

Inhibitory effects of *Euphorbia tirucalli* latex on murine B16/F10 melanoma cells and lung metastasis

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Abstract. *Euphorbia tirucalli* (*E. tirucalli*) is a tropical and subtropical plant that produces a latex which is used for several purposes. The components of *E. tirucalli* latex include triterpenes, diterpenes and steroids. The aim of the present study was to evaluate the effects of diluted *E. tirucalli* latex on murine B16/F10 melanoma cells and lung metastasis. For this purpose, an *in vitro* study was first performed, in which B16/F10 cells were treated with diluted (1/2 to 1/11,192) *E. tirucalli* latex. In a second study, B16/F10 melanoma cells were inoculated into the tail vein of mice to generate lung metastases; the mice then received 0.467 µg of latex diluted in 200 ml saline by gavage for 14 days. A significant decrease in B16/F10 cell viability was observed using the MTT assay at 24 and 48 h after treatment with *E. tirucalli* latex. In addition, a significant decrease in the volume fraction occupied by B16/F10 metastatic colonies in the lungs was observed in mice treated with *E. tirucalli* latex. These results confirm the antineoplastic effects of diluted *E. tirucalli* latex.

Introduction

Euphorbia tirucalli (*E. tirucalli*), commonly referred to as 'aveloz', is a tropical and subtropical ornamental plant, traditionally used in folk medicine for the treatment of syphilis,

asthma, rheumatism, arthritis (1) and cancer (2-7). This plant most likely originated from tropical East Africa, and is reportedly endemic in a number of African countries; it may also be found in southern Europe, Asia and the Americas, where it is used for decorative as well as medicinal purposes (8). *E. tirucalli* is widely cultivated in Brazil, particularly in the north and northeast regions (9).

E. tirucalli produces a latex that is a vesicant and is commonly used as a remedy against several diseases. However, most of its medicinal properties are reported informally and there appears to be little scientific evidence to validate them (8). The main substances present in *E. tirucalli* latex are cyclotirucanenol (triterpene), diterpene ester, steroids and tirucallicine (diterpene) (8). Its active components include euphol, euphorbol, euphorone, isoeuphoral, taraxasterol, tirucalol, citric acid, glucose, kameferol, malic acid, sapogenin acetate and succinic acid (10,11). Recently, Palharini *et al* (12) investigated eutirucallin, a lectin with antitumor and antimicrobial properties. Some of these active components have been reported to have biological activities, such as preventive anticancer activity, antitumor, antimutagenic, antibacterial, laxative, antiseptic, disinfectant, anti-inflammatory, antistrep-tococcal, antiparasitic, antiulcer, analgesic, antiasthmatic and expectorant properties, as well as anticancer effects against specific types of cancer of the breast, lung, cervix, esophagus and mouth (13).

Valadares *et al* (5) first reported that the extract of *E. tirucalli* modulated myelopoiesis and reduced the growth of mouse Ehrlich ascites tumor. Wang *et al* (14) demonstrated that euphol inhibited the growth of T74D human breast cancer cells and reduced the levels of cyclin A and B1 expression, which corresponded to the decreased distribution of cells in the S and G2/M phases, respectively. These results indicated that euphol is an active ingredient of *E. tirucalli* that exerts anticancer effects, possibly by arresting cell cycle progression of cancer cells. Santos *et al* (13) also demonstrated that *E. tirucalli* extract inhibited the growth of Ehrlich ascites tumor. Furthermore, euphol inhibited the growth of human gastric cancer cells by modulating apoptosis mediated by ERK1/2 and cyclin D1 (7).

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The aim of the present study was to investigate the *in vitro* and *in vivo* effects of diluted *E. tirucalli* latex on B16/F10 melanoma cells.

Materials and methods

E. tirucalli latex. *Euphorbia tirucalli* Lineu (Euphorbiaceae) was obtained from the plant resource center Sabor da Fazenda (São Paulo, Brazil) and was certified by the ECOCERT body (<http://www.brazil.ecocert.com/index/>). In addition, a sample of pressed plant was obtained, dried in an oven, fixed on a standard-size paperboard accompanied by a label containing information on the plant and the collection site, and stored in the herbarium of the Department of Botany of the Institute of Biosciences of the University of São Paulo, Brazil (<http://www.ib.usp.br/en/botany-welcome.html>).

All the *E. tirucalli* latex required for the experiment was collected directly from the stems; latex drops were collected from the plant stem in a sterilized glass beaker.

Dilution of *E. tirucalli* latex in saline solution was performed according to its popular use, namely 9 drops (0.0934 g) in 1 liter of saline solution. A total of 5 μ l of this solution, containing 0.467 μ g of *E. tirucalli* latex (for an animal weighing 25 g) was transferred to 200 μ l (q.s.) and this volume was administered to each mouse. This quantity of latex is equivalent to that recommended in popular medicine for a person weighing 70 kg (1.3 g).

Immediately after the preparation, the diluted latex saline solution of *E. tirucalli* and the control saline solution were stored at 4°C.

Culture of B16/F10 cells. B16/F10 murine melanoma cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (50 IU/ml), streptomycin (50 mg/ml) and L-glutamine (2 mmol/l). The bottles were kept in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and 95% air under temperature-controlled conditions.

In vitro experiment

Evaluation of cell proliferation with the MTT assay. On day 1, B16/F10 murine melanoma cells were plated on three flat-bottomed 96-well plates at a density of 3x10³ cells/well (150 μ l of DMEM/well). DMEM was supplemented with fetal bovine serum, penicillin (50 IU/ml), streptomycin (50 mg/ml) and L-glutamine (2 mmol/l). A stock solution of latex in DMEM at a concentration of 0.1037 μ g/ μ l was prepared. Serial dilutions were prepared using DMEM (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1,024, 1/2,048, 1/5,096 and 1/11,192). On day 2, 100 μ l/well of each dilution of the stock solution was added to each well. The amount of latex per dilution is shown in Table I. The control group was treated with 100 μ l/well DMEM.

After 24, 48 and 72 h, 10 μ l tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Amresco, LLC, Solon, OH, USA) was added to both the control and experimental wells. The salt was added to each well of the plates to be metabolized by viable cells, and incubated for 3 h in a humidified chamber at 37°C in an atmosphere of 5% CO₂ and 95% air under temperature-controlled conditions.

Table I. Amount of latex corresponding to each dilution present in each well of the 250- μ l plate.

Dilution	Latex amount (μ g) per well	Latex concentration (ng/ μ l) per well
1/2	5.185	82.96
1/4	2.5925	41.48
1/8	1.2962	20.74
1/16	0.6481	10.37
1/32	0.3240	5.185
1/64	0.1620	2.5925
1/128	0.0810	1.29625
1/256	0.0405	0.648125
1/512	0.0202	0.324063
1/1,024	0.0101	0.162031
1/2,048	0.0051	0.081016
1/5,096	0.0025	0.040508
1/11,192	0.0013	0.020254

Subsequently, the cells were centrifuged at 2,755 x g for 10 min, and the supernatant was discarded. The formazan crystals were dissolved with 100 μ l/well DMSO. The reading was performed in a Multiskan EX (Thermo Fisher Scientific, Inc., Waltham, MA, USA) ELISA reader at 570 nm. The experiments were carried out in six replicates.

In vivo experiment

Experimental animals. A total of 14 C57BL/6 male mice, aged 2 months and weighing 25-30 g, were used in the experiments. The mice were obtained from the Animal Facility of the Department of Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo and were kept under the following environmental conditions: 12-h light/dark cycle, temperature 22±2°C and relative humidity 45-65%. During the experimental period, the animals remained in the animal facility, and had access to water and balanced feed *ad libitum*. The experimental protocols were approved by the Committee on Ethics in Animal Use (CEUA) of the School of Veterinary Medicine and Animal Science of the University of São Paulo (SVMAS-USP, process no. 3410250216) and the School of Medicine of the University of São Paulo (FM-USP, process no. 043/16).

Inoculation of B16/F10 melanoma cells into the tail vein for the development of lung metastasis in mice. Cell suspensions containing murine melanoma B16/F10 cells (100 μ l containing 5x10⁵ cells/animal) were inoculated into the tail vein of 6-month-old C57BL/6 male mice (15-17). The animals were treated with *E. tirucalli* latex daily by gavage (0.467 μ g/25 g in 200 μ l), diluted as 9 drops of latex in 1 liter of saline solution, or saline solution for 14 days (control), starting 1 week after inoculation. At the end of the experiment, the animals were euthanized with an overdose of intraperitoneal ketamine and xylazine solution (doses >100 mg/kg ketamine and 10 mg/kg xylazine were used), and death was confirmed by verifying the lack of respiratory, cardiac and nervous functions. The lungs

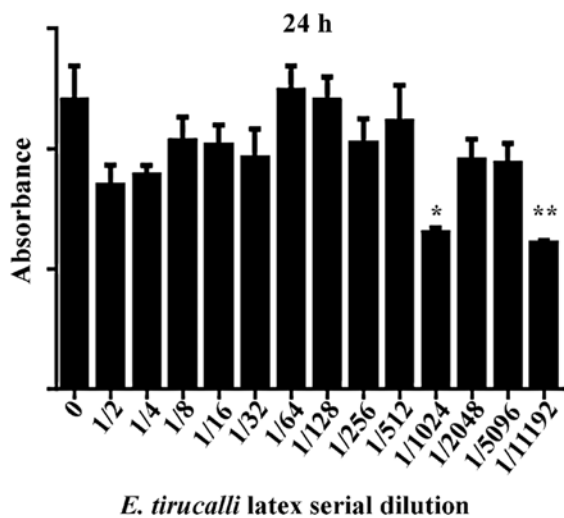


Figure 1. Cell viability analysis by the MTT method after 24 h of treatment with different dilutions of *E. tirucalli* latex. Decreased cell viability was observed at serial dilutions of 1/1,024 and 1/11,192. Column 0 represents only melanoma cells. The Kruskal-Wallis statistical test was used to compare the results. N=6 replicates; *P<0.05 and **P<0.01. *E. tirucalli*, *Euphorbia tirucalli*.

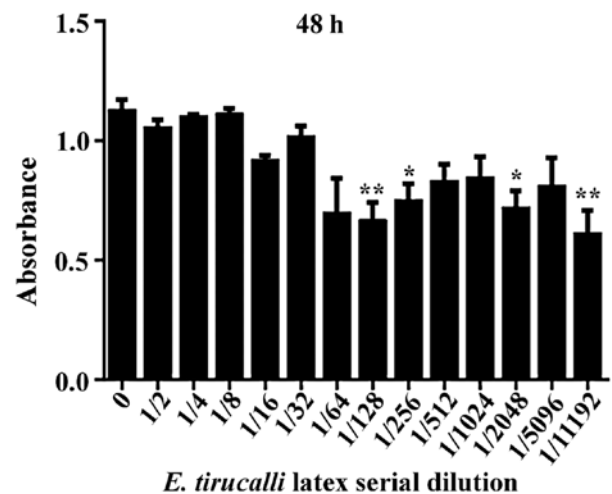


Figure 2. Cell viability analysis by the MTT method after 48 h of treatment with different dilutions of *E. tirucalli* latex. Decreased cell viability was observed at serial dilutions of 1/128, 1/256, 1/2,048 and 1/11,192. Column 0 represents only melanoma cells. The Kruskal-Wallis statistical test was used to compare the obtained results. N=6 replicates; *P<0.05 and **P<0.01. *E. tirucalli*, *Euphorbia tirucalli*.

were removed and fixed in buffered formalin solution (10%) for histopathological and morphometric examination. Representative samples of the lungs, liver, kidneys and spleen were fixed in 10% formalin, embedded in paraffin wax and the 5- μ m histological sections were routinely processed and stained with hematoxylin and eosin (H&E) for histopathological analysis.

Quantification of the volumetric fraction of lungs occupied by metastatic melanoma. The volumetric fraction occupied by the lung colonies of the melanoma group and the *E. tirucalli* latex-treated melanoma group was calculated by the dot-counting method (18). The lungs were fixed in 10% formalin and embedded in paraffin. A total of 15 non-consecutive, randomly selected histological sections of the lungs of each animal (7 animals from the melanoma group and 7 animals from the *E. tirucalli* latex-treated melanoma group) were prepared.

The histological slides were photographed under the Leica M165C stereo microscope (Leica Microsystems, Inc.), using the image capture system composed by the Leica DFC 290 camera and the LAS V4.1 program in the Laboratory of Stochastic Stereology and Chemical Anatomy (LSSCA-USP). Next, the images covering the whole area of the histological section were superimposed by a grid of points where all the points that covered the lung section were counted, and the points that covered only the pulmonary melanoma metastases were registered. The process was repeated 5 times for each image by rotating the grid. The percentage of points that coincided with the metastases in all sections was calculated in relation to the total number of points that coincided with the lung section, and it corresponded to the proportion of volume occupied by the nodules in relation to the total lung tissue.

Statistical analysis. To compare cell viability in cell cultures treated with *E. tirucalli* latex, the Kruskal-Wallis statistical test was used. Subsequent comparisons were performed by

the Dunn's post hoc test. To evaluate the volumetric fraction of lungs occupied by metastatic melanoma, the Student's t-test was applied, and data are expressed as mean \pm standard deviation. Both the statistical tests and the assembly of the graphs and tables were performed with Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) and GraphPad InStat version 3.10. P-values <0.05 were considered to indicate statistically significant differences.

Results

Evaluation of cell viability following treatment of murine B16/F10 melanoma cells with *E. tirucalli* latex. To investigate the effect of *E. tirucalli* latex treatment on murine B16/F10 melanoma cells, different dilutions (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1,024, 1/2,048, 1/5,096 and 1/11,192) were used. The cell viability was evaluated by the MTT assay at 24, 48 and 72 h.

In this series of experiments, cell incubation at different dilutions of *E. tirucalli* for 24 h reduced cell viability at the dilutions of 1/1,024 (46.58%) and 1/11,192 (52.67%) (P<0.05; Fig. 1).

Incubation of the cells with different dilutions of *E. tirucalli* for 48 h reduced cell viability at the dilutions of 1/128 (47.67%), 1/256 (41.83%), 1/2,048 (43.17%) and at 1/11,192 (49.83%) (P<0.0001; Fig. 2).

However, incubation with different dilutions of *E. tirucalli* latex for 72 h did not significantly reduce cell viability any further (P>0.05; Fig. 3).

Evaluation of the effects of *E. tirucalli* treatment on the development of B16/F10 melanoma metastasis. For this experiment, it was examined whether treatment with *E. tirucalli* could also reduce the number of lung metastases. A total of 100 μ l of B16/F10 murine melanoma cell suspension were inoculated into the tail vein of C57BL/6 mice (5×10^5 cells/animal).

After 14 days of treatment with *E. tirucalli*, all mice were euthanized and necropsies were performed. Macroscopically,

Table II. Volume fraction of lung occupied by B16/F10 melanoma metastatic colonies in *E. tirucalli* latex-treated or untreated C57Bl6 mice.

Groups	Number of animals	Mean (%) \pm SD
Untreated mice	7	65.7 \pm 15.4
<i>E. tirucalli</i> -treated mice	7	13.8 \pm 7.5
P-value ^a		0.0001

^aMann-Whitney U test. SD, standard deviation.

the lungs of the control group had numerous melanoma nodules, whereas the lungs of the mice treated with *E. tirucalli* exhibited markedly smaller numbers of metastatic nodules (Fig. 4).

Quantification of the volumetric fraction occupied by metastatic melanoma. The results of the quantification of the volumetric fraction occupied by metastatic melanoma foci in *E. tirucalli* latex-treated (MT) or -untreated (M) C57Bl6 mouse lungs are summarized in Table II. While in control mice 35 \pm 18% of the lungs were occupied by melanoma, only 10.5 \pm 7.7% of the lung area was occupied by melanoma in *E. tirucalli*-treated mice. The difference was statistically significant (Student's t-test, $P=0.024$; Figs. 5 and 6).

Histopathological analysis. No microscopic alterations were observed in the histological sections of the liver, kidneys and spleen of mice treated with diluted *E. tirucalli* latex; in the lungs, small areas of congestion and hemorrhage were observed (control group). These results indicate that the *E. tirucalli* latex does not cause toxicity to mice at the abovementioned dilutions.

Discussion

Treatment modalities popular in folk medicine, mostly medicinal plants, may be of therapeutic value in a number of diseases. However, they must be approached with caution and thoroughly investigated in order to objectively evaluate their efficacy and safety (19).

The objective of the present study was to scientifically evaluate the effects of *E. tirucalli* latex on B16/F10 murine melanoma cells *in vitro* and *in vivo*. Only little information is currently available in the literature on *E. tirucalli* latex and, to the best of our knowledge, this is the first such study on melanoma, a highly lethal cancer affecting humans and animals.

In the *in vitro* experiment, it was observed that *E. tirucalli* latex significantly reduced the viability of B16/F10 murine melanoma cells at high dilutions. Silva *et al.* (20) demonstrated that euphol, a major component of *E. tirucalli* latex, exerted cytotoxic effects on several human cell lines.

In fact, dilutions of *E. tirucalli* latex were effective in reducing melanoma cell viability (as detected by MTT assay) after 24 and 48 h of treatment, but not after 72 h. In addition, the higher dilutions of *E. tirucalli* were consistently more effective in reducing the viability of tumor cells. These findings indicate

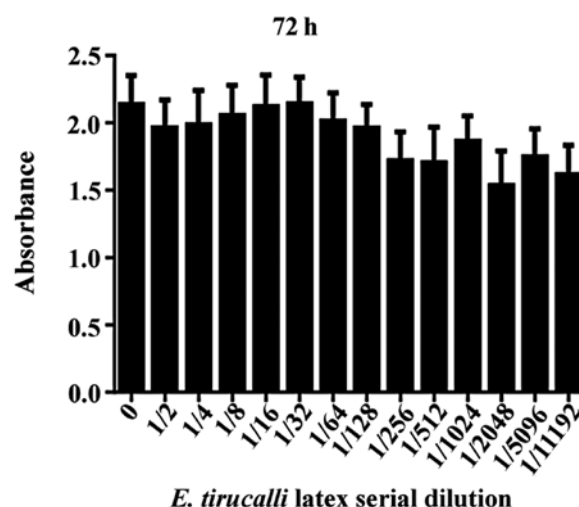


Figure 3. Cell viability analysis by the MTT method revealed no significant decrease in cell viability in any of the serial dilutions after 72 h of treatment with *E. tirucalli* latex. Column 0 represents only melanoma cells. The Kruskal-Wallis statistical test used to compare the obtained results. N=6 replicates. *E. tirucalli*, *Euphorbia tirucalli*.

that the effectiveness of *E. tirucalli* latex against melanoma cells was time-dependent (therefore, it must be administered daily or every other day in order to be effective), and its action apparently occurs at low doses, or possibly at doses that effectively reach the tumor cells inside the tumor mass when the diluted plant latex is administered orally. However, this hormetic effect must be further investigated.

Although *in vitro* studies are informative, when isolated, they cannot predict the *in vivo* efficacy of new therapies. These must be tested either in laboratory animals, or, optimally, in well-conducted clinical trials. In the present study, an *in vivo* experiment was conducted to confirm the efficacy of the *in vitro* results.

Metastasis is characteristic of malignant tumors, and is considered as the main cause of death among cancer patients. Therefore, we investigated the effectiveness of *E. tirucalli* latex in controlling metastasis in mice. Spontaneous metastasis mouse models are rare; therefore, in the present study, mice were inoculated with tumor cells through the tail vein to generate lung metastases (15-17) in order to evaluate the possible effect of *E. tirucalli* latex on metastatic growth. It was observed that the 14-day treatment with diluted *E. tirucalli* latex significantly reduced the volume fraction of the mouse lungs occupied by metastatic melanoma nodules, indicating that it is important to further evaluate the effect of this popular dilution of *E. tirucalli* on other tumors and in the context of other treatment protocols.

Furthermore, when the vital organs of the mice were microscopically examined, it was verified that *E. tirucalli* latex, at the popularly used dilution of 9 drops in 1 liter of saline solution, was not associated with adverse histopathological changes in the liver, kidneys or spleen. The absence of histopathological alterations indicates lack of toxicity of *E. tirucalli* at the commonly used dilution.

The experiments performed using B16/F10 melanoma cells (*in vitro* and metastasis assays) revealed consistent antineoplastic effects, confirming the efficacy of *E. tirucalli*

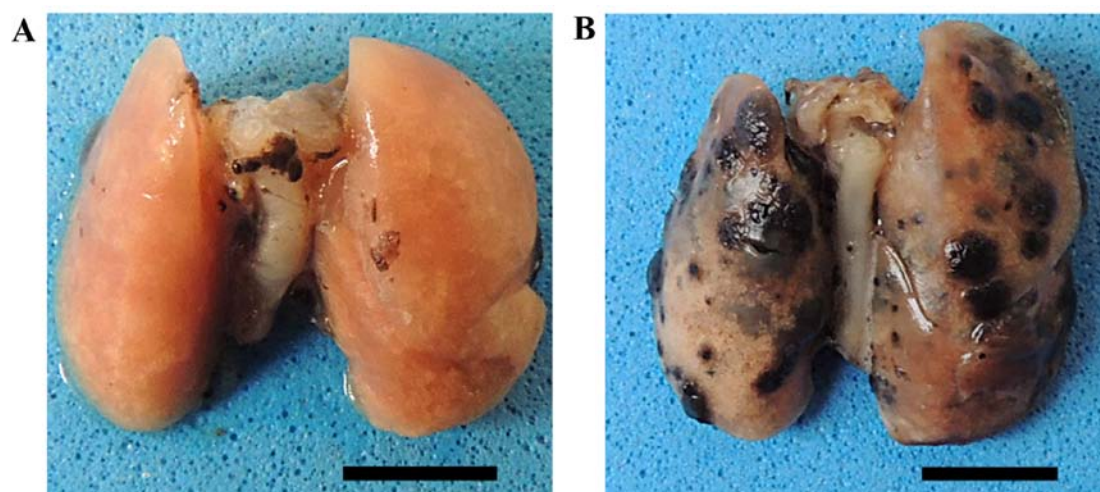


Figure 4. Macroscopic examination of the lungs of C57BL/6 mice that received tail vein injection of B16/F10 melanoma cells. (A) In the *E. tirucalli*-treated group, melanoma cell colonies were observed, but they were smaller and fewer compared with the (B) control group, which received no *E. tirucalli*. Bar, 10 mm.

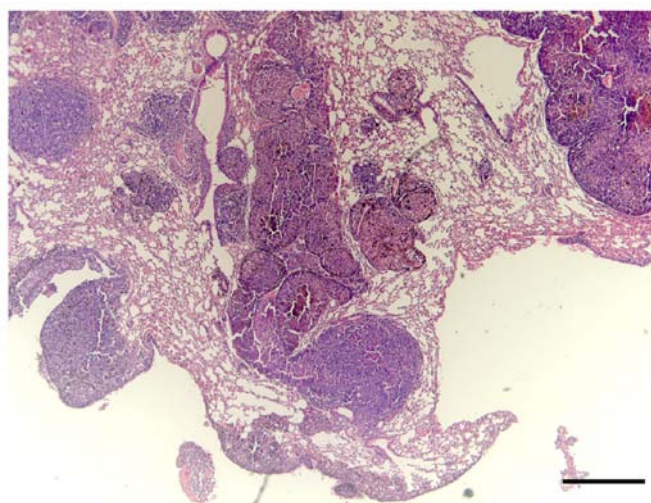


Figure 5. Photomicrograph of a histological section from the lung of a C57BL/6 mouse that was inoculated through the tail vein with B16/F10 melanoma cells. The presence of melanoma cell colonies in the pulmonary parenchyma is observed. Control group. Hematoxylin and eosin staining. Bar, 500 μ m.

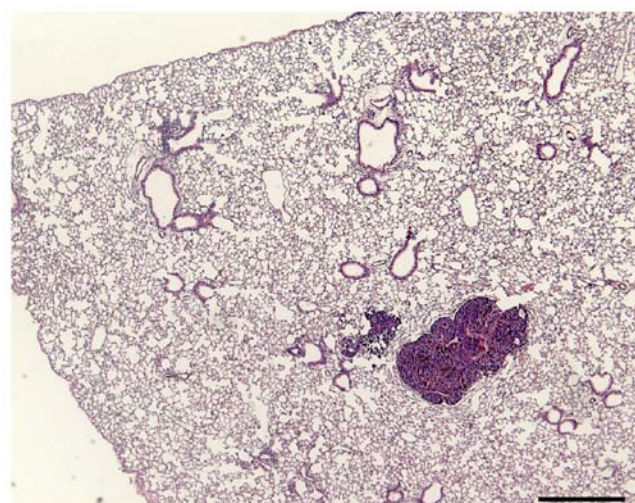


Figure 6. Photomicrograph of a histological section from the lung of a C57BL/6 mouse that was inoculated through the tail vein with B16/F10 melanoma cells and received treatment with 0.467 μ g *E. tirucalli* latex in 200 μ l saline solution. Preserved pulmonary alveoli and small-sized colonies of metastatic melanoma cells are observed. Hematoxylin and eosin staining. Bar, 500 μ m. *E. tirucalli*, *Euphorbia tirucalli*.

latex against B16/F10 murine melanoma. These anticancer effects are most likely associated with euphol (7,14) and/or eutirucallin (12). However, the exact mechanisms underlying the effects of diluted *E. tirucalli* latex require further investigation.

In conclusion, the goal of any experimental study is to test hypotheses which, if scientifically proven, may be used to improve the quality of life and the survival of patients. In the present study, *E. tirucalli* latex has shown promising anti-neoplastic properties that warrant further validation in clinical trials.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

RLB performed the *in vitro* and animal studies. DPADP obtained the *E. tirucalli* plant, standardized the harvesting and dilution of the latex, and helped in the *in vitro* experiments. IIMDF, MKN, MPDG and COMSG helped in the *in vitro* studies with melanoma cells, including the statistical analysis. CMCM and NQH helped in the *in vivo* experiments. MLZD and FJHB mentored the study, supervised the students and reviewed the manuscript. All the authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

The experimental protocols were approved by the Committee on Ethics in Animal Use (CEUA) of the School of Veterinary Medicine and animal Science of the University of São Paulo (SVMAS-USP, process no. 3410250216) and the School of Medicine of the University of São Paulo (FM-USP, process no. 043/16).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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