Bioactivity of Biflorin, a Typical *o*-Naphthoquinone Isolated from *Capraria biflora* L.

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Z. Naturforsch. **60 c**, 394–398 (2005); received October 27/December 27, 2004

Capraria biflora L. (Scrophulariaceae) is a perennial shrub widely distributed in several countries of tropical America. The present work verified the cytotoxic and antioxidant potential of biflorin, an *o*-naphthoquinone isolated from *C. biflora* collected in the northeast region of Brazil. The cytotoxicity was tested on three different animal cell models: mouse erythrocytes, sea urchin embryos and tumor cells, while the antioxidant activity was assayed by the thiocyanate method. Biflorin lacked activity on mouse erythrocytes as well as on the development of sea urchin eggs, but strongly inhibited the growth of all five tested tumor cell lines, especially the skin, breast and colon cancer cells with IC_{50} of 0.40, 0.43 and 0.88 μg/ml for B16, MCF-7 and HCT-8, respectively. Biflorin also showed potent antioxidant activity against autoxidation of oleic acid in a water/alcohol system.

Key words: Capraria biflora, Biflorin, Cytotoxicity

Introduction

Capraria biflora L. (Scrophulariaceae) is a perennial shrub distributed in North and South America. Its leaves are used to treat pain, fever, flu, vomiting, childbirth recovery, diarrhea, hemorrhoids, rheumatism, and swelling (Correia, 1984), while the roots have antibacterial properties (Serpa, 1958). Phytochemical investigations of this plant species led to the isolation and characterization of biflorin [6,9-dimethyl-3-(4-methyl-3-pentenyl)naphtha[1,8-*bc*]-pyran-7,8-dione], an antibacterial *o*-naphthoquinone, from its roots (Lima *et al.*, 1958; Fonseca *et al.*, 2003), and two iridoids, harpagide and caprarioside, and the insecticidal sesquiterpenoids caprariolides A, B, C and D from the aerial parts (Heinrich and Rimpler, 1989; Collins *et al.*, 2000).

Pharmacological studies with *C. biflora* leaves aqueous extract showed potent cytotoxic, analgesic and anti-inflammatory activities (Nascimento *et al.*, 1984; Acosta *et al.*, 2003a, b). Biflorin, on the other hand, is an antibiotic compound, strong active against Gram-positive and alcohol-acid resistant germs, with no other described bioactivity (Lima *et al.*, 1958). Since the *o*-naphthoquinones constitute a promising group of antitumor compounds (Driscoll *et al.*, 1974), the aim of the present study was to evaluate the cytotoxic potential of biflorin and also its oxidative properties. The cytotoxic activity was measured as the ability to inhibit the sea urchin eggs development, tumor cell lines proliferation and to cause lysis of mouse erythrocytes. The oxidative properties were accessed by the inhibition of autoxidation of oleic acid.

Materials and Methods

Isolation of biflorin

Biflorin was isolated from the roots of *C. biflora* collected in a plantation at Fortaleza, Ceará, Brazil. A voucher specimen (# 30848) is deposited at the Prisco Bezerra Herbarium (EAC), Department of Biology, Federal University of Ceará.

The air-dried powdered roots (450 g) were twice extracted with $CHCl_3$ to give 4.6 g of the extract after evaporating the solvent under reduced pressure. The residue (4.6 g) was chromatographed over silica gel, using gradient mixtures of 0 to 100% EtOAc/petroleum ether as eluent. Fractions were pooled according to thin-layer chromato-

0939–5075/2005/0500–0394 $ 06.00

graphic (TLC) analysis. From combined fractions 10–26 eluted at 30:70 (v/v) a precipitate was obtained, filtered and recrystallized from petroleum ether to provide biflorin (100 mg). The structure of this compound (Fig. 1) has been determined by spectroscopy means, including one and two dimensional NMR spectral analysis such as COSY, HMQC, HMBC, physical properties and comparison with data from literature (Fonseca *et al.*, 2003).

Cytotoxicity against tumor cell lines

The cytotoxicity of biflorin was tested against CEM and HL-60 (human leukemias), B16 (murine melanoma), HCT-8 (human colon) and MCF-7 (human breast cancer) cell lines obtained from the National Cancer Institute, Bethesda, MD, USA. The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin, and incubated at 37 °C with a 5% CO_2 atmosphere. For experiments, the cells were plated in 96-well plates (10^5 cells/well for adherent cells or 0.5×10^5 cells/well for suspended cells in 100 μl of medium). After 24 h, biflorin (0.39 to 25.0 μg/ml) dissolved in DMSO 1% was added to each well and the cells were incubated for 72 h. Control groups received the same amount of DMSO. Doxorubicin (Doxolem®, Zodiac Produtos Farmacêuticos S/A, Brazil) and etoposide (Etosin®, Asta Medica Ltda, Brazil) were used as positive control. The 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-*2H*-tetrazolium bromide (MTT) to a purple formazan product (Mosmann, 1983). At the end of the incubation, the plates were centrifuged, and then the medium was replaced by fresh medium (200 μl) containing 0.5 mg/ml MTT. 3 h later, the MTT formazan product was dissolved in 150 μl DMSO, and the absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada). The drug effect was quantified as the percentage of control absorbance of reduced dye at 550 nm.

Antimitotic activity on sea urchin eggs

The assay was performed following the method described by Jimenez *et al.* (2003). Adult sea urchins (*Lytechinus variegatus*) were collected at Lagoinha beach, on the northeastern coast of Brazil. Gamete elimination was induced by injecting 3.0 ml of 0.5 M KCl into the urchin's coelomic cavity. For fertilization, 1 ml of a sperm suspension (0.05 ml of concentrated sperm in 2.45 ml of filtered sea water) was added to every 50 ml of egg solution. The assay was carried out in 24-multiwell plates. Biflorin was added immediately after fecundation (within 2 min) to get concentrations of 1, 3, 10, 30 and 100 μg/ml in a final volume of 2 ml. Doxorubicin and etoposide were used as positive control. At appropriate intervals, aliquots of 200 μl were fixed in the same volume of 10% formaldehyde to obtain first and third cleavages and blastulae. 100 eggs or embryos were counted for each concentration of test substance to obtain the percentage of normal cells.

Hemolytic assay

The test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mM $CaCl_2$, following the method described by Jimenez *et al.* (2003). Biflorin was tested at concentrations ranging from 3.9 to 1000.0 μg/ml. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectrophotometrically as the absorbance at 540 nm.

Antioxidant assay: Autoxidation of oleic acid

The oxidation of oleic acid [9(*Z*)-octadecenoic acid], a monounsaturated fatty acid that is the major constituent of triglycerides, was monitored for 6 d, as described by Masuda *et al.* (1992). Briefly, biflorin (2 mg) in 4.0 ml of EtOH, 4.1 ml of oleic acid (2.53%) diluted in EtOH, 8.0 ml of phosphate buffer (50 mM, pH 7) and 3.9 ml of distilled water were placed in a vial (50.0 ml) with a screw cap and placed in a oven at 40 °C in the dark. Oxidation of oleic acid was monitored by the following method: to 0.1 ml of this sample, 9.7 ml of EtOH (75%), 0.1 ml of ammonium thiocyanate (30%) and 0.1 ml of ferrous chloride (0.2 mM) diluted in HCl (3.5%) were added to the reaction mixture, and after exactly 3 min the absorbance was measured at 500 nm, for each day during 6 d.

Statistical analysis

Data are presented as mean ± S.E.M. The IC_{50} values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA).

Results and Discussion

A cell culture provides an important tool to study the cytotoxicity of compounds with potential therapeutic activity (Pailard *et al.*, 1999). The present study evaluated the bioactivity of biflorin, an *o*-naphthoquinone isolated from *C. biflora*, in mouse erythrocytes, developing embryos of sea urchin, tumor cell lines and the oleic acid oxidant assay.

Biflorin (Fig. 1) as well as the chemotherapeutic agents inhibited the proliferation of the five cell lines in a dose-dependent manner analyzed through the MTT assay (Fig. 2). Moreover, it presented a stronger activity against the solid tumor cells (B16, MCF-7 and HCT-8), with higher potency than that of etoposide in HCT-8 cells, as confirmed through the IC_{50} values (Table I). In a previous work, Nascimento *et al.* (1984) demonstrated that the extract of the roots of *C. biflora* was strongly cytotoxic against KB cells with an IC_{50} value of 3 μg/ml. Present data suggests that this activity could be related to the presence of biflorin in the roots of *C. biflora.* According to the literature, pure compounds could be considered satisfactorily to warrant further studies as an antineoplastic drug when present an IC_{50} value lower than 1 μg/ml or 1 μM (Pessoa *et al.*, 2000). Thus, biflorin could be considered a promising antitumor compound.

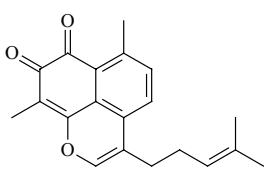

Fig. 1. Molecular structure of biflorin [6,9-dimethyl-3-(4-methyl-3-pentenyl)naphtha[1,8-*bc*]-pyran-7,8-dione] isolated from *Capraria biflora.*

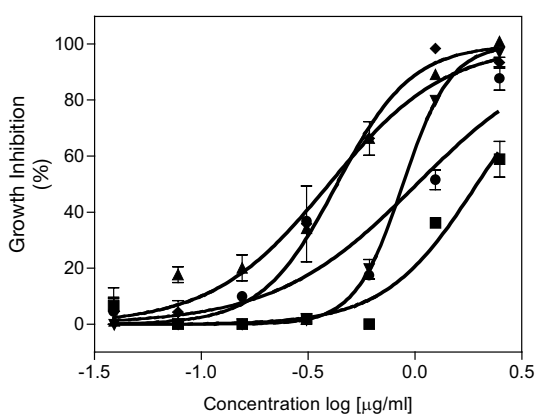

Fig. 2. The effect of biflorin on CEM (●), HL-60 (■), B16 (▲), HCT-8 (▼) and MCF-7 (◆) cell growth analyzed by the MTT assay after 72 h of incubation. Data are presented as mean ± S.E.M. of 3 experiments. The curves were obtained by non-linear regression.

In the sea urchin eggs assay, biflorin did not inhibit the development of the embryos even at the highest concentration tested (100 μg/ml), differently of what was observed for both of the tested chemotherapeutic agents (Table II). Generally, cytotoxic substances tested in the sea urchin eggs and tumor cells have shown to be active in both assays, although compounds may present a higher IC_{50} value in the sea urchin eggs than in tumor cells, as observed herein for doxorubicin and etoposide (Montenegro *et al.*, 2004).

The study of alterations in sea urchin egg development is a suitable model for detecting cytotoxic, teratogenic and antineoplastic activities of new compounds (Jacobs and Wilson, 1986). This assay method can detect such selective agents as DNA and RNA synthesis inhibitors, protein synthesis inhibitor and inhibitors of microtubule assessment (Jacobs and Wilson, 1986; Fusetani, 1987). Thus, the selectivity of biflorin towards mammallian cells suggests a cycle-dependent activity or inquires about a mechanism of action that would involve a specific structure of pathway different to that described for the tested antineoplastic drugs.

To verify whether biflorin cytotoxicity is related to membrane disruption, the ability to induce lyses of mouse erythrocytes was investigated and no membrane damage was found (data not shown).

The cytotoxicity of naphthoquinones has been extensively described in the literature (Driscoll *et al.*, 1974; Silva *et al.*, 2002). Lapachol [2-hydroxy-3-(3-methyl-2 butenyl)-1,4-naphthoquinone], extracted from Pau d'arco (family Bignoneaceae), is one of the most studied naphthoquinones with cytotoxic, antitumor, antibiotic, antimalarial, mollucicidal, larvicidal, cercaricidal, trypanocidal, anti-inflammatory and anti-ulceric properties (Driscoll *et al.*, 1974; Lagrota, 1978; Carvalho *et al.*, 1988; Oliveira *et al.*, 2002). And according to several authors, oxidative stress leading to DNA damage and alkylation of nuclear cellular nucleophiles are the two major mechanisms of quinone cytotoxicity (Bolton *et al.*, 2000).

To determine the oxidative properties of biflorin, the oxidation of oleic acid was assayed by the thiocyanate method but biflorin showed anti-

Cell line	Biflorin IC_{50} [μg/ml (μM)] CI 95% [μg/ml]	Etoposide IC_{50} [μg/ml (μM)] CI 95% [μg/ml]	Doxorubicin IC_{50} [μg/ml (μM)] CI 95% [μg/ml]
B16	0.40 (1.20)	0.11 (0.19)	0.03 (0.05)
	1.42–1.91	0.07–0.18	0.02–0.04
MCF-7	0.43 (1.30)	> 5.80 (9.85)	0.20 (0.34)
	1.29–1.77		0.17–0.24
HCT-8	0.88 (2.80)	0.11 (0.18)	0.04 (0.07)
	4.73–12.5	0.08–0.15	0.03–0.05
HL-60	1.95 (6.30)	0.01 (0.02)	0.02 (0.03)
	1.26–2.69	0.01–0.02	0.01–0.02
CEM	1.02 (3.30)	0.03 (0.05)	0.02 (0.03)
	1.24–1.96	0.02–0.04	0.01–0.02

Table I. Inhibitory effect on cultured cell growth of biflorin, etoposide and doxorubicin (positive controls) of 5 different tumor cell lines. Data are presented as IC_{50} values and their 95% confidence interval (CI 95%) is obtained by nonlinear regression.

Table II. Antimitotic activity of biflorin, doxorubicin and etoposide (positive controls) on sea urchin (*Lytechinus variegatus*) eggs development. Data are presented as IC_{50} values and their 95% confidence interval (CI 95%) for first and third cleavages and blastulae obtained by nonlinear regression.

Substance	1st cleavage IC_{50} [μg/ml (μM)] CI 95% [μg/ml]	3rd cleavage IC_{50} [μg/ml (μM)] CI 95% [μg/ml]	Blastulae IC_{50} [μg/ml (μM)] CI 95% [μg/ml]
Biflorin	> 100.00 (324.60)	> 100.00 (324.60)	> 100.00 (324.60)
Doxorubicin	6.28 (10.83)	0.34 (0.59)	0.54 (0.93)
	4.34–9.09	0.16–0.73	0.27–1.07
Etoposide	7.85 (13.33)	0.30 (0.51)	0.85 (1.44)
	1.21–48.84	0.20–0.46	0.53–1.37

oxidant activity against autoxidation of oleic acid in a water/alcohol system until 4 d of incubation while in the fifth and sixth day, it was not so efficient (Fig. 3). This data strongly suggest that the cytotoxicity of biflorin was not related to the generation of reactive oxygen species.

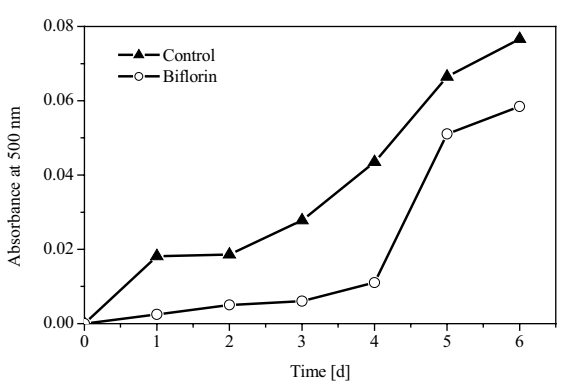

Fig. 3. Effect of biflorin on autoxidation of oleic acid. Oxidation of oleic acid was assayed by the thiocyanate method described in Materials and Methods.

In conclusion, biflorin is a strong cytotoxic agent against tumor cell lines, but with no activity on sea urchin eggs. Different from observed with other cytotoxic naphthoquinones, biflorin does not induce oxidative stress. Further studies are already in progress for the elucidation of the mechanism of cytotoxic activity of biflorin against the tumor cells, and also to verify whether this cytotoxicity is also observed against normal human cells.

Acknowledgements

We wish to thank CNPq, CAPES, Instituto Claude Bernard, FUNCAP, Banco do Nordeste and FINEP for the financial support in the form of grants and fellowship awards. The authors also thank the National Cancer Institute (Bethesda, MD, USA) for the donation of the tumor cell lines used in this study. The authors thank Silvana França dos Santos and Maria de Fátima Texeira for technical assistance.

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