

Original Research Article

Ehrlich ascites tumor-bearing mice treated with aqueous ethanol plant extract from *Euphorbia tirucalli* showed signs of systemic toxicity

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Abstract

Purpose: To evaluate the antitumor effect of a latex extract from *Euphorbia tirucalli* Linn. (Euphorbiaceae) and its toxicity.

Methods: Aqueous ethanol and petroleum ether extracts were obtained through maceration. Maximum tolerated dose was determined in healthy mice. Antitumor activity was measured in Ehrlich ascites tumor-bearing mice treated with the extract through intraperitoneal injection (62.5, 125 or 250 mg/kg) every 48 h (four doses). Efficacy was assessed by weight gain, abdominal circumference, volume of ascitic fluid and packed tumor cells, tumor cell viability and survival. Toxicity indicators were serum glucose, triglycerides, total proteins, activity of alanine and aspartate aminotransferases and mass of heart, spleen, kidney and liver. A hemolysis assay was also performed.

Results: Doses of 62.5 and 125 mg/kg caused no antitumor activity, while 250 mg/kg dose reduced weight gain (3-fold), abdominal circumference and volume of ascitic fluid (> 50 %) and packed cells (50 %), but lowered tumor cell viability (40 %). However, mice treated with the extract survived for a shorter time than control mice. Furthermore, the 250 mg/kg dose caused cardiac atrophy, splenomegaly and fasting hyperglycemia. The extract caused hemolysis, and the half-maximal effective concentration (EC₅₀) was 1.6 (0.9 – 2.7) mg/mL.

Conclusion: *Euphorbia tirucalli* extract inhibits Ehrlich ascites tumor in mice, but the therapeutic dose is also harmful to non-tumor tissues.

Keywords: *Euphorbia tirucalli*, Ehrlich ascites tumor-bearing mice, Antitumor, Toxicity, Cardiac atrophy, Splenomegaly

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INTRODUCTION

Some ethnobotanical articles reported that people from different communities and rural workers in Brazil have used the latex of

Euphorbia tirucalli Linn. (Euphorbiaceae) for the traditional treatment of cancer. In most cases, the recommendation is to dilute some drops of latex into water and take it orally [1-3]. In Brazil, *Euphorbia tirucalli* (*E. tirucalli*) is known as

“aveloz”. This plant is originally from Africa and is found in many tropical regions, grown as an evergreen plant containing latex in the aerial parts [–1-4]. There are also some claims that the latex is used on external warts [3].

Some investigations evaluating the antitumor activity of *E. tirucalli* support its potential for the treatment of cancer. However, literature is still lacking *in vivo* data and safety data. One study has shown that euphol, a triterpene from *E. tirucalli* inhibited the growth of gastric cancer cells by ERK 1/2-mediated apoptosis [5]. *In vivo*, one preliminary report postulated that Ehrlich tumor-bearing mice had myelosuppression and increased numbers of spleen granulocyte-macrophage colony forming units. The treatment of these animals with *E. tirucalli* aqueous ethanolic extract (125, 250 or 500 mg/kg) through gavage daily for five days stimulated marrow myelopoiesis and reduced spleen colony formation [6].

Other studies showed that *E. tirucalli* is toxic [4,7-11]. It contains 4-deoxyphorbol ester, which can suppress the immune system and promote tumor growth. The endemicity of Burkitt's lymphoma coincides with profusion of *E. tirucalli* in tropical Africa [4]. The latex is extremely irritating to the skin and mucosa [8]. The oral administration of one drop of latex for 18 weeks caused splenomegaly in healthy mice [9]. A recent study demonstrated that eutirucallin, a lectin from the latex presents proinflammatory properties [10]. The aqueous extract from *E. tirucalli* caused cytotoxicity, genotoxicity and changes in antioxidant gene expression in human leukocytes [11].

With this background, it is evident that the internal administration of products from *E. tirucalli* must be evaluated deeper. Even when it is considered an antitumor activity, when toxicity can be used with therapeutic purposes, this cannot be useful if it is not controllable and directed preferably against tumor cells. The aim of the current study was to enhance the understanding of the antitumor activity and toxicity of *E. tirucalli*.

EXPERIMENTAL

Plant material

The aerial parts of *E. tirucalli* were collected between June and July 2013 in the western region of Santa Catarina, Brazil, and authenticated by Professor Jasper J Zanco, from

Universidade do Sul de Santa Catarina (Unisul). A voucher specimen (no. R-SRS50033) was deposited at Herbarium *Laelia purpurata* of the same University. This work complied with the rules governing biodiversity rights [12].

Extraction

The aerial parts of *E. tirucalli* consist of fleshy branches containing latex. Therefore, aerial parts were milled fresh and kept in shake-assisted maceration (1:2 weight/volume) for 2 days, repeated 3 times. An aqueous ethanol extract was made using water:ethanol (1:8). Another extract was made in parallel using petroleum ether. All solvents were Vetec® ACS grade reagents. The solvents were eliminated under reduced pressure. Extraction performances were calculated in mass of dried extract (g) and yield (%), taking into consideration the mass of the starting material [13].

Animals

The study was conducted in compliance with international (NIH) guidelines [14] and had the approval of the ethics committee of Unisul (no. 13.027.4.03.IV). Male Balb/c mice (20 ± 5 g) were housed under controlled conditions in the Laboratory of Neurosciences from Unisul, having food and water *ad libitum*. Before experiments, mice were fasted for 6 h. Animals were allowed to acclimatize (12 h light-dark cycle, 22 ± 2°C, 60 % relative humidity) for at least 5 days before the experiments.

Determination of maximal tolerated dose

Groups of healthy mice (n = 6) were treated through intraperitoneal injections (i.p.) with up to four doses of extract (50, 100, 250, 350 or 450 mg/kg) given every 48 h to determine the maximum tolerated dose by verifying the occurrence of death, hair erection, aggressiveness, dullness, inactivity and loss of appetite [15].

Tumor induction and treatment

Ehrlich carcinoma (5 × 10⁶ cells) was inoculated into the abdomen of another set of animals [16]. Inoculation day was considered “day zero” when animals were weighed and the abdominal circumference was measured. Twenty-four hours later, animals were divided into 4 groups (n = 12/each), including a control group treated (i.p.) with saline (50 µL) and 3 test-groups which received aqueous ethanol extract (i.p.) at 62.5, 125 or 250 mg/kg. Four doses were administered in total. Each dose was given every 48 h.

Evaluation of antitumor activity

On day 10, mice were weighed and the abdominal circumferences were measured one more time. Half of each group was euthanized (n=6), and the ascitic fluid was collected in graduated Falcon™ conical tubes. Tubes were centrifuged at 5.000 g (5 min) to measure packed tumor cells volume [16]. Viability of tumor cells was assessed through the trypan blue assay [17]. Cells were counted by a TC10 counter (Bio-Rad, USA). Inhibition of tumor growth (ITG) was calculated with basis on abdominal circumference change (AC) using Equation 1 [18]:

$$\text{ITG (\%)} = [\text{AC (test-group)} \times 100 / \text{AC (control)}] - 10 \dots \dots \dots (1)$$

Evaluation of mice survival

The remaining animals of each group (n=6) were monitored on a daily basis to evaluate survival using Kaplan-Meier method [19].

Determination of toxicity

The mass of heart, spleen, kidneys and liver from mice treated with the antitumor effective dose of extract (250 mg/kg) and mice from the control group (n = 6) was measured in a Shimadzu AUJ 200 analytical balance (Japan) [15]. Blood samples were collected for estimating serum glucose, triglycerides, total proteins and activity of aspartate and alanine - aminotransferases (AST and ALT) using kits Labtest® (Brazil).

Hemolysis assay

Hemolytic test was performed in triplicate using 96-well plates [20]. Each well received saline (100 µL 0.85 % NaCl and 10 mM CaCl₂). Then, 100 µL of saline containing extract (0.08 - 2.5 mg/mL) were added. The negative control

(hemolysis 0 %) contained only saline. Triton X-100 (20 µL) was added as positive control (100 % hemolysis). Each well received a 2 % suspension of mouse erythrocytes. After incubation at room temperature (30 min) and centrifugation (5.000 g for 5 min), hemoglobin was quantified at 540 nm. The half maximal effective concentration (EC₅₀) and 95 % confidence intervals were calculated using Graph Pad Prism (San Diego, USA).

Statistical analysis

Data are presented as mean ± standard deviation or confidence intervals. Statistical analysis was carried out using Graph Pad Prism software, analysis of variance (ANOVA) and Bonferroni test. *P* < 0.05 was taken as statistically significant.

RESULTS

The yield of aqueous ethanol and petroleum ether extractions was 0.86 ± 0.05 and 0.14 ± 0.02, respectively. Thus, the extraction yield using water-ethanol was much higher (6-fold) than the petroleum ether extraction. Therefore, the aqueous ethanol extract was selected for screening for antitumor activity and toxicity.

Figure 1 shows the mortality of healthy mice following a first, second, third and fourth administration of extract. The maximum tolerated dose was 250 mg/kg, which caused neither mortality nor behavior changes.

Considering the treatments of Ehrlich ascites tumor-bearing mice, Figure 2 (A) shows that the weight gain in mice from the saline-treated control group was 12.8 ± 3 g, which is a weight gain of more than 50 % in comparison to the initial measurement.

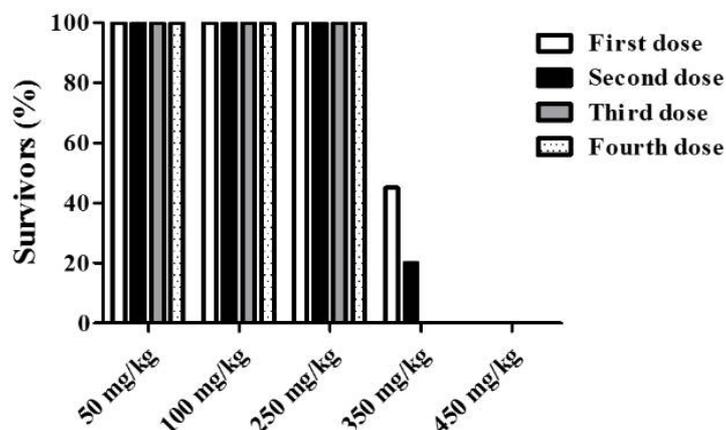


Figure 1: Mortality of healthy mice receiving doses of *Euphorbia tirucalli* aqueous ethanol extract every 48 h through intraperitoneal injections (n=6/each group)

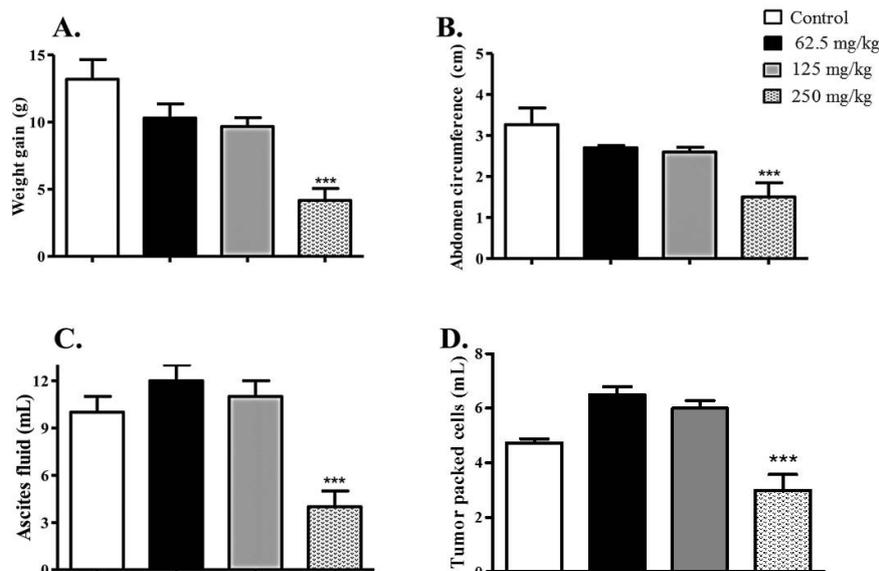


Figure 2: Weight gain (A), abdomen circumference (B), volume of ascitic fluid (C) and packed tumor cells (D) in Ehrlich ascites tumor-bearing mice treated with *Euphorbia tirucalli* aqueous ethanol extract (62.5, 125 or 250 mg/kg) and the saline-treated control. Data are as means \pm SD (n=12 in A and B, whereas n=6 in C and D). ***statistical difference compared to the control ($p < 0.01$)

There were no differences in weight gain between the control and the groups dosed with extract at 62.5 and 125 mg/kg. The group dosed at 250 mg/kg had a weight gain of 3.6 ± 1.8 g, showing a strong reduction compared to the control group.

trend was observed for the volume of ascitic fluid (Figure 2 C). The packed cells volume in mice from the control was 4.7 ± 0.1 mL, this value was 2.9 ± 0.2 mL in mice treated with extract at 250 mg/kg.

Figure 2 also shows data of abdominal circumference (B), volume of ascitic fluid (C) and packed tumor cells (D). Again, no differences were observed between the control and mice treated with either 62.5 or 125 mg/kg. Mice treated with 250 mg/kg showed an abdominal circumference determined at 1.5 ± 0.6 cm on average, which means a reduction close to 50 % compared to the control (Figure 2 B). A similar

Figure 3 (A) shows tumor cell viability. Only the treatment done with extract at 250 mg/kg reduced the number of viable tumor cells compared to the control. Therefore, if taken together the above results suggest an antitumor activity for the extract at 250 mg/kg. The inhibition of tumor growth was calculated at $> 40\%$. This corroborates claims for the antitumor activity of *E. tirucalli* [1-3].

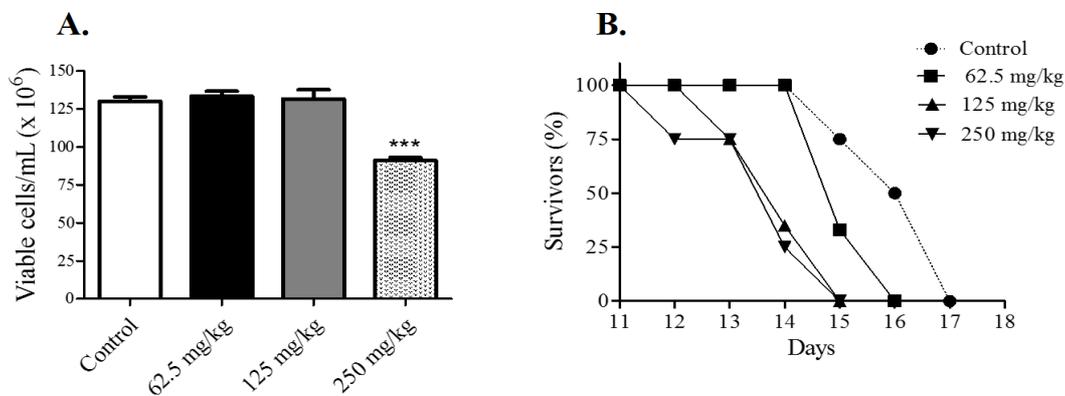


Figure 3: Number of viable tumor cells in mice treated with *Euphorbia tirucalli* aqueous ethanol extract. Data are means \pm SD (n=6/each group). ***statistical difference compared to the control ($p < 0.01$) (A). Lifespan of Ehrlich tumor-bearing mice treated with the extract at 62.5, 125 or 250 mg/kg and the saline-treated control (n=6/each group) (B)

Figure 3 (B) shows the percentage of survivors after treatments. Increase in the area under the curves indicated an increase of survival. The smallest areas corresponded to mice treated with the extract at 125 and 250 mg/kg. The area was slightly larger when mice were dosed at 62.5 mg/kg. However, all mice treated with extract survived a shorter time than mice from the control.

Figure 4 shows the mass of organs of mice treated with extract at 250 mg/kg and the control. Figures 4 (A) and 4 (B) show that the mass of

kidneys and liver were not affected. Figures 4 (C) and 4 (D) show evidences of cardiac atrophy and splenomegaly in mice receiving the extract.

Some serum parameters were measured to evaluate the toxicity of extract given at 250 mg/kg (Figure 5). No differences were verified neither in terms of triglycerides (5 A), total proteins (5 B) nor activity of AST and ALT (5 C/D). However, mice treated with extract had fasting hyperglycemia compared to the control (Figure 5 E).

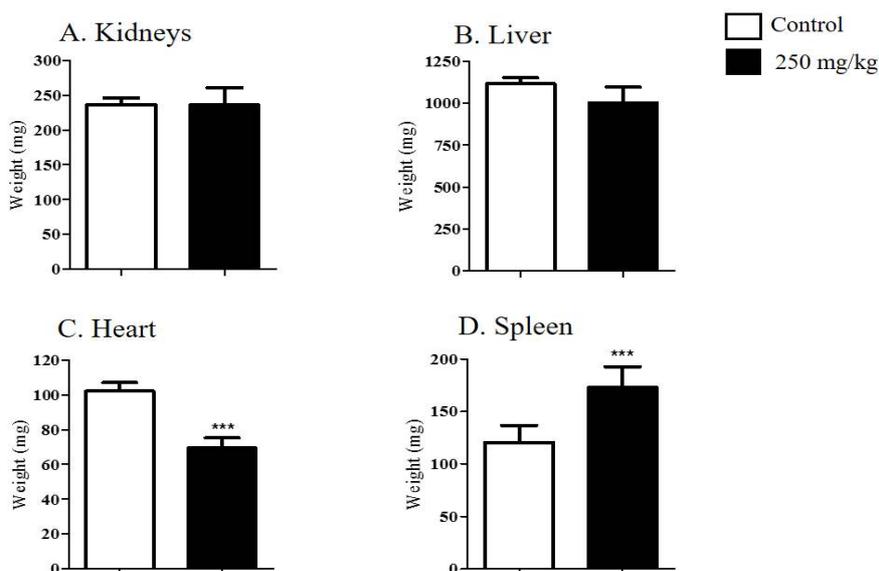


Figure 4: Mass of organs of Ehrlich tumor-bearing mice treated with *Euphorbia tirucalli* aqueous ethanol extract at 250 mg/kg (intraperitoneally) (closed bars). Saline-treated control group (opened bars). Data are means \pm SD (n=6/each group). ***statistical difference compared to the control ($p < 0.01$)

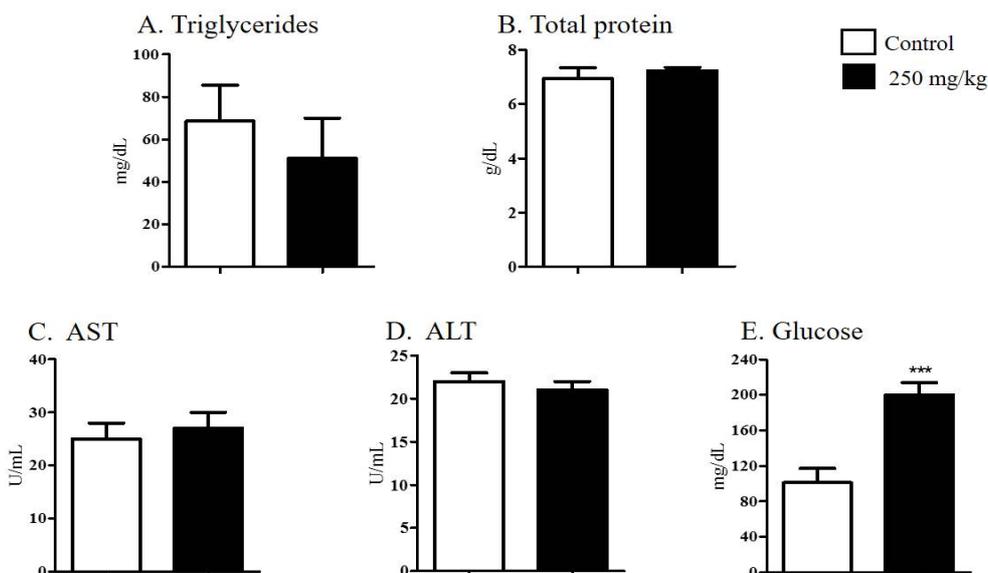


Figure 5: Serum triglycerides (A), total proteins (B), activities of aspartate AST (C) and alanine-aminotransferases ALT (D) and glycemia (E) in Ehrlich tumor-bearing mice treated with *Euphorbia tirucalli* aqueous ethanol extract (250 mg/kg, intraperitoneally) (closed bars). Saline-treated control (opened bars). Data are means \pm SD (n=6/each group). ***statistical difference ($p < 0.01$)

Figure 6 shows that *E. tirucalli* aqueous ethanol extract caused concentration-dependent hemolysis. The EC₅₀ value was 1.6 (0.9 – 2.7) mg/mL.

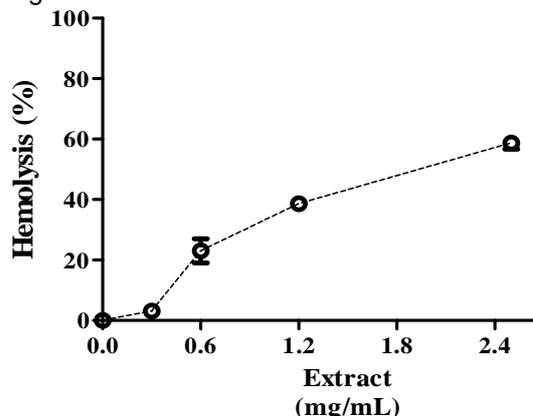


Figure 6: Hemolysis caused by *Euphorbia tirucalli* aqueous ethanol extract. Data of three independent experiments performed in triplicates

DISCUSSION

Some ethnobotanical studies and data of *in vitro* experiments suggest usefulness of *E. tirucalli* for cancer treatment [1-3,6]. The plant latex is toxic [4,8-11]. To be worthy therapeutically, this toxicity had to be evaluated when considering internal administration.

The overall data of the first phase of this investigation suggested an antitumor action of *E. tirucalli* extract administered at 250 mg/kg, capable to kill tumor cells (Figure 3A). That caused reduction of volumes of ascitic fluid and packed tumor cells (Figure 2). The ascitic fluid volume gives information of requisition and nutritional status of tumor and availability of nutrients, whereas the packed tumor cells volume represents the tumor size itself [21].

This investigation started by evaluating healthy mice to determine the maximum tolerated dose of extract administered intraperitoneally (250 mg/kg) (Figure 1). Initially, 250 mg/kg was considered safe because healthy mice did not die and had no behavioral changes. Figure 1 shows that doses above 250 mg/kg induced toxicity (> 50 % of death after a single dose at 350 mg/kg within 24 h). Therefore, a range of doses (62.5 to 250 mg/kg) was tested for antitumor activity.

Lower doses (62.5 and 125 mg/kg) were ineffective. Only 250 mg/kg caused tumor growth inhibition. Nevertheless, data showed that the effect was poorly selective and compromised other organs. All tumor-bearing mice treated with the extract survived a shorter time than mice

from the control. Dose of 250 mg/kg was safe in healthy animals (Figure 1), but this was the threshold of safety because it weakened tumor-bearing mice. These mice had cardiac atrophy, splenomegaly (Figure 4 C/D) and fasting hyperglycemia (Figure 5E).

Some drugs used for cancer are tricky because they can cause cardiotoxicity [22]. A cardiac atrophy normally represents a secondary consequence resulting from a larger systemic phenomena on [23]. It is possible that in this case it was induced by the extract.

Splenomegaly can also indicate a limitation for the treatment. In principle, the enlargement of the spleen can be associated with a variety of etiologies (infectious, neoplastic or congestive). When these causes are ruled out, splenomegaly is normally a response to hyperfunction in any disorder that usually involves abnormal erythrocytes being destroyed in the spleen [24]. The interpretation of data suggesting splenomegaly in the current study had to consider that the experiment was done in tumor bearing mice. Data suggested that tumor-bearing mice treated with the extract from *E. tirucalli* at 250 mg/kg had enlarged spleens compared to the control group (Figure 4D). Previously, Varrichio *et al* [9] reported that healthy mice treated with latex from *E. tirucalli* had splenomegaly.

Irrespective of whether splenomegaly was associated with the toxicity of extract, it was also verified if the extract was able to cause hemolysis. Compounds possessing biological activity may not be useful if they cause hemolysis. The hemolytic assay may reveal some information about the toxicity mechanism [25]. Data showed that *E. tirucalli* extract caused hemolysis (Figure 6).

The final evidence showing that the effective dose of the extract was poorly selective was because treated mice had fasting hyperglycemia (Figure 5E). In toxicology, fasting hyperglycemia is used as a marker to screen compounds with toxic effects on pancreatic beta cells [26].

CONCLUSION

E. tirucalli aqueous ethanol extract administered intraperitoneally inhibits the growth of Ehrlich ascites tumor in mice, but only at doses that are also harmful to non-tumor tissues. Further studies may be required to explore how the extract is developed to therapeutic doses without toxic effects on normal cells.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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