ELSEVIER

Journal of Ethnopharmacology 78 (2001) 129–132

Journal of ETHNO-PHARMACOLOGY

www.elsevier.com/locate/jethpharm

Biological activity of *Guatteria cardoniana* fractions

A. Paredes [a], M. Hasegawa [b], F. Prieto [b], J. Mendez [b], M. Rodríguez [b], M. Rodríguez-Ortega [a,*]

[a] *Laboratorio de Leprología y Patología Experimental, Instituto de Biomedicina, Facultad de Medicina, Universidad Central de Venezuela, Caracas 1010A, Venezuela*

[b] *Laboratorio de Productos Naturales, Centro de Química Orgánica, Escuela de Química, Facultad de Ciencias, Universidad Central de Venezuela, Caracas 1010A, Venezuela*

Accepted 17 July 2001

Abstract

Methanolic extract of leaves and twigs of *Guatteria cardoniana* R.E. Fries (Annonaceae), a plant from the Venezuelan rain forest, was separated in alkaloid rich fractions and their biological effect on baby hamster kidney (BHK) cell line was studied. The initial plant extract (FA) induced cell proliferation, cytotoxicity as well as antiviral activity, depending on the concentration used. Further separation of this methanolic extract allowed us to separate these biological activities. The fraction with the highest antiviral activity (F7) was chromatographed and three of the nine alkaloid-rich fractions obtained, retained this activity. One of them ($F_7$11) exhibited the highest inhibitory effect against a neurotropic Sindbis virus (NSV). © 2001 Published by Elsevier Science Ireland Ltd.

Keywords: Annonaceae; *Guatteria cardoniana*; Antiviral; Sindbis virus

1. Introduction

Guatteria cardoniana R.E. Fries is a plant, which grows in the Venezuelan rain forest, and belongs to the Annonaceae family, one of the largest families of tropical plants (Leboeuf et al., 1982). Recently, phytochemical and pharmacological studies of this family have increased due to the discovery of new natural products with diverse biological activities. Indigenous people from Bolivia use *Guatteria foliosa* as insect repellent and a new antiparasitic aporphine alkaloids have been isolated from this plant (Mahiou et al., 1994). More recently an antimalarial constituent has been obtained from G*uatteria amplifolia* (Weniger et al., 2000). Ethanolic extract of *Annona muricata* (Annonaceae) had been reported to inhibit the cytopathic effect of Herpes simplex virus 1 (Padma et al., 1998).

As part of our research on plant secondary metabolites as antiviral agents, we separated different alkaloid-rich fractions from leaves and twigs of *G. cardoniana*, to study their effect on baby hamster kidney (BHK) cells, as well as their inhibitory effect on the replication of a neuro-adapted strain of Sindbis (neurotropic Sindbis virus (NSV)), which is an Alphavirus belonging to the Togaviridae family.

2. Materials and methods

2.1. Plant material

Leaves and twigs of *G. cardoniana* were collected in the rain forest along the Cataniapo riverside near Puerto Ayacucho City, Amazon State, Venezuela. The plant was identified by Professor Anibal Castillo of the Biology School, Faculty of Sciences, Universidad Central de Venezuela and a voucher specimen is preserved at the National Herbarium of Venezuela (VEN) with the collection number 3360 *AC*.

2.2. Cells and virus

BHK-21 cells were obtained from the National Health Institute of Caracas, Venezuela. Dr Guadalupe

* Corresponding author. Tel.: +58-212-8608840; fax: +58-212-8611258.

E-mail address: mrodri@tel.net.ve (M. Rodríguez-Ortega).

0378-8741/01/$ - see front matter © 2001 Published by Elsevier Science Ireland Ltd.
PII: S0378-8741(01)00315-4

Guzman (Virology Department of the Pedro Kouri Institute, Havana, Cuba) kindly provided BHK-21 clone 15 cells. The neurovirulent strain (NSV) of Sindbis virus was donated by Dr Dianne Griffin (Medical School, John Hopkins University, Baltimore, USA). BHK-21 cells were grown in Eagle's minimal essential medium (MEM) supplemented with 5% inactivated fetal bovine serum (FBS), 2 mM glutamine and 40 μg/ml gentamicine (growth medium). The cells were maintained at 37 °C and 5% CO_2.

NSV was grown in BHK-21 cells with maintenance medium (MEM, 1% FBS, 2 mM glutamine and 40 μg/ml gentamicine). After infection, viral supernatants were purified. Viral titer was obtained by plaque assay (Kurokawa et al., 1995). NSV yielded a titer of 5.25×10^8 pfu/ml. The virus stocks were stored at -70 °C.

2.3. Extract and fraction

Dried leaves and twigs (800 g) of *G. cardoniana* were extracted and fractioned. Seven fractions (FA, FA_1, FA_2, FA_3 FB, FB_1, FB_2) were chromatographed on a silica gel column and eluted with polarity-increasing mixtures of CH_2Cl_2–MeOH. Fraction FA (2.5 g), was further separated on a silica gel column to yield seven fractions (F1–F7). The highest antiviral activity was found in fraction F7. Therefore, it was further chromatographed through a silica gel column, eluted with CH_2Cl_2 and nine fractions (F_70, F_72, F_74, F_77, F_711, F_714, F_717, F_723, and F_724) were obtained. Stock solutions for biological assays were prepared by dissolving each of the fractions obtained in DMSO and stored at 4 °C.

2.4. Biological tests

2.4.1. Cytotoxicity assay

Cell viability was determined by a modified MU (3-(4,5 dimethylthiazol-2-yl) 2,5 diphenyl-tetrazolium bromide) assay. Briefly, cells were adjusted to 4×10^4 cells per l00 μl in growth medium and seeded in a 96-well plate. After 24 h, cells were incubated with serial dilutions of each extract in maintenance medium, MTT 10 μl (10 mg/ml) was added to each well 24 h later, and plates were read at 570 nm after 18 h (Shearman et al., 1994).

2.4.2. Antiviral activity assay

Cells were seeded and stabilized in the same conditions as the cytotoxic assay. BHK cells were infected with NSV at a multiplicity of infection (MOI) of 0.1 pfu per cell. The infections were carried out in the presence or absence of different concentrations of extracts. After 24 h, the viability of the cells was determined by MTT assay as described before.

2.4.2.1. Plaque assay. BHK clone 15 at a final concentration of 2.5×10^5 cell was seeded in 24-wells plates. Viral suspension and the extracts were incubated for 1 h at 37 °C, then 50 μl of each solution were added to the cells to obtain a MOI of 0.02 pfu per cell and 10 μg/ml of extracts final concentration (Kurokawa et al., 1995).

2.4.2.2. Data analysis. All experiments were performed in triplicate, and each experiment was reproduced a minimum of three times. The 50% toxicity concentrations (IC50) and 50% effective concentrations (EC50) were calculated using the mean of the values of each concentration. The IC50 was estimated by linear regression analysis. The therapeutic indices (TI) were calculated as follows: IC50/EC50 (Zembower et al., 1998). Variance analysis on virus replication in the presence of the different fractions was evaluated. A $P < 0.05$ value was defined as statistically significant.

3. Results

The seven fractions obtained from the silica gel column showed a distinctive pattern of cellular responsiveness on BHK cells (Table 1). Thus, we found that FB_1 and FB_2 were able to induce cell growth in concentrations higher than 18 and 11 μg/ml, respectively, and were innocuous at lower concentrations. FA_1, FA_2, FA_3 and FB were innocuous in the wide range of concentrations tested. On the contrary, FA showed the three different biological activities (innocuous, proliferation and cytotoxicity) depending upon the concentrations used. In preliminary experiments FA, FA_1 and FA_3 showed an antiviral activity against a neurovirulent strain of the Sindbis virus (Table 2).

When FA was further chromatographed on a silica gel column, seven fractions (F1–F7) were obtained. We could separate the three biological effects (innocuous, proliferation and cytotoxicity) in different fractions at different concentrations. Thus, F1 and F2 showed the proliferating activity at low concentration (0.2–3.3 μg/ml). F3, F4, F5 and F6 were cytotoxic at concentrations higher than 42 μg/ml and the antiviral activity was rescued in fractions F2 and F7, the last one being the most effective, inhibiting NSV replication with a TI of 17 (Table 2). A high alkaloid presence was detected in F5. However, this fraction does not show antiviral activity.

A further F7 purification gave nine fractions. All of them were innocuous at the concentration tested (0.2–50 μg/ml). Three of the fractions obtained (F_711, F_714, F_717) could inhibit NSV replication using the colorimetric assay, F_714 and F_717 displayed an antiviral activity at higher concentrations; therefore, their TI is quite low (Table 2). In addition, we studied the antivi-

Table 1
Biological activity of fractions and sub-fractions of *G. cardoniana*

G. cardoniana	Innocuous[a] (μg/ml)	Cytotoxicity[b] (μg/ml)	Proliferation[c] (μg/ml)
Fractions			
FA	6.25–51	>51	0.2–6
FA_1	0.2–293	>293	
FA_2	0.2–558	>558	
FA_3	0.2–117	>117	
FB	0.2–112	>112	
FB_1	0.2–18		>18
FB_2	0.2–11		>12
FA sub-fractions			
F1	3.13–333	>333	0.2–3
F2	3.13–232	>232	0.2–3
F3	0.2–43	>43	
F4	0.2–49	>49	
F5	0.2–63	>63	
F6	0.2–49	>49	
F7	0.2–115	>115	
F7 sub-fractions			
F_70–F_724	0.2–50		

[a] Innocuous, 50–110% cellular viability.
[b] Cytotoxicity, <50% cellular viability.
[c] Proliferation, >110% cellular viability.

ral effect of these fractions, using an inhibition plaque assay. By this method, the difference in the plaque forming units between the viral control and fractions F7, F_711 and F_714 is statistically significant, the best fraction was F_711 with a frequency (F) of 31.69 and $P = 0.000493$ (Fig. 1). Also, the difference between F7 and F_711 is statistically significant, $F = 5.79$ and $P = 0.042789$, however, the difference between F7 and F_714 is not significant ($F = 1$; $P = 0.34$) when used at 10 μg/ml, these results are in agreement with the ones obtained in the colorimetric assay where F_711 showed a higher therapeutic index compared with F_714 and F_717 (Table 2).

4. Discussion

Different *Guatteria* species have shown an antiparasitic activity (Mahiou et al., 1994; Weniger et al., 2000). The present study was carried out to evaluate the biological activity of fractions obtained from *G. cardoniana* focusing in the antiviral activity against Sindbis virus. The distinct biological activity shown by a crude extract of leaves and twigs from *G. cardoniana* was separated in different fractions. F_711, F_714 and F_717 were the fractions where the antiviral activity was obtained. Due to the fact that F_714, F_717 are eluted subsequently to F_711, it is possible that the compound(s) accountable for the antiviral activity in these fractions are the same or/and with similar chemical structure to the ones responsible for this activity in F_711 fraction. F_711 showed a higher percent of inhibition in the colorimetric assay, with a TI of 20.48, by plaque assay this fraction is able to inhibit the NSV replication in approximately 45%. We are in the process of further purification, isolation and identification of the compound(s) that are responsible for the inhibition of NSV replication in these fractions. The present work is the first report of fractions from genera *Guatteria* with antiviral activity.

Table 2
Anti-NSV activity of fractions and sub-fractions of *G. cardoniana*

G. cardoniana	IC50 (μg/ml)	EC50 (μg/ml)	TI (IC50/EC50)
Fractions			
FA	50.83	2.48	20.50
FA_1	292.94	18.18	16.11
FA_3	116.51	55.91	2.08
FA sub-fractions			
F2	232.14	27.5	8.44
F7	114.53	6.73	17.08
F7 sub-fractions			
F_711	249.92	12.20	20.48
F_714	342.27	60.63	5.64
F_717	254.79	50.79	5.01

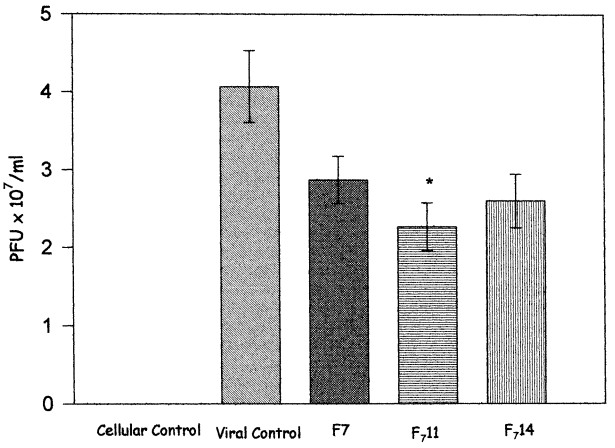

Fig. 1. Inhibition of NSV infection by *G. cardoniana* subfractions (10 μg/ml) using plaque assay. *, $P = 0.042789$.

Acknowledgements

The authors are grateful to Professor Anibal Castillo for the plant material botanical identification and to Dr Rafael Barges for the statistical analyses. This research was supported by CONICIT project No G-97000627.

References

Kurokawa, M., Nagasaka, K., Hirabayashi, T., Uyama, S., Sato, H., Kageyama, T., Kadota, S., Ohyama, H., Hozumi, T., Namba, T., Shiraki, K., 1995. Efficacy of traditional herb medicines in combination with Acyclovir against Herpes simplex virus type 1 infection in vitro and in vivo. Antiviral Research 27, 19–37.

Leboeuf, M., Cavd, A., Bhaunik, P.K., Mukherjee, B., Mukherjee, R., 1982. The phytochemistry of Annonaceae. Phytochemistry 21, 2783–2813.

Mahiou, V., Roblot, F., Hocquemiller, R., Cavé, A., Rojas de Arias, A., Inchausti, A., Yaluff, G., Fournet, A., Angelo, A., 1994. New aporphine alkaloids from *Guatteria foliosa*. Journal of Natural Products 7, 890–895.

Padma, P., Pramod, N.P., Thyogarajan, S.P., Khosa, R.L, 1998. Effect of the extract of *Annona muricata* and *Petunia nyctaginiflora* on Herpes simplex virus. Journal of Ethnopharmacology 61, 81–83.

Shearman, M.S., Ian Ragan, C., Iversen, L.L., 1994. Inhibition of PC12 cell redox activity is a specific early indicator of the mechanism of β-amyloid-mediated cell death. Proceedings of National Academy of Science USA 91, 1470–1474.

Weniger, B., Aragon, R., Deharo, E., Bastida, J., Codina, C., Lobstein, A., Antón, R., 2000. Antimalarial constituents from *Guatteria amplifolia*. Pharmazie 55, 867–868.

Zembower, D.E., Lin, Y.-M., Flavin, M.T., Chen, F.-C., Korba, B.E., 1998. Robustaflavone, a potential non-nucleoside anti-hepatitis B agent. Antiviral Research 39, 81–88.