Production of an Antiproliferative Furanoheliangolide by *Lychnophora ericoides* Cell Culture

Pierre Alexandre dos Santos,[a] Maria Fernanda Castro Amarante,[a,b] Ana Maria Soares Pereira,[b] Bianca Bertoni,[b] Suzelei Castro França,[b] Cláudia Pessoa,[c] Manoel Odorico de Moraes,[c] Letícia Veras Costa-Lotufo,[c] Marcio Roberto Pinho Pereira,[d] and Norberto Peporine Lopes*,[a]

[a] *Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo; Via do Café S/N, CEP 14.040–903, Ribeirão Preto-SP, Brazil:* [b] *Departamento de Biotecnologia Vegetal, Universidade de Ribeirão Preto; 14096–380, Ribeirão Preto-SP, Brazil:* [c] *Faculdade de Medicina, Universidade Federal do Ceará, Fortaleza; Ceará, 60430–160, Brazil: and* [d] *Centro de Ciências da Saúde, Universidade de Fortaleza; Ceará, 60430–160, Brazil.*

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This work reports for the first time the production a furanoheliangolide (goyazensolide) by plant cell culture. Monitoring of the goyazensolide metabolism revealed that the maximum production occurred during the lag phase of the *Lychnophora ericoides* callus culture. The antiproliferative activity of obtained goyazensolide was evaluated against seven cancer cell lines using MTT assay. The results revealed a potent cytotoxic activity for the furaheliangolide with IC_{50} values in the range of 0.06 μg/ml for CEM leukemia cells to 0.75 μg/ml for B16 melanome cells.

Key words *Lychnophora ericoides*; plant cell culture; antiproliferative effect; sesquiterpene lactone; goyazensolide

Lychnophora ericoides Mart. is a Brazilian Asteraceae from the central "cerrado" region of Brazil.[1)] This species is popularly known as "falsa arnica" or "arnica da serra" and it is used in folk medicine as an analgesic or as an anti-inflammatory agent.[1)] Previous phytochemical investigations carried out on various parts of the plant (including the roots, aerial parts, glandular extracts from the leaves) have described the occurrence of steroids, triterpenes, flavonoids, furanoheliangolides and lignans.[2—4)] The lignan cubebin, isolated from the roots showed *in vivo* analgesic activity, but no significant anti-inflammatory or antipyretic effects.[3)] On the other hand, furanoheliangolides may modulate the anti-inflammatory process by *in vitro* inhibition of the transcription factor NF-kβ selectively alkylating its p65 subunit.[5)] The most potent furanoheliangolide normally belongs to the goyazensolide class of compounds. The intramolecular stereospecific Michael addition of this moiety produces the eremantholide, normally resulting in the loss of anti-inflammatory activity.[5)] Centratherin and goyazensolide (Fig. 1) isolated from glandular extracts of the plants are the most potent anti-inflammatory members (by *in vitro* NF-kβ inhibition) of this class of molecules.[4,5)] NF-kβ inhibition (by the selective alkylation) could be related to oncogenesis, triggering cell proliferation and inhibiting apoptosis, which also make this transcriptional factor an interesting target in anticancer therapy.[6)]

Commercial exploitation of "arnica da serra" within the native areas of Brazil has resulted in a devastating decline of the plant populations. Now the Brazilian Botanical Society has included this plant species in the list of Brazilian plants that are likely to become extinct.[7)] Recently, germination and propagation experiments showed a low development, by a complex symbiosis system, between *L. ericoides* plantlets and some endemic genus of micorrize.[8)] An alternative to preserve the population of "arnica da serra" for further chemical and biological studies is an *in vitro* culture development. In addition, the goyazensolide moiety exhibit a very complex and characteristic cycles made by plant enzymes and are very hard to obtain by synthesis.[9)] Based on this previous information, the aims of the present work were to obtain a callus culture of *L. ericoides*, to study the production of the previously isolated compounds and also to evaluate the antiproliferative effects toward tumor cell lines.

Experimental

General Experimental Procedures All solvents were redistilled. Merck silica gel 60 (230—240 mesh) and silica gel 60 GF_{254}, were used for vacuum flash chromatography and thin layer chromatography. Gas chromatography (Hewlett Packard Series II) was conducted using an HP-1 column, detector temperature of 300 °C, injector temperature of 250 °C and Hydrogen as the carrier gas. HPLC (Shimadzu LC-6A) was performed using a UV detector (266 nm) and a Shim-Pack ODS (5 μm) column. IR spectra were obtained on a Nicole Protégé 460. The ^{1}H-NMR spectra were recorded at 300 MHz and the ^{13}C-NMR spectra at 75 MHz (Bruker DPX-300). ESI-MS analyses were performed at low-resolution on a Quattro-LC instrument (Micromass, Manchester, U.K.).

Plant Material Achenes of *L. ericoides* were collected from Ibiraci, Minas Gerais, Brazil in February 1999, and were identified by João Semir, University of Campinas, Brazil, where a voucher specimen is deposited (NPL-122).

Establishment and Maintenance of Callus Culture Achenes surface were disinfected with 1% benomil (w/v) for 24 h under continuous agitation and then immersed in 70% ethanol (v/v) for 1 min. They were then kept in 0.5% calcium hypochlorite (w/v) for 30 min and inoculated. The MS basal medium was supplemented with 3% sucrose (w/v). The medium was solidified with 0.2% Phytagel and pH adjusted to 6.0 before autoclaving at 121 °C for 20 min. The obtained plantlets were maintained for three months on MS medium with 3% sucrose (w/v), α-naphthaleneacetic acid (2.0 mg/l) and 6-benzylaminopurine (4.0 mg/l). The plantlets were transferred to fresh medium under sterile conditions in intervals of 30 d. Leaf explants from plantlets were placed on MS agar medium containing 3% of sucrose, and combination 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BA), α-naphthaleneacetic acid (NAA), indolacetic acid (IAA), zeatin and citocin. The matrix combination was done using concentrations between 0.1 to 1.0 mg/l to the followed pairs: 2,4-D and BA, 2,4-D and NAA, 2,4-D and IAA, 2,4-D and zeatin, 2,4-D and citocin, BA and NAA, BA and IAA, NAA and IAA, to induce callus formation. To define the influence of 2,4-D concentration a second experiment was performed using 2.0 and 3.0 mg/l. The callus that exhibited the presence of furanoheliangolide in IR analysis were maintained by subculturing every 15 d to a fresh medium over 6 months at

* To whom correspondence should be addressed. e-mail: npelopes@fcfrp.usp.br

25 °C under 16 h photoperiod. Callus growth was determined by measuring fresh weight and expressed as a percentage of total growth.

Extraction and Isolation The lyophilized calli (38.83 g) of *L. ericoides* were powdered and extracted with MeOH at room temperature and produced 4.0 g of crude extract. The extract was submitted to vacuum-liquid chromatography, eluted with CH_2Cl_2 and MeOH to yield 3.2 g and 0.25 g of material respectively. The CH_2Cl_2 fraction was analyzed by IR, ^{1}H- and ^{13}C-NMR, that indicate the presence of a mixture of stigmasterol, campesterol and β-sitosterol as the major compounds. To confirm the presence of the steroides the CG analysis (co-injection) were performed as previous described in the quantification of steroides in *guaco* callus using Supelco standars (Bellefonte, PA, campesterol lot: LA 89298; β-sitosterol lot: LA 81076 and stigmasterol lot: LA 89840).

The methanolic fraction was submitted to preparative HPLC (ODS-Shimadzu, 5.0×250 mm column, MeOH–H_2O 50 : 50, λ=280 nm, flow 8 ml/min) producing the sesquiterpene lactone goyazensolide (1.5 mg). Goyazensolide was a white crystal. IR ν_{max} (film) cm^{-1}: 3400—3100 (OH), 1771 (C=O, lactone), 1705 (C=O, ester), 1654 (C=C), 1588 (C=C), 1300 (C–O), 1100 (C–O). ^{1}H-NMR (300 MHz, $CDCl_3$) 5.80 (1H, s, H-2), 6.28 (1H, dt, *J*=1.6, 3.0 Hz, H-5), 5.34 (1H, m, H-6), 3.79 (1H, m, H-7), 4.55 (1H, dt, *J*=2.5, 1.6 Hz, H-8), 2.50 (1H, dd, *J*=1.6, 13.8 Hz, H-9α), 2.32 (1H, dd, *J*=2.1, 13.8 Hz, H-9β), 6.23 (1H, d, *J*=3.1 Hz, H-13a), 5.47 (1H, d, *J*=2.5 Hz, H-13b), 1.54 (3H, s, H-14), 4.40 (3H, dd, *J*=1.7, 2.9 Hz, H-15), 6.01 (1H, dq, *J*=1.0, 2.0 Hz, H-3′a), 5.55 (1H, dq, *J*=1.5, 2.0 Hz, H-3′b), 1.83 (3H, t, *J*=1.5 Hz, H-4′). ESI-MS $[M+H]^+$ 361 and $[M+Na]^+$ 383.

Steroid Quantification by Gas Chromatography The friedelanol internal standard (I.S.) was added (9 μg) to the lyophilized cells (20 mg) following extraction with 5 ml of CH_2Cl_2 (three times). The solvent was evaporated to dryness and the extract dissolved in CH_2Cl_2 (125 μl). Gas chromatography was performed on a Hewlett Packard (Wilmington, DE, U.S.A.) Series II GC using an HP-1 column (30 m×0.25 mm, 0.25 μm), FID detector at 300 °C, manual injector at 250 °C with hydrogen as the carrier gas (linear velocity 39 cm/s). Column oven temperature conditions were as follows: initial temperature 250 °C was held for 12 min then increased by 6 °C/min to 280 °C, where it was held constant for 15 min. Two microliters of the samples were injected on the column, at a split rate of 1 : 60. Supelco standards (Bellefonte, PA, campesterol lot: LA 89298; β-sitosterol lot: LA 81076 and stigmasterol lot: LA 89840) were applied to prepare the Calibration graphs. All the analysis were performed in triplicate and the the means were used to express the production.

Goyazensolide Quantification by HPLC To lyophilized cells (50 mg) were added 9 μg of internal standard (16α-(1′,2′-dihidroxy-1′-methylpropyl)-eremantholide from our sesquiterpene bank of compounds, FCFRP-USP) following extraction with 5 ml of MeOH (three times). The solvent was evaporated to dryness and the extract dissolved in 150 μl of the mobile phase. Each fraction was centrifuged at 2500 ***g*** for 5 min and 10 μl was injected into the chromatographic system. The HPLC analyses were carried out at 22 °C on a Shim-Pack ODS column (4.5×250 mm, 5 μm particle size). The mobile phase for the analysis consisted of MeOH–H_2O gradient (30% MeOH to 60% MeOH in 15 min, 60% MeOH by 25 min, to 100% MeOH in 5 min) at a flow rate of 1 ml/min. All the analysis were performed in triplicate and the means were used to express the production.

Determination of Antiproliferative Activity The activity of the goyazensolide on cellular growth was evaluated against seven tumor cell lines (National Cancer Institute, Bethesda, MD, U.S.A.): B-16 (murine skin), HCT-8 (human colon), MCF-7 (human breast), SF-268 (human nervous system), PC-3 (human prostate), CEM and HL-60 (human leukemias). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C with 5% CO_2. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a purple formazan product.[10] For experiments, cells were plated in 96-well plates (10^5 cells/well for adherent cells or 3×10^5 cells/well for suspended cells in 100 μl of medium). After 24 h, goyazensolide (0.04 to 2.5 μg/ml) dissolved in DMSO (1%) was added to each well and incubated for 3 d (72 h). Control groups received the same amount of DMSO. Doxorubicin and etoposide were used as positive controls. Thereafter, the plates were centrifuged and then, the medium was replaced by fresh medium (200 μl) containing 0.5 mg/ml MTT. Three hours later, the MTT formazan product was dissolved in 150 μl DMSO, and absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada). Drug effect was quantified as the percentage of control absorbance of reduced dye at 550 nm.

Results and Discussion

Initial experiments with explants from mature leaves of *L. ericoides* showed no cellular proliferation and a high level of contamination by microorganisms. Calli were induced from explants from plantlet leaves obtained by *in vitro* achenes germination. The influence of auxins, NAA, IAA, BA and 2,4-D and their combination with zeatin and cinetin were evalutated. The results showed that it was possible to induce cellular proliferation and callus formation by adding 2,4-D, between 0.1 to 1 mg/l to the medium. Combination of 2,4-D (1 mg/l) and BA (0.1 mg/l) also result in callus formation. We also observed that higher doses of 2,4-D (2.0, 3.0 mg/l) are less effective in inducing callus in this tissue. The NAA, IAA and cinetin and zeatin showed no influence in the callus formation at the assayed doses (0.1 to 1.0 mg/l). After 30 d of culture, 2,4-D plus BAP showed the best growth for production of friable calli and it was the only culture media that exibit the typical absortion from furanheliangolide in IR spectra. These lines were subcultured to a fresh medium [MS supplied with 2,4-D (1 mg/l) and BA (0.1 mg/l)] each 15 d over 6 months. After this time the yield of growth was determined by fresh weight. No significant growth of the calli was observed until the 6th day and then exponential growth was observed until the 24th day (2.5 ratio of growth). Part of the calli were powdered and extracted with MeOH. The IR spectrum of the dried methanolic extract showed again the absorptions at 1771 cm^{-1} (γ-lactone), 1705 and 1588 cm^{-1} (furanone), which are indicative of a furanoheliangolide.[2,4] Supported by the IR spectrum the phytochemical investigation was developed to look for the furanoheliangolides. The extract was partitioned and the apolar fraction afforded the steroids stigmasterol, campesterol and β-sitosterol. The compound structures were determined by spectroscopy evidence and by GC analysis with co-injection with Supelco standards (see Experimental). After the purification steps the polar fraction yielded a white crystal that was analysed by IR, ^{1}H-NMR and ESI-MS. The IR spectra showed the same characterist signals (for furanoheliangolides) as previously observed in the crude extract. The ESI-MS showed the presence of the $[M+H]^+$ 361 and $[M+Na]^+$ 383 that indicate the presence of the anti-inflammatory sesquiterpene lactone goyazensolide (Fig. 1), previously isolated from *L. ericoides* glandular extract.[4] Finally the analysis of the ^{1}H-NMR spectrum (see Experimental) confirmed the goyazensolide structure by comparison with the literature data.

For quantification of the isolated steroids during the calli growth, a GC method was developed using friedelanol as an internal standard (I.S.). The results demonstrated a linear relationship between the analyts peak rate areas and concentrations. The linearity of the data was demonstrated by the regression coefficient obtained (r>0.999, Table 1). The quantification of the goyazensolide was performed using a method developed for HPLC, using 16α-(1′,2′-dihidroxy-1′-

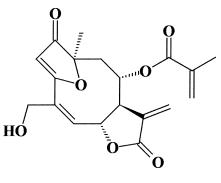

Fig. 1. Structure of the Goyasenzolide

Table 1. Calibration Graphs for Sesquiterpene Lactone Goyazensolide Analysis by HPLC and Sterols Analysis by GC

Analyte	Regression line	r	Concentration (μg/ml)
Goyazensolide	$Y=3.32925+1.09324x$	0.9999	3.75—600
Stigmasterol	$Y=0.00384+0.00875x$	0.9966	3.125—50
β-Sitosterol	$Y=0.00412+0.01113x$	0.9982	3.125—50
Campesterol	$Y=0.0008575+0.01173x$	0.9987	3.125—50

Y=peak area of analyte/peak area internal standard (for steroid analysis was fridelanol for goyazensolide was 16α-(1′,2′-dihidroxy-1′-methylpropil)-eremantholide), x=mass injected, r=correlation coeficient. Concentration express the range of linearity.

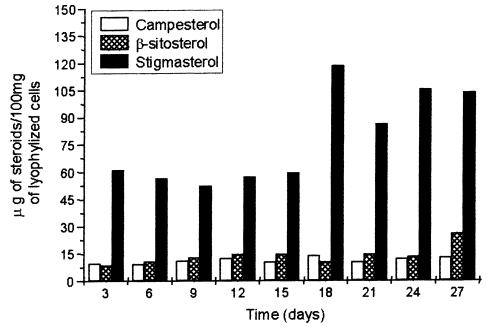

Fig. 2. Production of Steroids by *Lychnophora ericoides* Callus Culture

The reproducibilty was between 1 and 2% for the 3 repetitions.

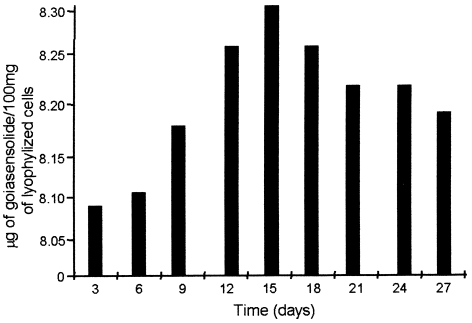

Fig. 3. Production of Goyazensolide by *Lychnophora ericoides* Callus Culture

The reproducibilty was between 1 and 2% for the 3 repetitions.

methylpropyl)-eremantholide as I.S. The results obtained for goyazensolide also showed strong linearity with a good regression coefficient (Table 1).

The major steroid production started in the lag phase with the maximum production occurring during the 18th day and then, their concentrations remained constant (Fig. 2). As expected, the production of the sesquiterpene lactone (Fig. 3) was observed to be lower than the steroids (Fig. 2). Biosynthesis of goyazensolide started at the beginning of the lag phase with the maximum during the 15th day and started to decrease as shown in Fig. 2. The decrease in the goyazensolide accumulation could be related to several chemical possibilities including the cyclisation to eremantholide moiety.

To evaluate the anticancer potential of goyazensolide, seven tumor cell lines were treated with increasing concentrations of the isolated compound (0.04 to 2.5 μg/ml) for 72 h. The goyazensolide activity was further compared to doxorubicin and etoposide. As shown in Table 2, goyazensolide was more active than etoposide on human adherent

Table 2. Antiproliferative Effects of Goyasenzolide Obtained from Callus Culture of *Lychnophora ericoides* on Tumor Cell Lines

Cell line	Goyazensolide IC_{50} μg/ml (μM)	Etoposide IC_{50} μg/ml (μM)	Doxorubicin IC_{50} μg/ml (μM)
HL-60	0.076 (0.211)	0.007 (0.012)	0.016 (0.028)
	0.005—0.113	0.005—0.009	0.013—0.019
CEM	0.060 (0.167)	0.030 (0.051)	0.018 (0.031)
	0.052—0.069	0.024—0.037	0.015—0.022
MCF-7	0.421 (1.169)	>5.886 (10.0)	0.203 (0.350)
	0.341—0.520		0.170—0.241
HCT-8	0.106 (0.294)	0.112 (0.190)	0.037 (0.064)
	0.099—0.113	0.070—0.181	0.028—0.049
B16	0.748 (2.078)	0.109 (0.185)	0.030 (0.052)
	0.638—0.876	0.080—0.149	0.024—0.036
PC3	0.602 (1.672)	>5.886 (10.0)	0.240 (0.414)
	0.556—0.652		0.213—0.269
SF-268	0.699 (1.942)	2.530 (4.298)	0.114 (0.196)
	0.642—0.761	1.547—4.138	0.112—0.184

Data are presented as IC_{50} values and 95% confidence interval obtained by non-linear regression for leukemia (HL-60 and CEM), breast (MCF-7), colon (HCT-8), murine skin (B-16), prostate (PC3) and Central Nervous System (SF-268) cancer cells from three independent experiments. Doxorubicin and etoposide were used as positive controls.

cells, but was weaker than doxorubicin. The IC_{50} ratio (goyazensolide/doxorubicin) varies from 2.0 on MCF-7 cells to 6.1 on SF-268 cells.

Sesquiterpene lactones form one of the largest group of plant-derived antitumor compounds.[11,12] However, this is the first report on the antiproliferative activity of furanoheliangolides possessing the goyazensolide moiety. This functional group seems to add to the activity, since the reported compounds showed cytotoxicity compared two clinically used chemotherapic drugs.

From these results, it is concluded that it is possible to obtain a sesquiterpene lactone from a callus culture of *Lychnophora ericoides*. This study demonstrates a successful alternative to help preserve the population of this endemic species. Furthermore, furanoheliangolides belonging to the goyazensolide class may be a promising group of antitumor compounds.

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References

1) Cerqueira M. B. S., Souza J. T., Júnior R. A., Peixoto A. B. F., *Ciênc. Cult.*, **39**, 551—553 (1987).
2) Borella J. C., Lopes J. L. C., Vichnewski W., Cunha W. R., Herz W., *Biochem. Syst. Ecol.*, **26**, 671—676 (1998).
3) Borsato M. L. C., Grael C. F. F., Souza G. E. P., Lopes N. P., *Phytochemistry*, **55**, 809—813 (2000).
4) Sakamoto H. T., Flausino D., Castellano E. E., Stark C. B. W., Gates P., Lopes N. P., *J. Nat. Prod.*, **66**, 693—695 (2003).
5) Rüngeler P., Castro V., Mora G., Gören N., Vichnewiski W., Pahl H. L., Merfort I., Schmidt T., *J. Bioorg. Med. Chem.*, **7**, 2343—2352 (1999).
6) Lin A., Karin M., *Semin. Cancer Biol.*, **13**, 107—114 (2003).
7) Sociedade Botânica do Brasil, "Centuria Plantarum Brasiliensium Extintionis Miniata," Sociedade Botânica do Brasil, 1992, p. 175.
8) Lopes N. P., *Pesquisa FAPESP*, **64**, 42—44 (2001).
9) Fischer N. H., *Recent Adv. Phytochem.*, **24**, 161—167 (1991).
10) Mosmann T., *J. Immunol. Meth.*, **16**, 55—63 (1983).
11) Picman A. K., *Biochem. System. Ecol.*, **14**, 255—281 (1986).
12) Konish T., Shimada Y., Nagao T., Okabe H., Konoshima T., *Biol. Pharm. Bull.*, **25**, 1370—1372 (2002).