Comparative study of *Tilia x viridis* extract and isolated compounds on the proliferation of two lymphoma cell lines.

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**ABSTRACT**

Several lymphoma T cell lines differ in membrane superficial markers and do not respond to the same drugs and compounds. *Tilia x viridis* (Bayer) Simonk nothosubspecie moltkei (Dippel) Xifreda is a plant widely distributed in Argentina. The aim of this work was to study comparatively the effects of dichloromethane extract and isolated compounds on BW 5147 and on another T lymphoma cell line EL-4, evaluating principally proliferation and viability. On BW 5147 cells, dichloromethane extract exerted antiproliferative action, presented cytostatic effect at low concentrations and cytotoxic action at high concentrations. Monoterpenes exerted antiproliferative action being the most potent limonene; low concentrations of them were cytostatic and high concentrations were cytotoxic. On EL-4 lymphoma, dichloromethane extract and monoterpenes exerted proliferative action, and none of them affected cell viability. It is extremely important to note that depending on tumor cell type the same extract or compounds can elicit different actions.

**Keywords:** *Tilia x viridis*, dichloromethane extract, monoterpenes, BW 5147, EL-4.
**Introduction**

Several lymphoma T cell lines differ in membrane superficial markers and do not respond to the same drugs and compounds (Ralph, 1973). On the other hand, some plants are used in popular medicine for the treatment of several diseases. In this sense, *Tilia* species have been used for many years in Europe to treat anxiety and as a psychological depressor (Font Quer, 1976) but also, the extracts have been used for the treatment of colds, bronchitis, fever, inflammations and influenza infections. In this regards, the tea prepared with *Tilia* flowers is listed in German Pharmacopeia (Thomson, 1987). *Tilia x viridis* (Bayer) Simonk nothosubspecies moltkei (Dippel) Xifreda is a plant widely distributed in Argentina. In a previous study, it was shown that a dichloromethane extract of *Tilia x viridis* presented antiproliferative action on a lymphoma cell line (BW 5147). Moreover, one fraction rich in coumarins and other rich in monoterpenes were obtained from dichloromethane extract. The fraction most active against tumor cells was the fraction containing monoterpenes including limonene (Manuele et al, 2008). The aim of this work was to study comparatively the effects of dichloromethane extract and isolated compounds (monoterpenes such as limonene, α pinene and β pinene) on BW 5147 and on another T lymphoma cell line EL-4, evaluating principally proliferation and viability.

**Experimental**

*Tilia x viridis* (Bayer) Simonk nothosubspecies moltkei (Dippel) Xifreda flowers were collected in the province of Buenos Aires in January and authenticated by Dr Gustavo Giberti (IQUIMEFA-CONICET). A voucher specimen is deposited at Museum of Pharmacobotanic, Faculty of Pharmacy and Biochemistry, University of Buenos Aires. To prepare the dichloromethane extract (DM), dried flowers (9g) were macerated twice overnight with 200 ml of dichloromethane, filtered, evaporated and the residue used for the experimental studies. Moreover, the monoterpenes were quantified by gas chromatography (Manuele et al 2008).

Monoterpenes: Limonene, α-pinene and β-pinene were purchased in Sigma-Aldrich company (San Diego, USA)

Cell culture conditions

The lymphoma T cell lines (BW 5147 and EL-4) were obtained from Institute fur Virologie und Immunobiologie der Universitat Wurzburg, Germany. Cells were cultured at optimal concentrations of 3x10^5 cells/ml in RPMI 1640 medium (Sigma, San Diego, USA) supplemented with 10 % fetal calf serum, 2 mM glutamine and antibiotics (Sigma, San Diego, USA) (Anesini et al, 1996).

**Proliferation assays**

The effect on proliferation of different concentrations (1, 10, 100, 1000 and 10000 µg/ml) of DM and monoterpenes was evaluated. Cell proliferation was assayed by the uptake of tritiated thymidine ([³H]TdR). Cells were cultured during 24 h and then pulsed with [³H]TdR (20 Ci/mmole) for the last 6 h as previously described (Anesini et al, 1996). Results were expressed as dpm and as percentage of proliferation inhibition respect to control.

**Viability assay**

Cell viability of treated and untreated cells were determined by the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, San Diego, USA). Briefly, tumor lymphocytes (3x10^5 cells/ml) were incubated alone or in presence of different concentrations of DM or monoterpenes during 24 h. After incubation, 100 µl of RPMI 1640 containing 10 µl of MTT (5 mg/ml) (Sigma, CA, USA) were added. The purple formazan formed was solubilized after 4 h of incubation by the addition of acidic isopropanol. The absorbance was measured using a microplate reader (Microplate Reader Benchmark. Bio-Rad) at 570 nm. Untreated cells were used as control of viability (100%) and results were expressed as % of viability relative to control (Davicino et al, 2008).

**Statistic analysis**

Data are the average of duplicate samples performed by triplicate. The data were recorded as mean value ± standard error of mean. One way analysis of variance was performed by ANOVA followed by comparisons with Dunnett test. A p ≤ 0.05 was considered statistically significant.

**Results and discussion**

On BW 5147 cells, DM extract exerted antiproliferative action (EC50 µg/ml: 4.8± 2), presented cysostatic effect at low concentrations and cytotoxic at high concentrations. Monoterpenes exerted antiproliferative action
being the most potent limonene (EC$_{50}$ µg/ml: 35± 2, β pinene: 114.8± 8, α pinene: 1100± 10), low concentrations of them were cytostatic and high concentrations were cytotoxic (Figure 1 and Table 1).

On EL-4 lymphoma, DM and monoterpenes exerted proliferative action, and none of them affected cell viability. The maximum stimulatory concentration for the extract was 100µg/ml (Figure 2). On the other hand, monoterpenes were found in low concentrations in DM (Figure 3).

On BW 5147, monoterpenes could contribute to the antiproliferative activity of high concentrations of DM, at low concentrations of DM other compounds more active should exert the effect or monoterpenes could have a synergistic action. On EL-4 cells, the stimulatory action on cell proliferation could be related to limonene, to the synergistic effect of all monoterpenes or to the presence of other kind of compound. It is extremely important to note that depending on tumor cell type the same extract or compounds can elicit different actions.

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References
-Font Quer R. (1976), Plantas medicinales. El dioscórides renovado; Labor; Barcelona, España.
**Figure 1.** Effect of DM and monoterpenes on proliferation (A, C) and viability (B, D) of BW5147 cell line. Results represent the Mean ± SEM of three experiments made by triplicate. *P< 0.05, **p<0.01 significantly differences between basal and treated groups.
Figure 2. Effect of DM and monoterpenes on proliferation (A, C) and viability (B, D) of EL-4 cell line. Results represent the Mean ± SEM of three experiments made by triplicate. *P< 0.05, **p<0.01 significantly differences between basal and treated groups.
Figure 3. Chromatogram of DM of *Tilia x viridis* extract. The chromatogram was obtained by gas chromatography.
Table 1. EC<sub>50</sub> of *Tilia* extract and monoterpenes on the antiproliferative action in BW 5147 lymphoma cells.

<table>
<thead>
<tr>
<th>Additions</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td>DM extract</td>
<td>4.8 ± 2</td>
</tr>
<tr>
<td>α-pinene</td>
<td>1100 ± 10</td>
</tr>
<tr>
<td>β-pinene</td>
<td>114.8 ± 8</td>
</tr>
<tr>
<td>Limonene</td>
<td>35 ± 2</td>
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