

Article

Evaluation of *Euphorbia tirucalli* L. immunomodulatory and Antitumor Activity

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ABSTRACT

Euphorbia tirucalli L. is commonly used to treat various pathologies, including cancer. The objective of this study was to evaluate the effect of a crude extract of *E. tirucalli* on the development of Ehrlich solid tumors and immune function. We prepared an extract by macerating the aerial parts of the plant with 80% ethanol (1:10 g/v) for 15 days. Its effects on tumor development were assessed in male Swiss mice (n=10). The mice were injected subcutaneously with 10⁶ tumor cells and then treated by gavage daily with the extract (33.33, 67, and 133.34 mg kg⁻¹) or saline (0.5% ethanol) for 30 days. The treatment had no toxic effect and did not reduce tumor growth. However, the weight of tumor mass was lowest in the mice treated with extract at 67 mg kg⁻¹. Immunomodulatory activity was evaluated in mice (n=8), with and without tumors, treated with the extract (67 mg kg⁻¹), under the same conditions as described above for 7 and 14 days. Lymphoproliferation, NK activity and IL-12, TNF- α , IFN- γ , and IL-10 levels did not differ significantly between the groups. However, IL-4 levels were reduced in the *E. tirucalli* treated groups at 7 and 14 days when compared to the controls. We concluded that the extract is not toxic and cannot inhibit Ehrlich solid tumor development. Its immunomodulatory activity involves its ability to modulate IL-4 levels.

Keywords: *Euphorbia tirucalli* L.; aveloz; Ehrlich tumor; immunomodulation.

RESUMO

Euphorbia tirucalli L. é empregado popularmente para o tratamento de diversas patologias, inclusive o câncer. O objetivo deste trabalho foi avaliar o efeito do extrato bruto da planta sobre o desenvolvimento do tumor sólido de Ehrlich (EHR) e sobre o sistema imunológico. O extrato foi preparado com as partes aéreas da planta maceradas com etanol 80% (1:10 g/v, 15 dias). A análise do desenvolvimento tumoral, em camundongos Swiss (n=10; machos) inoculados subcutaneamente com 10⁶ células EHR e tratados (via intragástrica, diariamente) com o extrato (33,33, 67 ou 133,34 mg kg⁻¹) ou solução salina (0,5% etanol) durante 30 dias, demonstrou que o tratamento não apresentou efeito tóxico e não reduziu o crescimento do tumor, sendo o extrato de 67 mg kg⁻¹ o que apresentou o menor valor da massa tumoral. A atividade imunomoduladora foi avaliada em camundongos swiss (n = 8; machos), portadores do tumor ou não, e tratados com 67 mg kg⁻¹ do extrato, nas mesmas condições, durante 7 e 14 dias. A linfoproliferação, atividade NK e a produção de IL-12, TNF- α , IFN- γ e IL-10 não apresentaram diferença significativa entre os grupos, mas a produção de IL-4 foi



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reduzida no grupo Aveloz e Aveloz + EHR, aos 7 e 14 dias, em comparação aos controles. Concluímos que o extrato não é tóxico, porém não foi capaz de inibir o desenvolvimento tumoral, e a sua ação imunomoduladora foi concentrada na modulação da IL-4.
Palavras-chave: *Euphorbia tirucalli* L.; aveloz; tumor de Ehrlich; imunomodulação.

1. Introduction

For thousands of years, medicinal plants have been used as alternative and complementary therapies for various diseases (Dutra et al. 2016). Pharmacological products extracted from medicinal plants are popular worldwide; more than 80% of the drugs currently used in modern medicine are compounds derived from natural products, including herbal medicines, microbes, and bioactive compounds (Dutra et al. 2016; Gahamanyi et al. 2021). Brazil has the largest biodiversity in the world, with over 45,000 plant species, which is 20%–22% of the total plant species (Santos et al. 2013; Dutra et al. 2016). Although natural products are an active area of research in Brazil, with more than 10,000 articles published between 2011 and 2013, few herbal medicines have been developed and approved by the Brazilian Regulatory Agency (*Agência Nacional de Vigilância Sanitária* [ANVISA]) (Dutra et al. 2016). There is great potential for Brazilian research in the bioprospecting sector (Dutra et al. 2016).

Euphorbia tirucalli L. (family Euphorbiaceae) is a shrub or small tree endemic to tropical areas with pencil-like branches from which the vernacular name “pencil tree” is derived (Duong et al. 2019). In Brazil, *E. tirucalli* is known as “aveloz” (Varricchio et al. 2008; Filho et al. 2013). Although *E. tirucalli* is native to Africa and America, it is also found in Brazil, mainly in the state of Amazonas, in the Northeast region, and the coast of São Paulo state (Silva et al. 2007; Khaleghian et al. 2010). *E. tirucalli* is used as an ornamental house plant (Shlamovitz et al. 2007; Ernst et al. 2015) and has medicinal properties against warts, cough, sexual impotence, hemorrhoids, epilepsy, cancer, syphilis, asthma, parasitosis, rheumatism, and earaches (Khaleghian et al. 2010; Duong et al. 2019).

Studies of different *E. tirucalli* extracts identified several bioactive compounds, including triterpenes, sterols, phenolic compounds, protease inhibitors, and lectins, to which its antibacterial, molluscicidal, antiherpetic, anti-mutagenic, anticarcinogenic, wound healing, antioxidant, and antifungal activities were attributed (Silva et al. 2007; Santos et al. 2013; Filho et al. 2013; Araújo et al. 2014; Oliveira et al. 2014; Palharini et al. 2017; Le et al. 2021; Milhm et al. 2022). Regarding its anticarcinogenic activity, Valadares et al. (2006) and Santos et al. (2016) observed that treatment with an ethanol extract could modulate the immunological response of animals with Ehrlich ascites tumors, reducing tumor growth and increasing survival. Myelosuppression, extramedullary hematopoiesis, and high levels of prostaglandin E₂, induced during the development of Ehrlich tumors, were reduced in treated animals, suggest its immune system regulating effect (Valadares et al. 2006). Ehrlich tumor is a female mouse breast adenocarcinoma that is widely used as an experimental model to study therapeutic drugs, as its development is similar to that of human tumors (Feitosa et al. 2021). In addition, Ehrlich tumor cells modulate the immune function of animals, escaping the immune response, making it an interesting model to study the immunopathology of tumors (Feitosa et al. 2021).

The objective of this study was to evaluate the effect of an ethanol extract from the aerial parts of *E. tirucalli* on the development of Ehrlich solid tumors and immune function. To this end, lymphoproliferative activity, cytokine production and natural cytotoxic activity of spleen cells were measured in mice. To the best of our knowledge, this is the first study to evaluate the immunomodulatory effects of *E. tirucalli* on Ehrlich solid tumors.



2. Experimental

The study was divided into two experimental phases. In the first phase, we evaluated the antitumor potential of an ethanol extract obtained from the aerial parts of *E. tirucalli* L. plants *in vivo*. Model mice (n=10) were generated by subcutaneous inoculation with 1×10^6 Ehrlich tumor cells. After 24 h, the mice were treated daily with three different doses of the extract (33.33, 67, and 133.34 mg kg⁻¹) or vehicle (buffered saline with 0.5% ethanol) by gavage for 30 days. After 30 days, the tumor masses were removed and weighed to assess tumor development. Based on the results of this experiment, we chose a dose of 67 mg kg⁻¹ to evaluate the immunomodulatory effects. In the second phase of the experiment, mice (n=8) with and without Ehrlich tumors were treated with *E. tirucalli* extract (67 mg kg⁻¹) or vehicle daily by gavage for 7 and 14 days. Then, the animals were sacrificed by cervical dislocation, and their immunological function was assessed by measuring the lymphoproliferative and cytotoxic activity of spleen cells, and *in vitro* cytokine production.

2.1. Animals

Male ~50-day-old non-isogenic Swiss mice were obtained from the Central Animal Facility of the Federal University of Mato Grosso, Cuiabá, MT. The mice were housed in polypropylene boxes with autoclaved shavings for bedding. Mice were provided feed (Nuvital, Purina, Campinas, São Paulo, Brazil) and water *ad libitum* and maintained under a 12 h light/dark cycle at 22°C ± 2°C and 10% ± 5% humidity. For the tumor experiments, mice were inoculated subcutaneously, in the right flank, with Ehrlich tumor cells or buffered saline. They were treated daily by intragastric gavage with 0.1 mL of extract or vehicle solution (buffered saline containing 0.5% ethanol) per animal. At 24 h after the last treatment, the mice were sacrificed by cervical displacement. All animal experiments were performed in accordance with international recommendations for animal management and experimental use. The experimental protocol was approved by the Ethics Committee on Animal Use (*Comitê de Ética no Uso de Animais*) of the Federal University of Mato Grosso (Protocol No. 23108.702149/13-0).

2.2. *Euphorbia tirucalli* L. extract preparation

The aveloz plant (*E. tirucalli* L.) was collected at “rua A1 19 Setor Industrial Norte, CEP: 78550-352, (S = 11° 51' 07.1", W = 055° 29' 55.0"), Sinop, MT”. The dry plant material was deposited in the Biological Collection of the Southern Amazon (*Acervo Biológico da Amazônia Meridional*) by the person in charge of the Monique Machiner herbarium (specimen number 6150). To prepare the extract, the aerial parts of the plant were dried in a forced circulation greenhouse (Ethik Technology model 400-7D; Vargem Grande Paulista, São Paulo, Brazil) at 40°C for 15 days. The dried plant material was crushed into a powder using a homemade blender (Philips-Walita, Amsterdam, The Netherlands). The dried crushed material was mixed in 80% ethanol at a ratio of 1:10 in Erlenmeyer flasks and incubated at room temperature for 15 days. The solvent was changed every 5 days. Then, the ethanol solution was filtered, evaporated (Fisatom model 558; Perdizes, São Paulo, Brazil) at 40°C and 60 rpm, and then incubated in a forced circulation greenhouse at 40°C for 10 days. Next, the obtained extract was stored in a desiccator for 2 days. Three aliquots were obtained by serial extraction. An initial weight of 6 grams was diluted with 70 mL of saline solution containing 0.5% ethanol. Samples were stored at -22°C until use.

2.3. Ehrlich tumor cell suspension preparation

Ehrlich tumor cells were provided by Rondon Tosta Ramalho, Ph.D. at the Federal University of Mato Grosso do Sul, Campo Grande, Brazil and inoculated intraperitoneally (ascitic form) into Swiss mice every 7 days. Tumor cell suspensions were prepared in sterile PBS and diluted to a final concentration of 1×10^7 viable



cells mL^{-1} . Mice were inoculated subcutaneously in the right flank (0.1 mL per animal). Cell viability, as assessed by the Trypan Blue dye exclusion method, was at least 70%.

2.4. Spleen cell suspension preparation

Spleen cell suspensions were obtained by teasing spleens on a sterile fine nylon screen in Roswell Park Memorial Institute (RPMI) 1640 medium (Cultilab, Campinas, SP, Brazil). Cell suspensions were centrifuged at 1,500 rpm for 10 min and resuspended in 1 mL of RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil).

2.5. Cytotoxicity measurement by Trypan Blue exclusion

The toxic effect of *E. tirucalli* extract was assessed by determining the viability of spleen cells and Ehrlich tumor cells. A spleen cell suspension was prepared from two male Swiss mice according to the protocol in section 2.4. One animal with an Ehrlich ascites tumor was used to prepare tumor cells. The cells (4×10^6 cells mL^{-1}) were seeded in triplicate (50 μL /well) in a 96-well flat bottomed microculture plate, and the effect of serial dilutions of the extract was tested (0.25 mg mL^{-1} to 1 mg mL^{-1}). The control group received 50 μL of complete medium. The plates were incubated for 24 h at 37°C under 5% CO_2 . After incubation, cell viability was assessed using the Trypan blue exclusion assay in a Neubauer chamber. Cell viability was calculated according to the following formula: viable cells (%) = [(number of viable cells)/(number of viable cells + number of dead cells)] \times 100.

2.6. Lymphocyte proliferation assay

Cell suspensions (prepared as described in section 2.4) containing 4×10^6 cells mL^{-1} were seeded in 96-well microculture plates (50 μL /well). Concanavalin A (ConA, 3.5 $\mu\text{g mL}^{-1}$; Sigma Aldrich) was added to each well, and the plates were cultured for 72 h at 37°C under 5% CO_2 . A colorimetric MTT-based assay was used for non-radioactive quantification of cell proliferation to evaluate the response of spleen cells to the mitogen (Cell Proliferation Kit I; Roche Diagnostics, Mannheim, Germany). The optical density at 630 nm was read in a Thermo plate TP-reader. The lymphoproliferative activity of spleen cells was calculated according to the following formula: % lymphoproliferation = $(\text{OD}_{\text{Con A}} - \text{OD}_{\text{basal}})/(\text{OD}_{\text{basal}}) \times 100$.

2.7. Yac.1 target cell suspension preparation

The Yac.1 mouse lymphoma cell line, an NK-sensitive tumor cell line, was provided by Rio de Janeiro Cell Bank. Aliquots of Yac.1 cells were cultured in complete medium at 37°C under 5% CO_2 for 7 days. The cells were centrifuged and resuspended in RPMI containing 1% FBS to a concentration of 1×10^6 cells mL^{-1} .

2.8. Assessment of cytotoxic activity

Cytotoxicity was evaluated using a non-radioactive colorimetric method based on the quantification of lactate dehydrogenase (LDH) activity (Cytotoxicity Detection Kit; Roche Diagnostics, Mannheim, Germany). Mononuclear cells were obtained by centrifuging a suspension of spleen cells on a Ficoll-Hypaque gradient (Sigma-Aldrich, St. Louis, MO, USA), and incubation in glass Petri dishes for 90 min at 37°C to remove adherent cells. Non-adherent cells were gently recovered from the dishes and resuspended in RPMI supplemented with 1% FBS, and the cell density was adjusted to 1×10^7 cell mL^{-1} . Next, 100 μL of the non-adherent cell suspension (effectors) was seeded into a U-bottomed 96-well microtiter plate. Each well contained 100 μL of the target cell suspension (Yac.1) at a concentration of 1×10^6 cell mL^{-1} (effector to target ratio 50:1). Target cells were lysed by adding 100 μL of Triton X solution (Sigma-Aldrich, St. Louis, MO, USA).



Spontaneous lysis of Yac.1 cells was assessed by incubating cells with RPMI supplemented with 1% FBS. A background control was prepared with RPMI supplemented with 1% FBS but without cells. After 4 h of incubation at 37°C under 5% CO₂, the plate was centrifuged for 10 min at 1500 rpm. A 50 µL aliquot of the supernatant was carefully removed from each well and transferred into a 96-well flat-bottomed microtiter plate (Nunc A/S; Roskilde, Denmark), and LDH activity was quantified by adding 50 µL of a Diaphorase/NAD⁺ mixture and a dye solution containing iodotetrazolium chloride. Sodium lactate was added to each well and incubated for 30 min at room temperature in the dark. The absorbance was read at 492 nm with a microplate reader (Thermo plate TP-reader). Cytotoxicity was determined as the percentage of cell lysis and was calculated according to the following formula: cytotoxicity (%) = [(absorbance of target and effector cells – absorbance of effector cells) – absorbance of spontaneous lysis control]/(absorbance of maximal lysis control – absorbance of spontaneous lysis control) × 100. Data are presented as means ± standard deviation. Before calculating cytotoxicity, the mean of the background control was subtracted from all absorbance values.

2.9. Cytokine measurement in culture supernatant

Cytokines in culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA; eBioscience, San Diego, CA, USA) according to the manufacturer's guidelines. IL-4, IL-10 and IFN-γ levels were measured in the supernatant of cell cultures stimulated with ConA (3.5 µg mL⁻¹) for 24 h. TNF-α and IL-12 were measured in cells stimulated with *Staphylococcus aureus* formalized cells (SAC; 1:5,000) for 48 h.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad InStat software (San Diego, CA, USA). One-way analysis of variance (ANOVA) and Tukey-Kramer tests were applied. Differences were considered significant when the p value was less than 5% (p<0.05).

3. Results and Discussion

3.1. Evaluation of *in vitro* cytotoxicity

We first evaluated the *in vitro* cytotoxic effect of *E. tirucalli* extract on normal and tumor cells using the Trypan blue exclusion assay. The cell viability assessment showed that, at the tested concentrations, the extract had no direct toxic effect on Ehrlich tumor cells (Control: 87.24 ± 10.22, 1 mg mL⁻¹ extract: 70.04 ± 15.70, 0.5 mg mL⁻¹ extract: 88.59 ± 8.21, and 0.25 mg mL⁻¹ extract: 83.58 ± 9.65) or total spleen cells (Control: 82.49 ± 6.39, 1 mg mL⁻¹: 73.01 ± 10.02, 0.5 mg mL⁻¹: 70.7 ± 10.27, and 0.25 mg mL⁻¹: 67.59 ± 9.29) (Figure 1).

Based on the number of living cells, we observed that extract treatment significantly reduced the number of tumor cells (Control: 18.67 ± 8.24, 0.25 mg mL⁻¹: 10.00 ± 3.85) and spleen cells (Control: 42.67 ± 8.24, 0.5 mg mL⁻¹: 26.50 ± 11.06; Figure 1). However, quantification of the number of dead tumor cells (Control: 2.50 ± 2.07, 1 mg mL⁻¹: 4.33 ± 1.97, 0.5 mg mL⁻¹: 1.50 ± 1.05, and 0.25 mg mL⁻¹: 2.17 ± 1.33) and spleen cells (Control: 9.00 ± 3.52, 1 mg mL⁻¹: 9.33 ± 2.66, 0.5 mg mL⁻¹: 10.83 ± 4.12, and 0.25 mg mL⁻¹: 14.00 ± 4.52) indicated that the treatment did not have a significant effect when compared to the control group (Figure 1). As the extract had no direct toxic effect on cells, we then evaluated the effect on tumor development.

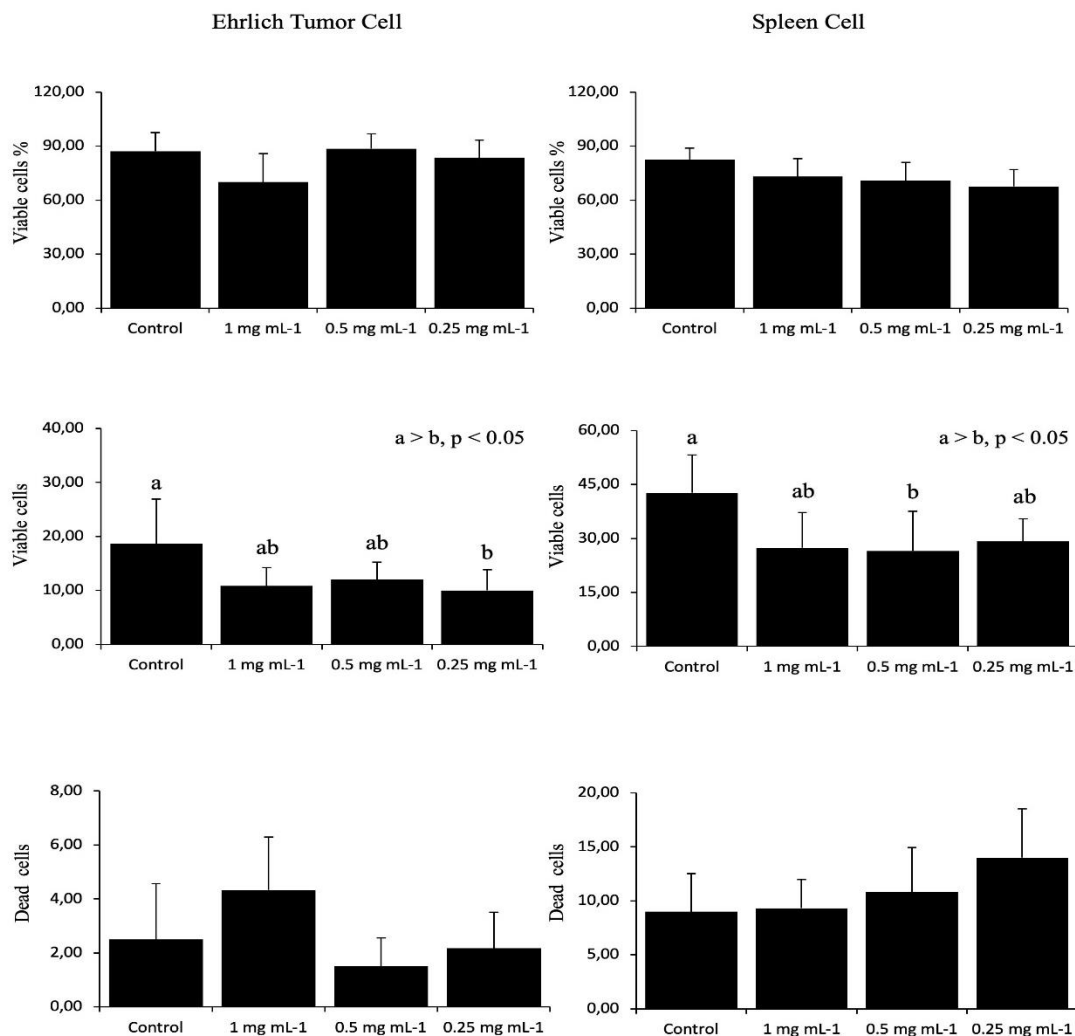


Figure 1. *In vitro* viability assessment of cells treated with different concentrations of *Euphorbia tirucalli* L. ethanolic extract for 24 h. Cell viability was determined by Trypan blue exclusion assay in triplicate. The control consisted of RPMI + 20% FBS. Values are the mean percentage \pm standard deviation or mean absolute number \pm standard deviation. Letters indicate comparisons of the treated groups to the control.

3.2. Evaluation of the effect of *E. tirucalli* extract on Ehrlich tumor development

In the first phase of the experiment, we evaluated the development of Ehrlich subcutaneous tumors in model mice treated with different doses of *E. tirucalli* extract for 30 days. The results, presented in Figure 2, showed that the extract did not significantly alter tumor development when compared to the untreated control group (Control: 0.93 ± 0.72 , 33.33 mg kg^{-1} : 1.58 ± 1.04 , 67 mg kg^{-1} : 1.29 ± 1.08 , and $133.34 \text{ mg kg}^{-1}$: 1.40 ± 0.87). *E. tirucalli* extract also did not affect the body weight of the animals at the end of the experiment (Control: 43.58 ± 3.02 , 33.33 mg kg^{-1} : 43.12 ± 3.55 , 67 mg kg^{-1} : 44.68 ± 5.95 , and $133.34 \text{ mg kg}^{-1}$: 44.95 ± 2.68), the absolute weight (Control: 0.38 ± 0.11 , 33.33 mg kg^{-1} : 0.54 ± 0.22 , 67 mg kg^{-1} : 0.52 ± 0.31 , $133.34 \text{ mg kg}^{-1}$: 0.48 ± 0.18) or the relative weight of the spleen (Control: 0.86 ± 0.41 , 33.33 mg kg^{-1} : 1.07 ± 0.41 , 67 mg kg^{-1} : 1.21 ± 0.91 , $133.34 \text{ mg kg}^{-1}$: 1.25 ± 0.50). These results demonstrate that *E. tirucalli* extract is not toxic to animals, as it did not alter cell viability (Figure 1) or animal weight (Figure 2). However, it did not inhibit tumor development (Figure 2).



In previous studies, extracts obtained from the latex or aerial parts of *E. tirucalli* did not induce systemic toxic effects in treated animals (Filho et al. 2013; Brunetti et al. 2019; Martins et al. 2020). Developmental toxicity studies conducted in rats also showed no toxicity (Silva et al. 2007). Oliveira et al. (2014) did not observe differences in the viability of human leukocytes treated with an aqueous extract and latex preparation from *E. tirucalli* L. compared to the negative control. *In vitro* experiments using a murine macrophage cell line (RAW 264.7) yielded similar results (Souza et al. 2019).

Effects on tumor cells likely depend on the cell type, extract concentration, and exposure time (Choene and Motadi 2016). Paz et al. (2020) assessed the cytotoxic effects of diluted *E. tirucalli* latex on human melanoma cells after 48 and 72 h of treatment. Like in our study, treatment with 1 mg mL⁻¹, 0.5 mg mL⁻¹, and 0.25 mg mL⁻¹ for 24 h did not affect tumor cell viability (Paz et al. 2020). Studies conducted in murine melanoma cells (B16F10) also showed the absence of an inhibitory effect of *E. tirucalli* latex after 24, 48, and 72 h of exposure (Brunetti et al. 2019). In contrast, *E. tirucalli* extracts effectively inhibited the growth of two breast cancer cell lines (MCF-7 and MDA-MB 231), inducing cell cycle arrest in G0/G1 phase by promoting p21 overexpression (Choene and Motadi 2016). *E. tirucalli* latex also had a cytotoxic effect on gastric adenocarcinoma tumor cells (Souza et al. 2019) and Hep-2 larynx squamous cell carcinoma cells (Franco-Salla et al. 2016).

Some products derived from the *E. tirucalli* plant can be toxic (Sumba et al. 2003; Shlamovitz et al. 2007; Mannucci et al. 2012; Machado et al. 2016). Direct eye contact with latex has been reported to cause ocular toxicity, ranging from keratoconjunctivitis to blindness (Shlamovitz et al. 2007). Similarly, cardiac dysrhythmias, pulmonary edema, seizures, and death due to accidental ingestion has also been described (Shlamovitz et al. 2007). *E. tirucalli* L. contains a 4-deoxyphorbol ester that is closely related to the tumor promoting substance 12-O-tetra-decanoylphorbol-13-acetate (TPA) (Mannucci et al. 2012). Some studies suggest that *E. tirucalli* is genotoxic (Machado et al. 2016) and is cofactor for Burkitt's lymphoma, which is endemic in Africa, by reactivating the lytic cycle of Epstein-Barr virus and inducing genetic alterations, leading to overexpression of the MYC oncogene and resulting in polysomies and chromosomal translocations, which contribute to malignant transformation (MacNeil et al. 2003; Mannucci et al. 2012). These characteristics could explain the observed effects on the development of Ehrlich tumors.

Another important observation is that although *E. tirucalli* extract did not alter cell viability or the absolute number of dead cells, it did reduce the absolute number of viable cells (at 0.5 mg mL⁻¹ for spleen cells and 0.25 mg mL⁻¹ for Ehrlich tumor cells). Trypan Blue dye does not stain living cells with intact cell membranes (Adan et al. 2016). However, dead cells with damaged membranes can absorb the dye, which turns them blue (Adan et al. 2016). The Trypan Blue dye exclusion method cannot distinguish necrosis from apoptosis (Strober, 2015; Adan et al., 2016). Thus, the reduction in the absolute number of living cells could reflect irreversible changes in cell function that result in cell death (Elia et al. 1993; Adan et al. 2016). These changes include autophagy (Silva et al. 2018), induction of apoptosis by caspase-3 activation and cell cycle regulatory proteins, such as p27^{kip1} and proteins involved in ERK1/2-pathway, as previously described for *E. tirucalli* (Li et al. 2012; Wang et al. 2013).

It is possible that, at the doses used in this study, *E. tirucalli* extract was not toxic but altered cell functions that contribute to tumor development. Thus, we selected 67 mg mL⁻¹ as the dose for the second phase of the study since this was the intermediate dose and it resulted in a reduction in average tumor weight in the first phase of the study (Figure 2).

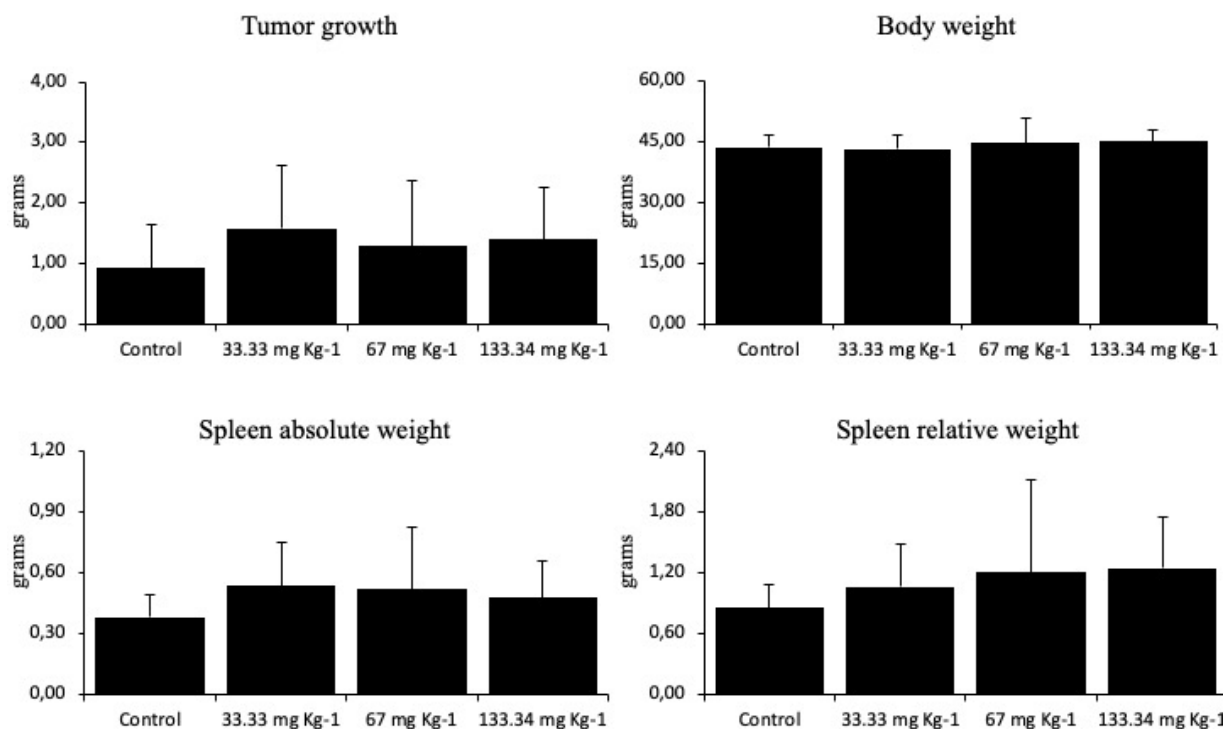


Figure 2. Evaluation of tumor development in mice treated with different doses of *Euphorbia tirucalli* L. ethanolic extract by gavage daily for 30 days. Values are the mean weight (g) ± standard deviation.

3.3. Lymphoproliferative activity evaluation

Figure 3 shows spleen cell lymphoproliferation in response to the mitogen ConA. *E. tirucalli* extract increased lymphoproliferation when compared to the tumor (EHR) group at 7 days (EHR: 36.376 ± 23.162 , *E. tirucalli*: 94.028 ± 29.687). However, no effect was observed compared to the control (Control: 60.897 ± 23.348). No significant differences were observed between experimental groups after 14 days of treatment (Control: 112.202 ± 62.9 , EHR: 153.143 ± 68.026 , Aveloz: 163.502 ± 98.627 , EHR + Aveloz: 151.061 ± 38.017).

The mitogenic effect of *E. tirucalli* has been described (Llanes-Coronel et al. 2011). The plant increased the proliferation of peripheral blood mononuclear cells, mainly CD3⁺ cells, without accessory cells (Llanes-Coronel et al. 2011). In addition, an aqueous solution of *E. tirucalli* latex promoted neo-angiogenesis when applied to the chorioallantoic membranes of fertilized chicken eggs (Bessa et al. 2015).

Although in our study, *E. tirucalli* treatment did not result a significant difference in lymphocyte proliferation compared to the control, our findings corroborate those of Lin et al. (2012), who showed that compounds present in *E. tirucalli* extract can modulate the cell cycle and regulatory proteins, which could promote cell proliferation or apoptosis. The proliferation-inducing compound in the *E. tirucalli* extract could be a phorbol ester, which can elicit inflammation and trigger NF- κ B expression (Machado et al. 2016). The intensity of the proliferative response seems to be related to an apoptosis-inducing mechanism through activation of death receptors (activation-induced cell death) (Llanes-Coronel et al. 2011; Choene and Motadi 2016). This might explain both the reduction in the number of viable cells in the cytotoxicity assay and the increased lymphoproliferation.

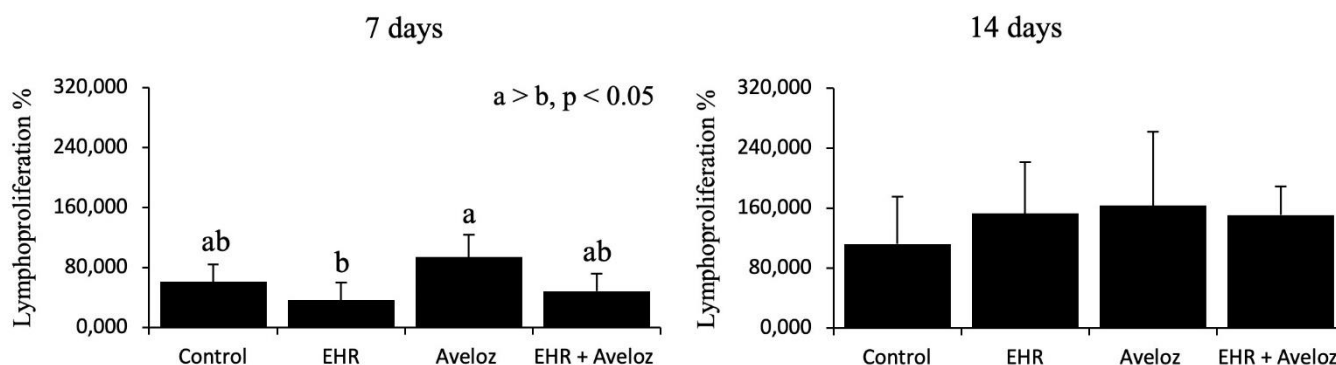


Figure 3. Lymphoproliferative activity of total spleen cells from mice treated with 67 mg kg⁻¹ of *Euphorbia tirucalli* L. ethanolic extract daily by gavage for 7 and 14 days. Values are the mean \pm standard deviation.

3.4. Cytotoxic activity evaluation

Cytotoxic activity was measured by a LDH colorimetric assay using Yac.1 cells as target cells. Yac.1 cells are derived from a mouse with T-cell lymphoma (Sarker and Zhong 2014; Li et al. 2019). The cell line was obtained by inoculating Moloney leukemia virus into a newborn A/Sn mouse and is susceptible to lysis by NK cells (Sarker and Zhong 2014; Li et al. 2019). Thus, it is widely used to detect the cytotoxic activity of mouse NK cells (Sarker and Zhong 2014; Li et al. 2019). The spleen is the largest lymphoid organ with the highest lymphocyte throughput (Sojka et al. 2014; Liezmann et al. 2012). It is the organ where cell pooling and unnecessary cell removal occur, and it has regulatory effects on a wide variety of immune cells, including NK cells (Sojka et al. 2014; Liezmann et al. 2012). Thus, the cytotoxic activity detected in this experiment is likely related to NK cells, since the animals had not been previously stimulated with Yac.1 cells.

The results of the cytotoxic activity assay showed that *E. tirucalli* treatment did not significantly alter the response of NK cells after 7 days (Control: 14.81 \pm 16.63, EHR: 28.16 \pm 16.62, Aveloz: 18.52 \pm 4.24, EHR + Aveloz: 10.34 \pm 11.68) or 14 days of exposure (Control: 2.48 \pm 1.40, EHR: 18.71 \pm 13.19, Aveloz: 11.52 \pm 6.32, EHR + Aveloz: 14.93 \pm 6.50) (Figure 4).

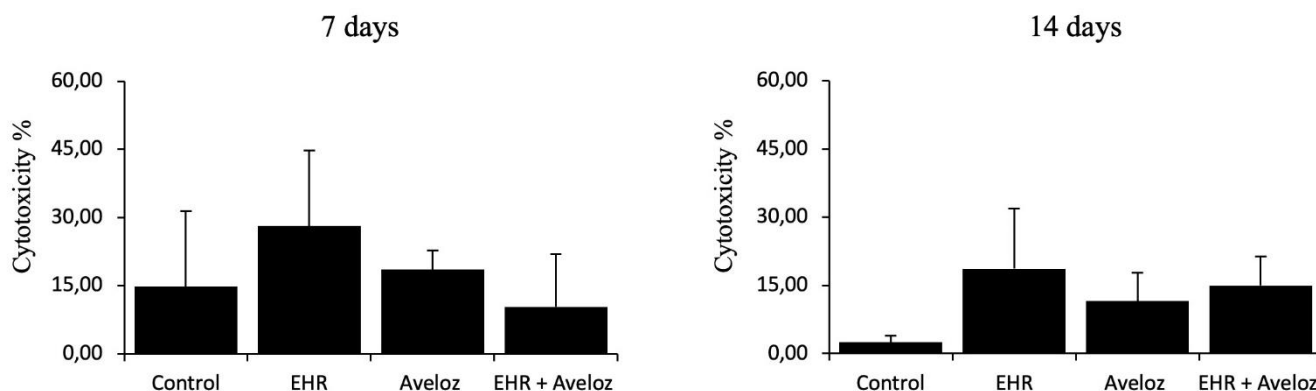


Figure 4. Natural cytotoxic activity of non-adherent spleen mononuclear cells from Swiss mice (n=8) treated with *Euphorbia tirucalli* L. ethanolic extract by gavage daily for 7 and 14 days. Control animals were treated with buffered saline containing 0.5% ethanol. The results are expressed as a percentage of the mean (referred to different groups) \pm standard deviation. Animals carrying EHR tumors were inoculated subcutaneously with 1×10^6 Ehrlich tumor cells. Yac.1 cells were used as target cells at a 1:50 ratio.



3.5. Cytokine determination in cell culture supernatant

Cytokine production was evaluated *in vitro* by collecting the supernatant from a total splenocyte cell culture after stimulation with ConA (24 h) or SAC (48 h). The results are shown in Figures 5 and 6, and the values are expressed in pg mL⁻¹. Our findings showed significantly higher levels of IL-12 and IFN- γ in stimulated cell cultures. However, there was no difference in production capacity among experimental groups under basal and stimulated conditions. TNF- α (Figure 5) and IL-10 (data not shown) did not differ significantly among the studied groups. Basal IL-4 production by splenocytes in the control group (68.240 ± 16.529) was quite high after 7 days of treatment and differed significantly from the levels in the Aveloz (20.793 ± 21.572), EHR (13.014 ± 0.629) and EHR + Aveloz groups (12.940 ± 0.363). At 14 days, the IL-4 level in EHR + Aveloz group stimulated with mitogen (21.685 ± 7.776) were lower than the level in the control groups (Control + ConA: 55.572 ± 30.623 , Aveloz + ConA: 47.499 ± 18.040). Similar to IL-12 and IFN- γ , IL-4 production by stimulated splenocytes differed from basal cell production at 7 days and 14 days (Figure 6).

IL-4 is one of the main cytokines involved in the differentiation of Type 2 T-Helper (Th2) lymphocytes, which are characteristic of a specific humoral immune response (Mirlekar 2022). IL-4 is released by basophils, mastocytes, and Th2 itself (Mirlekar 2022). IL-4 plays a different role in the tumor microenvironment, where it functions as a regulator of tumor cell survival, proliferation, and migration (Mirlekar 2022). It also has an immunosuppressive effect, as it reduces the cytotoxic activity of T lymphocytes (Tc) and NK cells and antitumor activities (Mirlekar 2022).

IL-4-induced tumor rejection is thought to occur via a biphasic mechanism (Li et al. 2009). In the first phase, IL-4 induces the accumulation of myeloid precursor cells, promoting their differentiation to granulocytes, which directly inhibit tumor cell expansion (Li et al. 2009). In the second phase, Tc cells induce complete tumor rejection (Li et al. 2009). IL-4 also inhibits tumor-induced angiogenesis (Li et al. 2009). Thus, the high levels of IL-4 in phase 1 activate NK and NKT cells through a Stat-6-dependent and Stat4- and IL-12-independent mechanism (Morris et al. 2006). This induces the production of IFN- γ , which, in sufficient quantities, stimulates the differentiation of Th1 antitumor cells and restrains the Th2 response elicited by IL-4 (phase 2) (Morris et al. 2006). In addition, IL-4 can act synergistically with IL-12, triggering the production of IFN- γ and TNF- α by NK cells (Naume et al. 1993).

In this study, we observed that *E. tirucalli* extract reduced IL-4 production (in the Aveloz and EHR + Aveloz groups) after 7 and 14 days of treatment. However, no differences were found in TNF- α , IL-12, IL-10, and IFN- γ levels. Although there was no statistical difference in tumor weight, exposure to the extract appeared to favor the development of Ehrlich tumors. This effect may be a consequence of unbalanced IL-4 production, which may interfere with the induction of an antitumor immune response, as demonstrated by the absence of NK activity. Ibrahim et al. (2019) observed the same response profile in rats treated with an extract from the aerial parts of *E. tirucalli*, i.e., increased production of IL-4 and TNF- α and IFN- γ levels similar to those of the controls.

Our results corroborate previously published data, indicating the complex effects of the compounds present in the latex and aerial parts of *E. tirucalli*, which have both pro- and anti-tumor activities. Another important aspect to consider is the seasonal and geographical variation in the chemical composition of the extracts and the effects of the separate compounds in relation to the combined effect of all the substances present in the crude extract (Silva et al. 2011; Paz et al. 2020).

Recently, a diterpene isolated from *Euphorbia peplus* L called Picato[®] (ingenol mebutate) was approved by the US Food and Drug Administration for the topical treatment of actinic keratosis, a common disease mainly affecting the elderly caused by cumulative ultraviolet exposure that can lead to invasive squamous cell carcinoma



(Berman 2012; Ernst et al. 2015). Thus, conducting further studies to characterize the compounds in *E. tirucalli* extract, identify their biological effects and mechanisms, and better understand their effects on tumor and non-tumor cells may yield more medically useful compounds.

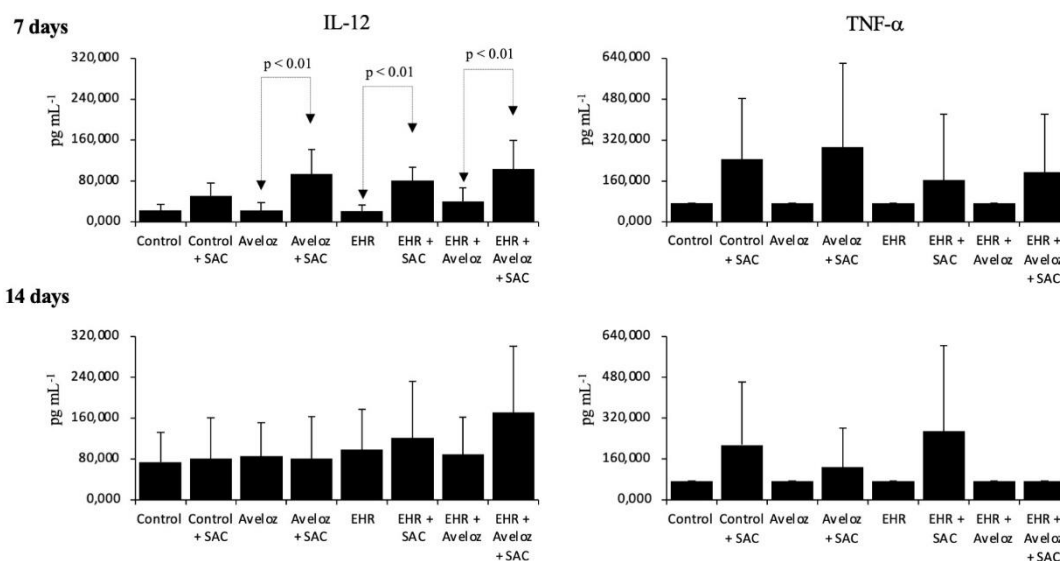


Figure 5. Measurement of IL-12 and TNF- α levels in the supernatant of a culture of total splenocytes from mice treated with 67 mg kg⁻¹ *Euphorbia tirucalli* L. ethanolic extract by gavage daily for 7 and 14 days. Values are the mean \pm standard deviation.

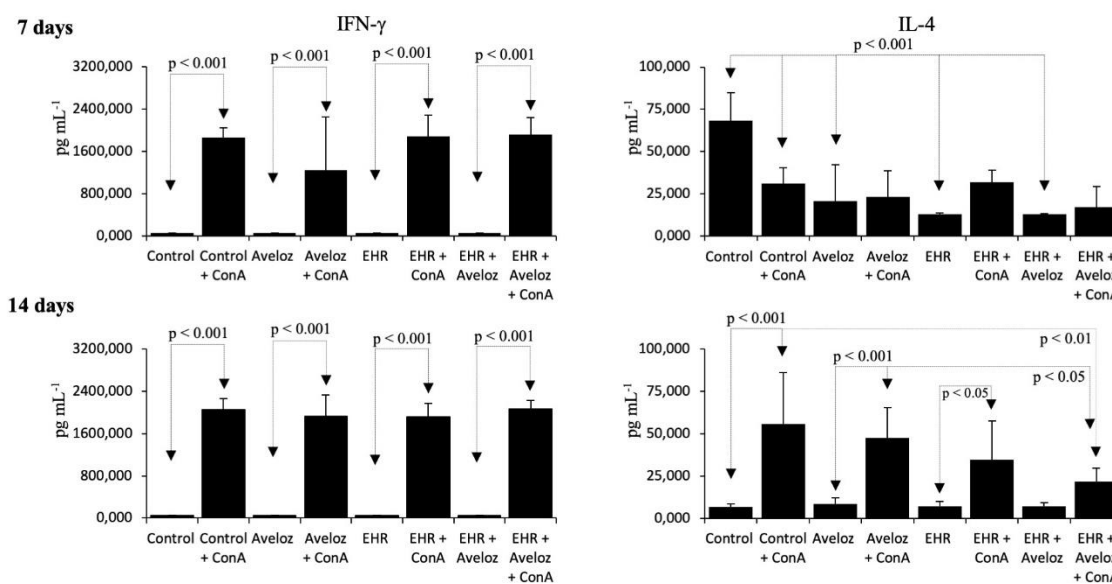


Figure 6. Measurement of IFN- γ and IL-4 production in the supernatant of a culture of total splenocytes from mice treated with 67 mg kg⁻¹ of *Euphorbia tirucalli* L. (*tirucalli*) ethanolic extract by gavage daily for 7 and 14 days. The values are the mean \pm standard deviation.



4. Conclusions

This study demonstrated that an ethanolic extract of the aerial parts of *E. tirucalli* L. does not have toxic effects *in vitro* and *in vivo*, as it did not alter the viability of mouse spleen cells and Ehrlich tumor cells in culture, or the body weight and spleen weight of mice treated for 30 days. The extract did not inhibit the development of Ehrlich subcutaneous tumors after 30 days of treatment. The only detected immune system-related effect was a reduction in IL-4 production.

Declaration of Interest statement

The authors state that there are no conflict of interests to declare.

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References

- Araújo KM, Lima A, Silva JN, Rodrigues LL, Amorim AGN, Quelemes PV, Santos RC, Rocha JÁ, Andrades EO, Leite JRSA, Mancini-Filho J, Trindade RA. 2014. Identification of phenolic compounds and evaluation of antioxidant and antimicrobial properties of *Euphorbia tirucalli* L. *Antioxidants*. 3: 159-175.
- Berman B. 2012. New developments in the treatment of actinic keratosis: focus on ingenol mebutate gel. *Clinical, Cosmetic and Investigational Dermatology*. 5: 111–122.
- Bessa G, Melo-Reis PR, Araújo LA, Mrué F, Freitas GB, Brandão ML, Silva Júnio NJ. 2015. Angiogenic activity of látex from *Euphorbia tirucalli* Linnaeus 1753 (Plantae, Euphorbiaceae). *Brazilian Journal of Biology*. 75: 752-758.
- Brunetti RL, Paz DPA, Fonseca IIM, Nagamine MK, Mori CMC, Grande MPD, Queiroz-Hazarbassanov N, Salles-Gomes COM, Dagli MLZ, Hernandez-Blázquez FJ. 2019. Inhibitory effects of *Euphorbia tirucalli* látex on murine B16/F10 melanoma cells and lung metastasis. *Molecular and Clinical Oncology*. 11: 511-516.
- Choene M, Motadi L. 2016. Validation of the antiproliferative effects of *Euphorbia tirucalli* extracts in breast cancer cell lines. *Molecular cell Biology*. 50: 115-128.
- Duong TH, Beniddir MA, Genta-Jouve G, Nguyen HH, Nguyen DP, Nguyen TAT, Mac DH, Boustie J, Nguyen KPP, Chavasiri W, Pogan PL. *Fitoterapia*. 135: 44-51.
- Dutra RC, Campos MM, Santos ARS, Calixto JB. 2016. Medicinal plants in Brazil: pharmacological studies, drug discovery, challenges and perspectives. *Pharmacological Research*. 112: 4-29.
- Ernst M, Grace OM, Saslis-Lagoudalis CH, Nilsson N, Simonsen HT, Ronsted N. 2015. *Journal of Ethnopharmacology*. 176: 90-101.



- Feitosa IB, Mori B, Teles CBG, Costa AG. 2021. What are the immune responses during the growth of Ehrlich's tumor in ascitic and solid form? *Life Sciences*. 264: 118578.
- Filho ENS, Santos OJ, Filho AKDB, Rocha AA, Silva RC, Santos RHP, Santos RAP. 2013. Evaluation of the use of raw extract of *Euphorbia tirucalli* L. in the healing process of skin wounds in mice. *Acta Cirúrgica Brasileira*. 28: 716-720.
- Franco-Salla GB, Prates J, Cardin LT, Santos ARD, Silva Jr WA, Cunha BR, Tajara EH, Oliani SM, Rodrigues-Lisoni FC. 2016. *Euphorbia tirucalli* modulates gene expression. In larynx squamous cell carcinoma. *BMC Complementary and Alternative Medicine*. 16: 136.
- Gahamanyi N, Munyaneza E, Dukuzimana E, Tuyiringire N, Pan CH, Komba EVG. 2021. Ethnobotany, ethnopharmacology, and phytochemistry of medicinal plants used for treating human diarrheal cases in Rwanda: a review. *Antibiotics*. 10: 1231.
- Ibrahim EH, Kilany M, Mostafa OMS, Shaker KH, Alshehri M, Alsyaad KM, Alshehri A, Khan KA, Qasim M, Kotb N, Alahmari AS, Ghramh HA, Dajem SM. 2019. TH1/TH2 chemokines/cytokines profile in rats treated with tetanus toxoid and *Euphorbia tirucalli*. *Saudi Journal of Biological Sciences*. 26: 1716-1723.
- Khaleghian A, Riazi GH, Ghafari M, Rezaie M, Takahashi A, Nakaya Y, Nazari H. 2010. Effects of inganen anticancer properties on microtubule organization. *Pakistan Journal of Pharmaceutical Sciences*. 23(3): 273-278.
- Le NTM, Cuong DX, Thinh PV, Minh TN, Manh TD, Duong TH, Minh TTL, Oanh VTT. 2021. Phytochemical screening and evaluation of antioxidant properties and antimicrobial activity against *Xanthomonas axonopodis* of *Euphorbia tirucalli* extracts in Binh Thuan Province, Vietnam. *Molecules*. 26: 941.
- Li Z, Wu Y, Wang C, Zhang M. 2019. Mouse CD8+NKTlike cells exert dual cytotoxicity against mouse tumor cells and myeloid-derived suppressor cells. *Cancer Immunology, Immunotherapy*. 68: 1303-1315.
- Liezmann C, Stock A, Peters EMJ. 2012. Stress induced neuroendocrine-immune plasticity. *Dermato-Endocrinology*. 4: 271-279.
- Lin MW, Lin AS, Wu DC, Wang SSW, Chang FR, Wu YC, Huang YB. 2012. Euphol from *Euphorbia tirucalli* selectively inhibits human gastric cancer cell growth through the induction of ERK1/2-mediated apoptosis. *Food and Chemical Toxicology*. 50: 4333-4339.
- Lin Z, Chen L, Qin Z. 2009. Paradoxical roles of IL-4 in tumor immunity. *Cellular & Molecular Immunology*. 6(6):415-422.
- Llanes-Coronel DS, Gámez-Díaz LY, Suarez-Quintero LP, Páez LJ, Torres F, Echeverri F, Ponte-Sucre A, Patiño PJ, Trujillo-Vargas CM. 2011. New promising Euphorbiaceae extracts with activity in human lymphocytes from primary cell cultures. *Immunopharmacology and Immunotoxicology*. 33(2): 279-290.
- Machado MM, Oliveira LFS, Zuravski L, Souza R, Fischer P, Duarte JÁ, Rocha MO, Güez CM, Boligon AA, Athayde ML. 2016. Evaluation of genotoxic and cytotoxic effects of hydroalcoholic extract of *Euphorbia tirucalli* (Euphorbiaceae) in cell cultures of human leukocytes. *Annals of the Brazilian Academy of Sciences*. 88: 17-28.



- MacNeil A, Sumba OP, Lutzke ML, Moormann A, Rochford R. 2003. Activation of the Epstein-Barr virus lytic cycle by the latex of the plant *Euphorbia tirucalli*. *British Journal of Cancer*. 88: 1566-1569.
- Mannucci S, Luzzi A, Carugi A, Gozzetti A, Lazzi S, Malagnino V, Simmonds M, Cusi MG, Leoncini L, Bosch CAVD, Falco GD. 2012. EBV reactivation and chromosomal polysomies: *Euphorbia tirucalli* as a possible cofactor in endemic Burkitt lymphoma. *Advances in Hematology*. 2012: 11 pages.
- Martins CG, Appel MH, Coutinho DSS, Soares IP, Fischer S, Oliveira BC, Fachi MM, Pontarolo R, Bonatto SJR, Fernandes LC, Iagher F, Souza LM. 2020. Consumption of látex from *Euphorbia tirucalli* L. promotes a reduction of tumor growth and cachexia, and immunomodulation in Walker 256 tumor-bearing rats. *Journal of Ethnopharmacology*. 255: 112722.
- Milhm ACP, Bonet LFS, Aiub CAF, Junior CLS. 2022. Biochemical characterization and phytotoxic activity of protein extract from *Euphorbia tirucalli* L. *Journal of Ethnopharmacology*. 285: 114903.
- Mirlekar B. 2022. Tumor promoting roles of IL-10, TGF- β , IL-4, and IL-35: its implications in cancer immunotherapy. *SAGE Open Medicine*. 10: 1-15.
- Morris SC, Orekhova T, Meadows MJ, Heidorn SM, Yang J, Finkelman FD. 2006. IL-4 induces in vivo production of IFN- γ by NK and NKT cells. *The Journal of Immunology*. 176: 5299-5305.
- Naume B, Gately MK, Desai BB, Sundan A, Espevik T. 1993. Synergistic effects of interleukin 4 and interleukin 12 on NK cell proliferation. *Cytokine*. 1: 38-46.
- Oliveira LFS, Fuentefria AM, Klein FS, Machado MM. 2014. *Brazilian Journal of Microbiology*. 45: 1349-1355.
- Palharini JG, Richter AC, Silva MF, Ferreira FB, Pirovani CP, Naves KSC, Goulart VA, Mineo TWP, Silva MJB, Santiago FM. 2017. Eutirucallin: a lectin with antitumor and antimicrobial properties. *Frontiers in Cellular and Infection Microbiology*. 7: 9 pages.
- Paz DPA, Nagamine MK, Grande MPD, Leite JVP, Sobreira FMG, Bacchi EM, Dagli MLZ. 2020. Inhibitory effects of *Euphorbia tirucalli* Lineu (Euphorbiaceae) diluted látex on human and canine melanoma cells. *Evidence-based Complementary and Alternative Medicine*. 4093206, 5 pages.
- Santos OJ, Carvalho-Júnior FF, Sauaia-Filho EN, Santos RHP, Santos RAP, Barbalho WG. 2013. Gastric healing process with raw extract of *Euphorbia tirucalli* L.: study in rats. *ABCD Arquivos Brasileiro Cirurgia Digestiva*. 26(4): 256-259.
- Santos OJ, Filho ENS, Nascimento FRF, Junior FCS, Fialho SEM, Santos RHP, Santos RAP, Serra ICPB. 2016. Use of raw *Euphorbia tirucalli* extract for inhibition of ascitic Ehrlich tumor. *Revista Colégio Brasileiro Cirurgia*. 43: 18-21.
- Sarker MM, Zhong M. 2014. Keyhole limpet hemocyanin augmented the killing activity, cytokine production and proliferation of NK cells, and inhibited the proliferation of Meth A sarcoma cells in vitro. *Indian Journal of Pharmacology*. (46): 40-45.



- Shlamovitz GZ, Gupta M, Diaz JA. 2009. A case of acute keratoconjunctives from exposure to latex of *Euphorbia tirucalli* (pencil cactus). *The Journal of Emergency Medicine*. 36: 239-241.
- Silva ACP, Faria DEP, Borges NBES, Souza IA, Peters VM, Guerra MO. 2007. Toxicological screening of *Euphorbia tirucalli* L.: developmental toxicity studies in rats. *Journal of Ethnopharmacology*. 110: 154-159.
- Silva RMRJ, Teixeira DF, Sampaio ALF, Leitão TCA. 2011. Analysis of in vivo activity of high dilutions of *Euphorbia tirucalli* L. in human melanoma cells. *International Journal of High Dilution Research*. 10(36): 183-193.
- Silva VAO, Rosa MN, Miranda-Gonçalves V, Costa AM, Tansini A, Evangelista AF, Martinho O, Carloni AC, Jones C, Lima JP, Pianowski LF, Reis RM. 2018. Euphol, a tetracyclic triterpene, from *Euphorbia tirucalli* induces autophagy and sensitizes temozolomide cytotoxicity on glioblastoma cells. *Investigational New Drugs*. 15 pages.
- Sojka DK, Plougastel-Douglas B, Yang L, Paqk-Wittel MA, Artyomov MN, Ivanova Y, Zhong C, Chase JM, Rothman PB, Yu J, Riley JK, Zhu J, Tian Z, Yokoyama WM. 2014. Tissue resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. *Elife*. 3: e01659.
- Souza LS, Puziol LCP, Tosta CL, Bittencourt MLF, Ardisson JS, Kitagawa RR, Filgueiras PR, Kuster RM. 2019. Analytical methods to access the chemical composition of an *Euphorbia tirucalli* anticancer latex from traditional Brazilian medicine. *Journal of Ethnopharmacology*. 237: 255-265.
- Valadares MC, Carrucha SG, Accorsi W, Queiroz MLS. 2006. *Euphorbia tirucalli* L. modulates myelopoiesis and enhances the resistance of tumour-bearing mice. *International Immunopharmacology*. 6: 294-299.
- Varricchio MCBN, Pereira C, Sales F, Gomes T, Daudt E, Aquino CL, Barbosa GM, Gomes N, Pyrrho AS, Hobaica PEM, Branco MC, Kuster R, Holandino C. 2008. Chronic toxicological effects of high diluted solutions of Aveloz (*Euphorbia tirucalli* L.) on healthy mice: a preliminary study. *International Journal of High Dilution Research*. 7(25): 174-178.
- Wang L, Wang G, Yang D, Guo X, Xu Y, Feng B, Kang J. 2013. Euphol arrests breast cancer cells at the G1 phase through the modulation of cyclin D1, p21 and p27 expression. *Molecular Medicine Reports*. 8: 1279-1285.