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Leishmanicidal and Trypanocidal Activities of Acetogenins Isolated from *Annona glauca*

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The dichloromethane extract of seeds of *Annona glauca* (Annonaceae) was active against three strains of *Leishmania* species. Nine known acetogenins were isolated and identified and then evaluated *in vitro* against *Leishmania* species and the bloodstream forms of *Trypanosoma cruzi*. Annonacin A and goniothalamicin showed activity against *Leishmania*, and glaucanisin, squamocin, annonacin A and annonacin against *Trypanosoma cruzi* reducing the parasites by 78%, 67%, 71% and 85%, respectively. © 1998 John Wiley & Sons, Ltd.

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INTRODUCTION

The Annonaceous acetogenins are bioactive natural compounds, they are known to exhibit interesting biological activities including cytotoxic, insecticidal and antiprotozoal activities (Cavé *et al.*, 1997; Fang *et al.*, 1993; Rupprecht *et al.*, 1990).

Previous studies on extracts containing acetogenins have described activities against parasites such as *Entamœba histolytica, Trichomonas vaginalis* and helminths (*Nippostrongylus brasiliensis* and *Molinema dessetae*) (Bories *et al.*, 1991). The leishmanicidal and trypanocidal activities of these compounds have been reported against *Leishmania donovani* (Cavé *et al.*, 1987), the parasite of visceral leishmaniasis by acetogenins isolated from *Annona senegalensis*, and against *Trypanosoma brucei* (Sahpaz *et al.*, 1994).

This present investigation was carried out to evaluate the leishmanicidal and trypanocidal activities of *Annona glauca* crude extracts and nine acetogenins isolated from the dichloromethane extract.

MATERIALS AND METHODS

General experimental procedures. Optical rotations were determined on a Schmidt-Haensch Polartronic I polarimeter. UV spectra were obtained on a Philips PU 8720 spectrometer. IR spectra were measured on a Perkin-Elmer 257 spectrometer. The ^{1}H-NMR and ^{13}C-NMR spectra ($CDCl_3$) were obtained with Bruker AC-200 or AC-400 instruments at 200 and 50 MHz or at 400 and 100 MHz, respectively. EIMS and CIMS (methane) were performed on a Nermag R10-10C spectrometer. HPLC analytic analyses were performed with a Waters 501 pump, a Waters 991 spectrophotometer (214 nm) and a Waters WISP automatic injector on a μBondapak C_{18} prepacked column (10 μm, 8 × 100 mm), elution with MeOH–$H_2$0 at various gradients at a flow rate of 1 mL/min. Preparative HPLC was carried out with a Millipore-Waters (Milford MA, USA) system equipped with a 590 pump, a SSV injector, and a 484 UV detector (214 nm), on a μBondapak C_{18} prepacked column (10 μm, 25 × 100 mm), elution with MeOH–$H_2$0 at various gradients at a flow rate of 10 mL/min.

Plant material. Seeds of *A. glauca* were collected in September 1994 in Senegal by D. Fall and authenticated by Professor A. Le Thomas, Museum National d'Histoire Naturelle, Paris.

Extraction and isolation. The dried pulverized seeds (680 g) were macerated with MeOH. The MeOH extract was diluted with 10% volumes of water and submitted to liquid–liquid partition with hexane, leading to 24 g of a concentrated extract. The hydromethanol phase was extracted with CH_2Cl_2. The concentrated CH_2Cl_2 extract (14 g), containing acetogenins (Kedde +), was fractionated by flash chromatography on silica-gel 60 and eluted with solvents of increasing polarity leading to several fractions. Two of which were chromatographed over a silica-gel 60 H column and elution by a mixture CH_2Cl_2–AcOEt–MeOH (90:8:3) affording four acetogenins (see Fig. 1). Glaucanisin (**1**), rolliniastatin-2 (**2**) and squamocin (**3**) were obtained as pure fractions. HPLC was used to verify the purity of them (Fujimoto *et al.*, 1988; Pettit *et al.*, 1989; Waechter *et al.*, 1995). Glaucafilin (**4**) was

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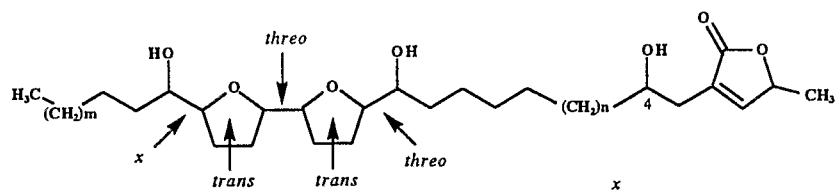
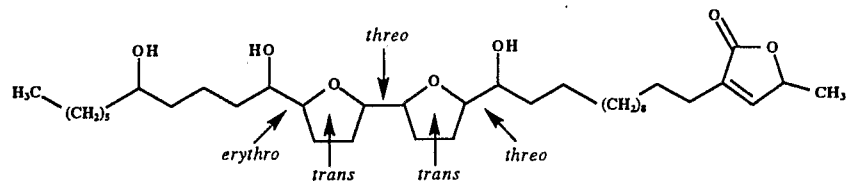
obtained as an almost pure fraction and was further purified by HPLC (Waechter *et al.*, 1997). One fraction, chromatographed equally on a silica-gel 60 H column and elution by a mixture CH_2Cl_2–AcOEt–MeOH (90:8:3) afforded two acetogenins. Molvizarin (**5**) and parviflorin (**6**) were further purified by preparative HPLC (Cortes *et al.*, 1991; Ratnayake *et al.*, 1994). Another fraction was chromatographed over a silica-gel 60 H column; elution by a mixture CH_2Cl_2–AcOEt–MeOH (80:15:5) afforded two acetogenins. Annonacin A (**7**) and annonacin (**8**) were obtained as pure fractions proved by HPLC (Lieb *et al.*, 1990; McCloud *et al.*, 1987). The last studied fraction was chromatographed over a silica-gel 60 H column and elution by a mixture CH_2Cl_2–AcOEt–MeOH (80:13:7) afforded goniothalamicin (**9**) as a pure fraction (HPLC) (Alkofahi *et al.*, 1988). Compounds **1–9** showed identical spectral data to those previously reported for these compounds.

Leishmanicidal activity. Cultures of *Leishmania* spp were obtained from IICS (Instituto de Investigaciones en Ciencias de la Salud, Asuncion) and identified by isoenzyme analysis. Three strains of *Leishmania* were used during these investigations: *L. braziliensis* (MHOM/BR/75/M 2903), *L. amazonensis* (IFLA/BR/67/PH8) and *L. donovani* (MHOM/IN/83/HS-70)) grown at 22°C in Schneider's Drosophila medium containing 20% fetal bovine serum. Compounds were dissolved in 5 µL of dimethyl sulphoxide (DMSO), then in medium and placed in microtitre plates in triplicate. The minimal amount (µg) of compound to inhibit the growth of *Leishmania* sp was evaluated after 48 h by optical observation on a drop of each cell culture with a microscope by comparison with control cells and with reference drugs (*N*-methylglucamine antimonate and pentamidine). The maintenance, cultivation and isolation of promastigote-stage parasites have been described in detail elsewhere (Fournet *et al.*, 1994). Pentamidine was used as a positive control (Fournet *et al.*, 1993).

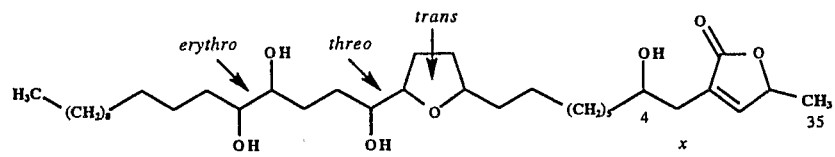
Trypanocidal activity. Albino mice infected with *Trypanosoma cruzi* strain, 7 days after infection were used. Blood was obtained by cardiac puncture using 3.8% sodium citrate as anticoagulant in a 7:3 blood/anticoagulant ratio. The parasitaemia in infected mice ranged between 1×10^5 to 5×10^5 parasites per millilitre. Plant extracts and isolated compounds were dissolved in cold DMSO to a final concentration of 250 µg/mL. Aliquots of 10 µL of each extract of different concentrations (4, 20, 40, 100 and 250 µg/mL) were mixed in microtitre plates



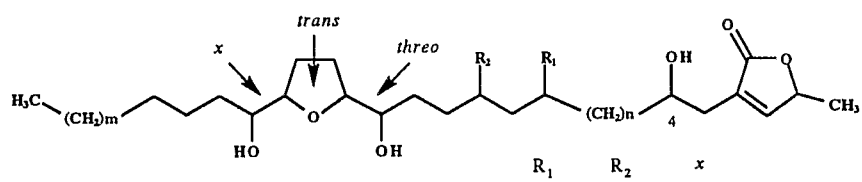
Glaucanisin (**1**) :	n = 4	m = 13	*erythro*
Rolliniastatin-2 (**2**) :	n = 6	m = 11	*erythro*
Molvizarin (**5**) :	n = 4	m = 11	*erythro*
Parviflorin (**6**) :	n = 4	m = 11	*threo*



Squamocin (**3**)



Glaucafilin (**4**)



			R_1	R_2	x
Annonacin A (**7**) :	n = 5	m = 8	OH	H	*erythro*
Annonacin (**8**) :	n = 5	m = 8	OH	H	*threo*
Goniothalamicin (**9**) :	n = 3	m = 10	H	OH	*threo*

Scheme 1. Acetogenins from *Annona glauca*.

Table 1. *In vitro* activity of *A. glauca* crude extracts and acetogenins towards three strains of promastigote forms of *Leishmania* spp (IC_{100} μg/mL)

Extract or compound	*L. braziliensis* (2903)	*L. amazonensis* (PH-8)	*L. donovani* (HS-70)
Hexane extract	>100	>100	>100
Dichloromethane extract	25	25	25
Glaucanisin (1)	25	25	25
Rolliniastatin-2 (2)	25	25	25
Squamocin (3)	25	25	25-
Glaucafilin (4)	25	25	25
Molvizarin (5)	>100	>100	>100
Parviflorin (6)	>100	>100	>100
Annonacin A (7)	10	10	10
Annonacin (8)	25	25	25
Goniothalamicin (9)	5	5	5
Pentamidine	5	5	5

with 100 μL of infected blood containing different parasite concentrations (1×10^5 and 10^6 parasites per mL). Infected blood and infected blood containing gentian violet at 250 μg/mL were used as controls. The plates were shaken for 10 min at room temperature and kept at 4°C for 24 h. Each solution was microscopically observed at 400x, placing a 5 μL-sample on a slide and covering it with a 22 × 22 mm coverglass for parasite counting (Rojas de Arias *et al.*, 1994; Schempler, 1978).

Cytotoxic activity on KB and Vero cells. The extracts, fractions and isolated compounds were routinely evaluated for brine shrimp lethality test (BST) (Meyer *et al.*, 1982). The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5)-diphenyltetrazolium bromide) tests for cytotoxicity to cell lines KB and Vero were performed as previously described (Fleury *et al.*, 1984). The IC_{100} (μg/mL) value was defined as the concentration of sample that achieved a 100% reduction of viable cells with respect to the control.

RESULTS AND DISCUSSION

The hexane extract of *A. glauca* did not present a significant activity against *Leishmania* promastigotes, whereas the dichloromethane extract had an IC_{100} value of 25 μg/mL (Table 1). The same activity was found with the isolated compounds: glaucanisin (**1**), rolliniastatin-2 (**2**), squamocin (**3**), glaucafilin (**4**) and annonacin (**8**). Molvizarin (**5**), a C_{35} acetogenin and its isomer, parviflorin (**6**), did not show activity. Annonacin A (**7**) and goniothalamicin (**9**) were quite active towards *Leishmania* promastigotes, showing IC_{100} values of 10 and 5 μg/mL respectively.

Some structure–activity relationship trends of these compounds can be considered. The width of the alkyl chain of the bis-THF acetogenins (compounds **1, 2, 3, 5** and **6**) seems to influence the weak leishmanicidal activity of these compounds. In effect, molvizarin (**5**) and parviflorin (**6**), two C_{35} bis-THF acetogenins, were inactive against *Leishmania* spp, but glaucanisin (**1**), rolliniastatin-2 (**2**) and squamocin (**3**), C_{37} bis-THF acetogenins, presented an IC_{100} value of 25 μg/mL. Neither the sub-type of lactone (1a or 1b) nor the number of hydroxy groups seems to be implicated in the activity of these molecules (see Fig. 1) (Cavé *et al.*, 1997).

The compounds **4, 7, 8** and **9** are C_{35} mono-THF acetogenins (sub-group 1b) with four hydroxy groups, these compounds presented IC_{100} values of 25, 10, 25 and 5 μg/mL, respectively against *Leishmania* spp. The difference in activity between **8** and **9** and on the other hand between **7** and **8** is probably due to the position of THF-ring and its relative configuration. Considering these results, it is not easy to establish a structure–activity relationship, nevertheless the mono-THF acetogenins seem to be more leishmanicidal than the bis-THF acetogenins.

The hexane extract from *A. glauca* did not present an activity against the bloodstream forms of *Trypanosoma cruzi,* but some acetogenins, glaucanisin (**1**), squamocin (**3**), annonacin A (**7**) and annonacin (**8**) produced a reduction of the parasites of 78%, 67%, 71% and 85% (Table 2). This level of activity is considered as

Table 2. *In vitro* activity of *A. glauca* crude extracts and acetogenins on bloodstream forms of *Trypanosoma cruzi* (IC_{100} μg/mL)

Extract or compound	Reduction of the parasite number in infected murine blood (%)
Hexane extract	31
Glaucanisin (1)	78
Rolliniastatin-2 (2)	13
Squamocin (3)	67
Glaucafilin (4)	0
Molvizarin (5)	41
Parviflorin (6)	35
Annonacin A (7)	71
Annonacin (8)	85
Goniothalamicin (9)	32
Gentian violet	100

Table 3. Cytotoxicities of extracts of *A. glauca*

Extract	BST[a] (LC_{50}) (μg/mL)	VERO[b] (LC_{100})	KB[c] (LC_{100})
Hexane extract	0.04	5	1.5
Dichloromethane extract	<0.04	5×10^{-5}	5×10^{-5}
Methanole extract	0.5	-	-

[a] Brine shrimp test; [b] Epithelial cells from monkey kidney; [c] Human nasopharyngeal carcinoma.

significant (Croft *et al.*, 1988). Alone, annonacin A (**7**) proved to be active in the bioassay against *Leishmania* spp. and *T. cruzi.* The dichloromethane extract showed high cytotoxicity (IC_{100} 5×10^{-5} μg/mL) and the hexane extract a weak toxicity (IC_{100} 1.5–5 μg/mL) against KB and Vero cell lines (Table 3). These results are coherent, since the dichloromethane extract contained the acetogenins **1–9.**

In conclusion, the results obtained in this study suggest that it would interesting to continue the biological investigations of some acetogenins against *Trypanosoma cruzi,* using an animal or cellular model. More experiments, especially with a larger number of compounds (position and configuration isomers) may be helpful to elucidate precise structure–activity relationships.

REFERENCES

Alkofahi, A., Rupprecht, J. K., Smith, D. L., Chang, C.-J., and McLaughlin, J. L. (1988). Goniothalamicin and annonacin : bioactive acetogenins from *Goniothalamus giganteus* (Annonaceae). *Experientia* **44**, 83–85.

Bories, C., Loiseau, P., Cortes, D., Myint, S. H., Hocquemiller, R., Gayal, P., Cavé, A., and Laurens, A. (1991). Antiparasitic activity of *Annona muricata* and *Annona cherimolia* seeds. *Planta Med.* **57**, 434–436.

Cavé, A., Hocquemiller, R., and Laprévote, O. (1987). Utilisation d'acétogénines en thérapeutique en tant que substances antiparasitaires. *Brevet (France)* N° 4689 23 (25 August 1987).

Cavé, A., Figadère, B., Laurens, A., and Cortes, D. (1997). *Progress in the Chemistry of Organic Natural Products : Acetogenins* from *Annonaceae.* ed. by *W. Herz, G. W., Kirby R. E. Moore, W. Steglich* and *Ch. Tamm* vol. **70**, 81–288. Springler-Verlag, Wien, New York.

Cortes, D., Myint, S. H., and Hocquemiller, R. (1991). Molvizarin and mortrilin : two novel cytotoxic bis-tetrahydrofuranic γ-lactone acetogenins from *Annona cherimolia. Tetrahedron* **47**, 8195–8202.

Croft, S. L., Walker, J. J., and Gutteridge, W. E. (1988). Screening of drugs for rapid activity against *Trypanosoma cruzi* trypomastigotes *in vitro. Trop. Med. Parasitol.* **39**, 145–148.

Fang, X.-P., Rieser, M. J., Gu, Z. M., Zhao, G. X., and McLaughlin, J. L. (1993). Annonaceous acetogenins:an updated review (and appendices). *Phytochem. Anal.* **4**, 27–67.

Fleury, C., Cotte, J., and Quero, A. M. (1984). Evaluation de la cytotoxicité d'un antiseptique par une microméthode photométrique. *Pathologie Biologie* **32**, 628–630.

Fournet, A., Angelo Barrios, A., and Muñoz, V. (1994). Leishmanicidal and trypanocidal activities of Bolivian medicinal plants. *J. Ethnopharmacol.* **41**, 19–37.

Fournet, A., Muñoz, V., Roblot, F., Hocquemiller, R., Cavé, A., and Gantier, J. C. (1993). Antiprotozoal activity of dehydrozaluzanin C, a sesquiterpene lactone isolated from *Munnozia maronii* (Asteraceae). *Phytother. Res.* **7**, 111–115.

Fujimoto, Y., Eguchi, T., Kakinuma, K., Ikekawa, N., Sahai, M., and Gupta, Y. K. (1988). Squamocin, a new cytotoxic bistetrahydrofuran containing acetogenin from *Annona squamosa. Chem. Pharm. Bull.* **36**, 4802–4806.

Lieb, F., Nonfon, M., Wachendorff-Neumann, U., and Wendisch, D. (1990). Annonacins and annonastatin from *Annona squamosa. Planta Med.* **56**, 317–319.

McCloud, T. G., Smith, D. L., Chang, C.-J., and Cassady, J. M. (1987). Annonacin, a novel, biologically active polyketide from *Annona densicoma. Experientia* **43**, 947–949.

Meyer, N. B., Ferrigni, N. R., Putnam, J. E., Jacobsen, L. B., Nichols, D. E., and McLaughlin, J. L. (1982). Brine shrimp : a convenient general bioassay for active plant constituents. *Planta Med.* **45**, 31–34.

Pettit, G. R., Riesen, R., and Leet, J. E. et al. (1989). Isolation and structure of rolliniastatin-2 : a new cell growth inhibitory acetogenin from *Rollinia mucosa. Heterocycles* **28**, 213–217.

Ratnayake, S., Gu, Z. M., and Miesbauer, L. et al. (1994). Parvifloracin and parviflorin : cytotoxic bistetrahydrofuran acetogenins with 35 carbons from *Asimina parviflora* (Annonaceae). *Can. J. Chem.* **72**, 287–293.

Rojas de Arias, A., Inchausti, A., Ascurra, M., Fleitas, N., Rodriguez, E., and Fournet, A. (1994). *In vitro* activity and mutagenicity of bisbenzylisoquinolines and quinones against *Trypanosoma cruzi* trypomastigotes. *Phytother. Res.* **8**, 141–144.

Rupprecht, J. K., Hui, Y.-H., and McLaughlin, J. L. (1990). Annonaceous acetogenins : a review. *J. Nat. Prod.* **53**, 237–278.

Sahpaz, S., Bories, C., and Loiseau, P. et al. (1994). Cytotoxic and antiparasitic activity from *Annona senegalensis* seeds. *Planta Med.* **60**, 538–540.

Schempler, B. R. (1978). Estudos experimentais de quimioprofilaxia de transmissao de doença de Chagas por transfusao sanguinea. *Rev. Patol. Trop.* **7**, 55–111.

Waechter, A.-I., Hocquemiller, R., Laurens, A., and Cavé, A. (1995). Glaucanisin, a new acetogenin from *Annona glauca. Nat. Prod. Lett.* **6**, 133–138.

Waechter, A.-I., Hocquemiller, R., Laurens, A., and Cavé, A. (1997). Glaucafilin, an acetogenin from *Annona glauca. Phytochemistry* **44**, 1537–1540.