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In vitro antiproliferative activity of 3 H-spiro [1-benzofuran-2,1'-cyclohexane] derivatives.

[Actividad antiproliferativa *in vitro* de derivados de 3 H-spiro [1-benzofuran-2,1'-cyclohexano**]**]

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Abstract
The *in vitro* effect of the resinous exudate of *Heliotropium filifolium*, of the 3 H-spiro[1-benzofuran-2,1 '-cyclohexane] derivative called filifolinol **1,** isolated from the resin and the semi-synthetic compounds filifolinone **2** and filifolinoic acid **3**, obtained from filifolinol **1**, were evaluated on the proliferation of an immortalized cell line, UCHT1, derived from rat thyroid. We evaluated the effect of these compounds on UCHT1 cell growth parameters by calculating doubling time; and toxicity using the LIVE/DEAD™ *in vitro* test. The results showed that the resin is not active, while filifolinone **2**, filifolinoic acid **3** and filifolinol **1** produced a significant inhibition of cell doubling time, in concentrations equal or greater than 50, 25 and 75 µM, respectively. The LIVE/DEAD test showed no significant toxicity at these concentrations, compared to cultures kept in absence of compounds. These results suggest a possible cytostatic effect of these compounds, and could therefore constitute potential alternatives for antineoplasic therapy.

Keywords: aromatic geranyl derivatives, antitumoral activity, antiproliferative activity, *Heliotropium filifolium*, resinous exudates, filifolinol.

Resumen
Se evaluó el efecto *in vitro* de la resina aislada desde *Heliotropium filifolium* y del derivado 3 H-spiro[1-benzofuran-2,1'-cyclohexano] llamado filifolinol **1**, obtenido desde este exudado resinoso y los compuestos semi-sintéticos filifolinona **2** y ácido filifolinoico **3**, obtenidos a partir de filifolinol **1**, sobre la proliferación de la línea celular inmortal, UCHT1, derivada de tumor de tiroide de rata. Evaluamos el efecto de estos compuestos en el desarrollo celular de UCHT1 a través de los parámetros tiempo de doblaje y citotoxicidad usando el test LIVE/DEAD™ *in vitro*. Los resultados mostraron que la resina no presentó actividad y que filifolinona, ácido filifolinoico y filifolinol producen una inhibición significativa del tiempo de doblaje celular, en concentraciones iguales o superiores a 50, 25 y 75 µM, respectivamente. El test LIVE/DEAD no mostró toxicidad significativa en comparación con los cultivos mantenidos en ausencia de compuestos. Estos resultados sugieren un posible efecto citostático de estos compuestos y por lo tanto, constituirían alternativas potenciales para terapia antineoplásica.

Palabras Clave: derivados aromáticos geranilados, actividad antitumoral, actividad antiproliferativa, *Heliotropium filifolium*, exudado resinoso, filifolinol.

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INTRODUCTION

The species *Heliotropium filifolium* (Miers) Reiche (Heliotropiaceae) is a native Chilean bush of particular ecological interest, as it grows in arid lands under extreme environmental conditions. Like many plants of *Heliotropium* spp., *Heliotropium filifolium* produces from glandular trichomes, a resinous exudate that covers the leaves and stem (Urzúa *et al*., 2000). In search of an explanation of the role of this resin, it has been proposed that they could constitute the first line of protection against predators. This protection could be due both to a mechanical effect, associated with a sticky character which causes them to retain predators (Eigenbrode *et al*., 1996) and to a chemical protection due to the presence of secondary metabolites that exhibit antibacterial, antiviral, antifungal, antifeedant and antioxidant activities (Lissi *et al*., 1999; Urzúa *et al*., 2001; Villarroel *et al*., 2001; Torres *et al*., 2002, 2008; Mendoza *et al*., 2008; Modak *et al*., 2003, 2004a, 2004b, 2007, 2009a, 2009b, 2010). In general, the presence of the resinous exudates is associated with a defense mechanism of the plant. The resin produced by *Heliotropium filifolium* is characterized by the presence of a mixture of flavonoids and 3 H-Spiro[1-benzofuran-2,1'-cyclohexane] derivatives where the main compound is called filifolinol **1** (Torres *et al*., 1994; Paduch *et al*., 2007). This compound is the first example of a 3 H-spiro[1-benzofuran-2,1'-cyclohexane] derivative in this species, representing 50% of the total mass of the resin. We have now synthesized two compounds from filifolinol **1,** named filifolinone **2** (Torres *et al*., 2002) and filifolinoic acid **3** (Modak *et al*., 2004b) , for the study of the biological properties of these compounds.

Although, *Heliotropium* species are not used as medicinal plants because they contain high concentration of pyrrolizidine alkaloids, and therefore may cause intoxication, the components isolated from the resins may have interesting biological properties that can be exploited (Sharma *et al*., 2009).

In a previous work, we have demonstrated that these compounds show antiviral activity on five viral species of importance in human diseases: Polio sabin, Herpex simplex type 1 and 2, Junin and Respiratory syncitial (Torres *et al*., 2002); where filifolinol **1** exhibited the strongest activity, in particular against Polio. Further, these compounds have been studied as inhibitors of the Andes variety of Hantavirus, where the results have shown antiviral activity and inhibition of its replication in Vero E6 tumoral cells, a model where Hantavirus can replicate and induce apoptosis (Torres *et al*., 2008; Modak *et al*., 2004b).

On the other hand, filifolinyl senecionate, a natural derivative of filifolinol **1,** exhibited an inhibitory effect on infectious pancreatic virus replication. This compound inhibited viral genomic RNA synthesis, suggesting that the ester may interact with the viral RNA during the viral cycle (Modak *et al*., 2010).

Also, the extract of *Heliotropium filifolium*, filifolinol **1** and the semi-synthetic derivative filifolinoic acid **3** , reduced mycelial growth of the phytophathogenic fungus *Botrytis cinerea* and the extract of *Heliotropium filifolium* and filifolinol **1** delayed the germination of conidia of this fungus (Mendoza *et al*., 2008).

The antimicrobial properties of filifolinol **1** and several natural esters obtained from the extract of *Heliotropium filifolium* were tested against Gram positive and Gram negative bacteria. Some of them proved to be active against Gram positive, but inactive against Gram negative bacteria. In searching for structure-activity relationships from the obtained MIC values, lipophilicity was shown to be an important variable (Urzúa *et al*., 2008).

Cancer is one of the major causes of death worldwide, and there is a general belief that research in this area is needed to develop new compounds for anticancer therapy. In this regard, recent studies have shown that phytochemicals can modify the growth and proliferation of tumoral cells. These studies have resulted in the discovery of several new phytochemicals that posse's cancer preventive effects, such as isothiocyanates from cruciferous vegetables, polyphenols from green and black tea, and flavonoids from soybeans (Kwon *et al*., 2007).

Based in the aforementioned facts, we have now studied the antiproliferative activity of these compounds (see structures in Fig. 1) using an *in vitro* assay developed with the immortalized cell line UCHT1, established at the ICBM, Faculty of Medicine of the University of Chile. This study considered two tests: the evaluation of the doubling time to determine antiproliferative/cytostatic activity, and the LIVE/DEAD™ assay valuate for cytotoxic effects of these compounds.

MATERIALS AND METHODOS

Plant Material

Heliotropium filifolium (Miers) Reiche was collected in October 2006 in the North of Vallenar, IV region,

Chile, 28°45' S, 70°49' W. A voucher specimen was deposited in the Herbarium of the Faculty of Biological Science of the Catholic University of Chile (ST-2214 SSUC).

Extraction and isolation of the natural compounds and resin

The resinous exudates were extracted by immersion of fresh plant material in dichloromethane for 30 s at room temperature. The extract was concentrated to a sticky residue (6.3 % w/w). The extract was purified by column chromatography (silica gel) using a hexane-AcOEt step gradient, to obtain filifolinol **1**.

Synthesis of filifolinone 2 **and filifolinoic acid** 3

Filifolinone **2** was obtained from filifolinol **1,** by conventional oxidation with CrO_3 and purified by column chromatography (silica gel) using benzene-AcOEt (Torres *et al*., 2002). Filifolinoic acid **3** was obtained from filifolinol **1** by basic hydrolysis with NaOH, followed by purification with crystallization (Modak *et al*., 2004b).

PREPARATION OF TEST SAMPLES

Stock solutions of 10 mg/mL were prepared with the test compound **1-3** and the resin using dimethylsulfoxide (Merck) as solvent. From these solutions were obtained the test dilutions.

TISSUE CULTURE

To study antitumoral *in vitro* activity, we used the UCHT1 cell line, obtained from a thyroid tumor of an adult Fisher 344 rat, and established according to the protocol described by Caviedes and Stanbury (1976). In vitro, UCHT1 cells exhibit a homogeneous epithelial morphology, and they express thyroid function. In the exponential growth phase, the cell line has a doubling time of approximately 24 hrs. This cell line is tumorigenics, inducing large tumors when grafted into isogenic animals.

The cells were thawed from a liquid nitrogen tank in a water bath at 37° C in 1 min, and were then seeded onto 10 cm diameter Petri dishes. The culture media was composed of a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F12 Nutrient mixture (DME/Ham F12, 1:1, Sigma Chemical Co., St. Louis, MO), pH 7.4, supplemented with sodium bicarbonate 1 g/L, gentamicin 40 mg/L, 15 mM HEPES, 10% adult bovine serum, 5% fetal bovine serum. The cells were kept in an incubator at an atmosphere of 5% CO_2 a temperature of 37° C and 100% humidity. Media was renewed every three days. Cells were passaged upon confluence, in a 1:5 ratio.

Determination of doubling time.

To assess doubling time, cultures with 50% confluence were used, at least 24 h after seeding. 5 fields were selected and marked in each dish, and the cells in each field were counted under a phase contrast microscope. The doubling time was estimated after 24 hrs, using the following formula:

$$TD = (Tfin - T\ in) \times \log2/(LogNt1 - logN0)$$

Where TD is the doubling time (hrs); Tfin is the time of the final count (hrs); Tin is the time of the initial count (hrs); Nt1 = Final count; N0 is the initial count.

The cells were then washed and the compounds were added, in concentrations of 0, 25, 50, 75 and 100 μM. Recounts of the previously marked fields were carried out in triplicate, yielding a recount of 15 fields per condition. Recounts were carried out at 24, 48 and 72 h after the addition of the compounds.

Determination of citotoxicity

To determine the effect of the compounds on cell toxicity, we used the LIVE/DEAD technique (Invitrogen, Carslbad, CA). Briefly, toxicity on UCHT1 cells was evaluated at 24, 48 and 72 h of incubation in each compound and the resin. UCHT1 cells cultured in coverslips were washed in PBS 1X (8 g/L NaCl, 0.4 g/L KCl, 0.02 g/L Na_2HPO_4, pH 7.4). Later, the coverslips were incubated with 100 μL calcein-AM 2μM and 100 mL of propidium iodide solution (0.5 mg/mL in PBS with 0.1% sodium azide, pH approximately 7.4) in PBS for 45 m at 37º C. The coverslips were then washed in PBS, and mounted on microscope slides. The cells were observed in a Carl Zeiss microscope equipped with fluorescent optics, using an emission filter of 588 nM. Live cells become loaded with calcein, giving a characteristic green fluorescence, whereas the nuclei of dead cells exhibited red fluorescence. Labeled cells were counted for each condition, and cytotoxicity was estimated by the ratio of dead vs live cells.

Data analysis

All data were expressed as mean ± SEM values. The statistical significance between groups was assessed using ANOVA for multiple comparisons, and Student's unpaired *t* test.

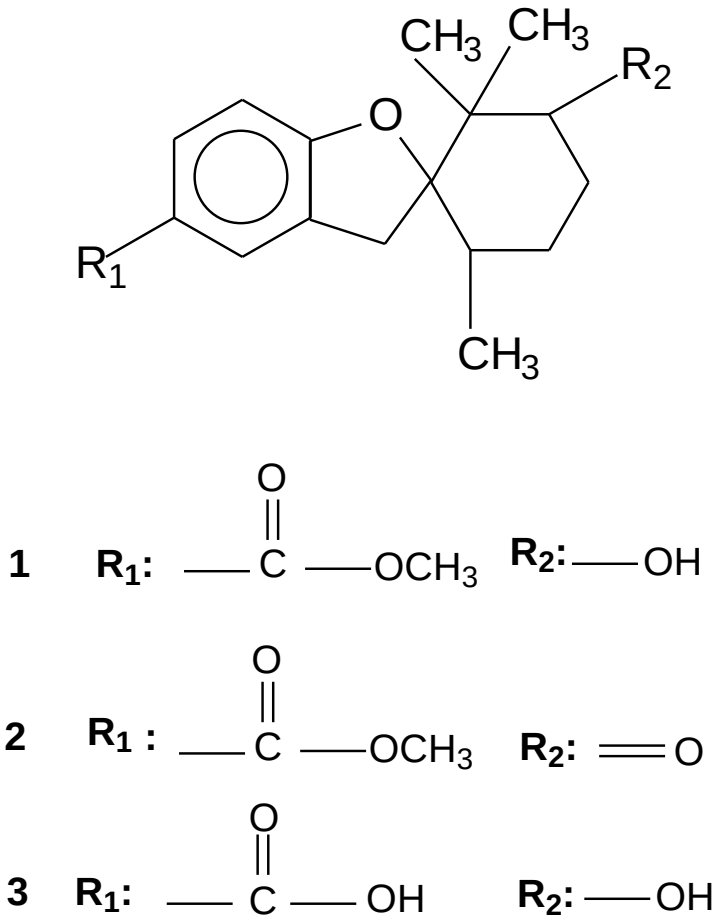

Figure 1: Structures of the natural and semi-synthetic compounds tested as inhibitors of the time of the doubling cellular. The groups added to the base molecule yield the different compounds tested. **1** filifolinol **2** filifolinone **3** filifolinoic acid.

RESULTS AND DISCUSSION

Figure 1 shows the structures of the natural and semi-synthetic compounds tested. The base molecule is presented with the corresponding groups added to yield the different compounds.

We tested the effect of these 3 H-spiro[1-benzofuran-2,1'-cyclohexane] derivatives and the resin, in growth parameters and toxicity of the tumoral cell line UCHT1. The effect of the compounds filifolinol **1,** filifolinone **2** and filifolinoic acid **3** in the doubling time of UCHT1 cells is shown in figure 2. The resin does not show any significant activity. The results correspond to independent experiments carried out in triplicate. This study showed that all pure compounds inhibited cell proliferation, as expressed by significant increases in doubling time at different concentrations (25 µM to 100 µM). Indeed, figure 2(A) shows that treatment with filifolinol **1** 75 µM induced a significant increase in the proliferation time of almost 6-fold that of control cultures ($p < 0.05$), although the doubling time was again reduced at 100 µM. Filifolinone **2** also exhibited an antiproliferative effect at 50 µM concentration (figure 2B), expressed in a 2-fold increase in doubling time. At higher concentrations, the decrease in the cell proliferation remained relatively constant. On the other hand, when comparing the doubling time between the three higher concentrations of this compound (50, 75 and 100 µM), no significant differences were observed ($p > 0.01$), and the activity remained constant. Regarding filifolinoic acid **3**, the antiproliferative effect was significant from concentrations as low as 25 µM ($p <$

0.01) (figure 2C). However, in concentrations of 50 µM or higher, the effect was greater, reaching at least 2-fold increase in doubling time.

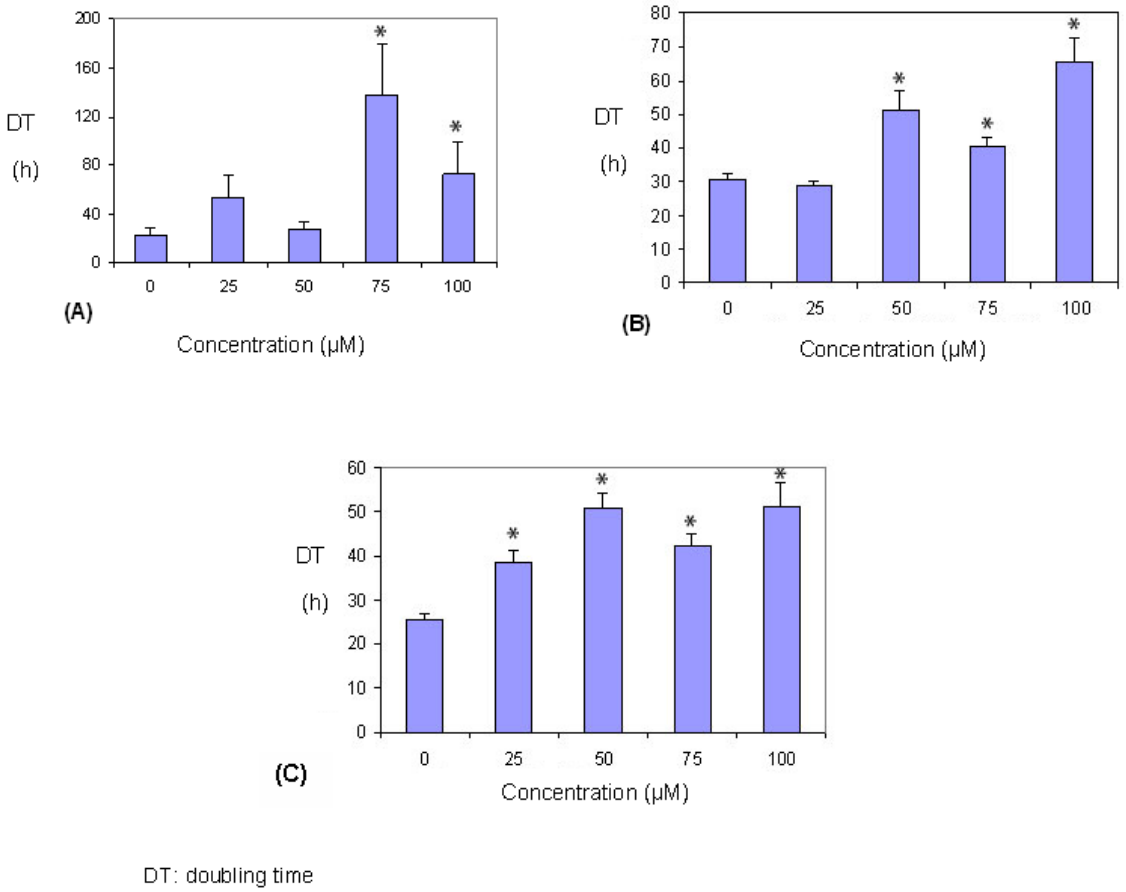

Figure 2: Effect in the time of doubling on UCHT1 cells treated with compounds obtained from resin of *Heliotropium filifolium* and derivatives semi-synthetic. The concentrations of the compounds are expressed in µM. The results correspond to the mean ± EE of the values of three independent experiments in triplicate. (A) Cells treated with different concentrations of filifolinol *****p < 0.05 respect to control. (B) Cells treated with different concentrations of filifolinone *****p < 0.01 respect to control. (C) Cells treated with different concentrations of filifolinoic acid *****p < 0.01 respect to control.

Therefore, we performed both studies in parallel. In this regard, filifolinol **1** proved to be quite toxic at the concentrations used (see table 1). In order to relate the effect on doubling time of filifolinol **1** and at the same time account for its inherent toxic effects at the concentrations noted, we corrected the determinations of doubling time, dividing by the corresponding values of cell vitality (expressed as percentage of remaining, living cells) at a given concentration of the compound. Interestingly, the corrected values indicated a progressive increase in doubling time, reaching values of up to 100-fold at 75 µM. Values so corrected were 0.3 (control), 1.0 (25 µM), 1.7 (50 µM) and 23.2 (75 µM). On the other hand, at the concentrations used for calculation of doubling times, filifolinone **2** and filifolinoic acid **3** exhibited no toxicity.

There are currently no data in the literature pertaining to the effect of these compounds as inhibitors of tumoral cell proliferation. However, several molecules derived from plants are being used as antitumorals. Among them are terpenes, which have been studied extensively. For example, the triterpenoids oleanolic acid and ursolic acid have been shown to act at various stages of tumor development to inhibit tumor initiation and promotion, as well as inducing tumor cell differentiation and apoptosis. In the two-stage mouse skin carcinogenesis model, the protective effect of oleanolic acid against 12-O-teradecanoye phorbol-13-

acetate induced carcinogenesis is associated with inhibition of aberrant gene expression. Oleanolic acid derivatives are also effective for acute myeloid leukemia by inducing apoptosis of tumor cells. These triterpenoids and their derivatives emerge as a new class of chemotherapeutics, as they are effective in inhibiting angiogenesis, as well as the invasion and metastasis of tumor cells (Liu, 2005). Also, ursolic acid inhibits proliferation and stimulates apoptosis in HT29 cells (Andersson *et al*., 2003). Apparently, the acid character is important in the antiproliferative activity, which could explain the strong effect of filifolinoic acid **3** at low concentrations. Another example is the triterpene betulinic acid. This compound isolated from *Flueggea virosa* was tested on two cancer cells lines, adriamycin-sensitive erythroleukemia cells (K562) and adriamycin-resistant erythroleukemia cells (K562/Adr), exhibiting a high antiproliferative activity in both cell types (Monkodkaew *et al*., 2009).

Concentration (μM)	Time (h)	Filifolinol			Filifolinone			Filifolinoic acid		
		Mean	*EE*	*n*	*Mean*	*EE*	*n*	*Mean*	*EE*	*n*
0	24	100.0	0.00	6	100.0	0.00	3	100.0	0.00	8
25		95.8	4.16	6	100.0	0.00	6	100.0	0.00	8
50		5.6	5.5	9	100.0	0.00	6	100.0	0.00	8
75		0.0	0.0	6	100.0	0.00	3	100.0	0.00	8
100		9.53	7.1	7	100.0	0.00	3	100.0	0.00	8
0	48	79.1	9.35	10	100.0	0.00	6	-	-	0
25		54.8	17.3	7	100.0	0.00	6	99.12	0.88	6
50		16.7	16.7	6	100.0	0.00	6	100.0	0.00	3
75		5.9	3.8	6	100.0	0.00	3	100.0	0.00	3
100		0.0	.0.0	6	100.0	0.00	3	98.35	1.31	7
0	72	94.17	4.16	6	100.0	0.00	6	100.0	0.00	1
25		5.6	5.5	9	100.0	0.00	6	100.0	0.00	3
50		25.4	12.5	8	100.0	0.00	6	100.0	0.00	3
75		17.71	9.5	8	100.0	0.00	3	100.0	0.00	2
100		3.6	3.3	7	100.0	0.00	3	100.0	0.00	3

Table I. Results obtained of the analysis of the cytotoxicity on UCHT1 cells treated with compounds obtained from resin of *Heliotropium filifolium* and derivatives semi-synthetic **1**-**3**. The concentrations of the compounds are expressed in μM. The results correspond to the mean ± EE. The mean correspond to the average of alive cells expressed in percentage.

The present results suggest that the compounds described herein affect the cellular mitotic process and therefore can be potential antineoplasic agents. Also, UCHT1 cells appear as an adequate model to screen effects on cell proliferation of such compounds. Further studies, in particular related to cell biology of UCHT1 cells, could shed light on cell mechanisms involved in the effects described, and could help in the identification of therapeutic targets.

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