

RESEARCH ARTICLE

# New promising *Euphorbiaceae* extracts with activity in human lymphocytes from primary cell cultures

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## Abstract

**Context:** *Euphorbiaceae* plants exhibit anti-inflammatory and immunomodulatory properties.

**Methods:** We evaluated the activity of 14 extracts from seven *Euphorbiaceae* plants on primary immune cell cultures from healthy individuals. Peripheral blood mononuclear cells (PBMC) were exposed to the extracts w/o phytohaemagglutinin A or cycloheximide as agents that induce proliferation or apoptosis in PBMC, respectively.

**Results:** We found that five up to 14 *Euphorbiaceae*'s extracts had the ability to modulate at least one of the immune parameters evaluated in this study. However, only the latex extracts of *Euphorbia cotinifolia* and *Euphorbia tirucalli* strongly induced both proliferation and apoptosis in PBMC. These extracts were further subfractioned by silica gel column chromatography. Two subfractions with enhanced activity in comparison to the crude extracts were obtained. Although these subfractions induced proliferation on both CD3<sup>+</sup> and CD3<sup>-</sup> cells, the most prominent effects were observed in the former subpopulation. Interestingly, the subfraction from *E. tirucalli* induced lymphocyte proliferation without the need of accessory cells; this ability was not inhibited by the carbohydrates D-galactose and  $\alpha$ -Methyl-D-Mannopyranoside.

**Conclusions:** Altogether, these results reveal the presence of novel candidates within the *Euphorbia* plants to induce proliferation and apoptosis in human lymphocytes, mainly in CD3<sup>+</sup> T cells.

**Keywords:** Natural Products, immunomodulation, apoptosis, cytotoxicity, lymphoproliferation

## Introduction

The family *Euphorbiaceae* consists of monoecious or dioecious plants including shrubs, herbs, trees, and cactus growing in different altitudes, preferentially in tropical areas. They are grouped in almost 8000 species, sorted in 317 genera representing the family of angiosperms with the highest number of species around the world.<sup>(1)</sup> Because these plants exhibit anti-inflammatory properties, great effort has been made to identify *Euphorbiaceae*-derived therapeutic targets against different human diseases.<sup>(2–5)</sup> However, the pleiotropic activities of *Euphorbiaceae* plants are likely related with their immunomodulatory properties. They have been shown to directly interfere with mechanisms of innate

and adaptative immunity in different models. For example, using a cell proliferation-based screening assay with mouse interleukin-10 (IL-10)-dependent cell lines, flavonoids obtained from *E. lunulata* exhibit IL-10-like activity.<sup>(6)</sup> Ehrlich ascites tumor-bearing mice receiving intraperitoneally dehydrocrotonin, a diterpene isolated from *C. cajucara*, show increased survival presumably due to an improvement in the cytotoxic activity of murine natural killer cells *in vitro*. Moreover, a biopolymeric fraction of *E. tirucalli* decreases the number of peripheral blood T-lymphocytes and reduces the intracellular expression of IL-2 and interferon- $\gamma$  when orally administrated to mice.<sup>(7)</sup>

On the other hand, *Euphorbiaceae* plants have the ability to interfere with the action of molecules with

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known immune effects. The latex from *C. lechleri*, for example, modulates in a dose-dependent manner, the phagocytic activity of human monocytes previously stimulated with lipopolysaccharide (LPS) from *Escherichia coli in vitro*.<sup>(8)</sup> This extract, also, inhibits the proliferation of mice splenocytes stimulated with Concavalin A.<sup>(8)</sup> Moreover, Pepluanone, a terpene isolated from *E. peplus*, reduces the production of nitric oxide, prostaglandin E<sub>2</sub> and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) on LPS-stimulated murine macrophages due to the inhibition of the NF- $\kappa$ B activation.<sup>(9)</sup> Finally, stem extracts from *Sapium glandulatum* inhibit the proliferation of phytohaemagglutinin A (PHA)-stimulated peripheral blood mononuclear cells (PBMC).<sup>(10)</sup>

In the present study, we demonstrate that specific subfractions of *E. cotinifolia* and *E. tirucalli* greatly induce proliferative or apoptotic activity in PBMC mainly in CD3<sup>+</sup> cells. Interestingly, these cells do proliferate in the presence of the *E. tirucalli* subfraction without the need of accessory cells, suggesting that this subfraction directly activate T-lymphocytes. More interestingly, the activity of the *E. tirucalli* subfraction is not inhibited in the presence of D-galactose or  $\alpha$ -Methyl-D-mannopyranoside suggesting that its active compounds have no affinity for these carbohydrate residues in surface receptors of immune cells. Taken together, our findings describe novel *Euphorbia*-derived candidates capable to modulate human lymphocyte function *in vitro*. Additional studies are necessary to evaluate the efficacy of these extracts *in vivo* and identify their active compounds.

## Methods

### Plant collection

The plants included in this study were selected based on ethnobotanical criteria from the Andean region of Colombia. A scientist from the Group of Organic Chemistry of Natural Products classified the plants taxonomically. The latex or leaves from *Caryodendron orinocense*, *Codiaeum variegatum*, *E. cotinifolia*, *E. pulcherrima*, *E. tirucalli*, *H. crepitans*, and *P. niruri*, were collected during 2005 and 2006 from several farms belonging to the University of Antioquia, located in San Pedro, Barbosa, Porce and Cauca, Colombia. Voucher specimens (*C. orinocense* A94316, *C. variegatum* 142566, *E. cotinifolia* 2707, *E. pulcherrima* 6715, *E. tirucalli* 6797, *H. crepitans* 142565 and *Phyllanthu niruri* 6743) were deposited in the University of Antioquia Herbarium.

### Extract preparation

Milled leaves from *Caryodendron orinocense*, *C. variegatum*, *E. pulcherrima*, *H. crepitans*, and *P. niruri*, (500 g each) were extracted by percolation with 6 L of methanol during 3 days. After filtration and solvent evaporation under vacuum, each residue was dissolved in water and extracted with hexane:dichloromethane:methanol (HDM) (2:1:1 v/v). At the end, soluble and insoluble fractions with a yield of ~90–120 mg of dried residue were

obtained. Endogenous latex was not eliminated from leaves. A modified chemical procedure was performed to obtain latex extracts. Shortly, latex from *C. variegatum* (~15 mL) was directly collected from the plant by detaching leaves and then, stored at 0°C until further separation. Subsequently, the latex was extracted with HDM and finally with methanol. The latex from *E. cotinifolia* and *E. tirucalli* were extracted with ethyl acetate and dried in a rotoevaporator (yield of ~230–280 mg of dried residue). Afterwards, latex from *E. cotinifolia* was further separated in its either HDM-soluble or insoluble fractions. Finally, latex fractions were partitioned by silica gel column chromatography with the solvent system C<sub>6</sub>H<sub>14</sub>:EtOAc using a gradient of increasing polarity. Several fractions were collected, and then combined according to their thin-layer chromatography profiles to obtain three to five fractions.

The different extracts and chromatographic subfractions were dissolved with dimethylsulphoxide (DMSO) (Sigma, St Louis, MO) at concentrations of 40, 100, or 200 mg/mL depending on their solubility and stored in aliquots at -70°C until used. Endotoxin contamination was evaluated using the Limulus Amebocyte Lysate QCL-1000 kit from Lonza (Walkersville, MD) following the manufacturer instructions. All the extracts had less than 40 pg/mL of endotoxin in 100  $\mu$ g/mL of extract (data not shown).

### Isolation of PBMC and cell cultures

All the individuals included in this study were healthy adults, came from the Metropolitan Area of Medellin (Colombia) and gave their informed consent. The present study had the approval of the local Ethics Committee. PBMC were obtained from heparinized blood samples by centrifugation over Ficoll-Hypaque 1077 (Sigma). Viability of PBMC was determined by trypan blue exclusion. For cell culture, PBMC (1  $\times$  10<sup>6</sup> cells/mL) were resuspended in RPMI 1640 (Sigma) supplemented with 10% of heat-inactivated fetal bovine serum (Lonza, Walkersville, MD), 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin (Lonza, Walkersville, MD). When required, T-lymphocytes were obtained from PBMC by negative selection using beads with the T cell Negative Isolation Kit (DynaL-Biotech ASA, Oslo, Norway) following the manufacturer instructions. Cytotoxicity was evaluated using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) technique as previously described by Alley et al.<sup>(11)</sup> using five double dilutions of the plant extracts (from 32.25 to 1000  $\mu$ g/mL). To ensure that the cytotoxic effect of the extracts were not due to the toxicity of DMSO, the cytotoxic activity of DMSO was assessed simultaneously with MTT using a DMSO dilution corresponding to its final concentration in each of the extracts (data not shown). The percentage of cytotoxicity was calculated as [(A - B)/A]  $\times$  100, where A and B are the OD<sub>550</sub> of the MTT cell cultures from cells exposed to PHA (Sigma) in the absence or presence of the extracts, respectively.

For proliferation assays, triplicates from cells resuspended at 1  $\times$  10<sup>6</sup> PBMC/mL or alternatively, at 5  $\times$  10<sup>5</sup>

lymphocytes/mL were added into microtitre plates. Cells were incubated with either RPMI alone (unstimulated control) or five double dilutions of the plant extracts (from 6.25 to 100 µg/mL) w/o 10 µg/mL of PHA as mitogen (Sigma). To rule out the proliferative activity of DMSO, cells were exposed to dilutions corresponding to its final concentration in each extract. DMSO-exposed cells did not proliferate at those concentrations. When required, ten double dilutions of the subfractions (from 0.05 to 20 µg/mL) were also used to stimulate PBMC. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 66 h. Proliferation was measured by adding 1 µCi/well of [Methyl-<sup>3</sup>H] Thymidine (MP Biomedicals, Irving) for the last 18 h of incubation. Finally, cells were harvested onto glass fiber filters (Inotech Biosystems international Inc, Rockville, MD) using a Cell Harvester (Inotech Biosystems International Inc.) and resuspended in the High Flash point LSC cocktail (Perkin Elmer precisely, Shelton, CT). Thymidine incorporation was determined using a Plate Chamaleon Multilabel Reader (Hidex, Turku, Finland). Results were expressed as proliferation index (cpm of cells stimulated with PHA or extracts/cpm of cells from unstimulated cultures) or relative proliferation index (proliferation index from cells stimulated with PHA plus the extract/proliferation index from cells stimulated with PHA only).

#### Evaluation of the mitochondrial membrane depolarization as an early indicator of apoptosis in PBMC

PBMC at  $5 \times 10^5$  cells/mL were added into 48 wells plates with RPMI alone (unstimulated control) or five concentration of the plant extract (6.25–100 µg/mL) w/o of 200 µg/mL of Cycloheximide (Sigma). To rule out the apoptotic activity of DMSO, cells were exposed to dilutions corresponding to its final concentration in each extract. DMSO-exposed cells did not show any apoptotic events at those concentrations. Cultures were then incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 24 h. After incubation, cells were stained using 2.5 µL of DiOC<sub>6</sub> 4 µM (Invitrogen, Eugene, OR) and 2.5 µL of propidium iodide at 50 µg/mL (Sigma) and incubated for 25 min in the dark. Afterwards, the cells were centrifuged and resuspended in 200 µL phosphate-buffered saline. Flow cytometry was performed using the Becton Dickinson FACSORT (Becton Dickinson, San Jose, CA) and analyzed using Summit® v. 3.1 software (Cytomation, Fort Collins, CO). Results were expressed as apoptosis index (% of apoptotic cells using cycloheximide or extracts/% of apoptotic cells from unstimulated cultures) or relative apoptosis index (apoptosis index from cells exposed to the plant extract plus 200 µg/mL cycloheximide/apoptosis index of cell exposed to 200 µg/mL of cycloheximide only).

#### Proliferation assay using CFSE

The PBMC proliferative response to PHA and the selected subfractions was also evaluated using the carboxyfluores-

cein succinimidyl ester (CFSE) dilution assay (Molecular Probes, Eugene, OR). Briefly, 1 mL of CFSE 4 µM was added to  $4 \times 10^6$  PBMC for 15 min at 37°C in the dark. Subsequently, cells were washed twice and distributed at  $1 \times 10^6$  cells/well into 48 wells dishes for incubation with RPMI alone or either with 2.5, 5, 10, or 20 µg/mL of the subfractions or 5 µg/mL PHA for 120 h in a 5% CO<sub>2</sub> atmosphere. IL-2 (60 units/µL, Tecina Hoffmann-La Roche, Nutley, NJ) was added to PHA-stimulated cells and in some experiments, to cells stimulated with the subfractions for the last 72 h of culture. Subsequently, cells were stained with 5 µL Pe-Cy5 mouse anti-human CD3 (BD Pharmigen, San Jose, CA). Flow cytometry was performed using the Becton Dickinson FACSORT and analyzed using FlowJo version 7.8 for Macintosh (Tree Star Inc., Ashland, OR).

#### Proliferative PBMC assays with the subfraction EtL-1-5 using D-(+)-Galactose and α-methyl-Mannopyranoside

In an attempt to evaluate if the proliferative activity of EtL-1-5 upon PBMC was mediated by the binding of carbohydrate residues from surface cell receptors, as already described for several *Euphorbiaceae*-derived lectins,<sup>(12,13)</sup> we performed lymphoproliferation assays in the presence of D-(+)-Galactose (Sigma) and α-methyl-Mannopyranoside (Sigma). EtL-1-5 (5 µg/mL) or PHA (10 µg/mL) were preincubated with D-(+)-Galactose or α-methyl-Mannopyranoside at 8, 20, 40 µM in 96-well plates for 20 min at 37°C. Subsequently, PBMC ( $2.5 \times 10^5$  cells/well) were added to those wells and cell proliferation was measured by thymidine incorporation as previously described.

#### Protein quantification in the subfractions EtL-1-5

The amount of proteins in the subfraction was quantified using the *BCA*<sup>™</sup> *Protein Assay* kit (PIERCE, Rockford, IL) following the manufacturer instructions.

#### Statistical analysis

Results are presented as mean ± standard error (SEM). Statistical comparisons among three or more groups were performed using the Student-Newman-Keuls test, with a confidence level of 95 (SPSS 15 software, SPSS Inc., Chicago, IL). For the cytotoxicity MTT assays, cytotoxic concentration 50 (CC<sub>50</sub>) defined as the stimulus' concentration killing 50% of the cells, was obtained by adjusting the cytotoxic percentages versus doses to a polynomial curve using the software GraphPad Prism v. 4.0 for Windows (GraphPad Software, San Diego, CA). The corresponding CC<sub>50</sub> was then calculated by interpolation. Significant results had a *p* value < 0.05. A correlation analysis was also performed between the proliferation and apoptosis indexes of PBMC exposed to the different extracts. The Spearman index was calculated using the SPSS 15 Software.

## Results

**None of the 14 extracts evaluated showed a CC<sub>50</sub> below 100 µg/mL on PBMC, the greatest concentration used**

### to assess their biological effects

Plants belonging to the family *Euphorbiaceae* have been observed to exhibit intrinsic cytotoxic activity on different human cell lines.<sup>(14-16)</sup> Reports also indicate that *E. cotinifolia*, one of the plants included here, shows cytotoxicity to the HEP-2, HeLa, and CHO cell lines.<sup>(2)</sup> Therefore, we first evaluated the potential of the 14 extracts included in this study to kill PBMC *in vitro* (Table 1). Most of the extracts evaluated had a  $CC_{50}$  greater than 500  $\mu\text{g}/\text{mL}$ , which correspond to a dose fivefold higher than the maximum concentration used here to evaluate the extract's immunomodulatory activity on PBMC (100  $\mu\text{g}/\text{mL}$ ) (Table 1). The most cytotoxic extracts were the HDM-soluble latex extract of *C. variegatum* and the HDM-soluble leaves extract of *H. crepitans* ( $CC_{50}$  of 318.0  $\mu\text{g}/\text{mL}$  and 299.7  $\mu\text{g}/\text{mL}$ , respectively). However, their cytotoxic effect on PBMC was not related to the induction of apoptosis because PBMC exposed to these extracts do not exhibit increased apoptosis index even at doses of 100  $\mu\text{g}/\text{mL}$  (apoptosis index = 1.14 and 1.17, respectively).

### Extracts from the latex of *E. cotinifolia* and *E. tirucalli* showed the most prominent effects on PBMC

To further analyze the activity of the extracts upon PBMC, we evaluated the ability of the 14 extracts (from 6.25 to 100  $\mu\text{g}/\text{mL}$ ) to directly induce proliferation or apoptosis. Table 1 shows the proliferation and apoptosis indexes of PBMC incubated with the extracts at representative doses. We found that only the latex extracts of *E. cotinifolia* (EcL), and *E. tirucalli* (EtL) induced a significant increase in the proliferation and apoptosis indexes in PBMC. These two extracts also induced TNF- $\alpha$  secretion of PBMC at the evaluated doses (data not shown). Increased albeit no significant effects on PBMC proliferation and apoptosis were observed also with the leaves extracts of

*E. pulcherrima* and *H. crepitans*. Also, HDM-insoluble leaves extracts of *Caryodendron orinocensis* and HDM-soluble leaves extracts of *C. variegatum* induced minor positive effects in either apoptosis or proliferation of PBMC *in vitro*, respectively (Table 1).

In view of these results, we performed PBMC proliferative and apoptosis dose-response analysis using EcL and EtL (Figure 1). As positive controls for these experiments, PBMC were exposed to PHA or cycloheximide for proliferation or apoptosis assays, respectively. We observed that EcL stimulated the proliferation and apoptosis of PBMC at almost the same level with all the concentrations evaluated in this study (Figure 1A). In the case of EtL, the maximum effects on proliferation or apoptosis events of PBMC were observed at doses of 50  $\mu\text{g}/\text{mL}$  and 100  $\mu\text{g}/\text{mL}$ , respectively (Figure 1B).

After evaluating the direct effect of crude extracts upon PBMC proliferation and apoptosis, we analyzed whether a correlation was observed between proliferation and apoptosis indexes of PBMC stimulated with the same crude extract at concentrations ranging from 6.25 to 100  $\mu\text{g}/\text{mL}$ . For the analysis, the extracts were separated in three groups, they are *Codiaeum spp.*, *Euphorbia spp.* and others genus (*Caryodendron spp.* and *Hura spp.*). Interestingly, proliferation indexes from cells exposed to increasing concentrations of the extracts from the *Euphorbia* genus correlated with the apoptosis of these cells ( $R^2$  values of 0.295;  $p < 0.01$ ; data not shown). These results suggest that fluctuations in the proliferation indexes of PBMC stimulated with these extracts, does predict fluctuations in apoptosis indexes of these cells exposed to the same extract. On the other hand, pair wise comparisons between the proliferation and apoptosis indexes of PBMC exposed to increasing concentrations of the extracts either from *Codiaeum spp.* or other genus did

Table 1. Direct biological activity of *Euphorbiaceae* plants extracts upon PBMC

Plant	Part used	Solubility in HDM <sup>1</sup>	$CC_{50}$ ( $\mu\text{g}/\text{mL}$ )	Proliferation index <sup>2</sup>	Apoptosis index <sup>3</sup>
<i>Caryodendron orinocensis</i>	Leaf	Insoluble	658.3	0.82 $\pm$ 0.10	1.49 $\pm$ 0.50
		Soluble	704.6	1.04 $\pm$ 0.17	0.98 $\pm$ 0.17
<i>Codiaeum variegatum</i>	Leaf	Insoluble	654.0	1.14 $\pm$ 0.48	0.49 $\pm$ 0.22
		Soluble	663.3	1.49 $\pm$ 0.54	0.69 $\pm$ 0.27
	Latex	Insoluble	>1000	1.38 $\pm$ 0.71	0.95 $\pm$ 0.38
		Soluble	318.0	1.36 $\pm$ 0.53	1.00 $\pm$ 0.12
<i>Euphorbia cotinifolia</i>	Latex	N/A <sup>4</sup>	978.1	6.53 $\pm$ 2.78 <sup>5</sup>	3.56 $\pm$ 1.83 <sup>6</sup>
<i>Euphorbia pulcherrima</i>	Leaf	Insoluble	>1000	1.15 $\pm$ 0.18	1.19 $\pm$ 0.25
		Soluble	>1000	1.62 $\pm$ 0.31	1.76 $\pm$ 1.18
<i>Euphorbia tirucalli</i>	Latex	N/A <sup>4</sup>	548.8	4.94 $\pm$ 0.99 <sup>5</sup>	2.19 $\pm$ 1.60 <sup>6</sup>
<i>Hura crepitans</i>	Leaf	Insoluble	590.0	1.92 $\pm$ 0.60	1.17 $\pm$ 0.55
		Soluble	299.7	0.85 $\pm$ 0.48	1.62 $\pm$ 1.45
<i>Phyllanthus niruri</i>	Leaf	Insoluble	554.0	0.97 $\pm$ 0.60	1.07 $\pm$ 0.20
		Soluble	543.9	1.28 $\pm$ 1.27	0.62 $\pm$ 0.18

<sup>1</sup>HDM: Hexane:Dichloromethane:Methanol (2:1:1).

<sup>2</sup>Proliferation index of PBMC at 25  $\mu\text{g}/\text{mL}$  of the extract.

<sup>3</sup>Apoptosis index of PBMC in presence of 12.5  $\mu\text{g}/\text{mL}$  of the extract.

<sup>4</sup>This plant extract was obtained by acetone extraction.

<sup>5</sup>Extract that significantly ( $p < 0.05$ ) increase the proliferation of PBMC.

<sup>6</sup>Extract that significantly ( $p < 0.05$ ) increase mitochondrial membrane depolarization (early indicator of apoptosis) of PBMC.

N/A: not applicable. Data expressed as the mean  $\pm$  SEM of three independent experiments.

not correlate ( $R^2$  values of 0.001 and 0.031, respectively; data not shown). Interestingly, when the three different *Euphorbia* species included in this study were evaluated separately, we found a significant inverse correlation between the proliferation and apoptotic activity of PBMC after stimulation with leaves extracts from *E. pulcherrima* ( $R^2$  values of 0.131;  $p < 0.01$ ; Figure 2B). These data support that extracts from *E. pulcherrima* have indeed effects in PBMC proliferation that predict a reduction in the apoptosis of these cells or viceversa being also potentially useful to manipulate lymphocyte function *in vitro*.

### Extracts from *Euphorbiaceae* plants have also the ability to modulate the biological activity of PHA and/or cycloheximide in PBMC

In addition to the evaluation of the direct biological activity of the extracts on PBMC, we also investigated if crude *Euphorbiaceae* extracts can interfere with the action of molecules with known immune effects, such as PHA or cycloheximide.<sup>(17-20)</sup> PHA is a lectin widely used to induce proliferation of T-lymphocytes *in vitro*. In turn, cycloheximide is an inhibitor of the protein synthesis, an event that triggers cell apoptosis. We demonstrated that three out of 14 extracts significantly modulated the biological activity of one of these compounds in PBMC. Although the HDM-insoluble leaves extract of *C. variegatum* was able to double the proliferative activity of PHA,

HDM-soluble latex extract of *C. variegatum* significantly decrease the proliferative activity of this molecule on PBMC (See Table 2). On the other hand, HDM-soluble leaves extract of *P. niruri* can modulate the response of PBMC to cycloheximide, enhancing its activity.

### Specific subfractions of EcL and EtL are responsible for the biological activity of the corresponding crude extracts on PBMC

After the evaluation of the immunomodulatory activity of the crude *Euphorbiaceae* extracts on PBMC, we proceed to subfractionate EcL and EtL, which were the extracts with the most prominent activity on proliferation and apoptosis of these cells. Previous to the isolation of the different subfractions by silica gel column chromatography, EcL extract was separated in either HDM-soluble or insoluble fractions (EcLS and EcLI). As depicted in Figure 3A, we observed that fractions EcLS-1-3 and EtL-1-5 were the main responsible subfractions for the PBMC proliferation activity of the crude extracts EcL and EtL, respectively. Additionally, they exhibited the most prominent effects in the apoptosis of these cells. A similar albeit minor effect was observed when cells were exposed to the subfraction EcLS-1-4. Interestingly, some of the subfractions retained the ability to induce only either proliferation or apoptosis on PBMC. For example, the

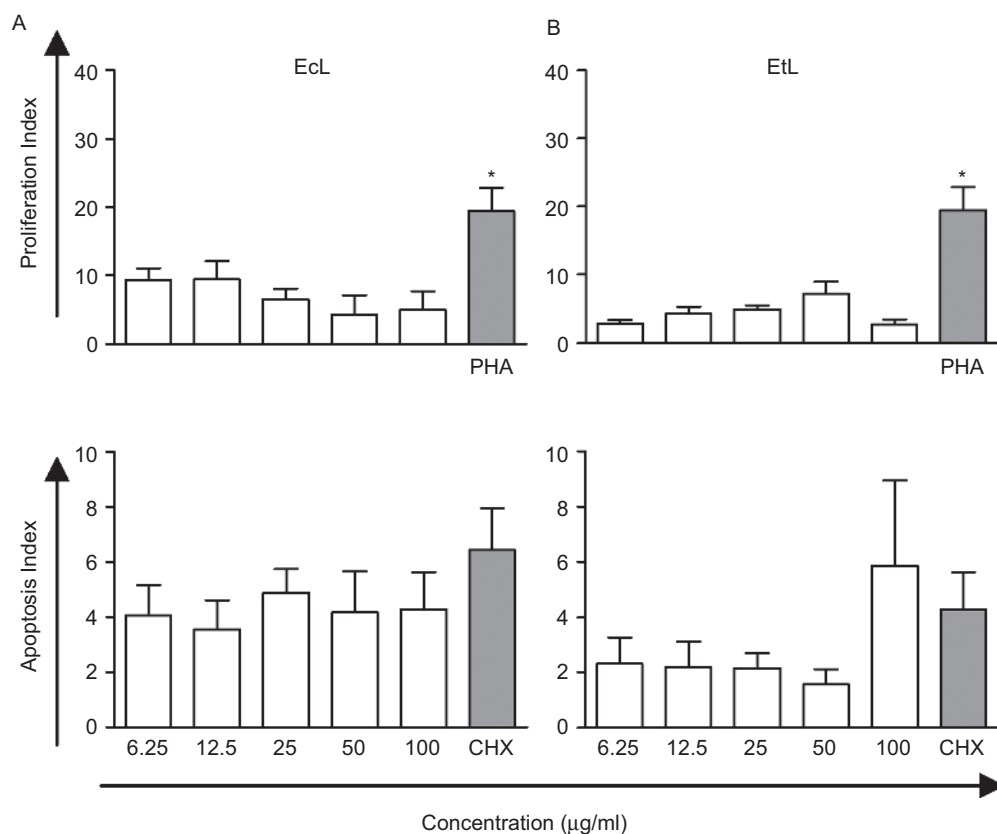


Figure 1. Dose-response curves of proliferation and apoptosis indexes from PBMC stimulated with the extracts EcL and EtL. Proliferation and apoptosis indexes of PBMC from three healthy donors stimulated with different concentrations of EcL (A) and EtL (C). PBMC were also stimulated with 10  $\mu\text{g}/\text{mL}$  of PHA or 200  $\mu\text{g}/\text{mL}$  of cycloheximide (CHX) as positive controls for proliferation or apoptosis, respectively.  $*p < 0.05$ .

subfractions EcLI-1-1 and EcLI-1-2 were only able to induce proliferation (a sevenfold increase compared to unstimulated cells) whereas subfractions EcLI-1-3 and EcLS-1-2 induced only apoptosis on PBMC.

The biological effect of the subfractions EcLS-1-3 and EtL-1-5 on PBMC was then compared to that from crude extracts at the same concentration, demonstrating that these two active subfractions greatly enhanced the immunomodulatory activity of the corresponding crude extract on these cells (Figure 3B). Specifically, EcLS-1-3 and EtL-1-5 showed a 5.87 and 2.35-fold increase in comparison to their crude extracts in their ability to induce proliferation of PBMC, respectively. Regarding to the induction

of apoptosis on PBMC, the subfraction EcLS-1-3 increase 6.6 times the activity of the crude extracts whereas the subfraction EtL-1-5 did not increase the ability to induce apoptosis in comparison to the crude EtL.

### The subfractions EcLS-1-3 and EtL-1-5 induce proliferation mainly in CD3<sup>+</sup> cells

To confirm the ability of the two most active subfractions from the *Euphorbiaceae* plants selected in this study to induce proliferation on PBMC, we performed the CFSE dilution assay using flow cytometry. PHA+IL-2-stimulated cells were the positive control for these experiments. In the CFSE assay, this stimulus induced proliferative

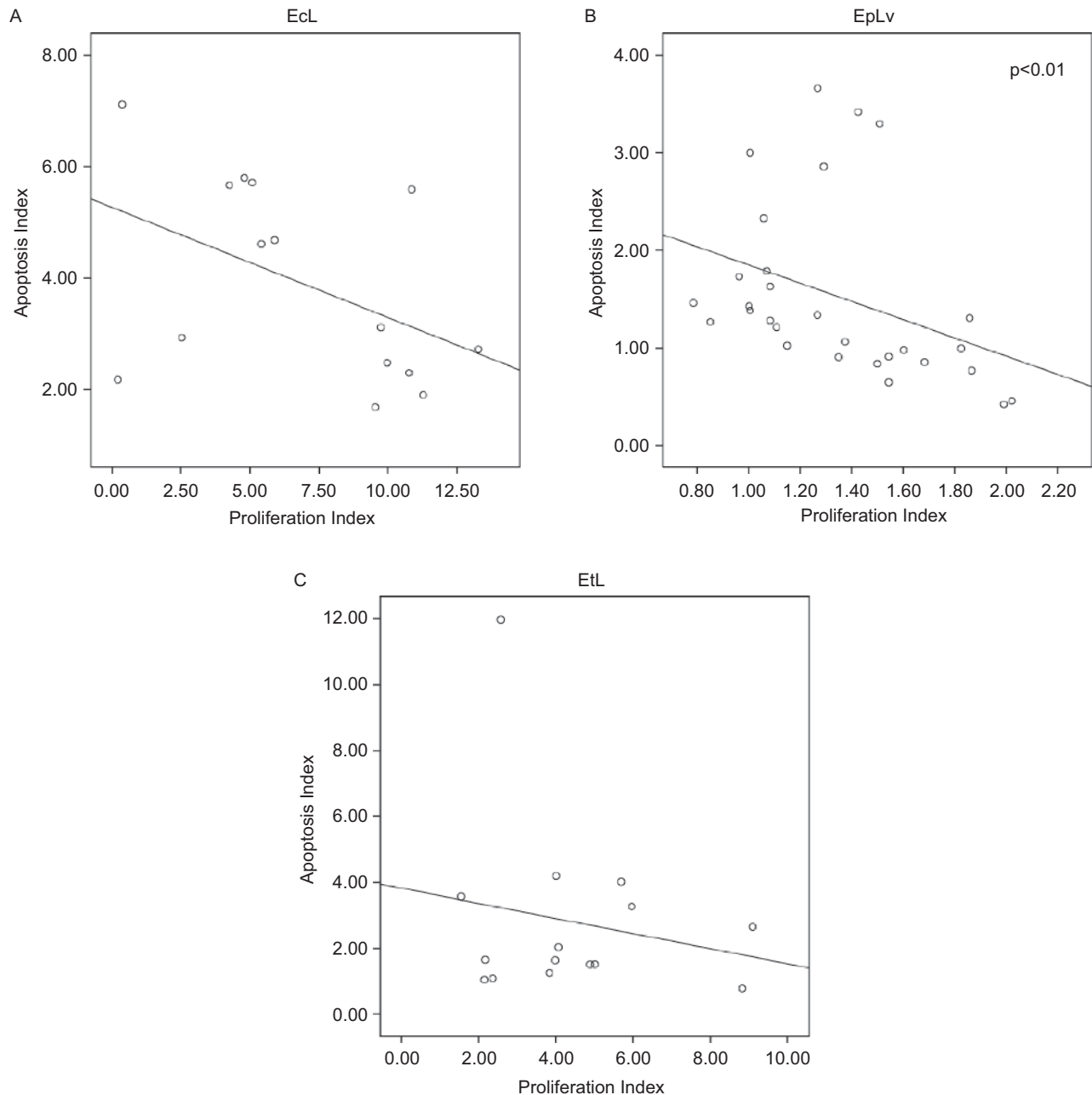


Figure 2. Correlation Analyses of proliferation versus apoptosis indexes of *Euphorbia*-exposed PBMC. Proliferation versus apoptosis indexes of PBMC from three different healthy donors stimulated with extracts (at 50  $\mu\text{g/mL}$  or 25  $\mu\text{g/mL}$ , respectively) from the latex of *E. cotinifolia* (EcL, A) or *E. tirucalli* (EtL, C) or leaves of *E. pulcherrima* (EpLv, B). Horizontal lines indicate data tendency.

responses exclusively on CD3<sup>+</sup> cells that exhibited four replication cycles (Table 3 and Figure 4). Additionally, 33.5% of the CD3<sup>+</sup> cells exposed to PHA+IL-2 from the original population of PBMC underwent proliferation at a rate of 1.68 divisions per cell. This corresponds to a 12-fold increase in the proliferation activity of PBMC when compared with unstimulated cells. Of note, the effects of PHA+IL-2 on the proliferation of CD3<sup>-</sup> cells were

negligible. Interestingly, the subfractions EcLS-1-3 and EtL-1-5 induced proliferation in CD3<sup>+</sup> as well as in CD3<sup>-</sup> cells from PBMC (Table 3 and Figure 4). In CD3<sup>+</sup> cells, EcLS-1-3 and EtL-1-5 induced two and three cell division cycles respectively, whereas in CD3<sup>-</sup> cells, two cell division cycles were observed. Moreover, we observed that EcLS-1-3 induced 11.6 and 14.2% CD3<sup>+</sup> or CD3<sup>-</sup> cells from PBMC to proliferate at a rate of 1.25 and 1.17 divi-

Table 2. Modulation of the biological activity of PHA or cycloheximide by *Euphorbiaceae* plants extracts in PBMC

Plant	Part used	Solubility in HDM <sup>1</sup>	Relative proliferation index <sup>2</sup>	Relative apoptosis index <sup>3</sup>
<i>Caryodendron orinocensis</i>	Leaf	Insoluble	0.91 ± 0.04	1.35 ± 0.17
		Soluble	0.81 ± 0.21	1.17 ± 0.27
<i>Codiaeum variegatum</i>	Leaf	Insoluble	2.12 ± 0.38 <sup>4</sup>	0.72 ± 0.25
		Soluble	1.42 ± 0.59	0.65 ± 0.17
	Latex	Insoluble	0.69 ± 0.20	1.16 ± 0.22
		Soluble	0.55 ± 0.13 <sup>5</sup>	0.88 ± 0.11
<i>Euphorbia cotinifolia</i>	Latex	N/A <sup>6</sup>	0.81 ± 0.37	1.40 ± 0.69
<i>Euphorbia pulcherrima</i>	Leaf	Insoluble	0.61 ± 0.17	1.20 ± 0.20
		Soluble	0.69 ± 0.25	1.39 ± 0.06
<i>Euphorbia tirucalli</i>	Latex	N/A <sup>6</sup>	0.71 ± 0.39	1.11 ± 0.63
<i>Hura crepitans</i>	Leaf	Insoluble	1.67 ± 0.29	1.02 ± 0.29
		Soluble	1.80 ± 1.01	0.83 ± 0.15
<i>Phyllanthus niruri</i>	Leaf	Insoluble	0.63 ± 0.12	1.26 ± 0.09
		Soluble	0.63 ± 0.51	1.41 ± 0.11 <sup>4</sup>

<sup>1</sup>HDM: Hexane:Dichloromethane:Methanol (2:1:1).

<sup>2</sup>Relative proliferation index of PBMC in presence of 10 µg/mL of PHA and 50 µg/mL of extract concentration.

<sup>3</sup>Relative apoptosis index of PBMC in presence of 200 µg/mL of cycloheximide and 12.5 µg/mL of extract concentration.

<sup>4</sup>Extract that significantly ( $p < 0.05$ ) enhance the activity of PHA or cycloheximide in PBMC.

<sup>5</sup>Extract that significantly ( $p < 0.05$ ) decrease the activity of PHA or cycloheximide in PBMC.

<sup>6</sup>This plant extract was obtained by acetone extraction.

N/A: not applicable. Data expressed as the mean ± SEM of three independent experiments.

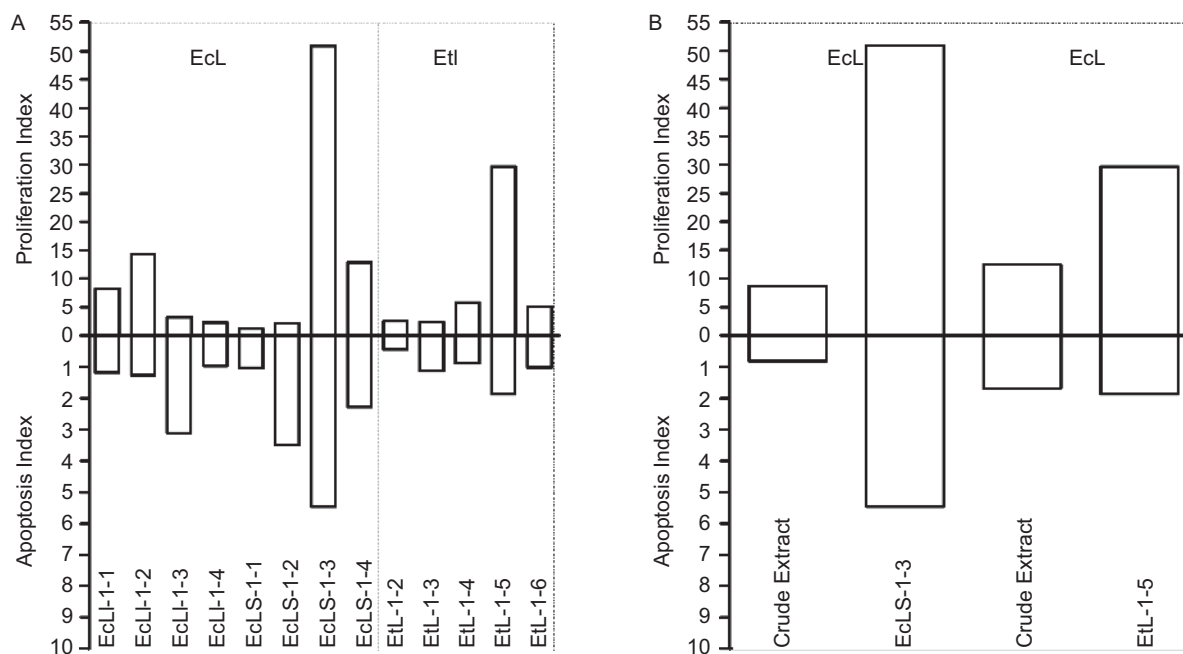


Figure 3. Immunomodulatory activity of *E. cotinifolia* (EcL) and *E. tirucalli* (EtL) latex subfractions upon PBMC. (A) Proliferation or apoptosis indexes of PBMC from one healthy donor cultured in the presence of 5 µg/mL of each subfraction; (B) comparison of the proliferation and apoptosis indexes of PBMC from one healthy donor stimulated with the crude extract or the most active subfraction of EcL and EtL.

sions per cell, respectively. Regarding to PBMC exposed to the subfraction EtL-1-5, 11.8 and 10.3% of the CD3<sup>+</sup> or CD3<sup>-</sup> cells underwent proliferation *in vitro* at a rate of 1.35 and 1.03 divisions/cell, respectively. However, the most marked proliferative effect was observed in CD3<sup>+</sup> cells because the ratio between the division indexes of cells w/o the subfractions (CE/SE, see Table 3) was 3.04 and 3.48 in CD3<sup>+</sup> cells compared to only 1.70 and 1.10 in CD3<sup>-</sup> cells exposed to EcLS-1-3 and EtL-1-5, respectively.

### Human purified T-lymphocytes exposed to EtL-1-5 proliferate in the absence of accessory cells

Taking into account that the subfractions EcLS-1-3 or EtL-1-5 induce proliferation mainly of CD3<sup>+</sup> cells (T-lymphocytes), we next selected EtL-1-5—the most easy-to-obtain and stable subfraction—to evaluate if proliferation could be also induced in isolated T-lymphocytes as compared with PBMC. As control of proliferation, we selected PHA because the proliferative activity of PHA

Table 3. Cell division of CD3<sup>+</sup> and CD3<sup>-</sup> cells from PBMC stimulated with EcLS-1-3 and EtL-1-5

Treatment	Cell division cycles		Percentage of divided cells <sup>1</sup>		Proliferation index <sup>2</sup>		CE/SE <sup>3</sup>	
	CD3 <sup>+</sup> cells	CD3 <sup>-</sup> cells	CD3 <sup>+</sup> cells	CD3 <sup>-</sup> cells	CD3 <sup>+</sup> cells	CD3 <sup>-</sup> cells	CD3 <sup>+</sup> cells	CD3 <sup>-</sup> cells
No stimulus	2	2	3.6	7.73	1.29	1.34		
PHA + IL-2	4	1	33.5	8.27	1.68	1	12.17	0.83
1.25 µg/mL EcLS-1-3	2	2	11.6	14.2	1.25	1.17	3.04	1.70
10 µg/mL EtL-1-5	3	2	11.8	10.3	1.35	1.03	3.48	1.10

<sup>1</sup>Percentage of cells which underwent cell division from the original PBMC population.

<sup>2</sup>Average of cell divisions that cells in replication underwent.

<sup>3</sup>CE/SE: Ratio between the division index of cells w/o stimulus.

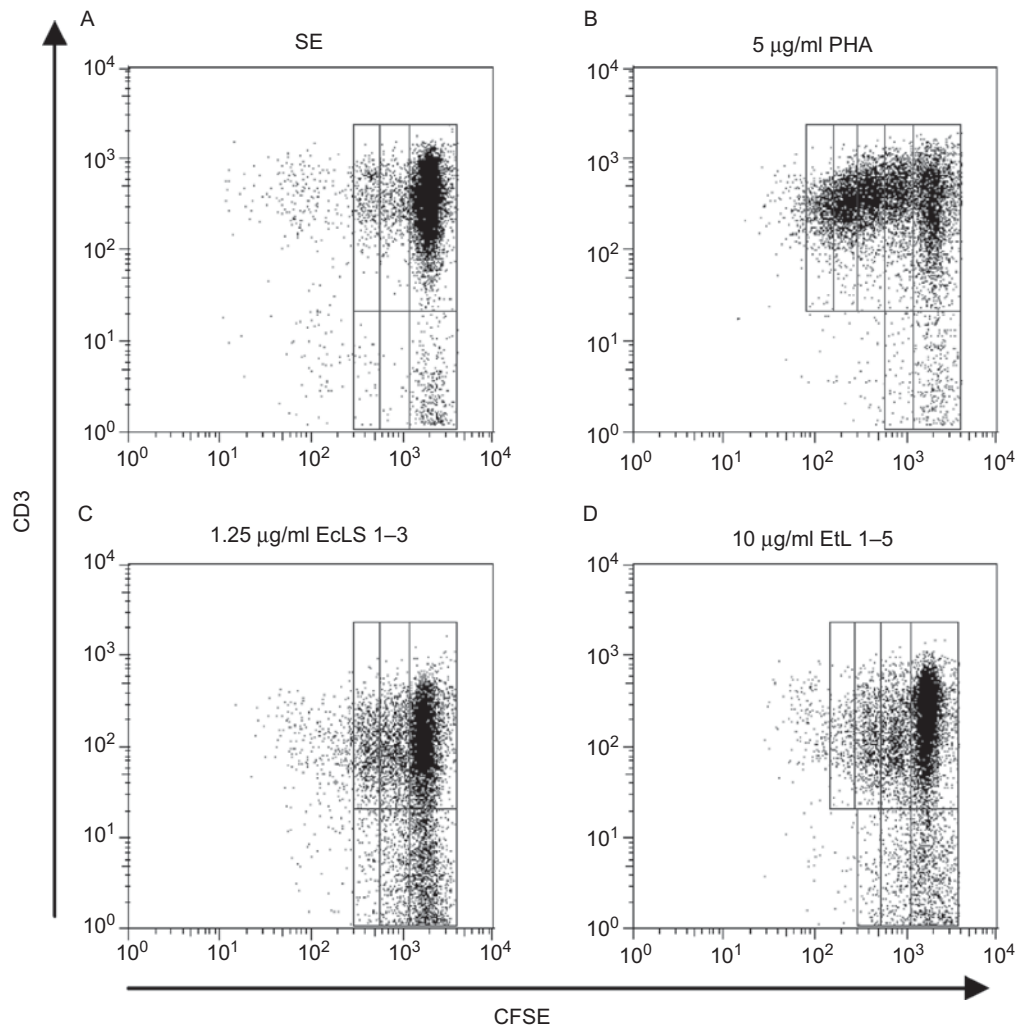


Figure 4. Proliferative activity of CD3<sup>+</sup> and CD3<sup>-</sup> cells stimulated with EcLS-1-3 and EtL-1-5 using the CFSE dye dilution assay by flow cytometry. Shown are the dot plots from PBMC stimulated with medium alone (A), 5 µg/mL of PHA (B), 1.25 µg/mL of EcLS-1-3 (C) or 10 µg/mL of EtL-1-5 (D). Squares indicate the proliferation cycles of PBMC upon stimulation. This result is representative of three different experiments using PBMC from healthy donors.



on human T-lymphocytes requires accessory cells.<sup>(18,21)</sup> As expected, the proliferation indexes of purified T cells were approximately four- to eight-fold lower as compared with proliferation indexes of PBMC after stimulation with PHA (Figure 5). Importantly, EtL-1-5 induced the same level of proliferation in purified T-lymphocytes and PBMC (Figure 5). These results suggest that the mechanism by which this subfraction induces proliferation on T cells is not mediated by the accessory cells present in the PBMC cell culture.

#### D-galactose and $\alpha$ -Methyl-D-Mannopyranoside did not inhibit the proliferation of PBMC exposed to EtL-1-5

Based on previous reports which indicate that lectins capable to bind carbohydrate residues in cell surface receptors are responsible for the proliferative capacity of extracts from *Euphorbiaceae* plants on immune cells,<sup>(12,13)</sup> we evaluated if two carbohydrate ligands (D-galactose and  $\alpha$ -Methyl-D-Mannopyranoside) commonly recognized by plant lectins<sup>(22,23)</sup> mediated the proliferative effects of EtL-1-5 on human PBMC. Cells were stimulated with PHA only as positive control for the thymidine incorporation assay. However, the proliferative activity of EtL-1-5 on human PBMC was not inhibited by any of the concentrations of D-galactose and  $\alpha$ -Methyl-D-Mannopyranoside evaluated here (Figure 6). However, these findings do not rule out the lectin-like activity of this subfraction as plant-derived lectins have also specificity for carbohydrates different to D-galactose and  $\alpha$ -Methyl-D-Mannopyranoside such as L-arabinose, D-fucose, Methyl- $\alpha$ -D-Mannoside, acetylgalactosamine and lactose.<sup>(22-24)</sup> Considering that lectins are mainly proteins, we then evaluate the presence of proteins in EtL-1-5 detecting levels of 13.49  $\mu$ g/mL of proteins at a concentration of 100  $\mu$ g/mL of the subfraction EtL-1-5.

## Discussion

The present study selected 14 extracts belonging to 7 plants from the *Euphorbiaceae* family based on ethnobotanic and phytochemical criteria to evaluate their immunomodulatory properties on human lymphocytes in primary cell cultures. Our results demonstrate that two of these extracts (from *E. cotinifolia* and *E. tirucalli*) had significant direct effects in PBMC proliferation and apoptosis and three additional extracts (from *C. variegatum* and *P. niruri*) modulated the biological activity of PHA or cycloheximide. Interestingly, when PBMC were exposed to leaves extract from *E. pulcherima*, minor albeit no significant effects on the proliferation and apoptosis of these cells were found and additionally, an inverse correlation between proliferation and apoptosis indexes was observed. The immunomodulatory activities of extracts from the latex of *E. cotinifolia* and *E. tirucalli* were greatly enriched mainly in two subfractions (ECLS-1-3 and EtL-1-5) of these plants. We also demonstrated that ECLS-1-3 and EtL-1-5 exert their activities mainly in CD3<sup>+</sup> cells. More importantly, further experiments with EtL-1-5 revealed that this subfraction does not require accessory cells to induce proliferation of T-lymphocytes *in vitro* and its activity is not inhibited by the carbohydrates D-(+)-Galactose and  $\alpha$ -methyl-mannopyranoside.

Because plants belonging to *Euphorbiaceae* family are highly toxic,<sup>(25)</sup> our preliminary experiments were focused on evaluating the cytotoxic activity of these extracts in PBMC. Interestingly, we found that the latex extract of *E. cotinifolia* was not cytotoxic to PBMC ( $CC_{50}$  = 978.1  $\mu$ g/mL). This finding differs from those reported by Betancur-Galvis *et al.*, who demonstrated a cytotoxic effect of stems and leaves extracts from this plant on the tumor cell lines HEp-2, HeLa, CHO and primary cultures of bovine fibroblasts ( $CC_{50}$  = 88.1 and 35.1  $\mu$ g/mL for HEp-2 cells,  $CC_{50}$  = 102.4 and 212.5  $\mu$ g/mL for HeLa cells,  $CC_{50}$  = 116.8

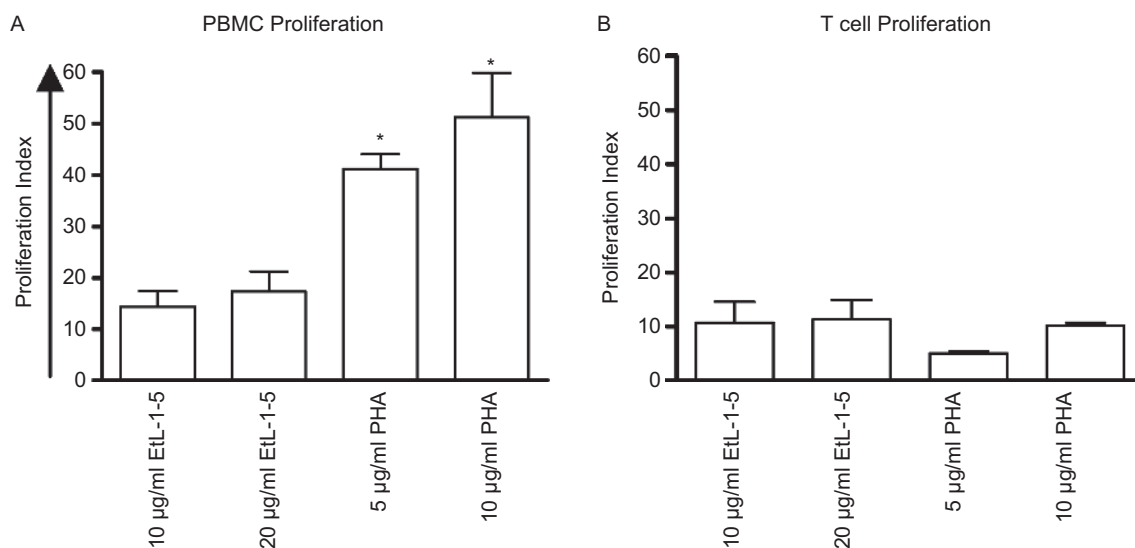


Figure 5. Proliferative indexes of PBMC or purified T-lymphocytes stimulated with the subfraction Et-1-5 using the tritium thymidine incorporation assay. (A)  $1 \times 10^6$  PBMC/mL or (B)  $5 \times 10^5$  T-lymphocytes/mL (94% purity) from two healthy donors. \* $p < 0.05$  indicating that proliferation index means from PBMC and T-lymphocytes stimulated with PHA are statistically different.

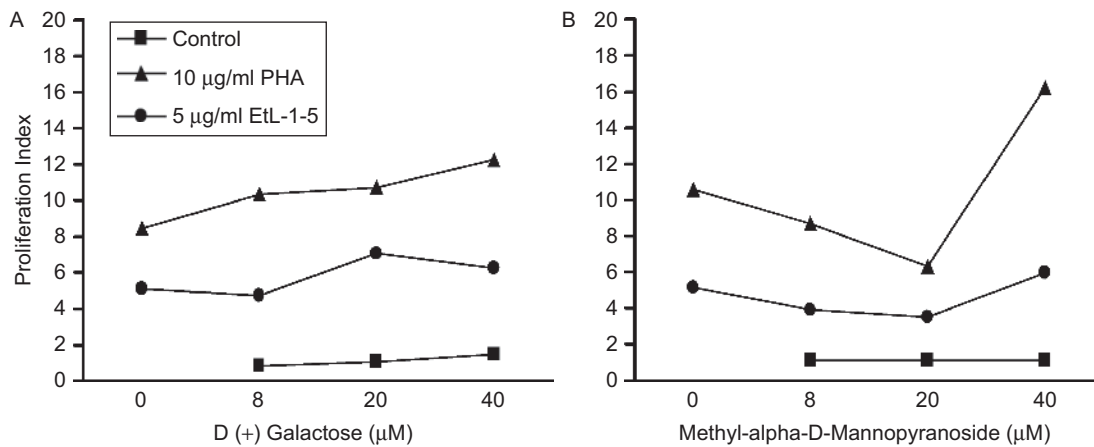


Figure 6. Proliferative indexes of PBMC in the presence of Et 1-5 after exposure to different carbohydrates. The ability of increasing concentrations of the carbohydrates D-(+)-Galactose (A) or  $\alpha$ -Methyl-D-Mannopyranoside (B) to inhibit the proliferative activity Et-1-5 upon PBMC was evaluated. PBMC were also stimulated with medium alone or 10  $\mu$ g/mL of PHA as negative or positive controls for proliferation, respectively. This result is representative of two independent experiments using PBMC from different healthy donors.

and 18.1  $\mu$ g/mL for CHO cells and  $CC_{50}$ =135.5 and 295.1  $\mu$ g/mL for bovine fibroblasts in the presence of steam or leaves extracts, respectively).<sup>(2)</sup> We postulate that the cytotoxic compounds of these plants are located in specific parts of their structure. However, our data did not reveal high cytotoxicity of the latex extracts of *E. tirucalli* in primary human lymphocyte cultures ( $CC_{50}$ =548.8  $\mu$ g/mL), although this latex exhibit high cytotoxicity in tumor cell lines.<sup>(26)</sup> In addition, Silva *et al.*, reported placenta size increase and leucopenia in 5–7 days post-coitus Wistar rats treated orally with latex aqueous extract of *E. tirucalli*.<sup>(27)</sup> Other possibility that may explain these results is that the cytotoxicity could be selective for tumor cell lines, a finding that, if confirmed, may postulate these plants as promising natural products for the treatment of some cancers. However, the activity of the extracts upon living cells may be altered by the presence of other cells or factors differentially produced according to the experimental settings. For this reason, the evaluation of the extracts toxicity should be performed in cell lines as well as in primary cultures and also *in vitro* as well as *in vivo*.

Regarding to the direct immunomodulatory activity of the extracts upon PBMC, the subfractions with the most prominent effects induced both proliferation as well as apoptosis in PBMC. A plausible mechanism to explain our results is that *Euphorbia*-mediated T-cell proliferation may stimulate a self-regulatory mechanism called activation-induced cell death (AICD) that results in apoptosis of proliferating T cells, hence preventing unlimited activation of adaptive immune responses.<sup>(28)</sup> During this process, only some activated T cells overcome pro-apoptotic signals to become memory T cells. Additional experiments are necessary to elucidate if AICD is implicated in the dual effect observed in PBMC upon exposure to the subfractions EcLS-1-3 and EtL-1-5.

Moreover, we also demonstrate that three out of 14 of the *Euphorbiaceae* extracts included in this study have the ability to interfere with the action of molecules as

PHA or cycloheximide. Interestingly, extracts from different parts of the plant (leaves vs. latex from *C. variegatum*) exert opposite effects on PHA-induced PBMC proliferation. The response of PBMC to cycloheximide was also modified by HDM-soluble leaves extract of *P. niruri*. Several compounds from the same plant may be differentially responsible for the opposite effects of the extracts from *C. variegatum* upon the PHA activity. These extracts could exert their interfering effects directly upon cells by modifying the function of surface receptors or intracellular signaling molecules which are crucial for the action of PHA or cycloheximide but also by direct chemical interaction with these molecules altering the affinity for specific cell ligands.

Although we still do not know which active compounds from the plants included in this study are responsible for their activity upon human PBMC, terpenoids, flavonoids, tannins, and lectins with immunomodulatory potential have been isolated from *Euphorbiaceae* plants. More specifically, some lectins have been purified from the latex of this family of plants<sup>(22,29–31)</sup> and the two more active subfractions obtained in our work are derived from latex. Additionally, previous work with lectins isolated from *H. crepitans*, *E. characias* L. and *E. marginata* indicates that they induce proliferation in T-lymphocytes in the absence of accessory cells,<sup>(22,31)</sup> as we observed for EtL-1-5. Based on these findings, we investigated if the ability of this subfraction to induce proliferation of human PBMC could be inhibited in the presence of the carbohydrates D-galactose and  $\alpha$ -Methyl-D-Mannopyranoside. The rationale behind these experiments is that these soluble carbohydrates would compete with carbohydrate residues present in surface T-cell receptors, blocking the cross-linking activity of EtL-1-5-containing lectins with specificity to D-galactose and  $\alpha$ -Methyl-D-Mannopyranoside. However, our evidence indicated that the immunomodulatory activity of EtL-1-5 upon human PBMC is not inhibited by these soluble carbohydrates. A wide range of carbohydrates should be tested considering that

previous studies demonstrate that lectins from *Euphorbia milii* and *Euphorbia heterophylla* have strong affinity for acetylgalactosamine and lactose.<sup>(24,32)</sup>

Taking together, our study demonstrates the potential of extracts from the *Euphorbiaceae* family and specifically, from the *Euphorbia* genus as immunomodulators. Our results also suggest the presence of several compounds within these plants with different effects in primary human immune cells. Additionally, we suggest a mechanism of action of the subfractions obtained from the extracts with the most potent effects on PBMC. A more comprehensive chemical characterization and immunological evaluation of these plants should be performed to establish the compounds of these subfractions with biological activity in order to determine their potential use as therapeutic or diagnostic tools in immunology.

## Conclusions

- Any of the extracts obtained from *Caryodendron orinocense*, *Codiaeum variegatum*, *E. cotinifolia*, *E. pulcherrima*, *E. tirucalli*, *H. crepitans* and *P. niruri* were cytotoxic (CC<sub>50</sub> >299 mg/ml) upon human PBMC in vitro.
- The latex extracts of *E. cotinifolia* (EcL), and *E. tirucalli* (EtL) induced a significant increase in the proliferation and apoptosis indexes of PBMC.
- A significant inverse correlation between the proliferation and apoptotic activity of PBMC was observed after stimulation with leaves extracts from *E. pulcherrima*.
- HDM-insoluble leaf extract of *C. variegatum* double the proliferative activity of PHA, while HDM-soluble latex extract of *C. variegatum* significantly decrease the proliferative activity of PHA on PBMC. On the other hand, HDM-soluble leaves extract of *P. niruri* can enhance the apoptosis of PBMC in response to cycloheximide.
- The subfractions EcLS-1-3 and EtL-1-5 greatly enhance the proliferation activity of the crude extracts EcL and EtL upon PBMC.
- The subfractions EcLS-1-3 and EtL-1-5 induce proliferation mainly in CD3<sup>+</sup> cells.
- EtL-1-5 induce proliferation upon human purified T-lymphocytes in the absence of accessory cells and its proliferative effects are not inhibited by the carbohydrates D-galactose and  $\alpha$ -Methyl-D-Mannopyranoside.

## Declaration of interest

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The authors declare that they have no competing interests.

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