

# Effect of crude latex from *Euphorbia tirucalli* on DMBA-induced carcinogenesis

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## Abstract

**Background:** The aim of the present study was to determine the action of crude latex from *Euphorbia tirucalli* dissolved in DMSO in animal models of DMBA-induced carcinogenesis.

**Methods:** Three groups of hamsters received a topical application on edge of the tongue: 1) 0.5% DMBA+crude latex from *E. tirucalli* in 10% DMSO; 2) 0.5% DMBA+10% DMSO; and 3) 0.5% DMBA+saline solution. Macroscopic and histopathological analyses of the tumors were performed and antibodies were quantified using ELISA after 13 and 20 weeks of treatment.

**Results:** No statistically significant association was found between the development of cancer of the tongue mucosa and the production of antibodies in the different groups.

**Conclusions:** Although the data were non-significant, crude latex from *E. tirucalli* seems to reduce the aggressiveness and size of lesions, but further tests are required.

**Keywords:** Carcinogenesis, DMBA, tongue, *Euphorbia tirucalli*

## Introduction

*Euphorbia tirucalli* (L.) is a native plant from Africa known popularly as “avelós” or “pencil tree” and is well adapted to the Brazilian Cerrado biome (savanna) [1,2]. The latex from this species is commonly used in traditional medicine [3] for the treatment of cancer [2,4-7]. This substance is also reported to have immunomodulatory [7,8], analgesic [9], angiogenic [10], antimicrobial [11], antimutagenic and antirheumatic [12] properties.

However, studies have reported the toxicity of this latex to skin and mucous membranes [13,14] due to the presence of phorbol esters in species of the family Euphorbiaceae [5,15]. As there is controversy regarding the anticarcinogenic effects and modulatory activity of the latex from *E. tirucalli* in cases of carcinogenesis [16-18,34], studies are needed to demonstrate its activity. Thus, the aim of the present study was to determine the carcinogenic and/or anticarcinogenic action of the crude latex from *E. tirucalli* *in vivo* in a model of carcinogenesis

induced by 9,10-dimethyl-1,2-benz-anthracene (DMBA) using histopathological analysis and an immunoassay.

## Materials and methods

### Sample and experimental groups

The use of animals in this study was in accordance with national and international legislation. The sample comprised 60 male and female hamsters (*Mesocricetus auratus*) aged 90 days with a mean body weight of 158.5 g. The animals were acquired from the animal housing facility of the René Rachou Research Center of the Oswaldo Cruz Foundation and acclimatized in the laboratory for 14 days at room temperature and humidity under a 12-hour light-dark cycle in plastic cages (60x50x22 cm) with free access to ration and water. Each cage was lined with wood shavings, contained a maximum of five animals and was cleaned twice per week. The animals were individually weighed at 3, 5, 7, 9, 11, 13, 15, 17, 19 and 20 weeks using an analytical scale (Gehaka®, model BG 440).

The hamsters were randomly divided into three groups of 20 animals each: 1) Euphorbia group -0.5% DMBA+crude latex from *E. tirucalli* in 10% dimethyl sulfoxide (DMSO); 2) DMSO group -0.5% DMBA+10% DMSO; and 3) DMBA group -0.5% DMBA+saline solution. The groups were subdivided based on evaluation times (13 and 20 weeks).

### Test preparations

The carcinogen DMBA (Sigma-Aldrich®, St. Louis, MO, USA) was diluted in acetone to obtain a solution with a concentration of 0.5% p/v. All days before application, the crude latex from *E. tirucalli* was collected and diluted in DMSO to obtain a solution with a concentration of 10% v/v.

### Experimental protocol

The substances were topically administered to the right lateral edge of the tongue using a N° 1 camel hair brush (Tigre®, San Paulo, Brazil). The application of DMBA was alternated every 24 hours with DMSO, crude latex from *E. tirucalli* and saline solution, depending on the test group. There was no application of any substance once a week. Each daily topical application consisted of four consecutive brushings. Excess substance was removed from the brush prior to the application to avoid accidental swallowing by the animals.

One day prior to euthanasia in each experimental period, a clinical analysis was performed of alterations of the lingual mucosa. The types of clinical alterations examined were based on the method proposed by Lima and Taveira [19], with modifications: diffuse erythematous or diffuse whitish lesions (alterations of a reddish or whitish coloration); white plaque, characterized by a flat, white, well-circumscribed elevation; diffuse whitish berry-shaped lesions (alterations in color and texture, with small rounded irregularities on an opacified surface); verrucous plaque, characterized by a flat circumscribed elevation with a papillomatous surface; exophytic lesion; nodular or tumor mass. Calipers (Jon®, São Paulo, Brazil) were used for the measurements of length (x axis) and width (y axis) of the plaque-type alterations. Measurements of the exophytic lesions also included depth (z axis). The area of the plaques and volume of the exophytic lesions were calculated as  $A=\pi.x/2.y/2$  and  $V=3/4.\pi.xyz$ , respectively.

At each evaluation time, ten hamsters from each group were anesthetized with xylazine (10 mg/kg of body weight) and ketamine (200 mg/kg of body weight) for euthanasia. After decapitation, the tongues were dissected and fixed in a 10% buffered formalin solution for 48 hours. The specimens were embedded in paraffin, cut, dehydrated in alcohol, cleared in xylol and stained with hematoxylin and eosin (HE).

The histological analysis was performed by a single examiner using an optical microscope (Olympus® BX 41, Shinjuku-Ku, Tokyo, Japan) at different magnifications. The histological alterations encountered were classified based on the presence and number of structural and cytological alterations [21]. The classification of the degree of squamous epithelial

dysplasia with the presence of structural and cytological alterations was based on the criteria established by Katz et al. [20] and Barnes et al. [21]. The degree of malignancy of squamous cell carcinomas was classified based on Johnson et al. [22]. The mean occurrence of structural and cytological alterations in the epithelium was calculated for squamous epithelial dysplasia. The histological area of the squamous cell carcinomas was calculated using the Motic Images Plus program, version 2.0 (Motic® China Group CO. LTD., Copyright® 2007, Guiyang, China).

### Evaluation of antibody production

In the initial phase of treatment, all animals were immunized with ovalbumin and submitted to further immunization seven days before euthanasia for the formation of antigen-specific antibodies. Serum samples were obtained and assessed using a standard enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated overnight at 4°C with 50 µg/mL of ovalbumin antigen per well in Na<sub>2</sub>CO<sub>3</sub> buffer, pH 9.6. The plates were washed with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and incubated overnight with PBS containing 5% casein solution and 0.01% azide (PBS-L), rewashed and incubated for 1 hour with dilutions of mouse antisera starting at 1/16 in 0.5% PBS-L solution, washed again and incubated with hamster anti-mouse globulins (anti-IgG) for 1 hour, rewashed and incubated with streptavidin (1:5000) and H<sub>2</sub>O<sub>2</sub> in the presence of orthophenylene diamine (OPD) in sodium citrate buffer, pH 5.0, for 10 minutes. The reactions were interrupted by the addition of 2 M of H<sub>2</sub>SO<sub>4</sub> and optical densities were read in an ELISA reader (Molecular Devices®, model SPECTRA MAX 190) with absorbance at 492 nm and 450 nm.

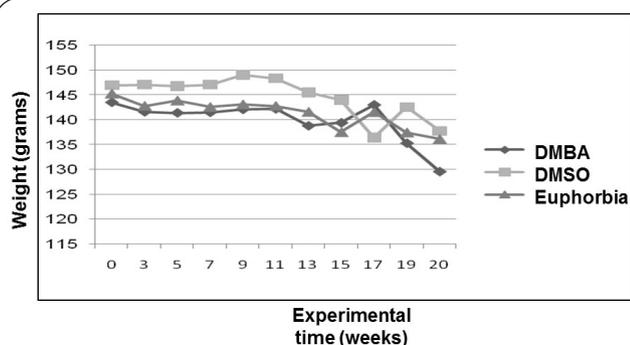
### Statistical analysis

The data were tabulated with the aid of the SPSS 17.0 program (SPSS® Inc, Chicago, Illinois, USA). Normality tests were performed prior to the application of statistical analyses. Repeated-measure one-way analysis of variance (ANOVA) was used to determine changes in the mean body weight of the animals. The chi-square test was used to compare the occurrence of clinical and histological alterations among the groups. For the immunological evaluation, the variance of the data and the confidence interval were determined using ANOVA with Dunnet's post hoc tests. For both tests, a p-value<0.05 was considered indicative of statistical significance.

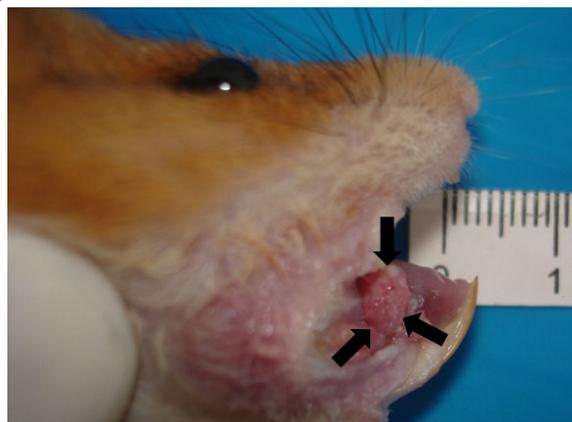
## Results

### Body weight

The mean body weight of the animals reduced throughout the course of the study. No linear progression in reduction occurred, as weight remained nearly constant the first 13 weeks and began to reduce in the following weeks. However, mean weight was higher in the DMSO and Euphorbia groups than the DMBA group (Figure 1).



**Figure 1. Weight (grams) X experimental time (weeks).**  
 Mean body weight of different groups during experimental periods. DMBA: 9,10-dimethyl-1,2-benz-anthracene; DMSO: dimethyl sulfoxide; Euphorbia: crude latex from *E. tirucalli*.



**Figure 2. Clinical alterations in lingual mucosa.**  
 Exophytic lesion (arrow) in group treated with crude latex from *E. tirucalli* after 20 weeks of treatment.

### Macroscopic clinical alterations

At the 13-week macroscopic analysis, most lesions were in the form of whitish plaque, whereas exophytic forms were prevalent at 20 weeks (Figure 2). During the experiment, a few animals died in the Euphorbia and DMSO groups: one animal died in the first four weeks and three died in the three last weeks.

### Histopathology of epithelium at 13 and 20 weeks

Three animals in the Euphorbia group (Week 12 and 18) and one animal in DMSO group (week 17) exhibited extensive cachexia and subsequently died. The animals that died were excluded from the clinical and cytological analysis of the lesions (Table 1). No statistically significant differences were found among the groups regarding different types of clinical and

cytological alterations or respective mean areas or volumes at the two evaluation times (Weeks 13 and 20).

Regarding lesions at 13 weeks, a greater occurrence of dysplastic lesions was found, especially mild dysplasia (13 cases; 44.8%) and moderate dysplasia (12 cases; 41.4%). The structural alterations found in cases of mild squamous epithelial dysplasia (SED) in the DMSO and Euphorbia groups were irregular epithelial stratification and a loss of polarity. The cytological alterations found were increases in the nucleus-to-cytoplasm ratio, number of nuclei and nucleus size and hyperchromasia. The presence of drop-shaped epithelial projections and increases in the number of atypical mitoses and white plaque characterized by a flat white well-circumscribed elevation and diffuse whitish berry-shaped lesions were only found in cases of mild SED in the DMBA group.

**Table 1. Distribution of histopathological aspects of lesions from DMBA-induced oral carcinogenesis on tongue of hamsters after 13 and 20 weeks of treatment.**

Histopathological descriptions	Experimental time							
	13 weeks (n=29)				20 weeks (n=27)			
	Euphorbia (n=9*)	DMBA (n=10)	DMSO (n=10)	p-value	Euphorbia (n=8†)	DMBA (n=10)	DMSO (n=9*)	p-value
Hyperplasia and hyperkeratosis with absence of atypia n(%)	1 (11.1)	1 (10.0)	--	0.374	--	--	--	--
SED - Mild n(%)	3 (33.3)	4 (40.0)	6 (60.0)	0.395	1 (12.5)	--	--	--
SED - Moderate n(%)	4 (44.4)	5 (50.0)	3 (30.0)	0.106	1 (12.5)	--	--	--
SED - Severe n(%)	1 (11.1)	--	--	--	--	1 (10.0)	1 (11.1)	0.632
SCC - Microinvasive n(%)	--	--	1 (10.0)	0.374	3 (37.5)	--	2 (22.2)	0.250
SCC - Mild n(%)	--	--	--	--	2 (25.0)	5 (50.0)	3 (33.3)	--
SCC - Moderate n(%)	--	--	--	--	--	3 (30.0)	1 (11.1)	--
SCC - Severe n(%)	--	--	--	--	1 (12.5)	1 (10.0)	2 (22.2)	--
SCC invasive	--	--	--	--	--	--	--	0.089

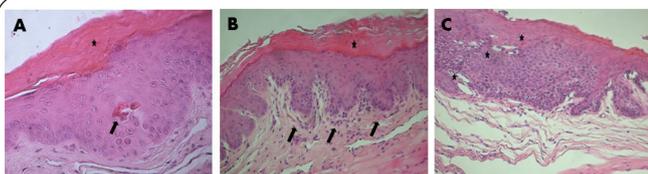
Chi-square test

\*death of 1 animal; †death of 2 animals; SED: squamous epithelial dysplasia; SCC: squamous cell carcinoma

In cases of moderate SED in the Euphorbia group, the structural alterations were irregular epithelial stratification and the cytological alterations were a loss of polarity, a change in cell size, an increase in nucleus size, hyperchromasia and pleomorphism. Irregular epithelial stratification with dyskeratosis and hyperkeratosis, denominated Darier's dysplasia, occurred in only one case (Figure 3A). The structural and cytological alterations for moderate SED in the DMSO and DMBA groups were irregular epithelial stratification, increases in the nucleus-to-cytoplasm ratio, number of nuclei and nucleus size and hyperchromasia. Drop-shaped epithelial projections and hyperkeratosis occurred in the DMBA group (Figure 3B).

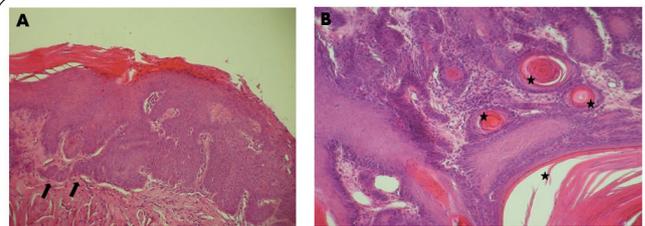
One case of hyperplasia and hyperkeratosis was found in the DMBA (10%) and Euphorbia (11.1%) groups at 13 weeks. The alterations were an increase in density in the stratum corneum and the number of cells in other extracts, with no morphological cell alterations. The occurrence of dysplastic lesions was lower in the Euphorbia group (eight cases; 88.8%) and similar in the DMBA and DMSO groups (nine cases each; 90%) (Table 1). However, only one case of severe SED was found in the Euphorbia group, with increases in the nucleus-to-cytoplasm ratio, number of atypical mitoses and nucleus size and number as well as hyperchromasia and total disruption of the epithelial layers (Figure 3C). A case of microinvasive squamous cell carcinoma (SCC) was found in the DMSO group, with superficial invasion of neoplastic epithelial cells to the connective tissue.

At Week 20, premalignant lesions were found in two cases (25%) in the Euphorbia group and one case in the DMBA (10%) and DMSO (11.1%) groups. In the Euphorbia group, the premalignant lesions were mild and moderate SED (12.5% each), with presence of irregular epithelial stratification, the loss of polarity, increases in the nucleus-to-cytoplasm ratio, nucleus size and number and hyperchromasia. In the DMSO and DMBA groups the lesions were severe SED and the structural and cytological alterations were irregular epithelial stratification, the loss of polarity, increases in the nucleus-to-cytoplasm ratio, number of nuclei and nucleus size and hyperchromasia.



**Figure 3. Histological alterations at week 13 in different groups.**  
**A:** Moderate squamous epithelial dysplasia (Darier type) with dyskeratosis (arrow) and hyperkeratosis (star) in Euphorbia group (H&E, x200); **B:** Moderate squamous epithelial dysplasia with presence of drop-shaped epithelial projections (arrow) and hyperkeratosis (star) in DMBA group (H&E, x100); **C:** Intense squamous epithelial dysplasia with total disruption of epithelial layers (star) in Euphorbia group (H&E, x200). H&E: hematoxylin and eosin.

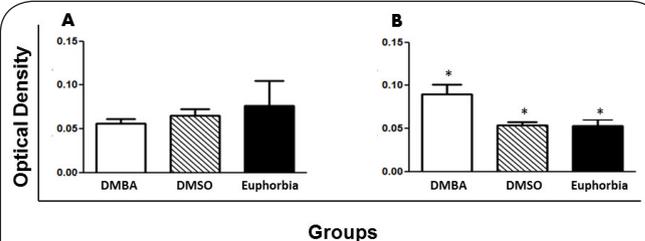
The Euphorbia group showed fewer malignant lesions at 20 weeks (75%) in comparison to the DMSO (88.9%) and DMBA (90%) groups (Table 1). In the DMSO and DMBA groups, cases of microinvasive SCC were found, with the occurrence of superficial invasion of neoplastic epithelial cells to the connective tissue adjacent to the epithelium (Figure 4A). In all groups, cases of small SCC were found, with epithelial projections toward the surface, whereas the invasive SCC exhibited exophytic growth and well-differentiated degrees of malignancy. The lesions exhibited invasion in all tissues, with an increased number of atypical mitoses and the presence of keratin pearls (Figure 4B). No statistically significant difference among groups was found with regard to the development of cancer in the oral mucosa at Weeks 13 and 20 ( $p > 0.05$ , chi-square test).



**Figure 4. Histological alterations at Week 20 in different groups.**  
**A:** Microinvasive carcinoma with superficial invasion to connective tissue (arrow) in DMSO group (H&E, x100); **B:** Squamous cell carcinomas with presence of keratin pearls (star) in DMBA group (H&E, x200). H&E: hematoxylin and eosin.

### Immunological evaluation

At Week 13, no statistically significant differences among groups were found regarding the production of antibodies (Figure 5A). At Week 20, the animals treated with the crude latex from *E. tirucalli* had a lower production of antibodies when compared to the DMBA group. However, this reduction was also found in the DMSO group (Figure 5B).



**Figure 5. Optical density of production of antibodies by different groups.**  
**A:** Production of antibodies in different groups after 13 weeks of treatment; **B:** Production of antibodies in different groups after 20 weeks of treatment. Data expressed as mean  $\pm$  SEM \* statistically significant ( $p < 0.05$ , ANOVA with Dunnett's post hoc test).

## Discussion

Cancer is the second most fatal form of disease behind cardiovascular disease [23]. The development of cancer is a multistep process with three distinct phases: initiation, promotion and progression [24]. The carcinogen DMBA is a chemical initiator and promoter of carcinoma in the oral mucosa [25] through production of free radicals (byproducts of its metabolism), leading to the damage of DNA, cell membranes and proteins as well as the dysregulation of anti-apoptotic mediators [26,27]. After the topical application of 0.5% DMBA in acetone for a period of 20 weeks, histopathological alterations similar to cancerous lesions in humans are found on the oral mucosa [25]. Other studies involving this methodology demonstrate this procedure to be effective in the evaluation of natural products using a carcinogenic model [25,28].

The crude latex from *E. tirucalli* is used in traditional medicine [3,5,29] for the treatment of several types of cancer [5,6]. In the present study, no significant differences were found between the different substances used regarding the anticarcinogenic effect on the oral mucosa after 13 and 20 weeks. However, reductions in the aggressiveness and size of the tumors were found in the *Euphorbia* group.

At Week 20, loss weight was found in all groups, but was more pronounced in the DMBA group. It is possible what this reduction is associated with the toxic action of the different substances applied, especially the carcinogen [30]. Lajolo et al. [30] found an increase in the weight of animals in the test groups when the application of DMBA was suspended.

The histopathological results showed that the topical application of crude latex from *E. tirucalli* promoted the reduction in the progression of oral carcinoma, as evidenced by the less aggressive nature of carcinoma in the *Euphorbia* group when compared the other experimental groups. In a carcinogenesis model in the peritoneal cavity of rats, Valadares et al. [2] found that the oral administration of an alcoholic extract of *E. tirucalli* promoted an increase in the production and functional activity of macrophages and granulocytes as well as a decrease in levels of prostaglandin E2, resulting in a decrease in tumors and the extended survival of the animals. It is probable that such a reduction in the progression of SCC is related to the chemical constitution of this latex. In other *in vitro* studies, euphol, which is a triterpene alcohol isolated from *E. tirucalli*, demonstrated high cytotoxicity to cancer cells. The authors suggest that euphol inhibits cyclins involved in the cell cycle and upregulates pro-apoptotic proteins [5,6,31].

Regarding the immunological evaluation, the results indicate that the crude latex from *E. tirucalli* does not exert a direct action on the humoral immune response. Recent studies have shown that this plant has an immunomodulatory effect via the stimulation of type 1 cytokine production (INF- $\gamma$  and TNF- $\alpha$  in leukocytes), which favors the cellular immune response [8]. It has also been shown that animals with induced tumors from EL4 or EG7 cell lines and rINF- $\gamma$  knockout or deficiency in the production of INF- $\gamma$  did not respond to

chemotherapy, exhibiting the normal production of this cytokine and its receptors when compared to the control group [32]. This demonstrates the importance of INF- $\gamma$  to an effective response to chemotherapy. It is therefore possible that the antitumor mechanism of action observed for *E. tirucalli* is through the stimulation of the cellular immune response. Furthermore, studies have shown the immunomodulatory effect of the solvent DMSO [33], which exerts a significant effect on the humoral immune response. Thus, the solvent itself favored the non-increase in antibody production in the presence of the latex.

## Conclusion

The crude latex from *E. tirucalli* seems decrease the aggressiveness and size of oral carcinomas, although no statistically significant differences were found in the development of cancer of the tongue mucosa or the production of antibodies in the different groups and at the different times assessed. The DMSO solvent may have interfered with the immunomodulatory action of the latex. Thus, further studies are needed to evaluate the immunomodulatory properties of the crude latex from *E. tirucalli*.

## List of abbreviations

ANOVA: Analysis of variance  
DMBA: 9,10-dimethyl-1,2-benz-anthracene  
DMSO: dimethyl sulfoxide  
DNA: Deoxyribonucleic acid  
EG7: Cell line OVA-transfected EL4 thymoma  
EL4: Cell Line murine lymphoblast from blood  
ELISA: Enzyme Linked Immuno Sorbent Assay  
ICT: Science and Technology Institute  
INF- $\gamma$ : Interferon gamma  
PBS-L: PBS containing 5% casein solution and 0,01% azide  
PBS-T: Phosphate-buffered saline containing 0.05% Tween-20  
OPD: Orthophenylene diamine  
SCC: Squamous Cell Carcinoma  
SED: Squamous epithelial dysplasia  
TNF- $\alpha$ : Tumor necrosis factor-alpha  
rINF- $\gamma$ : Receptor Interferon gamma  
UFVJM: Federal University of the Jequitinhonha and Mucuri Valleys

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

Authors' contributions	PRB	FDV	RLR	NLL	BAA	GEBAM
Research concept and design	✓	✓	--	--	--	✓
Collection and/or assembly of data	✓	✓	--	✓	--	--
Data analysis and interpretation	✓	✓	✓	✓	✓	--
Writing the article	✓	✓	--	--	--	--
Critical revision of the article	✓	✓	--	--	✓	✓
Final approval of article	✓	✓	--	--	✓	✓
Statistical analysis	--	--	✓	--	--	--

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