

Cytotoxic activity of the dichloromethane fraction from *Vernonia scorpioides* (Lam.) Pers. (Asteraceae) against Ehrlich's tumor cells in mice

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Abstract

Vernonia scorpioides has been widely used in Brazil to treat skin problems and chronic wounds, such as ulcers of the lower limbs and diabetic lesions. In the present study, we investigated the effect of a dichloromethane (DCM) fraction of *V. scorpioides* leaf extract on Ehrlich ascitic and solid tumor-bearing mice. The animals were treated once a day with the DCM fraction at a concentration of 5 mg/kg, administered *ip* during and after the development of the tumor. The lifespan, weight, number and type of leukocytes, number of tumor cells, volume of solid and ascitic tumors were measured. The development of the tumor with pre-treated tumor cells *in vitro* with the DCM fraction (5 mg/kg) was analyzed and the animals were sacrificed after 7 days. The DCM fraction (5 mg/kg) totally inhibited tumor development when in direct contact with tumor cells, and also ascitic tumor development with *in vitro* treatment or when administered *ip*, *in loco* (after 7 days). Animals treated with the DCM fraction increased their lifespan ca. 2 weeks and maintained their body weight for 30 days. When applied immediately after the inoculation of the tumor cells *in vivo*, it totally abolished tumor development, with tumor development only decreasing when treatment was started 3 days after the tumor challenge. These data suggest an antineoplastic activity of the fraction. Oral or *ip* administration of DCM fraction (5 mg/kg) for 7 days did not reduce the solid tumor volume. The cytotoxic activity described here differs from the conventional immune suppressing profile of standard chemotherapy because it increases neutrophil influx to the peritoneal cavity. These results show that, besides exhibiting a tumoricidal activity, the DCM fraction also exhibits inflammatory activity.

Key words

- *Vernonia scorpioides*
- Leaf extract
- Antitumoral
- Ehrlich ascitic tumor
- Neutrophils

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Introduction

Vernonia scorpioides (Lam.) Pers., Asteraceae, popularly known as piracá, enxuga or erva-de-São-Simão in Portuguese, is very common in Brazil, and usually grows in poor

and deforested neotropical soils (1). The topical application of the alcoholic extract of its fresh leaves is widely used to treat a variety of skin disorders, including chronic wounds such as ulcers of the lower limbs. Previous studies of the *V. scorpioides* crude extract

and its derived chloroform and hexane fractions have shown fungicidal activity (2), moderate bactericidal activity and mild wound healing effects (3).

There are an estimated 200 species of *Vernonia* in Brazil, some of which are traditionally ingested to treat gastrointestinal disorders (4), such as the fresh macerated leaves of *V. condensate*, commonly known as Indian Bitterness (fel-de-bugre, fel-de-índio or alumã in Portuguese). Recent studies focusing on the anti-inflammatory (5,6), antipyretic (7), anticancer (8-11), and antimalarial (12,13) activities of several *Vernonia* species have been published.

The genus *Vernonia* produces characteristic compounds such as sesquiterpene lactones, with several reported biological activities, such as fungistatic (14), and cytotoxic activities (15,16) and also acts as a smooth muscle relaxant (17). Some other compounds have been isolated from *Vernonia*, such as flavonoids (18), steroids (19) and polysaccharides (20).

In a previous study by our group (3), a pro-inflammatory profile was observed in healthy skin tissue, which prompted an investigation of effect of the crude extract on Ehrlich ascitic and solid tumors in mice. The promising antitumoral result preliminarily obtained with the crude leaf extract led us to partition it in order to obtain fractions of increasing polarity, namely: hexane, dichloromethane (DCM), ethyl acetate and aqueous fractions, tested at 200 mg/kg. Of these, the DCM fraction showed the highest cytotoxicity against tumor cells, especially when applied *in loco* to the developing tumor.

In the present study, we investigated the effect of the DCM fraction obtained after liquid-liquid partition of the crude hydroalcoholic extract of the *V. scorpioides* leaf on Ehrlich ascitic and solid tumor-bearing mice. The animals were treated with the DCM fraction at a concentration of 5 mg/kg once a day, administered intraperitoneally (*ip*) during and after tumor development. The lifes-

pan and weight (30 days), number and type of leukocytes, number of tumor cells, volume of the solid and ascitic tumors, and the development of the tumor with tumoral cells pre-treated *in vitro* with the DCM fraction were analyzed after 7 days of treatment.

Material and Methods

Extract, fractions and sample preparation

Flowers and fresh leaves (600 g) of the plant were macerated with 6000 mL ethanol for 7 days, in the absence of light, and the extract obtained was reduced to 1/6 of the initial volume, under vacuum, using a rotary evaporator (Quimis, São Paulo, SP, Brazil). Water (600 mL) was added to the crude extract obtained and the extract was submitted to liquid-liquid fractionation using solvents of increasing polarity. The fractions obtained were denoted: hexane (1.16 g), DCM (420 mg), ethyl acetate (560 mg), and water. After screening with all fractions, DCM showed high activity against the tumor cells (no tumor development was observed) and was selected for further investigation. An aliquot of the dry fractions was dissolved in saline using up to 2% Tween 80 and an ultrasonic bath (10 min) to help dissolution at the following concentrations: 200, 100, 50, 30, 15, and 5 mg/kg in a maximum final volume of 200 μ L.

The prepared aliquoted samples were frozen until the day of application.

The DCM fraction is a dark-green semi-solid material, its NMR spectra were recorded with a Bruker Avance 400 spectrometer (Rheinstetten, Germany).

Animals

Adult male inbred Swiss mice aged 8 weeks, maintained under standard environmental conditions, were used. The animals were fed a standard diet and received water *ad libitum*. The animals were used after an

acclimatization period of 7 days, and the experiments were conducted in accordance with the Univali Ethics Committee for the treatment of laboratory animals.

Ehrlich tumor

Ehrlich ascitic tumor (EAT) derived from a spontaneous murine mammary adenocarcinoma was maintained in the ascitic form by passages in syngenic Swiss mice by weekly transplantation of 5×10^6 tumor cells (*ip*). The ascitic fluid was removed by opening the belly and collecting all the fluid with a sterile syringe. Ascitic tumor cell counts were carried out in a Neubauer hemocytometer using the Trypan blue dye exclusion method. The animals used for the experiment received 200 μL of a suspension containing 5×10^6 tumor cells *ip* as described in Ref. 21.

Animal treatment

After *ip* implantation of tumor cells, seven groups of mice (6 mice per group) were treated with different concentrations of the DCM fraction (200, 100, 50, 30, 15, and 5 mg/kg, *ip*), the positive control group received 5-fluoro-uracil (5-FU) in 0.9% saline solution (20 mg/kg, *ip*) (22) and the negative control group received saline solution and 2% Tween *ip* in the same final volume. The body weights of the mice were measured daily until their death. The only animals to survive more than 30 days, those who received 5 mg/kg, were sacrificed 30 days after the beginning of the treatment.

Effect of the DCM fraction (5 mg/kg) on the ascitic tumor

Seven groups of 6 mice each were used. Treatment was started immediately after inoculation of the tumor cells. Group 1 received 5 mg/kg DCM fraction, *ip*, group 2 received 20 mg/kg 5-FU, *ip*, group 3 re-

ceived 0.9% saline, *ip*, group 4 received 5 mg/kg DCM fraction by gavage, and group 5 received 0.9% saline by gavage. Group 6 started to be treated with the DCM fraction, *ip*, 3 days after inoculation of the Ehrlich ascitic tumor cells, and group 7 started to receive 0.9% saline, *ip*, 3 days after implantation of the tumor cells. After 7 days, the mice were sacrificed and all the ascitic fluid was harvested for volume measurement and ascitic tumor cell count by the Trypan blue dye exclusion method.

Determination of the cytotoxicity of the DCM fraction (5 mg/kg) on Ehrlich ascitic tumor cells *in vitro*

EAT cells (5×10^6) were pre-treated *in vitro* with the DCM fraction for 15 min, and injected into the abdominal cavities of 6 mice (group 1). Group 2 received 5×10^6 EAT cells, *ip*, without pre-treatment. After 7 days, the mice were sacrificed and all the ascitic fluid was harvested for volume measurement and ascitic tumor cell count by the Trypan blue dye exclusion method.

Determination of the effect of the DCM fraction (5 mg/kg) on solid tumor development

Solid tumors were induced by injecting EAT cells, 5×10^6 /animal in 50 μL subcutaneously into the hind paw of 5 groups (6 mice/group) of Swiss albino mice. Group 1 was treated with the DCM fraction ($5 \text{ mg kg}^{-1} \text{ animal}^{-1}$) *ip* and group 2 was treated with the same dose of the DCM fraction by gavage. Group 3 received 5×10^6 EAT cells per animal pre-treated with DCM and did not receive any further treatment. Group 4 received 0.9% saline *ip*, and group 5 received 0.9% saline by gavage. The animals were treated with the DCM fraction daily for 10 days, with the exception of group 3. Footpad volume was measured using a digital pachymeter. All treatments were daily.

Analysis of peritoneal leukocytes

Six tumor-bearing mice per group were treated with 5 mg/kg of the DCM fraction, *ip*, the positive control group received 20 mg/kg 5-FU, *ip*, and the negative control group received 0.9% saline, *ip*. After 30 days, the leukocytes were harvested from the peritoneal cavity with 5 mL saline. The peritoneal fluid was centrifuged, re-suspended in 200 μ L saline, distributed (50 μ L) on 24-well flat-bottom plates containing round glass coverslips (15 mm) for 1 h at room temperature, processed histologically, and stained with Harry's hematoxylin for microscopic observation. A single blind microscopic evaluation of five fields per coverslip (N = 6) was performed using a reticule eyepiece with 100 quadrants. Based on nuclear shape, neutrophils, monocytes/macrophages and lymphocytes were counted by light microscopy at 100X magnification and the means for the five fields analyzed were calculated.

Morphological analysis of the spleen

The spleens from animals treated *ip* for 30 days with the DCM fraction (N = 4), from

tumor-bearing animals after 7 days of survival, and from normal mice were removed and processed histologically. Sections were stained with hematoxylin-eosin for microscopic observation.

Statistical analysis

Data are reported as means \pm SD and were analyzed statistically by the Dunnett test and the F-test. The level of significance was set at $P \leq 0.05$.

Results

Evaluation of the lifespan and body weight of the animals after treatment with different concentrations of the DCM fraction

Animals inoculated *ip* with 5×10^6 EAT cells were divided into 5 treated groups (N = 6) receiving different concentrations of the DCM fraction (5, 30, 50, 100, 200 mg/kg), negative control group (saline solution and 2% Tween) and a positive control group treated *ip* with 20 mg/kg 5-FU (22). As shown in Figure 1 (longevity of the animals), the mice treated with the highest concentration (200 mg/kg) survived for only 12 h, with longevity increasing proportionally with the subsequent decreasing concentrations. The animals that received 5 mg/kg of the DCM fraction survived the treatment and were sacrificed after 30 days. When the animals which had received higher doses were sacrificed, no tumor cells or ascitic fluid could be found in the abdominal cavities. Treatment with a 5 mg/kg dose of the DCM fraction caused a smaller loss of body weight during the 30-day period. A similar profile was observed for treatment with 5-FU. Conversely, the body weight of the mice treated with saline increased, and higher concentrations of the DCM fraction induced longer loss of body weight. For these groups (control and 15, 30, and 50 mg/kg DCM fraction), the difference between the initial and

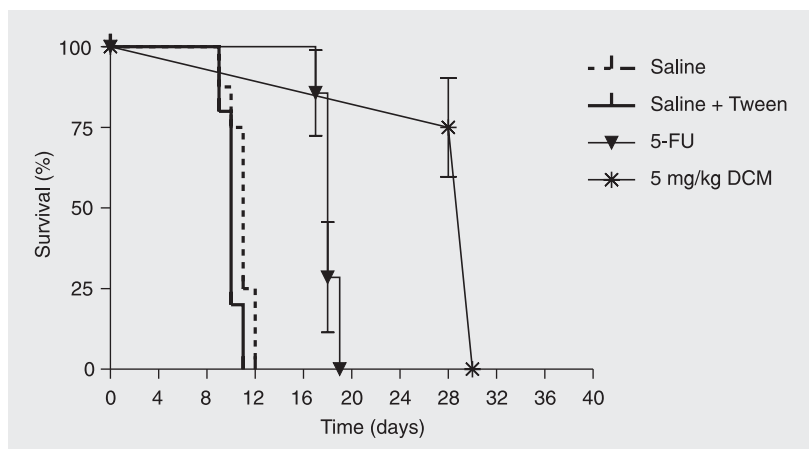


Figure 1. Kaplan-Meier survival curve of mice treated with saline, saline plus Tween, 5 fluoro-uracil (5-FU), or 5 mg/kg dichloromethane (DCM) fraction of the *Vernonia scorpioides* leaf extract (N = 6 in each group). Animals treated with the DCM fraction had a significantly longer lifespan than those treated with 5-FU ($P < 0.0001$, log-rank test).

final body weights was significant ($P < 0.05$; data not shown).

Antitumor activity of the DCM fraction immediately and after implantation of ascitic tumor cells

Figure 2A shows the effect of *ip* administration of the DCM fraction against tumor cells, and ascitic volume when treatment was initiated immediately after tumor cell implantation. The result observed was the suppression of 100% of the tumor cells and ascitic volume when the animals were treated immediately after the inoculation of EAT

cells, and around 80% tumor cells and 60% ($P < 0.001$) ascitic volume, when *ip* treatment was started 3 days after tumor development (Figure 2B). The control groups treated with saline showed a larger volume of tumor and tumoral cells.

In vitro cytotoxicity

When the EAT cells were pre-treated *in vitro* with 5 mg/kg DCM fraction for 15 min and injected into the abdominal cavity, there was no tumor development. This suggests that this fraction had a direct cytotoxic activity against EAT cells.

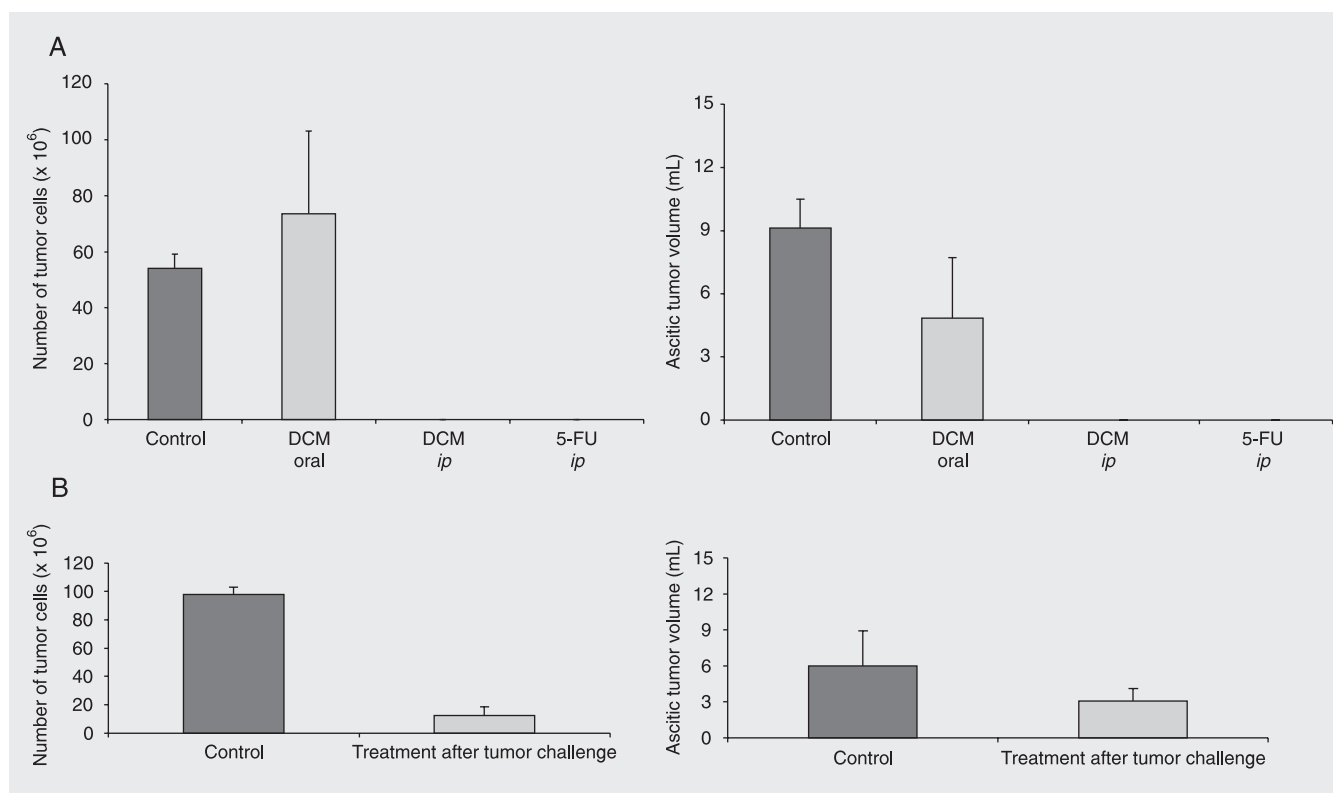


Figure 2. *A*, *In vivo* tumor cytotoxicity assay. The relative number of tumor cells and the ascitic volume were determined following daily oral and intraperitoneal (*ip*) administration once a day for 7 consecutive days of 5 mg/kg of the dichloromethane (DCM) fraction of the *Vernonia scorpioides* leaf extract, the standard reference drug 5-fluoro-uracil (5-FU, 20 mg/kg), and 0.9% saline (control) immediately after inoculation of 5×10^6 Ehrlich ascitic tumor cells. The mean values obtained from one experiment ($N = 6$ in each group) are presented. No significant difference in cell number was noted between the mice treated by gavage and the control group, as determined by analysis of variance using the F-test ($P \leq 0.05$). *B*, Effect of *ip* treatment with the DCM fraction after ascitic tumor challenge. Treatment was applied daily starting 3 days after the tumor challenge. The control group received 0.9% saline. The relative number of tumor cells and ascitic volume were calculated. The mean values obtained from one experiment ($N = 6$ in each group) are presented. A significant difference in number of cells was noted between the mice treated with the DCM fraction and the control group, as determined by analysis of variance using the F-test ($P \leq 0.05$).

Effect of DCM fraction on solid Ehrlich ascitic tumor development

Figure 3 shows the development of a solid tumor in the footpad after *ip* treatment with DCM. This fraction did not reduce the solid tumor volume when systemically inoculated, indicating that there was no indi-

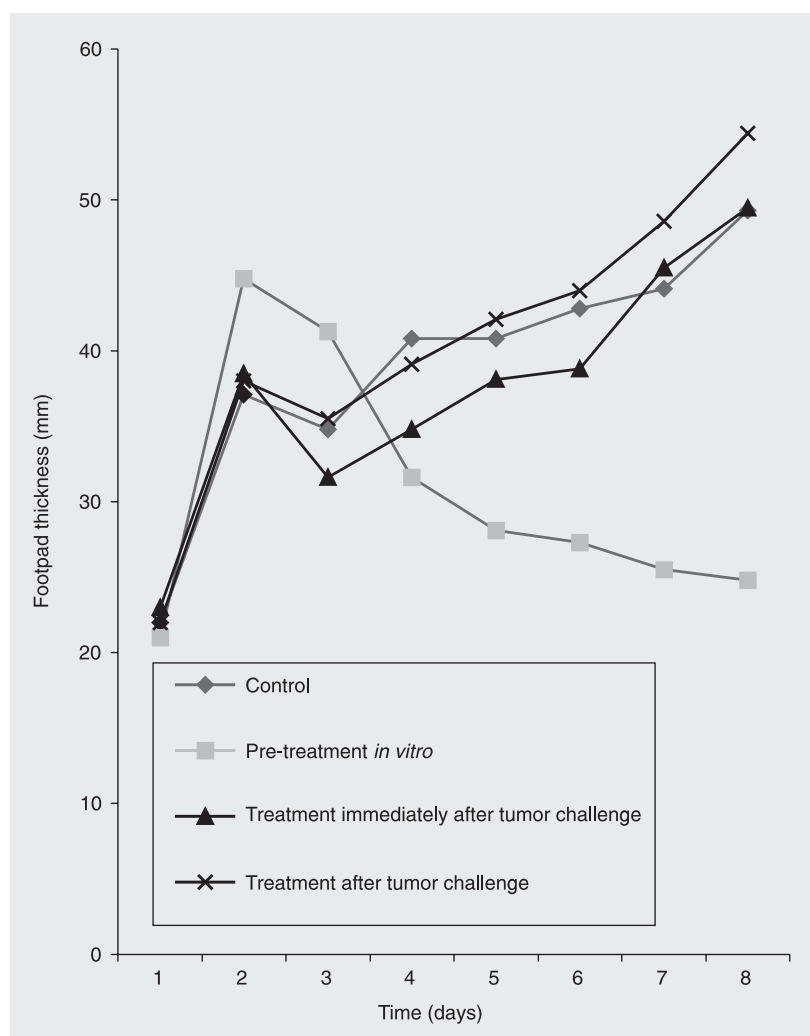


Figure 3. Effect of the dichloromethane (DCM) fraction of *Vernonia scorpioides* on solid tumor induced by Ehrlich's ascitic tumor (EAT) cells in mice. Tumor growth on the footpad was determined daily for 7 days following *ip* administration of the DCM fraction and 0.9% saline (control) immediately and 3 days after the tumor challenge. EAT cells pre-treated *in vitro* with the DCM fraction were also used for solid tumor induction. The mean values obtained from one experiment (N = 6 in each group) are presented. No significant difference was noted between the treated groups and the control group, as determined by analysis of variance via the F-test ($P \leq 0.05$). *In vitro* pre-treatment of the tumor cells with the DCM fraction inhibited tumor growth and after 3 days the solid tumor volume was significantly lower ($P \leq 0.05$, Dunnett test) than that of the saline-treated control.

rect effect of DCM, probably due to the fact that the active compounds did not reach the tumor. Footpad thickness was measured for a period of 10 days and was not reduced when the DCM fraction was inoculated immediately or 3 days after the injection of tumor cells into the animals' paws compared to control. When the tumor cells were pre-treated with the DCM fraction before implantation into the animals' footpad there was no solid tumor development.

Analysis of peritoneal leukocytes

Figure 4 shows the number of polymorphonuclear leukocytes present in the peritoneal cavity of EAT-bearing mice treated for 30 days with the DCM fraction and the standard reference drug 5-FU, compared with the number of cells present in the peritoneal cavity of normal mice treated with 0.9% saline. A significant difference was observed between the group treated with 5 mg/kg DCM fraction, the group treated with 5-FU, and normal mice as determined by analysis of variance via the F-test ($P \leq 0.05$). This profile is very different from the expected one, as shown by the lymphocyte count. The EAT-bearing mice treated with 0.9% saline died after 10 days.

The data in Figure 4 confirm the increased influx of leukocytes to the peritoneal cavity and illustrate the profile of inflammatory cells in each group. It was possible to compare the different types of leukocytes. Figure 4A shows the peritoneal cells, predominantly macrophages, from tumor-bearing mice treated with the DCM fraction for 30 days, Figure 4B shows a reduced number of leukocytes from tumor-bearing animals treated with 5-FU, and Figure 4C shows the results for control animals.

Morphological analysis of the spleen

Significant increases in spleen size were observed in the mice treated with the DCM

fraction compared to normal mice and mice that only received tumor cells. Histological examination of the spleens of mice treated with the DCM fraction demonstrated a significant increase in all white pulp areas, with an increased number of cells in the germinal center and lymphatic vessels.

Discussion

Plant-derived products such as vincristine, vinblastine, taxol, and many other substances in use today are excellent sources for the development of new anticancer chemotherapies. The *Vernonia* species belongs to the Asteraceae genus, which produces characteristic compounds such as sesquiterpene lactones with several biological properties, including anti-tumoral properties (23). In recent years, a significant number of papers have described the influence of these compounds on the growth of tumors. For example, parthenolide has been considered to be a cytotoxic or cytostatic drug and has been proposed as a complementary therapeutic drug in cancers with constitutively active NF- κ B, which mediates the expression of pro-metastatic, pro-angiogenic, anti-apoptotic, and multi-drug resistance genes (24).

Several studies focusing on the immunomodulatory effect of plant extracts have been conducted, showing a prominent role of extracts causing prophylactic growth inhibition of tumor cells (25,26). Among the *Vernonia* species, some cytotoxicity has been described for isolated sesquiterpene lactones from *V. cinerea* (15), *V. lasiopus* (16) and *V. amygdalina* (27). Some active *Vernonia* extracts have also been found, such as the water-soluble extract of edible *V. amygdalina* leaves (9), and the water extract from the roots of *V. kotschyana* (20) and *V. anthemintica* (10).

V. scorpioides has been studied in different biological tests and has shown cytotoxic effects in most of them. Previous studies

have shown that the DCM and hexane fractions from the extract have fungicidal and bactericidal properties (2). Also, many *Vernonia* species are used as trypanocidal (28), antiplasmodial (29) and anthelmintic agents (30).

The results presented here confirm the toxic properties of the DCM fraction, a dark-green semi-solid material rich in pigments that was characterized by infrared spectroscopy, showing strong absorption at ca. 1725 cm^{-1} , characteristic of the presence of lactones. The NMR spectra showed the presence of acetyl and carbonyl radicals as indicated by intense signals at 168-170 and 20 ppm. Also some hydroxyl/methoxyl derivatives (68 and 55 ppm, intense peaks) and double bonds (124-128 ppm) are probably present. Acetylated sesquiterpene lactones

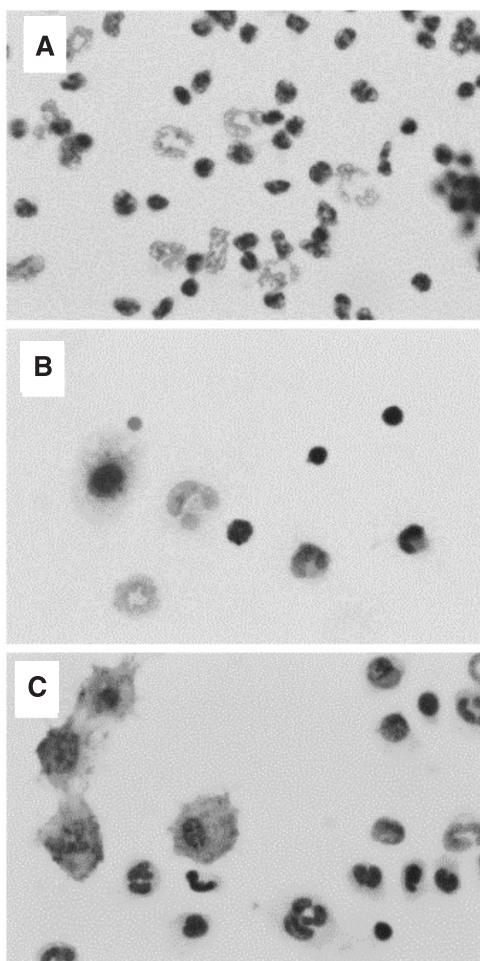


Figure 4. Mouse peritoneal leukocytes (hematoxylin staining). A, Tumor-bearing animals treated with the dichloromethane fraction of *Vernonia scorpioides* for 30 days. B, Tumor-bearing animals treated with 5-fluoro-uracil. C, Control saline-treated animals. Magnification = 100X.

are commonly found in *Vernonia* species (15).

The DCM fraction totally inhibited tumor development when in direct contact with tumor cells, as also demonstrated by *in vitro ip* administration, *in loco*, to the ascitic tumor. Inoculation of the DCM fraction (5 mg/kg) increased the animals' lifespan and maintained their body weight throughout the 30 days of treatment. When applied immediately after inoculation of the tumor cells *in vivo*, it totally abolished tumor development, and when treatment was started 3 days after the tumor challenge, tumor development was decreased, supporting the existence of a probable antineoplastic activity.

Treatment by the oral or *ip* routes did not reduce the solid tumor volume, suggesting that some enzymatic routes can deactivate the systemic route. Another property that may exacerbate this cytotoxic activity is the ability of the fraction to increase neutrophil influx into the peritoneal cavity, unlike the conventional immune-suppressing profile of standard chemotherapy. When the tumor developed in the ascitic form, daily inoculation of the DCM fraction led to an increase in

peritoneal leukocytes, and the tumor did not grow, with an increase in the animals' lifespan and maintenance of their body weight during the 30 days of treatment. These results show that, in addition to tumoricidal effects, the DCM fraction also has inflammatory activity. Sur et al. (31) showed that the seeds of *Trigonella foenum graecum* (Fenugreek) inhibited tumor growth by about 70% and also increased the influx of inflammatory cells to the peritoneal cavity. The present results have advanced our understanding of the antitumoral potential of the DCM fraction *V. scorpioides*. Investigations into the mechanism of action of the tumor-reducing activity and also of the compounds responsible for the activity of the DCM fraction are currently in progress.

Acknowledgments

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