Isolated Flavonoids against Mammary Tumour Cells LM2

Camila B. de A. Carli[a], Djamile C. de Matos[a], Flávia C. M. Lopes[a], Danielle C. G. Maia[a], Maristela B. Dias[a], Miriam Sannomiya[b], Clenilson M. Rodrigues[b], Marcio A. Andreo[b], Wagner Vilegas[b], Lucas L. Colombo[c], and Iracilda Z. Carlos[a,*]

[a] UNESP – São Paulo State University, Faculdade de Ciências Farmacêuticas, C. Postal 502, 14801–902, Araraquara, SP, Brazil. Fax: 55 16 33 01 65 59. E-mail: carlosiz@fcfar.unesp.br

[b] UNESP – São Paulo State University, Instituto de Química de Araraquara, C. Postal 355, 14801–970, Araraquara, SP, Brazil

[c] Buenos Aires University, Instituto de Oncologia, UBA, Buenos Aires, Argentina

* Author for correspondence and reprint requests

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The purpose of the present study was to investigate antitumour and anti-inflammatory activities of flavonoids isolated from *Byrsonima crassa, Davilla elliptica* and *Mouriri pusa*. The antitumour activity was measured by the MTT assay in murine mammary tumour cells (LM2) and the IC_{50} values of the flavonoids tested ranged from (31.5 ± 2.97) to (203.1 ± 5.9) μg/ml. The flavonoids **1** (myricetin-3-*O*-α-L-rhamnopyranoside) and **3** (quercetin-3-*O*-galactopyranoside) from *D. elliptica* were the most active ones against the tumour cells. The same samples were tested to determine the inhibition of the release of nitric oxide (NO) and of the tumour necrosis factor-alpha (TNF-α) in murine macrophages by the Griess and ELISA sandwich assay, respectively. Almost all the samples showed inhibitory activity to the release of NO but not of TNF-α. Of all substances tested, flavonoids **2** (quercetin) and **6** (myricetin) may show promising activity in the treatment of murine breast cancer by immunomodulatory and antiproliferative activities.

Key words: Flavonoids, Antitumour Activity, Cytokine

Introduction

Mouriri pusa Gardn. (Melastomataceae), *Davilla elliptica* St. Hill. (Dilleniaceae), and *Byrsonima crassa* Niedenzu (IK) (Malpighiaceae) are native trees of the savannah-like Brazilian cerrado (Sannomiya *et al*., 2004; Andreo *et al*., 2006; Santos *et al*., 2006). All of them are used in popular medicine for the treatment of gastrointestinal disturbances and inflammatory processes. Since antitumour activity is related to anti-inflammatory activity, the use of such traditional plants may show a possible antineoplasic activity.

In some tumour types over 70% of the mass of the tumour consists of infiltrating leukocytes. These tumour-associated leukocytes, especially macrophages, release angiogenic factors, mitogens, proteolytic enzymes and chemotactic factors, recruiting more inflammatory cells and sustaining tumour growth, invasion and angiogenesis (Yan *et al*., 2006). Macrophages constitute one of the main groups of phagocytes in the immunological system, and part of their effectiveness is due to their production of nitric oxide (NO) and cytokines. (Keil *et al.*, 1999). Research in the last few years has revealed, however, the involvement of NO in carcinogenesis through the stimulation of processes such as initiation, promotion, progression, metastasis and angiogenesis (Hofseth *et al*., 2003). The production of NO is regulated by cytokines such as tumour necrosis factor-alpha (TNF-α) and interferon-γ (IFN-γ) (Kovalovsk *et al*., 2000).

Experiments were performed to assess the antiproliferative and anti-inflammatory activity of flavonoids isolated from *B. crassa, D. elliptica* and *M. pusa*. The aim of the present study was to contribute to the discovery of potential phytopharmaceutics which, besides fulfilling an antiproliferative role, can also act as anti-inflammatory agents in the contention of tumours. This activity is not observed in treatments with the drug cisplatin, which is widely used in the treatment of breast tumours.

Experimental

Plant material

The extracts of the plants were prepared at the Instituto de Química, UNESP at Araraquara, Brazil under the guidance of Prof. Dr. Wagner Vilegas. Leaves of each species were used to produce the extracts with organic solvents as specified below. The extracts were stored in a freezer at –20 °C, forming part of a bank of extracts at the Instituto de Química.

D. elliptica: The air-dried and powdered leaves were extracted (three times) with chloroform, methanol and 80% aqueous methanol successively at room temperature. Solvents were evaporated at 60 °C under reduced pressure, affording the $CHCl_3$ extract, MeOH extract and MeOH/water extract. The MeOH extract was partitioned three times with a mixture of EtOAc/water (1:1 v/v). The isolation of the flavonoids **1–7** is described elsewhere (Rinaldo *et al*., 2006).

B. crassa: The air-dried and powdered leaves were extracted (three times) with chloroform, methanol and 80% aqueous methanol successively at room temperature. Solvents were evaporated at 60 °C under reduced pressure, affording the $CHCl_3$ extract, MeOH extract and MeOH/water extract. The MeOH extract was partitioned three times with a mixture of EtOAc/water (1:1 v/v). The EtOAc fraction was dissolved in 20 ml of a mixture consisting of 10 ml lower phase + 10 ml upper phase of the solvent system ethyl acetate/*n*-propanol/water (140:8:80 v/v/v). The flavonoid **8** was isolated as described previously (Sannomiya *et al*., 2004).

M. pusa: The air-dried and powdered leaves of *M. pusa* were extracted with dichloromethane (DCM) and methanol (MeOH) successively at room temperature. Solvents were evaporated at 60 °C under reduced pressure to yield the DCM extract and MeOH extract. A portion of the DCM extract was fractionated by silica gel column chromatography (Merck, Art. 7731, 35 cm × 3 cm i.d.) and eluted with a gradient of hexane/DCM. The flavonoid **9** was isolated as described previously (Andreo *et al*., 2006).

Animals

Swiss mice (6–8 weeks old, weighing 18–25 g) were maintained in a polycarbonate box at (23 ± 1) ºC, (55 ± 5)% humidity, 10–18 circulations/h and a 12-h light/dark cycle, with free access to water and chow (Purina). All animals were maintained and handled according to International Ethical Guidelines for the Care of Laboratory Animals (Faculty Ethics Committee #06/2005).

Peritoneal exudate cells

Thioglycollate-elicited peritoneal exudate cells (PEC) were harvested from Swiss mice in 5.0 ml of sterile phosphate-buffered saline (PBS), pH 7.4. The cells were washed three times by centrifugation at 200 × *g* for 5 min at 4 °C with 3.0 ml PBS. The cells were then resuspended in 1.0 ml RPMI-1640 culture medium (Sigma) containing $2 \cdot 10^{-5}$ M β-mercaptoethanol (Sigma), 100 U/ml penicillin (Sigma), 100 U/ml streptomycin (Sigma), 2 mM L-glutamine (Sigma), and 5% fetal bovine serum (Sigma). The medium with this composition was denoted complete RPMI-1640 (RPMI-1640-C) medium and was used for cell counts in a Neubauer chamber (Boeco, Hamburg, Germany). For the proposed tests, the cell suspension was adjusted to a concentration of $5 \cdot 10^6$ cells/ml.

LM2 cell line

The murine mammary tumour cells (LM2) were provided by Institute of Oncologia Angel H. Roffo, Buenos Aires, Argentina. This cell line is originated from a reproductive Balb/c female mouse and was maintained by subcutaneous passages (M2), from which the *in vitro* line was obtained (LM2). This is a very undifferentiated tumour which grows rapidly and forms metastases spontaneously (Hegyesi *et al*., 2007).

Assessment of cellular viability

The cell viability assay was assessed by a method based on the capacity of viable cells to cleave the tetrazolium ring present in MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], owing to the action of dehydrogenase enzymes in the active mitochondria, under the formation of purple formazan crystals (Mosmann, 1983).

The cells were resuspended in RPMI-1640 medium containing 5% fetal bovine serum, 100 IU/ml penicillin, 100 IU/ml streptomycin and 50 mM β-mercaptoethanol, and adjusted to $5 \cdot 10^6$ cells/ml. 100 μl of the suspension and 100 μl of pure flavonoids and cisplatin (*cis*-Pt) were added to each well of a microplate, where the peritoneal macrophages were incubated for 24 h. MTT

solution was added (100 μl) and the plate was incubated for 3 h at 37 ºC with 5% CO_2. Absorbance was read in a UV/Vis spectrophotometer at a wavelength of 540 nm, with a reference filter of 620 nm. The culture medium and cells alone were used as control, representing 100% viability of the macrophages.

Inhibition of NO production

Adherent PEC were resuspended in RPMI-1640-C medium and adjusted to $5 \cdot 10^6$ cells/ml. 100 μl of the suspension were placed in each well of a 96-well microplate and mixed with 100 μl of pure flavonoids and *cis*-Pt. The mixture was completed with 100 μl LPS (lipopolysaccharide from *Escherichia coli* O111:B4), and the plates were then incubated for 24 h at 37 ºC under 7.5% CO_2. LPS was used as positive control. The nitrite concentrations were indirectly measured by a quantitative colorimetric assay using the Griess reagent system: 50 μl aliquots of supernatant were added to 50 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine and 3% H_3PO_4), incubated at room temperature for 10 min, and the absorbance was measured at 540 nm in an Elisa microplate reader (Multiskan Ascent Labsystems, Helsinki, Finland). Samples were assayed in triplicate in four experiments and reported as μmol NO/$5 \cdot 10^5$ cells, measured from the standard curve (Green *et al.*, 1982).

Inhibition of TNF-α production

For inhibition of the TNF-α production, adherent PEC were stimulated with the isolated flavonoids and *cis*-Pt, together with LPS (1 μg/ml). After 24 h, the supernatants were removed, filter-sterilized, and stored at –80 °C until assayed by the immunoenzymatic assay (BD Pharmingen, San Diego, USA) according to the manufacturer's protocol. Results were expressed in pg/ml.

Antiproliferative activity

Cellular growth was quantified by the MTT assay. Cells were detached with trypsin, washed and transferred into 96-well microtiter plates at a concentration of $3 \cdot 10^4$ cell/ml. 190 μl of the tumour cell suspension were plated in the wells and incubated at 37 °C under 5% CO_2. The microtiter plates containing cells were pre-incubated for 24 h to allow stabilization prior to the addition of the samples. After this period, 10 μl of pure flavonoids were added and incubated for 48 h under the same conditions. Cisplatin was used as positive control. After culture, the medium was replaced with fresh medium containing 1 mg/ml of MTT. After 3 h, the medium was removed and 100 μl of isopropanol (Mallinckrodt) were added to solubilize the formazan crystals formed. The absorbance was measured at 540 nm with a reference filter of 620 nm in a spectrophotometer (Multiskan Ascent Labsystems). The 50% inhibitory concentration for cell growth (IC_{50}) was defined as the dose resulting in 50% reduction of tumour cell proliferation and was calculated with Microcal Origin (Mosmann, 1983).

Statistical analysis

Data are expressed as mean ± standard deviation (SD), and the Tukey test (Graphpad Instat 3.05) was used to determine the significance of the differences between the positive control and experimental groups.

Results and Discussion

It is well established that medicinal plants are useful sources of clinically relevant antitumour compounds (Cragg *et al.*, 1994). Furthermore, it is important to study anticancer drugs that act on the immune system by modulating the production of mediators that can control the growth of a tumour.

Analyzing the results for the production of NO, we observed that almost all the substances tested exerted immunosuppressive activity on the LPS-stimulated macrophages (Table I). Flavonoid **2** showed the strongest inhibition of NO production by macrophages (58.6%), but **3** (53.5%) and **8** (57.1%) also showed moderate inhibition of this mediator ($p < 0.001$). All samples showed statistical difference from the LPS control, except for **1** and **7** ($p > 0.05$). The standard drug *cis*-Pt showed no inhibitory activity on NO formation. Further reports have confirmed the role of NO in tumour carcinogenesis. NO synthase inhibitors prevent tumour-induced angiogenesis in mammary tumours (Jadeski and Lala, 1999), and therapy of cancer metastases can be accomplished by the inactivation of iNOS (Xie and Fidler, 1998). In our study, the flavonoids **2** ($p < 0.05$), **4** ($p < 0.05$) and **8** ($p < 0.001$) inhibited TNF-α production, an expected

Table I. Anti-inflammatory activity of the isolated flavonoids.

Flavonoid	Concentration	NO inhibition [μmol/5 · 10^5 cells]	TNF-α inhibition [pg/ml]
Davilla elliptica (MeOH extract)			
Myricetin-3-*O*-α-L-rhamnopyranoside (**1**)	25 μg/ml	66.4 ± 5.7 (7.2%)	202.4 ± 20.2 (13.0%)
Quercetin (**2**)	50 μg/ml	29.6 ± 5.2*** (58.6%)	148.2 ± 8.0* (36.3%)
Quercetin-3-*O*-galactopyranoside (**3**)	200 μg/ml	33.3 ± 3.3*** (53.5%)	167.3 ± 7.0 (28.2%)
Quercetin-3-*O*-arabinopyranoside (**4**)	200 μg/ml	55.4 ± 6.9** (22.6%)	159.4 ± 11.1* (31.5%)
Myricetin-3-*O*-β-galactopyranoside (**5**)	200 μg/ml	56.1 ± 2.9* (21.6%)	161.4 ± 40.9 (30.6%)
Myricetin (**6**)	200 μg/ml	51.5 ± 2.8*** (28.0%)	231.5 ± 20.3 (0.5%)
Quercetin-3-*O*-α-L-rhamnopyranoside (**7**)	25 μg/ml	65.9 ± 4.8 (7.9%)	216.5 ± 15.9 (7.0%)
Byrsonima crassa (MeOH extract)			
Amentoflavone (**8**)	100 μg/ml	30.7 ± 5.1*** (57.1%)	126.6 ± 25.1*** (45.6%)
Mouriri pusa (DCM extract)			
Quercetin-3-*O*-3-D-galactoside (**9**)	200 μg/ml	38.4 ± 2.8*** (46.3%)	216.6 ± 16.3 (6.9%)
Cisplatin (*cis*-Pt)	7.8 g/ml	71.5 ± 1.0 (0.1%)	232.7 ± 1.0 (0%)

Values represent mean ± SD; values in brackets represent percentage of inhibition when compared with LPS. NO and TNF-α inhibition by PEC in the presence of flavonoids and *cis*-Pt. Adherent cells (5 · 10^6 cells/ml) were incubated for 24 h with flavonoids and *cis*-Pt together with LPS (1 μg/ml). Cells incubated just with LPS were used as a positive control and cells in culture medium (RPMI-1640-C) as a negative control. For the proposed tests, the pure flavonoids and *cis*-Pt were tested at concentrations resulting in more than 70% cell viability.
*** $p < 0.001$; ** $p < 0.01$ and * $p < 0.05$ in comparison with LPS.

Table II. Antiproliferative activity of isolated flavonoids.

Flavonoid	IC_{50} [μg/ml]
Davilla elliptica (MeOH extract)	
Myricetin-3-*O*-α-L-rhamnopyranoside (**1**)	31.5 ± 2.7
Quercetin (**2**)	59.5 ± 4.4
Quercetin-3-*O*-galactopyranoside (**3**)	33.8 ± 4.0
Quercetin-3-*O*-arabinopyranoside (**4**)	43.9 ± 2.5
Myricetin-3-*O*-β-galactopyranoside (**5**)	35.7 ± 3.8
Myricetin (**6**)	56.3 ± 10.0
Quercetin-3-*O*-α-L-rhamnopyranoside (**7**)	203.1 ± 5.9
Byrsonima crassa (MeOH extract)	
Amentoflavone (**8**)	166.1 ± 2.0
Mouriri pusa (DCM extract)	
Quercetin-3-*O*-3-D-galactoside (**9**)	68.7 ± 11.9
Cisplatin (*cis*-Pt)	168.4 ± 3.9

Adherent cells (3 · 10^4/ml) were incubated for 24 h. The flavonoids and cisplatin were added to the cells and incubated for 48 h. Cells in culture medium (control) correspond to 100% viability. The cell viability was determined by the MTT assay as described previously (Mosmann, 1983).

effect of antitumour and anti-inflammatory activity. The flavonoid **8** showed the highest inhibition of TNF-α production by macrophages (45.6%). Almost all samples and *cis*-Pt showed no inhibition of the production of TNF-α by macrophages ($p > 0.05$). TNF-α acts as a tumour progression factor by modulating the tumour motility or the augmentation of adhesion molecule expression on the target organs (Gelin *et al.*, 1991). Previous studies have shown the role of TNF-α, IL-1 and IFN-γ in the NO production by macrophages (Liew, 1995). In the present research, the results of the inhibition of production of these mediators by all samples tested showed preferential inhibition of NO, compared to inhibition of TNF-α, probably due to the massive inhibition of cytokines such as IL-1 and IFN-γ, which are involved in the release of NO.

The flavonoids were also tested for their antiproliferative activities on the LM2 line of tumour cells. All flavonoids tested showed a better anti-

proliferative activity than *cis*-Pt, except for the flavonoid **7**. The flavonoid **1** showed the highest toxicity to LM2 tumour cells (IC_{50} 31.5 μg/ml), followed by **3** (IC_{50} 33.8 μg/ml) and **5** (IC_{50} 35.7 μg/ml), all obtained from *D. elliptica* (Table II). Although the mechanism of the tumour inhibition activity of flavonoids is not clear, they are reported to decrease the high glycolytic activity of Ehrlich's ascites tumour cells by inhibition of (Na^+,K^+)-ATPase in the plasma membrane (Pomilio *et al*., 1994). Flavonoids have been shown to be effective scavengers of reactive oxygen species (ROS), and they have been suggested to exhibit anticancer activities depending heavily on their antioxidant and chelating properties (Duthie and Dobson, 1999).

Of all the flavonoids tested, **2** and **6** show promising activity for mammary cancer treatment due to their immunomodulatory and antiproliferative activities. The results described here highlight the importance of conducting an in-depth study of the species of the Brazilian flora, and show the great potential of its biodiversity in the treatment of chronic inflammatory diseases such as cancer. Subsequent studies will be necessary to reveal the mechanism of anti-tumour action of the species studied here.

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