Short Research Article

In Vitro Cytotoxicity of Scopoletin Derived from *Eupatorium laevigatum* Lam.

ABSTRACT

Aims: *Eupatorium laevigatum* Lam. is commonly used as anti-inflammatory, antiseptic, anti-rheumatic, and in the treatment of colds and ulcers. The present study aimed to characterize the active fractions of the aerial parts of *E. laevigatum*, isolate its major constituents and to evaluate its cytotoxic effects against human tumor cells.

Methodology: Phytochemical analysis of the aerial parts of *E. laevigatum* detected the presence of flavonoids, saponins and coumarins. Nuclear magnetic resonance with carbon and hydrogen determined that coumarin to be scopoletin. The human cancer cell lines HT-29, NCI-H460, MCF-7 and RXF-393 were used to evaluate cytotoxicity through the sulforodamine B assay as well the evaluation of oxidative damage through the thiobarbituric acid reactive species assay.

Results: Our study has shown that *E. laevigatum* crude extract and chloroform, ethyl acetate and butanol fractions are not cytotoxic in the concentrations used (up to 100 µg/mL), but the coumarin scopoletin isolated from the aerial parts of *E. laevigatum* presented a cytotoxic effect against NCI-H460 and RXF-393 cells (IC_{50} value of 19.1 and 23.3 µg/mL, respectively). Scopoletin did not show any oxidative effect.

Conclusion: The coumarin scopoletin can be found in *E. laevigatum* and this compound induces cytotoxicity in NCI-H460 and RXF-393 cell lines. Moreover, it is suggested that the cytotoxic effect of scopoletin is no related to oxidative damage.

Keywords: Eupatorium, Antiproliferative, Coumarin, Oxidative damage

1. INTRODUCTION

Plants, fungus, insects, marine organisms and bacteria are important sources of biologically active substances. In economic terms, biodiversity usually transcends the boundaries of conventional industries, because it is a valuable source of biological data and very useful chemicals to discover innovative drugs [1]. Until now, a significant portion of cytotoxic agents used in treatments of human tumors is derived from natural products. The most successful examples are vinca alkaloids, anthacyclines, taxoids and camptothecin derivatives [2].

The *Eupatorium laevigatum* species of the genus *Eupatorium*, belonging to the Asteraceae family [3] is characterized by being a perennial shrub, 3-9 feet high, native throughout the tropical and subtropical America, from Mexico to the Northern Argentina [4]. This species is related to an economic and social potential because of its therapeutic use related to various diseases [5,6].

In South America (Argentina, Chile, Ecuador and Peru) *Eupatorium* species are commonly used as anti-inflammatory [3], antiseptic and anti-rheumatic [7,8,9,]. In Brazil, *E. laevigatum* is traditionally used to treat colds and ulcers. In the state of Rio Grande do Sul, southern Brazil, its popular use is related to potent effects as an abortifacient, laxative, regulating the menstrual cycle, from disease of poor prognosis, as well as colds and coughs [10].

Given the popular use of *E. laevigatum* related to its toxic effects, the purpose of this study was, using the technique of bio-driven fractionation, to characterize the active fractions, isolate its major constituents, and also to evaluate the cytotoxicity and oxidative damage against four types of human tumor cells: HT-29 (colon carcinoma), MCF-7 (breast carcinoma), NCI-H460 (non-small cell lung cancer), and RXF-393 (renal carcinoma).

2. MATERIAL AND METHODS

2.1 Plant material

The aerial parts of *Eupatorium laevigatum* (Asteraceae) were collected in the state of Rio Grande do Sul, Brazil. Herbarium specimens were prepared for identification and registration of plant material in the herbarium of the Botany Department of ULBRA by Professor Sergio A. L. Bordignon (HEREULBRA-3061). The material immediately after collection was selected and dried in an airy atmosphere, under direct light and then grounded in a knife mill.

2.2 Preparation of plant extracts and fractions

The air-dried and powdered aerial parts of *Eupatorium laevigatum* were first exhaustively extracted with methanol (1:10; plant/solvent) in a Soxhlet apparatus (5 x 48 h). The crude methanolic extract was evaporated to dryness in vacuum. Using the same method, a new amount *Eupatorium laevigatum* was subsequently extracted with chloroform, ethyl acetate and butanol. After, all the fractions were evaporated to dryness in vacuum at 50^{o} C.

2.3 Phytochemical analysis

The plant material was submitted to qualitative phytochemical screening to identify the main classes of active constituents. The phytochemical composition of *Eupatorium laevigatum* was determined according to the method described previously [11]. These reactions consist of colorimetric methods for the qualitative detection of flavonoids, tannins, anthraquinones, alkaloids, saponins, coumarins and cardiac glycosides.

2.4 Analysis and isolation of compounds

The fractions obtained from the different samples were submitted to thin layer chromatography using several eluent systems. Thus, it was possible to detect in the ethyl acetate fraction a major compound. This product was designated as P1 and isolated by preparative chromatography on silica gel, using as eluent chloroform-methanol (95/5; v/v). Subsequently, this product was isolated by preparative chromatography on silica gel and submitted for analysis by nuclear magnetic resonance carbon ($RMN^{13}C$) and hydrogen ($RMN^{1}H$).

2.5 Cell culture and maintenance

HT-29 human colon adenocarcinoma, NCI-H460 human non-small cell lung carcinoma, RXF-393 human renal cancer cell, MCF-7 human breast cancer were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI-1640 medium containing 10% (v/v) fetal calf serum and 2% (w/v) L-glutamine, at 37° C in a humidified atmosphere with 5% CO_2. Only the exponentially growing cell cultures with viability > 95% (confirmed by trypan blue exclusion) were used for experiments.

2.6 Cytotoxic analysis

Cells were seeded in microtiter plates consisting of 96 wells and stabilized for 24 hours. After they were treated for 72 hours with serial concentrations ranging from 0 to 100 mg/mL of crude extract, chloroform, ethyl acetate and butanol fractions or scopoletin. Cellular responses were determined using the sulforodamine B (SRB) assay, involving fixing with trichloroacetic acid, staining with SRB and a colorimetric evaluation at a wavelength of 540 nm [12]. Absorbance values were used to determine the potential for cell growth inhibition by the IC_{50} values (the minimum concentration required to inhibit 50% of cell growth). The antineoplastic agent etoposide was used as a positive control.

2.7 Evaluation of oxidative damage

The NCI-H460 human non-small cell lung carcinoma (more sensitive to treatment with scopoletin) was evaluated for the induction of oxidative damage by lipid peroxidation [13]. For this purpose, cells were treated with the IC_{50} dose of scopoletin (19.1 ± 2.4 µg/mL) for 72 hours. This experiment was performed in triplicate. After the treatment, cells were washed with PBS (0.1 M, pH 7.4), scraped from culture flasks in cold PBS and homogenized. The homogenates were immediately centrifuged (15,000 x g, 30 min, 4° C) and the supernatants used for the evaluation of lipid peroxidation and protein concentration determined by the Bradford method [14]. Oxidative damage by lipid peroxidation was estimated by the formation of thiobarbituric acid reactive species (TBARS), using as a standard, a solution of TMP (1,1,3,3-tetramethoxypropane). Briefly, each sample was added with trichloroacetic acid (TCA) and 15% thiobarbituric acid (TBA) 0.67%. The mixture was stirred, heated to 100°C for 30 min and cooled at room temperature. It was, then, centrifuged (3000 x g, 10 min) and the top fraction was quantified on a spectrophotometer at a wavelength of 532 nm. The concentration of TBARS obtained was expressed in nmol/mg of protein.

2.8 Statistical analyses

For the statistical analysis, paired Student's t-test was used. $p < 0.05$ was considered to be statistically significant. All analysis were performed with GraphPad Instat (version 3.05; GraphPad Software Inc.; San Diego, CA, USA)

3. RESULTS

3.1 Phytochemistry Analysis

Phytochemical analysis of aerial parts of *Eupatorium laevigatum* detected the presence of flavonoids, saponins and coumarins. Anthraquinones, tannins, alkaloids, cardiotonic glycosides, however, have not been found. Conducting the assay allowed us to determine the content of total flavonoids to be 2.74 g/dL of total phenolics and to be 5.52 g/dL of pyrogallol.

3.2 Structure Elucidation of *Eupatorium laevigatum* Major Compound

From the analysis by thin layer chromatography of the fractions obtained it was possible to detect in the ethyl acetate fraction a major compound. Subsequently, this product was isolated by preparative chromatography on silica gel and submitted for analysis by nuclear magnetic resonance carbon ($RMN^{13}C$) and hydrogen ($RMN^{1}H$). The isolated compound was identified as a coumarin. Based on the signals present in the spectra obtained from RMN13C and RMN1H (Table 1) and data from the literature [15] it is possible to identify the isolated compound (P1) as coumarin scopoletin.

Table 1: Spectral data of $RMN^{1}H$ e $RMN^{13}C$ of P1 and scopoletin.

Nº of carbon atoms in the molecule	RMN ^{1}H		RMN ^{13}C	
	P1	Scopoletin*	P1	Scopoletin*
2	-	-	161.0	161.5
3	6.2	6.3	112.9	113.4
4	7.4	7.6	142.8	143.3
5	6.8	6.9	106.9	107.4
6	-	-	143.5	144.0
7	-	-	150.3	150.2
7-OH	6.2	6.2	-	-
8	6.9	6.9	102.7	103.2
9	-	-	149.8	150.2
10	-	-	111.0	111.5
CH_3O	3.8	3.9	56.1	56.4

*Chemical data of the scopoletin (Vasconcelos et al., 1998) [17].

3.3 Cytotoxicity

The cytotoxic effects of the crude extract and the fraction from *E. laevigatum* was evaluated in four cancer cell lines (HT-29, NCI-H460, MCF-7, and RXF-393). The crude extract and the fractions did not demonstrate cytotoxicity in the cell lines tested (Table 2).

Table 2: Cytotoxicity of the crude extract, fractions of *E. laevigatum* and scopoletin (IC_{50} – µg/mL – average of three triplicates ± standard deviation).

Extract/Fraction	Cell lines			
	NCI-H-460	HT-29	MCF-7	RXF-393
Crude extratc	Inactive	Inactive	Inactive	Inactive
Fraction ethyl acetate	Inactive	Inactive	Inactive	Inactive
Fraction buthanolic	Inactive	Inactive	Inactive	97.6 ± 12.1
Scopoletin	19.1 ± 2.4	Inactive	Inactive	23.3 ± 1.5
Etoposide	0.3 ± 0.02	1.2 ± 0.9	3.4 ± 1.0	13.8 ± 2.7

Nevertheless, the effect of isolated coumarin scopoletin varied among the cell lines tested (Table 2). NCI-H460, was the most sensitive cell line (IC_{50} value of 19.1 µg/mL), whereas, RXF-393 demonstrated an IC_{50} value of 23.3 µg/mL. These IC_{50} values can be compared to the chemotherapeutic agent etoposide (Table 2).

3.4 Evaluation of the Oxidative Damage

The NCI-H460 human non-small cell lung carcinoma (more sensitive to treatment with scopoletin) was evaluated for the induction of oxidative damage by lipid peroxidation. TBARS assay did not show an increase in lipid peroxidation when compared to the control medium samples obtained from cells treated with IC_{50} (19.1 ± 2.4 µg/mL) of scopoletin (Figure 1). In cell samples, it was obtained values for the lipid peroxidation of TBARS of 2.5 nmol/mg of protein in the untreated control and TBARS of 3.2 nmol/mg of protein in treated cells. However, this difference was not statistically significant (Figure 1).

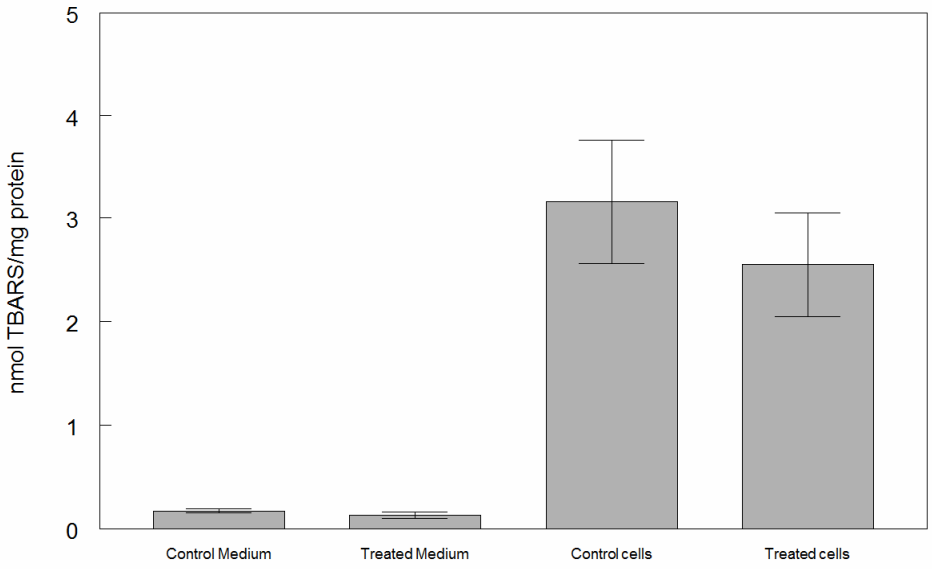

Figure 1: Average values of substances that react to thiobarbituric acid (TBARS) in the non-small cells lung carcinoma cellular lineage treated with H-460 scopoletin for 72 h. The results are expressed as average ± standard deviation (n = 9).

4. DISCUSSION

It is well established that plants are a useful source of clinically relevant antitumor compounds [1,2]. Coumarin scopoletin is a pharmacologically active agent, which has been isolated from several plant species such as *Erycibe obtusifolia*, *Aster tataricus*, *Foeniculum vulgare, Artemisia capillaris*, among others [16,17,18] and have shown cytotoxic effect on tumor lymphocytes [19], in the lymphocytic leukemia P-388 cell line [20], capability of inhibiting the proliferation of cancer cells, apoptosis induction in cases of benign prostatic hyperplasia, and the ability to produce these effects in human cancer cell lines [21,22]. Therefore, we evaluated the antitumor activity and oxidative damage of scopoletin obtained of *E. laevigatum* against four types of human cancer cell lines. To our knowledge this is the first time that scopoletin was identified in *E. laevigatum*.

The antiproliferative activity of the crude extract, fractions of *E. laevigatum* and scopoletin was evaluated in this study in four tumor cell lines, HT-29, NCI-H460, MCF-7, and RXF-393. The crude extract and fractions showed no cytotoxic activity. This can occur because the crude extract and its fractions present a small concentration of coumarin scopoletin, which is probably less than the amount needed to induce cytotoxicity. Others species belonging to the genus *Eupatorium* that also presents scopoletin [23,24], might, however, present this compound in a concentration high enough to induce cytotoxicity. As for coumarin scopoletin, the antineoplasic agent etoposide, showed different patterns of cytotoxicity on the cell lines tested. The comparison of the IC_{50} values demonstrated that the NCI-H-460 cells showed the highest sensitivity to scopoletin, when compared to the other cell lines. The cytotoxic activity observed in this study is consistent with a previous study, which reports that scopoletin showed different sensitivities in different tumor cell lines [21].

Coumarins have been described for several activities including anticancer, antioxidant, anti-inflammatory, analgesic and anticoagulant [25]. Recently this secondary metabolite has been reported to inhibit cell proliferation interfering with the function of microtubules, inhibiting the activity of metalloproteinases, blocking cell cycle, interfering with cell signaling and inducing oxidative damage [26].

Other studies shows that scopoletin may have antineoplasic effect by inducing apoptosis, such as the work performed by Liu et al. (2001), which showed that scopoletin inhibits the proliferation of PC3 prostate cancer cell line by inducing apoptosis.

Lipid peroxidation is an evidence of injury caused by free radicals in biological systems [27,28]. The level of lipid peroxidation may be estimated by determination of TBARS. In this regard, we evaluated the oxidative damage caused by scopoletin on NCI-H-460 cells (more sensitive), both in the culture medium and cells, and compared to their respective untreated controls. Our data suggest that the cytotoxic effect of scopoletin is not associated with oxidative damage. It has shown that at lower doses, the scopoletin can even act as antioxidant [29]. In recent studies, the antioxidant properties were found for some compounds of this chemical class of coumarins [30,31,32]. In the study conducted by Lin et al. (2008) the results suggest that the number of hydroxyl groups in the ring structure of coumarins is correlated with the effects of removal of reactive oxygen species.

The study performed by Tyagi et al. (2005) showed that the amino group is an effective substitute for the hydroxyl group to produce antioxidant and a dramatic inhibition of lipid peroxidation. It is demonstrated that orthodihydroxy and orthohydroxy-amino coumarins have higher antioxidant activity. When evaluating the ability of these coumarins protective against oxidative damage in a simple model membrane Morabito et al. (2010) describe 4-methylcoumarins can be considered as potential candidates for therapeutics for pathological conditions characterized by excessive production of free radicals.

5. CONCLUSION

In summary, the results of the current study detected the presence of scopoletin in *E. laevigatum* and that this compound induces cytotoxicity in NCI-H460 and RXF-393 cell lines. Although the findings of this study cannot provide a mechanistic explanation for this phenomenon, it is suggested that the cytotoxic effect of scopoletin is no related to oxidative damage. Further studies are needed to better understand the role that coumarin scopoletin plays as an anticancer drug.

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