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The crude latex of *Euphorbia tirucalli* modulates the cytokine response of leukocytes, especially CD4⁺ T lymphocytes

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Abstract: The plants of the Euphorbiaceae family, especially those of the genus *Euphorbia*, are frequently used by Brazilian folk communities to treat a wide variety of infectious, tumoral and inflammatory illnesses. Among the species of this genus, *Euphorbia tirucalli* L. is widely used in some Brazilian regions, such as the Jequitinhonha River Valley. There is evidence that the latex produced by *E. tirucalli* has antiviral and antitumor activities, but little is known about the mechanisms involved in these effects. It is likely that the mechanism for such activities involves leukocyte activation and cytokine production. In this work, we aimed to evaluate the production of type 1 (TNF- α and IFN- γ) and type 2 (IL-4 and IL-10) cytokines by circulating leukocyte subsets submitted to brief stimulation with the crude latex of *E. tirucalli*. Peripheral blood leukocytes of twenty healthy subjects were submitted to 4 h incubation with crude *E. tirucalli* latex diluted in dimethylsulfoxide. After the incubation period, the cells were stained with FITC-conjugated monoclonal antibodies specific to the cell surface receptors CD4, CD8 and CD14, and to PE-conjugated monoclonal antibodies specific to the cytokines TNF- α , IFN- γ , IL-4 and IL-10. The acquisition and analysis of data were performed by flow cytometry. The results showed a significant increase ($p < 0.05$) in the percentage of CD4⁺ T lymphocytes positive for the type 1 cytokines TNF- α and IFN- γ . Neutrophils and CD8⁺ T lymphocytes showed a mixed profile of cytokine production, characterized by an increase in the percentage of cells expressing IFN- γ , TNF- α , and IL-10. The data indicate a predominant type 1 cytokine response. The findings presented suggest that the effect popularly attributed to *E. tirucalli* usage may be attributed to its effect on the production of TNF- α and IFN- γ . However, the relationship between the *in vitro* and *in vivo* effects of *E. tirucalli* needs to be investigated.

Introduction

Plants from the Euphorbiaceae family, mainly from the *Euphorbia* genus, have been used by some Brazilian folk communities for the treatment of injuries, infectious illnesses, tumors and inflammatory diseases (Zhang et al., 2008; Yang et al., 2005; Uzair et al., 2009; Amirghofran et al., 2006; Fernandez-Arche et al., 2010). Regarding the high concentrations of terpenoid compounds in specific plants within this family, it is possible that some species have medicinal properties that can be exploited for therapeutic purposes (Pusztai et al., 2007; Lage et al., 2009).

Euphorbia tirucalli L., Euphorbiaceae, is

commonly used in popular medicine in the Jequitinhonha River Valley, one of the poorest regions of Minas Gerais, for therapeutic purposes to treat illness and tumors (Valadares et al., 2006; Betancur-Galvis et al., 2002; Lage et al., 2009). The *in vivo* antitumor effect of *E. tirucalli* has been demonstrated (Valadares et al., 2006), although the mechanisms responsible for such effect have not been investigated.

Cytokines are important mediators in the development of efficient and adequate immunological responses against pathogens and pathological processes. Studies have shown that plants from the *Euphorbia* genus can alter the molecular and cellular parameters of the immune system (Amirghofran et al., 2008; Singh

et al., 2006), suggesting that the therapeutic actions attributed to *E. tirucalli* could be the consequence of immune response changes evoked by the plant and not its direct action on the elements that promote the disease. In the present study, we investigated the *in vitro* effects of the crude latex of *E. tirucalli* on the production of the type 1 cytokines IFN- γ and TNF- α , and the type 2 cytokines IL-4 and IL-10 by human leukocyte populations.

Materials and Methods

Plant material

Euphorbia tirucalli L. (Euphorbiaceae) latex was collected from a specimen on the campus of the Universidade Federal do Vale do Jequitinhonha e Mucuri (UFVJM), located at MGT-367 road, km-583, 5000, Diamantina, Minas Gerais, Brazil (18°12.152'S, 43°34.667'W, 1.345 m altitude), in January of 2009. The plant was identified by Dr. F. N. Costa, Biological Sciences Department, UFVJM. The collected samples were deposited at the UFVJM herbarium under the registration number 1897. *E. tirucalli* latex was collected in the morning by incision at the base of a branch. The latex was diluted immediately in dimethylsulfoxide at 10% v/v. The quality of the solution was determined by homogeneity and the absence of precipitates.

Specific monoclonal antibodies used in flow cytometry analysis

The specific monoclonal antibodies (MoAbs) used were purchased from Pharmingen (San Diego, CA, USA), including anti-human IgG1-fluorescein isothiocyanate (FITC) clone 679•1Mc7, anti-human IgG_{2a}-phycoerythrin (PE) clone U7•27, anti-human CD4-FITC clone 13B8•2, anti-human CD8-FITC clone B9•11, and anti-human CD14-FITC clone M0P9. The PE-labeled (PE) anti-human cytokine MoAbs included anti-TNF- α (clone MOAB11), anti-IFN- γ (clone B27), anti-IL-4 (clone MP4-25D2), and anti-IL-10 (clone JES3-9D7).

Biological samples and short-term whole-blood culture

Leukocytes were obtained from the blood of twenty volunteers capable of blood donation with negative serology for relevant blood-borne pathogens, including HIV, hepatitis C virus, hepatitis B virus, syphilis and Chagas disease. Volunteers using corticosteroids or other immunosuppressive drugs were not considered for blood donation. Informed written consent was obtained from all participants. The study

was approved by the Ethical Committee at the UFVJM, Brazil (Protocol 02/009).

Blood samples were collected in vacuum tubes containing sodium heparin (Vacutainer; Becton Dickinson, San Jose, CA, USA), and 500 μ L aliquots were dispensed into individual 17 x 100 mm polypropylene tubes (Falcon 2059, Becton Dickinson, San Jose, CA, USA). Samples were incubated for 4 h at 37 °C in a 5% CO₂ humidified atmosphere in the presence of 500 μ L of RPMI-1640 (Gibco, Grand Island, NY, USA) and Brefeldin A (BFA) (Sigma Chemical Co., St Louis, MO, USA) at a final concentration of 10 μ g/mL. The stimulation with crude *E. tirucalli* latex was performed by the addition of 25 μ L of 10% crude *E. tirucalli* latex diluted in DMSO (EUP group) to the cultures. A solvent control, i.e., cells treated only with DMSO (25 μ L), was performed in parallel (DMSO group). After solvent or latex treatment, the cultures were treated with 2 mM ethylenediamine tetraacetic acid (EDTA) (Sigma Chemical Co.) and incubated at room temperature for 15 min.

Flow cytometric immunostaining for surface markers and intracellular cytokines

Samples were washed with 6 mL of phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.1% sodium azide (Sigma). Cells were centrifuged (600 x g, room temperature, 7 min) and then resuspended in PBS/BSA/sodium azide. Aliquots (200 μ L) were distributed into two 12 x 75 mm polystyrene tubes (Falcon 2052), and cells were individually stained with MoAbs directed against CD4, CD8, and CD14 for 30 min at room temperature in the dark. Stained samples were treated with 2 mL of FACS lysing solution (Becton Dickinson, San Jose, CA) by gentle vortexing. After re-incubation for an additional 10 min, the samples were centrifuged (600 x g, room temperature, 7 min), and the supernatant was discarded. The pellet was dissolved in 2 mL FACS permeabilizing solution, which contains PBS/BSA/sodium azide, and 0.5% saponin (Sigma), and was incubated in the dark for 10 min at room temperature. After incubation, the samples were washed twice in PBS/BSA/sodium azide. Then, 20 μ L of PE-labeled anti-cytokine MoAb were added to cells (20 μ L) in a 96-well U-bottom microplate (Thomas 9383-A90) for 30 min at room temperature. Cells were subsequently washed with 150 μ L of FACS permeabilizing solution followed by 200 μ L of FACS buffer. Samples were then fixed in 200 μ L of FACS fixing solution containing 10 g/L paraformaldehyde, 10.2 g/L sodium cacodylate and 6.63 g/L sodium chloride (Sigma) at pH 7.2, stored at 4 °C in the dark, and analyzed by flow cytometry within 24 h.

Flow cytometry

A total of 30000 events were acquired using a FACScan flow cytometer (Becton Dickinson) correctly set up to measure forward (FSC) and side (SSC) light scattering and FITC (FL-1) and PE (FL-2) fluorescence. CellQuest™ software was used for data acquisition and analysis. Identification of the lymphocyte subsets was performed by the dual-color immunophenotyping method using specific FITC-labeled anti-surface MoAbs and PE-labeled anti-cytokine MoAbs (Brito-Melo et al., 2006).

Initially, a lymphocyte scatter gate was set up, using FSC versus SSC dot plots, followed by the identification of cytokine-positive cells using FL-1/FITC-anti-cell marker versus FL-2/PE-anti-cytokine dot plots. Selective analysis of neutrophils was performed by establishing a specific scatter gate using the combination of anti-cell surface antigens and laser side scatter (SSC) to discriminate and gated the neutrophils as SSC^{high}CD14^{low+} cells. Following the selection of neutrophil population, the prevalence of cytokine-expressing cells was determined using quadrant statistics over FL2/anti-cytokine-PE dot plots. Analysis of cytokine⁺ monocyte was performed by double staining immunophenotyping using anti-CD14-FITC and anti-cytokine-PE labeled MoAbs. Monocytes were first selected as SSC^{low}CD14^{high+} cells, using FL1/anti-CD14-FITC versus SSC dot plots. Cytokine expression was then measured in terms of percentage of positive events within CD14^{high+} cells. All results were expressed as the percentage of cytokine-positive cells for the different gated leukocyte subpopulations analyzed in this study, selected as described above. The number of subjects displayed in the figures may differ due to methodological restrictions, including insufficient number of cells and autofluorescence interference in the flow cytometry data.

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered statistically significant when $p < 0.05$.

Results and Discussion

Plants from the Euphorbiaceae family have been reportedly used as medicinal plants because of their antiviral and antitumor properties (Kuo et al., 2006; Yan et al., 2008; Amirghofran, 2006; Yang et

al., 2005; Lage et al., 2009). However, the biological pathways involved in these processes have not been identified.

In this study, we demonstrated that the expression profiles of human peripheral blood leukocytes changed after their stimulation with crude *E. tirucalli* latex. As demonstrated in Table 1, lymphocytes predominantly expressed type 1 cytokines, and the IFN- γ and TNF- α expression levels in this cell population were, respectively, eleven and eight times higher than those of the control group. Studies have shown that phorbol esters obtained from *E. tirucalli* have an inflammatory effect in rat ears (Kinghorn, 1979). The CD4⁺ and CD8⁺ T lymphocyte subsets similarly presented increased expression of TNF- α and IFN- γ (Figures 1 and 2). CD4⁺ T lymphocytes are key cells involved in antiviral and antitumor immune responses. This cell population secretes cytokines that improve the recognition and cell-mediated elimination of virus-infected cells and tumor cells (Sasaki et al., 2010).

Table 1. Intracellular TNF- α , INF- γ , IL-4, and IL-10 expression profile of circulating leukocytes after *in vitro* short-time stimulation with *E. tirucalli* latex.

Peripheral leukocyte subsets	Cytokines	CC	DMSO	EUP
Neutrophils	TNF- α +	0.05±0.02	0.05±0.03	0.24±0.28 ^{a,b}
	IFN- γ +	0.16±0.05	0.15±0.08	0.50±0.29 ^{a,b}
	IL-4+	2.26±1.18	3.60±1.77	7.18±3.02 ^{a,b}
	IL-10+	0.17±0.16	0.11±0.12	0.17±0.11
Monocytes	TNF- α +	28.75±20.20	24.29±18.42	16.56±14.60
	IL-4+	4.27±2.40	4.26±2.42	6.38±3.22
	IL-10+	4.16±2.83	4.25±3.55	5.18±3.48
Lymphocytes	TNF- α +	1.05±2.39	0.38±0.28	8.59±3.75 ^{a,b}
	IFN- γ +	0.45±0.31	0.33±0.27	5.16±2.08 ^{a,b}
	IL-4+	0.56±0.27	1.19±0.91 ^a	1.05±0.73
	IL-10+	1.72±1.79	1.80±1.53	1.17±0.61

Data are presented as the mean percentage±SD for n=20. CC: control group, DMSO: solvent control group, EUP: *E. tirucalli* latex-stimulated group. Statistically significant differences are denoted by "a" for comparison with the CC group, and by "b" for comparison with the DMSO group.

The increase of type 1 cytokines mediated by plants of the *Euphorbia* genus was recently described. Singh et al. (2006) demonstrated that treatment with an ethanolic extract of *E. hirta* in allergic rats promoted an increase in the level of IFN- γ and decreased the severity of the allergic reactions. IFN- γ is an important type 1 cytokine because of its roles in macrophage and killer cell activation, in antigen presentation and cytotoxicity mediated by CD8⁺ T cells. Additionally, IFN- γ induces IgG₁ and IgG₃ production by B cells. Together, these immunological elements are able to efficiently eliminate

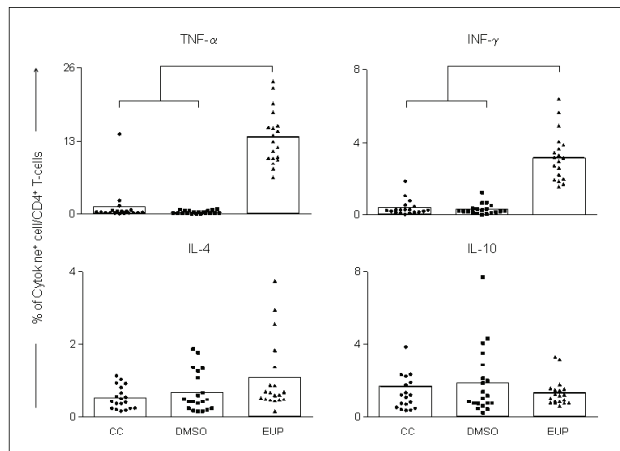


Figure 1. Intracellular TNF- α , INF- γ , IL-4 and IL-10 expression in peripheral blood CD4⁺ T lymphocytes after short-time stimulation *in vitro* with saline (CC), Dimethylsulfoxide (DMSO), and *E. tirucalli* latex (EUP group). The results are expressed as mean percentage+SD. Significant differences at $p < 0.05$ are identified by bars between evaluated groups.

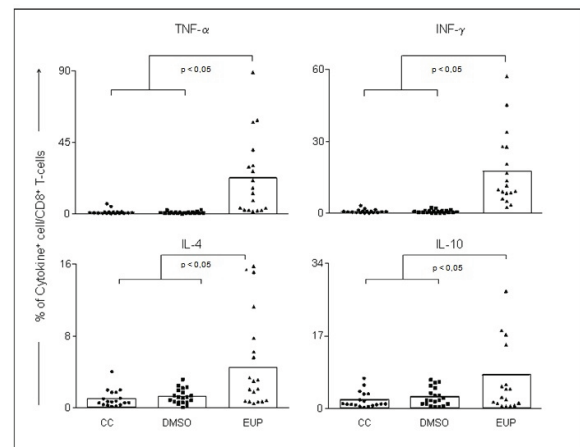


Figure 2. Intracellular TNF- α , INF- γ , IL-4 and IL-10 expression in peripheral blood CD8⁺ T lymphocytes after short-time stimulation *in vitro* with saline (CC), Dimethylsulfoxide (DMSO), and *E. tirucalli* latex (EUP). The results are expressed as mean percentage+SD. Significant differences at $p < 0.05$ are identified by bars between evaluated groups.

and destroy infected and tumor cells (Levy & Garcia-Sastre, 2001; Duffield, 2003; Snapper & Paul, 1987).

Euphorbia tirucalli extract diluted in saline was able to increase the survival of animals with ascitic tumors. Additionally, the hematopoiesis and spleen size of these animals returned to normal after treatment with the plant extract. However, this study neither conducted a cytokine analysis nor a detailed evaluation of the molecular mechanisms involved in the process (Valadares et al., 2006). In another report, treatment with plants from the *Euphorbia* genus changed the expression of multi-drug resistance (MDR) factors. These factors down-regulate the intracellular concentrations of drugs used in chemotherapy to improve tumoral resistance (Hohmann et al., 2002; Lage et al., 2009). It seems that some antitumor mechanisms are cytokine dependent primarily on IFN- γ (Stein & Walther, 1998). Ghiringhelli et al. (2009) demonstrated that animals injected with EL4 or EG7 tumor cells and deficient in IFN- γ production could not be effectively treated with chemotherapy, whereas the wild-type control group responded to treatment.

The increased percentages of CD4⁺TNF⁺ and CD8⁺TNF⁺ T cells support our hypothesis that bioactive compounds from *E. tirucalli* improve the type 1 immune response (Figures 1 and 2). TNF- α is a cytokine that activates improved phagocytosis mediated by macrophages and increases the expression of adhesion molecules on endothelial cells (Wajant, 2009; Apostolaki et al., 2010). Moreover, TNF- α may also be an effector molecule for the apoptosis of tumor or infected cells by binding to death domains that signal apoptotic events (Screaton & Xu, 2000).

Previous studies have demonstrated that plants

from the *Euphorbia* genus have biological activities that could be mediated by TNF- α . For example, a lectin extracted from *E. milii* latex induced leukocyte migration (Dias-Baruffi et al., 2000), and an active fraction of the *E. peplus* extract induced apoptosis in melanoma cells via TNF family receptor-dependent pathways (Gillespie et al., 2004).

Neutrophils presented an increase in IL-4, IFN- γ and TNF- α expression (Table 1). The increase of IL-4 expression by neutrophils, along with the increase of type 2 cytokine expression by CD8⁺ T lymphocytes, could modulate inflammatory responses. Type 2 cytokines inhibit the activation of inflammatory cells (Fiorentino et al., 1991). Monocytes did not contribute to the increase in cytokine production in either of the two profiles (Table 1).

Crude *E. tirucalli* latex extract is potentially useful in antitumor and antiviral treatments. Pre-clinical studies indicate that the plant has low toxicity in mice (Silva et al., 2007), and a considerable therapeutic index for antiviral activity (Betancur-Galvis et al., 2002). Our data suggest that the antitumor protection provided by *E. tirucalli* extract observed by Valadares et al. (2006) may be mediated by type 1 cytokine (TNF- α and INF- γ) expression in neutrophils and lymphocytes, particularly CD4⁺ T lymphocytes.

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