ^{13}C-NMR data (δ values, $CDCl_3$, *J* in Hz) for compounds **10** (500 MHz) and **13** (300 MHz).

analysed using a one-way ANOVA, followed by Dunnett's t-test. The significant difference was established for groups where the p value was lower than 0.05.

Cytotoxicyty assay: Compounds **10** and **13** were subjected to a cytotoxicity evaluation using nasopharyngeal (KB), breast (MCF-7) and colon (HF6) carcinoma cell lines. The cells were maintained in RPMI medium supplemented with 10% fetal bovine serum. Cell lines were cultured at 37 °C in an atmosphere of 5% CO_2 in air (100% humidity). The cell cultures at log phase were treated in triplicate with various concentrations of the test samples (0.16–20 µg/mL) and incubated for 72 h in the conditions described above. The cell density was determined by the NCI sulforhodamine method [16]. Results were expressed as the dose that inhibits 50% control growth after the incubation period (ED_{50}). The values were estimated from a semilog plot of the drug concentration against the percentage of viable cells. Campthotecin (Sigma) was included as a positive control.

Supporting information

Image of calluses emerging from hairy roots of *G. glauca* and HPLC chromatogram of compounds **10** and **13** are available as Supporting Information.

Results and Discussion

G. glauca undifferentiated tissue, originating from the hairy root cell line VYT was appropriate for cultivation, and abundant phytohormone-independent growth of calluses was observed in B5 solid nutrient medium, which were transferred to MS liquid medium in order to initiate cell suspension cultures. After 12 sequential subcultures the cell suspension line GgBa was successfully grown in the absence of phytohormones. The use of flasks with baffles proved effective for tissue disgregation. This cell line was selected for the analysis of genetic transformation events, as well as for kinetic growth and production of nor-friedelanes, be-

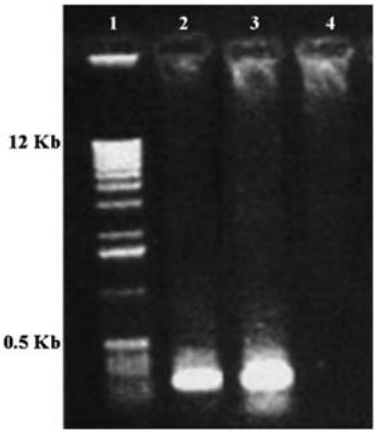

Fig. 2 PCR analysis *of G. glauca* transformed cell suspension culture GgBa. Lane 1: DNA marker; lane 2: positive control: PCR product from total DNA of *A. rhizogenes* showing amplification of a fragment of 248 bp of the *rol A* gene; lane 3: PCR product from DNA of GgBa cell suspension line in which the amplification of the 248 bp fragment of *rol A* was positive; lane 4: none PCR product from DNA of untransformed *G. glauca* cell line was observed.

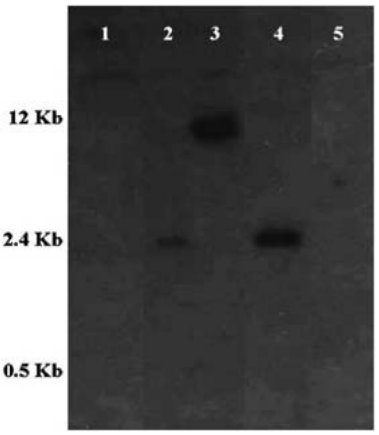

Fig. 3 Southern blot analysis of the transformed cell suspension line GgBa carried out using as a probe the PCR product of *rol A* gene amplified from *A. rhizogenes* DNA. Lane 1: DNA marker 1KB; lane 2: DNA from GgBa digested with *Eco* R1; lane 3: total DNA from *A. rhizogenes*; lane 4: DNA from *A. rhizogenes* digested with *Eco* R1; lane 5: untransformed *G. glauca* DNA digested with *Eco* R1.

cause of its degree of homogeneous growth, its viability and its high level of triterpene production (as observed by TLC).

Specific pairs of primers for the *rol A* and *rol C* genes were used for the PCR analysis (**Fig. 2**). This experiment indicated that the *rol A* gene was present in the transformed cell suspension line GgBa (**Fig. 2**). It is important to note that no amplification of *rol C* was observed in GgBa, as was in the case of the hairy root line VYT [7] from which GgBa has been derived. It is well known that low frequency rearrangements and deletions of T-DNA occur during long-term *in vitro* culture of stable transformed cell lines, which represent somaclonal variations [17, 18]. The absence of *rol C* in GgBa cultures may be explained by these rearrangements, in which a deletion of the gene occurred. The expression of only *rol A* in GgBa may promote callus formation, whereas the combined expression of *rol ABC* suppresses this morphogenetic behaviour, as has been recently reviewed for other cell cultures [19].

The 248 bp fragment of *rol A*, generated by PCR was used as a probe for Southern blot analysis (**Fig. 3**). DNA from the GgBa transformed cell line and from positive and negative controls was digested, using the restriction enzyme *Eco* RI. When digested GgBa DNA was probed with the *rol A* fragment, one 2.4 kbp hybridising band was identified (**Fig. 3**, lane 2). These data clearly indicated that the signal was due to the integration of the T-DNA into the plant genome.

The major compound (**13**) was isolated from the culture medium and totally purified by applying open column chromatographic procedures. Elucidation of its structure revealed that it represented a new nor-friedelane, related to the known galphimines (**1**–**9**) that constitute the sedative principles isolated from the aerial parts of wild specimens, as well as being related to the recently described glaucacetalins A–C (**10**–**12**). Analysis of the NMR data (^{1}H, ^{13}C, DEPTs 90 and 135, HH COSY, HSQC and HMBC) of compound **13** indicated the basic skeleton identified in all previously reported glaucacetalins [7]. The following structural features, common to all glaucacetalins were observed: one secondary methyl group at C-4 (Me-23, δ = 13.9/1.10); two tertiary methyl groups for Me-26 (δ = 23.3/1.54) and Me-28 (δ = 25.9/1.08); one methoxycarbonyl group at C-27 (δ = 3.49/51.8); one cyclic acetal at C-25 (δ = 5.34/101.7) and an attached methoxyl group at C-25 (δ = 3.36/55.7); one tertiary alchohol at C-18 (δ = 78.2); one exomethylene vinylic moiety (H_2-29: δ = 109.3/4.50 and 4.70); and two acetate groups linked at C-3 (δ = 2.08/21.5/170.7) and at C-7 (δ = 2.10/22.5/174.3). The acetal nature of compound **13** was confirmed by HMBC connectivity experiments, for which the acetal carbon C-25 (δ = 101.7) showed long-range correlations ($^3J_{CH}$) with the methoxy group centred at δ = 3.36. This chemical environment has the same feature of an anomeric carbon of the methyl glycopyranosides, in which the presence of a methyl group causes an inductive effect (-I) by *ca*. 7.0 ppm [20]. In the case of glaucacetalins series the only structures methoxylated at C-25 are glaucacetalins B (δ = 101.2) and D (δ = 101.3), in contrast with compounds A (δ = 94.2) and C (δ = 94.0) [7].

Glaucacetalin D (**13**) represents the least polar compound out of the glaucacetalin series, with reference to the TLC analysis, due to the acetylated positions at C-7 and C-3, and the presence of the methoxy groups at C-27 and particularly at C-25. A comparative analysis of the chemical shifts displayed in the case of compound **13** and the analogue compounds **10**–**12**, also corroborates its structural elucidation.

The results from a 32-day batch culture of the cell line GgBa growing in MS medium in the absence of phytohormones are displayed in **Fig. 4**. The maximum biomass concentration obtained on day 16 consisted of 23.3 g dry weight/L (DW/L), representing 21 times the initial dry weight. Cell viability ranged from 85 to 100% during the incubation period. Cell growth followed first-order kinetics with a mean specific growth rate (μ) of 0.13/d. During the time of culture, the pH registered was around 5 with a small decrease evident on day 18.

The transformed cell suspension line GgBa exhibited the capacity to synthesise compounds **10** and **14** that were previously identified in a hairy root culture [8], and was able to produce the novel compound **13**. These compounds were identified during various growth phases in either the cell suspension biomass or in the culture media, and employed to establish the kinetics of their production. Compound **14** obtained from cell biomass on day 24 exhibited a maximum production of 2.4 mg/g DW as well as a discontinuous pattern of accumulation during its time of culture. Kinetic production of **10** and **13** extracted from the media varied significantly over the culture period. Compound **13** was recovered, manifesting a maximum accumulation of 2.9 mg/L on day 26 along with a maximum productivity of 0.11 mg/L/d. The maximum yield of 2.7 mg/L for compound **10** was reached on day 26

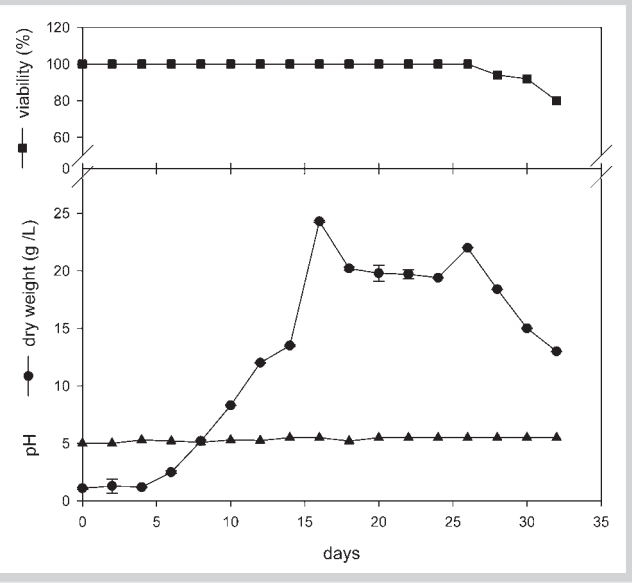

Fig. 4 Typical growth, pH and cell viability of the transformed cell suspension line GgBa of *G. glauca* grown in MS medium (n = 3 ± SD).

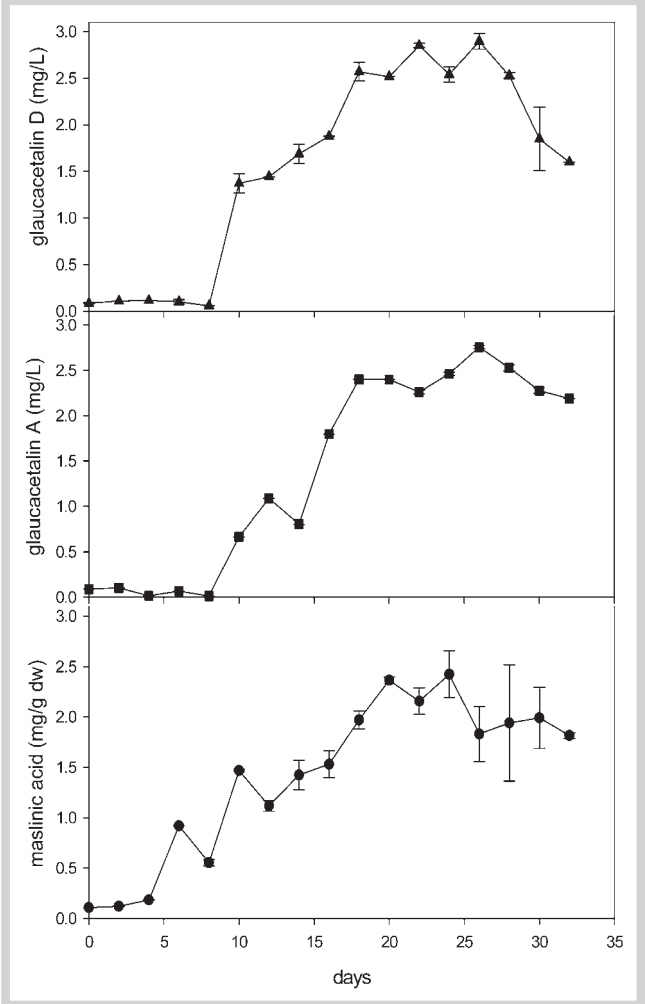

Fig. 5 Production of triterpenoids in transformed cell suspension culture of *Galphimia glauca*. Glaucacetalins A (**10**) and D (**13**) recovered from the culture medium, and maslinic acid (**14**) from biomass.

and maximum productivity was 0.10 mg/L/d (**Fig. 5**). When compared, the maximum yield of **10** for GgBa was similar to that obtained for the hairy root culture line (2.06 mg/L); and the maximum yield of **14** was higher in relation to the accumulation obtained for the hairy root line (0.43 mg/L) [8].

Stability was maintained in terms of the production of the glaucacetalins **10** and **13** by the transformed cell line GgBa, as corroborated by recording their accumulation on two different days (6 and 16) of batch cultures. This process was repeated in every subsequent subculture (performed monthly) throughout a one-year period (January 2008 – January 2009). The values for **10** fluctuated between 0.067 and 0.075 mg/L on day 6, and from 1.62 to 1.79 mg/L on day 16; while variations for **13** ranged from 0.083 to 0.1 mg/L on day 6, and from 1.53 to 1.87 mg/L, after 16 days of culture.

Although in an earlier publication [8], we described the production of glaucacetalins A–C (**10, 11** and **12**) in a hairy root culture of *G. glauca*, no glaucacetalin D (**13**) was produced in that culture. On the other hand, the glaucacetalin series have never been identified, either in untransformed cell suspension lines or in the wild plant material derived from *G. glauca*, indicating that the metabolic pathway for this type of compounds is produced in the transformed cultures of the species, rather than in the original plant state.

The information derived from the establishment of the cell line GgBa and the subsequent identification of the novel glaucatetalin D (**13**), will contribute to the elucidation of the metabolic pathway required to optimise the biotechnological production of bioactive nor-friedelanes from this species.

The sodium pentobarbital-induced hypnosis model measures the latency and duration of sleeping time in mice exposed to sedative compounds. **Fig. 6** shows that glaucacetalins A (**10**) and D (**13**) exhibited significant differences in terms of their levels of sedative effect, when the sodium pentobarbital-induced hypnosis test was applied [vehicle 24.0 ± 6.8%; diazepam 44.0 ± 6.3%; galphimine B 41.5 ± 8.5%, glaucacetalin A 34.7 ± 6.3%, glaucacetalin D 42.4 ± 5.8%;. $F = 9.07$, $p < 0.05$]. Glaucacetalin A (**10**) displayed moderate sedative activity when compared with the known values of sedative compounds such as diazepam and galphimine B (**1**). Glaucacetalin D (**13**) presented a sedative potency comparable to that of galphimine B (**1**), the main active compound present in the leaves of the wild plant. The weaker sedative activity observed from glaucacetalin A (**10**) in relation to its analogue glaucacetalin D (**13**), put in evidence the role of the methoxy group at C-25 for a higher activity. The results obtained in the elevated plus maze test showed that compound **10** and **13** did not posses anxiolytic activity when compared to diazepam.

This is the first report that demonstrates neuropharmacological actions from members of the glaucacetalin series.

Results obtained from the cytotoxic evaluation of **10** and **13** did not show any toxic effect against KB, MCF-7 and HF6 cancer cell lines ($ED_{50} > 4$ µg/mL). The ED_{50} (µg/mL) values obtained for campthotecin were 0.002 (KB), 0.006 (MCF-7), and 0.0009 (HF6). These results suggest that the glaucacetalins do not contribute to the previously reported cytotoxic action against a colon cancer cell line, obtained from *G. glauca* extracts [21].

What is remarkable in the cell line GgBa is a phytohormone-independent growth, genetic and metabolic stability, as well as the capacity to synthesise a novel sedative nor-friedelane. This

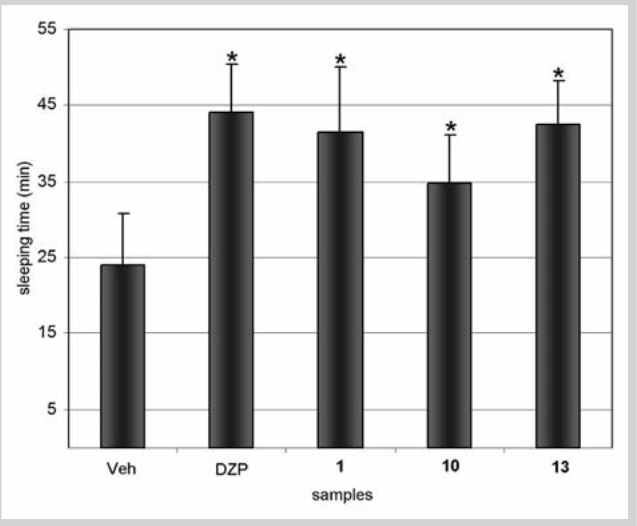

Fig. 6 Effect of administering 5 mg/100 g of galphimine B (**1**), glaucacetalin A (**10**), glaucacetalin D (**13**), control vehicle (Veh, 0.75 mL/100 g at 8% Tween 80 in saline) and diazepam (DZP, 0.1 mg/100 g) in the sodium pentobarbital-induced hypnosis model (thirty minutes subsequent to *i. p.* injection), taking sleeping time as the time elapsed between the disappearance and reappearance of the righting reflex. * $P < 0.05$ differences in relation to the vehicle on the ANOVA followed by post hoc Dunnett's t-test (mean ± S. D.).

new metabolic feature may be the consequence of genetic rearrangements that otherwise lead to the establishment of a new transformed cell suspension line.

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