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Cytotoxic activity of nepetin, a flavonoid from *Eupatorium ballotaefolium* HBK

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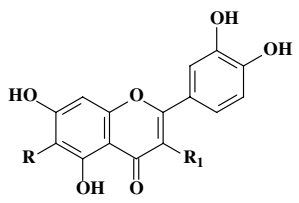
The present study evaluated the cytotoxic activity of nepetin and quercetin-3-*O*-glucoside, compounds isolated from the aerial parts of *Eupatorium ballotaefolium*. The antimitotic activity was determined as the ability to inhibit sea urchin eggs development and five tumor cells lines growth. Moreover, the activities of these compounds were compared to quercetin in the same models. Nepetin inhibited the proliferation of the five tumor cell lines, once quercetin-3-*O*-glucoside did not present any activity even at the highest tested concentration and quercetin only inhibited proliferation of the B16 cell line. On the sea urchin assay, nepetin and quercetin induced a dose-dependent inhibition on egg development, while quercetin-3-*O*-glucoside did not modify normal egg cleavage, even at the highest tested concentration (100 μg/ml).

1. Introduction

The genus *Eupatorium* (Asteraceae) is constituted by a taxonomically complex group of nearly 600 species distributed mainly in the tropical regions of the Americas (Maia et al. 2002). Several plants of this genus are widely used in folk medicine in different parts of the world due to their astringent, antirheumatic, antimicrobial, hepatoprotective, disinfectant and analgesic properties. They are used in the treatment of fever, headache, stomach ulcer, diarrhea and malaria (Lang et al. 2001; El-Seedi et al. 2002). Concomitantly, extracts of *Eupatorium* species have been shown several pharmacological activities, including hepatotoxic, cytotoxic, antiinflammatory, antioxidant and antigonorrhoeal effects (Mongelli et al. 2000; Kaushal et al. 2001). In fact, a number of bioactive compounds have been isolated of *Eupatorium* (Habtemarian 1998; El-Seedi et al. 2002). Thus, the genus seems to be a promising resource of new drugs and value-added products.



(**1**) R = OMe, R_1 = H
(**2**) R = H, R_1 = O-glucosyl
(**3**) R = H, R_1 = OH

In the course of our continuous search for cytotoxic agents from natural sources we have investigated extracts of plants from the Northeastern Brazilian flora. Since the chloroform fraction of the aerial parts *of E. ballotaefolium* showed cytotoxic activity, this was used as the rationale for the current study to identify compounds responsible for this effect. Herein we describe the isolation of nepetin (**1**) and quecertin-3-*O*-glucoside (**2**), as well as the study of their cytotoxic activity on tumor cell lines and on sea urchin eggs. The related compound, quercetin (**3**), previously isolated from *Lippia sidoides* (Costa et al. 2001) has been included in the biological tests for comparison purposes, since the two isolated compounds were quite similar to this well-known flavonoid (Middleton Jr et al. 2000).

2. Investigations, results and discussion

Nepetin (**1**) inhibited the proliferation of five tumor cell lines (Table 1), once quercetin-3-*O*-glucoside (**2**) did not present any activity even at the highest concentration tested. Quercetin (**3**) only inhibited the proliferation of B16 cell line with an IC_{50} of 8.54 μg/ml.

The study of alterations in sea urchin egg development is a suitable model for detecting cytotoxic, antimitotic and teratogenic activities of new compounds (Jacobs et al. 1981). Nepetin (**1**) and quercetin (**3**) induced a dose-dependent inhibition on egg development, while quercetin-3-*O*-glucoside did not modify normal egg cleavage, even at the highest tested concentration (100 μg/ml). The IC_{50} values are presented in Table 2.

The antitumor potential of flavonoids has been extensively discussed in the literature. Edwards et al. (1979) concluded

Table 1: Antiproliferative effects of nepetin (1), quercetin-3-*O*-glucoside (2) and quercetin (3) on tumoral cell lines

Substances	CEM μg/ml	HL-60 μg/ml	HCT-8 μg/ml	MCF-7 μg/ml	B-16 μg/ml
Doxorubicin	0.021	0.016	0.014	0.110	0.032
	0.016–0.026	0.013–0.019	0.011–0.018	0.097–0.116	0.024–0.045
(**1**)	8.24	6.42	7.18	14.18	9.42
	6.34–10.66	5.48–7.52	5.66–9.12	13.29–15.12	8.96–9.90
(**2**)	>25	>25	>25	>25	>25
(**3**)	>25	>25	>25	>25	8.24 (5.86–12.44)

Data are presented as IC_{50} values and 95% confidence interval obtained by non-linear regression for leukemias (HL-60 and CEM), breast (MCF-7), colon (HCT-8) and skin (B-16) cancer cells from 3 independent experiments.

Table 2: Antimitotic activity of nepetin (1), quercetin-3-*O*-glucoside (2) and quecetin (3) on sea urchin (*Lytechinus variegatus*) eggs development

Substances	1st cleavage μg/ml	3rd cleavage μg/ml	Blastulae μg/ml
Doxorubicin	6.3	0.3	0.5
	4.3–9.1	0.2–0.7	0.3–1.1
(**1**)	12.02	3.15	3.82
	9.00–16.06	2.08–4.78	2.69–5.44
(**2**)	>100.0	>100.0	>100.0
(**3**)	94.74	54.33	44.19
	85.19–105.4	47.59–62.03	25.05–77.95

Data are presented as IC_{50} values and 95% confidence interval obtained by non-linear regression for first and third cleavages, and blastulae from 3 independent experiments.

that flavonoids were not a promising group of antitumor compounds. On the other hand, Beutler et al. (1996) showed that, in fact, there are some structure-activity requirements for flavonoid cytotoxicity, at least when this activity is related to the inhibition of tubulin polimerization. According to several authors, the presence of a 3-methoxyl substitution increases the cytotoxicity of flavonoids (Beutler et al. 1996; Middleton Jr et al. 2000; Costa-Lotufo et al. 2003). Costa-Lotufo et al. (2003) described the cytotoxicity of kaempferol and isokaempferide using the same assays. These two flavonoids differ only by the presence of a methoxyl group on C-3 in isokaempferide instead of a hydroxyl group present in many flavon-3-ols like kaempferol, while neptin possesses a methoxyl group on C-6. Isokaempferide was at least three times more active than kaempferol against tumor cell lines, corroborating this theory at least when the cytotoxicity is determined on tumor cell lines. In the sea urchin assay, kaempferol and isokaempferide presented the same activity. The activity of nepetin (**1**) on tumor cell lines was stronger than that of kaempferol, but was weaker compared to isokaempferide and, in the sea urchin assay, nepetin was as active as kaempferol and isokaempferide. Thus, the presented data suggest that the presence of a methoxyl group on C-6 could also be related to an increased cytotoxicity of flavonoids.

3. Experimental

3.1. Extraction and isolation

Aerial parts of *E. ballotaefolium* were collected from their natural habitat on Meruoca Mountain, State of Ceará, in July 2001. The botanical material was authenticated by Prof. Edson P. Nunes of the Departamento de Biologia. A voucher specimen (# 27646) has been deposited at the Herbarium Prisco Bezerra (EAC), Universidade Federal do Ceará. The air-dried and powdered aerial parts of *E. ballotaefolium* (1.2 kg) were exhaustively extracted with *n*-hexane and EtOH at room temperature to give 21.6 g and 50.3 g of the respective extracts, after evaporation of the solvents under reduced pressure. The EtOH extract was fractionated over Si gel using $CHCl_3$, EtOAc and MeOH. The $CHCl_3$ fraction was repeatedly chromatographed over Si gel, eluting with *n*-hexane and *n*-hexane-EtOAc ($100:0 \rightarrow 0:100$) to yield nepetin (**1**, 469.4 mg). The EtOAc fraction was chromatographed on a Silica gel column eluted with $CHCl_3$, $CHCl_3$-EtOAC ($100:0 \rightarrow 0:100$), and MeOH to yield quercetin-3-*O*-glucoside (**2**, 27.8 mg). The structures of the compounds **1** and **2** were established by spectroscopic analysis and comparison with literature data (Wenkert and Gottlieb 1977; Agrawal 1989).

3.2. Cytotoxicity assays

Nepetin (**1**), quercetin-3-*O*-glucoside (**2**) and quercetin (**3**) were tested for cytotoxic activity on five tumoral cell lines: B-16 (murine skin cancer), HCT-8 (human colon cancer), MCF-7 (human breast cancer) CEM and HL-60 (leukemia cancer) cell lines (Children's Mercy Hospital, Kansas City, MO, USA) using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide assay (Mosmann 1983).
The sea urchin assay was performed essentially as described by Costa-Lotufo et al. (2002).

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