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Artigo

Cytotoxic activity of the sub-fraction 2125 from *Vernonia scorpioides* against Sarcoma 180 tumor cells in mice

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RESUMO: "Ação citotóxica da sub-fração 2125 de *Vernonia scorpioides* contra células do Sarcoma 180 em camundongos". O efeito da sub-fração SF-2125 obtida do extrato das folhas de *Vernonia scorpioides* foi investigado em camundongos portadores do tumor ascítico Sarcoma 180 (S180). Os animais foram tratados com SF-2125 na concentração de 5 mg/kg, administrada por vias intraperitoneal e intravenosa durante o desenvolvimento do tumor. O tratamento com SF-2125 5 mg/kg i.p. aumentou o tempo de vida dos animais, manteve seu peso corporal e o tumor na forma ascítica não teve desenvolvimento. O tratamento intravenoso não reduziu o volume do tumor.

Unitermos: Sarcoma 180, sub- fração 2125, *Vernonia scorpioides.*

ABSTRACT: The effect of the selected sub-fraction SF-2125 of the *Vernonia scorpioides* leaf extract on Sarcoma 180 (S180) ascitic tumor-bearing mice was investigated. The animals were treated with SF-2125 at a concentration of 5 mg/kg, administered intraperitoneally and intravenous during the development of the tumor. Treatment with SF-2125 5 mg/kg i.p. increased the lifespan of the animals, maintained their body and the ascitic tumor showed no development. Intravenous treatment did not reduce the tumor volume.

Keywords: Sarcoma 180, sub- fraction 2125, *Vernonia scorpioides.*

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 (Cabrera,1980). The genus *Vernonia* produces characteristic compounds such as sesquiterpene lactones, with several reported biological activities, such as fungistatic (Krishna Kumari et al., 2003), and cytotoxic activities (Kuo et al., 2003). Previous studies of the *Vernonia scorpioides* crude extract have shown moderate bactericide activity and mild wound healing effects (Leite et al., 2002; Biavatti et al., 2007). We observed a pro-inflammatory profile of the crude extract from *Vernonia scorpioides* in healthy skin tissue (Dalazen et al., 2005). In our previous work we investigated the effect of the dichloromethane fraction (DCM) from the crude extract on Ehrlich ascitic and solid tumors in mice. Dichloromethane fraction showed high cytotoxicity against tumor cells, especially when applied *in loco* to the tumor development (Pagno et al., 2006). This promising antitumoral result obtained with the DCM fraction led us to partition it, in order to obtain fractions of increasing polarity and the ethyl acetate sub-fraction (SF 2125) showed the highest cytotoxicity against Sarcoma 180 ascitic tumor cells when applied *in loco* to the tumor development.

MATERIAL AND METHODS

Extract, fractions and sample preparation

Flowers and fresh leaves (600 g) of the plant were macerated with 6,000 mL of ethanol for seven 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. To the crude extract

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obtained, water (600 mL) was added and the extract was submitted to liquid-liquid fractioning using solvents with increasing polarities. The respective fractions obtained were nominated: (HEX, 1.16 g), dichloromethane (DCM, 420 mg), ethyl acetate (EA, 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 chromatographic fractionation using silica flash (0.04-0.063 mm) and increasing gradient of hexane-ethyl acetate as eluent. The biomonitoring of expressive fractions obtained was done by dissolving aliquots in saline solution using up to 2% of Tween 80 with the aid of an ultrasonic bath (10 min) in the following concentrations: 200, 100, 50, 30, 15 and 5 mg/kg in a maximum final volume of 200 µL, and were frozen until the day of application**.**

The DCM fraction and the subsequent active sub fraction (SF 2125) were characterized by IR and NMR spectroscopy, and its spectra were recorded in a Bomem FT-IR (from UNIVALI) and in a BRUKER AVANCE 400 (NMR laboratory from Chemistry Department -UFSCar/SP), respectively.

Animals

Adult male inbred Swiss mice (8 weeks), weighing 20-30 g, maintained under standard environmental conditions, were used. They were fed with a standard diet and 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.

The S180 tumor cells were maintained in the ascitic form by passages in syngenic Swiss mice weekly, with transplantation of $5x10^6$ tumor cells intraperitoneally (ip.). The ascitic fluid was removed by opening the belly and collecting all the fluid using a sterile syringe. Ascitic tumor cell counts were carried out in a Neubauer hemocitometer, using the Trypan blue dye exclusion method. The animals used for the experiment received i.p. 200 µl of a suspension containing $5x10^6$ tumor cells, according to a previous study (Matsuzaki et al., 2003).

Treatment of animals

After intraperitoneally implanting the tumor cells, three groups of mice (8 mice per group) were treated with 5 mg/kg, intraperitoneal (i.p.) or intravenous (i.v.} in a vein from the tail, the positive control group received 5-fluoro-uracil (5-FU) in saline solution 0.9% (20 mg/kg i.p. or i.v.) (Christina et al., 2003) and the negative control group received saline solution 0.9% in the same via and final volume. The body weights of the mice were measured daily, until their death. The only animals to survive (those which received 5 mg/kg of SF-2125) were sacrificed 25 days after the start of the treatment.

Effect of SF-2125 on the ascitic tumor

Six groups of eight mice were used. Treatment was begun immediately after inoculation of the tumor cells. Group 1 received 5 mg/kg of SF-2125 i.p., group 2 received 5-fluoro-uracil (5-FU) 20 mg/kg i.p. and group 3 received saline 0.9% i.p. Group 4 received 5mg/kg of SF-2125 i.v., group 5 received 5-fluoro-uracil (5-FU) 20 mg/kg i.v. and group 6 received saline 0.9% i.v. After 7 days, the mice were sacrificed and all the ascitic fluid was harvested for volume measurement and ascitic tumor cell count using the Trypan blue dye exclusion method.

Determination of the cytotoxicity of SF-2125 on S180 cells in vitro

5×10^6 S180 ascitic tumor cells were pre-treated *in vitro* with saline solution (group 1), 5-FU 20 mg/L (group 2) or 5 mg/L of SF-2125 of *V. scorpioides* (group 3) for 15 minutes *in vitro,* and injected into the abdominal cavities of 8 mice. After 7 days, the mice were sacrificed and all the ascitic fluid was harvested for volume measurement and ascitic tumor cell count using the Trypan blue dye exclusion method.

Statistical analysis

The results were expressed as mean ± standard deviation. The statistical evaluation was carried out using the Dunnett's test and the F Test. The significance level was established at $p \leq 0.05\%$.

RESULTS

Evaluation of the lifespan and body weight of the animals after intraperitoneal and intravenous treatment with SF-2125

Animals inoculated with $5x10^6$ S180 cells i.p. were divided into 6 treated groups (n=8), receiving 5 mg/kg , the negative controls groups (saline solution) and the positive controls groups were treated with 5-fluoro-uracil (5-FU) 20 mg/kg. Mice were treated by intraperitoneal or intravenous via. Mice treated with the 5 mg/kg of SF-2125 i.p. survived all the treatment and were sacrificed after 25 days (Figure 1).

Treatment with a dose of 5 mg/kg SF-2125 determined less loss of body weight during the 10-day period (Figure 2).

Antitumor activity of SF-2125 immediately implantation of ascitic tumor cells

Figures 3 and 4 show the effect of intraperitoneal and intravenous administration of the SF-2125 against tumor cells, when treatment was initiated immediately

after the implantation of the tumor cells. The result observed was the suppression of 100% of the tumor cells and ascitic volume, when mice were treated with the sub-fraction by i.p. immediately after the inoculation of S180 cells. Intravenous treatment with SF-2125 did not reduce the tumor volume and number of tumor cells. The results found were similar to the saline treatment. Mice treated with 5-FU by both via did not develop ascitic tumor.

In vitro cytotoxicity

Figures 5 and 6 indicate that when the S180 tumor cells were pre-treated *in vitro* with 5 mg/kg SF-2125 or 20mg/kg of 5-FU for 15 minutes and injected into the abdominal cavity, there was no tumor development.

DISCUSSION

Many substances for anticancer chemotherapies in use today are plant-derived products, such as vincristine, vimblastine, taxol. We recently showed that when the tumor was developed in the ascitic form, daily intraperitoneal inoculation of DCM fraction from *Vernonia scorpioides* extract led to an increase in peritoneal leukocytes, and the tumor did not grow, increasing the animals' lifespan and maintaining the body weight during the 30 days of treatment. (Pagno et al., 2006). These results show that besides tumoricide effects, DCM fraction also has inflammatory activity. Our previous results confirmed the toxic properties of DCM fraction, which is characterized as a dark-green semi-solid material rich in pigments, and was characterized by infrared spectroscopy, showing strong absorption at ca 1725 cm^{-1} (Figure 7), characteristic of the presence of lactones. The ^{13}C NMR spectra (Figure 8) of the SF 2125 shown presence of acetyl and carbonyl radicals, due to the presence of intense signs at 168-170 and 20 ppm. Also some hydroxyl/methoxyl derivatives can be expected (68 and 55 ppm, intense peaks) and double bonds (124-128 ppm). Because of the strong presence of sesquiterpene lactones in this fraction, it was selected to perform this study. Acetylated sesquiterpene lactones are commonly found in *Vernonia* species (Kuo et al., 2003). The subfraction used in this experiment, SF2125, was obtained from DCM fraction, confirms the toxic properties of these compounds. In this study, we showed that the SF 2125, obtained from the chromatographic fractionation of the DCM fraction of *V. scorpioides* had the same activity showed by Pagno et al., 2006. Treatment with SF-2125 totally inhibited the development of the ascetic tumor S180 when in direct contact with tumor cells, as also demonstrated through *in vitro* intraperitoneal administration, *in loco*, to the ascitic tumor development. SF-2125 (5 mg/kg) inoculation increased the animals' lifespan

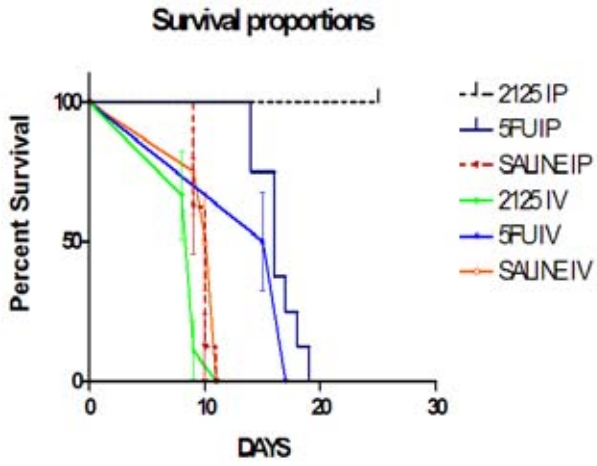

Figure 1. Kaplan-Meier survival curve of mice treated i.p. or i.v. with saline, 5-FU or 5mg/kg of SF-2125. Animals treated i.p. with the sub-fraction presented high lifespan (log-rank test $P<0.0001$).

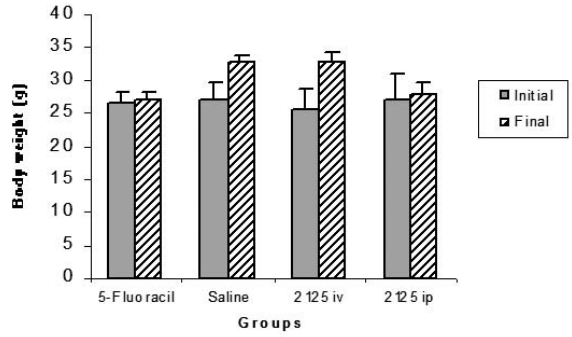

Figure 2. Initial body weight (first day of treatment) and final body weight (after 10 days) mice treated with saline (i.p.), 5-FU (i.p.) or 5mg/kg of SF-2125 i.p. and i.v. Mice treated with 5-FU and SF-2125 i.p. had low difference between initial and final body weight. SF-2125 i.v. and saline 0.9% treatment did not reduce tumor volume increasing the mice body weight. The difference of initial and final body weight was significant between the mice treated by i.v. and mice treated with saline, as determined by analysis of variance using F Test ($p \leq 0.05$).

and maintained the body weight during 30 days of treatment. When applied immediately after inoculation of the tumor cells *in vivo*, it totally abolished the tumor development, and when treatment began 3 days after the tumor challenge, the tumor development was decreased, sustaining a probable antineoplastic activity. The fact of the intravenous treatment did not reduce the ascitic tumor volume, suggest that some enzymatic route could be deactivating the systemic via, according with previous results, where mice treated by oral via with DCM fraction of *V. scorpioides* did not reduce Ehrlich´s ascitic or solid tumor (Pagno et al., 2006). Among the *Vernonia* species, some cytotoxicity has been described for isolated sesquiterpene lactones from *V. cinerea* (Kuo et al., 2003) and *V. lasiopus* (Koul et al., 2003). *Vernonia*

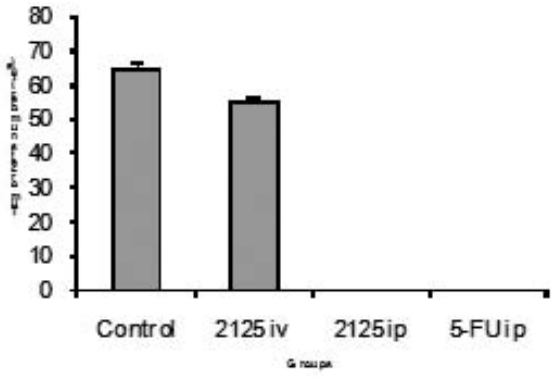

Figure 3

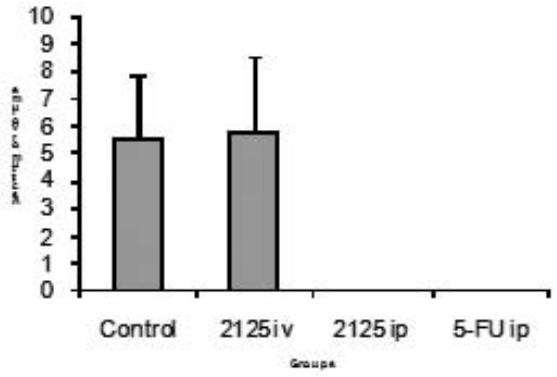

Figure 4

The relative number of tumor cells (Figure 3) and total ascitic tumor volume (Figure 4}following intravenous and intraperitoneal administration, once a day, with SF-2125 of *V. scorpioides*, the standard reference drug 5-fluoro-uracil, and saline 0.9% control, immediately after inoculation of the $5x10^6$ EAT cells and continuing for seven consecutive days. No significant difference in cell number and tumor volume between the mice treated by i.v. and the control group was noted, as determined by analysis of variance using F Test ($p \leq 0.05$).

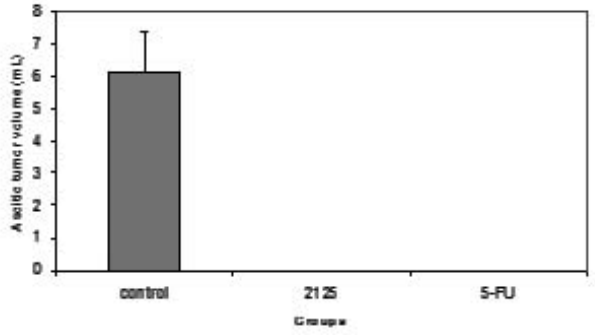

Figure 5

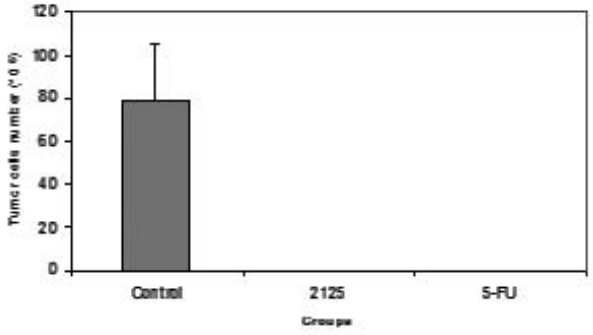

Figure 6

Total ascitic tumor volume (Figure 5) and viable tumor cells ($5x10^6$) (Figure 6) from the peritoneal cavity of the mice in the *in vitro* tumor neutralization assay. Tumor cells were pre-treated *in vitro* with saline, 5-FU or SF-2125 of *V. scorpioides* for 15 minutes and after were inoculated into the peritoneal cavity of the mice Control: Tumor cells pre-treated with saline 0.9%. The total ascitic volume of the animals was harvested after 7 days. The mean values obtained (n=8) are presented.

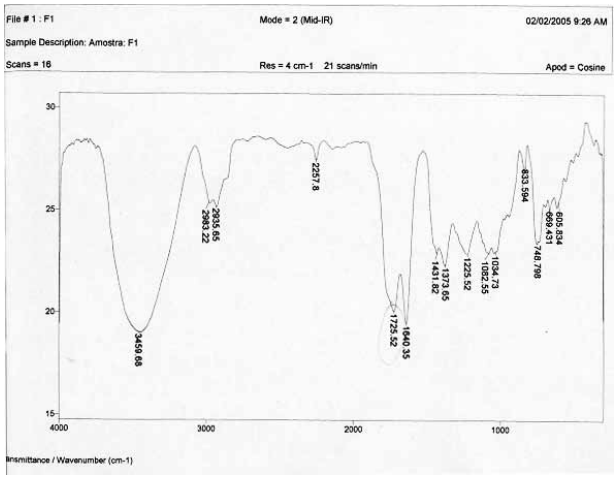

Figure 7. IR spectrum of DCM fraction.

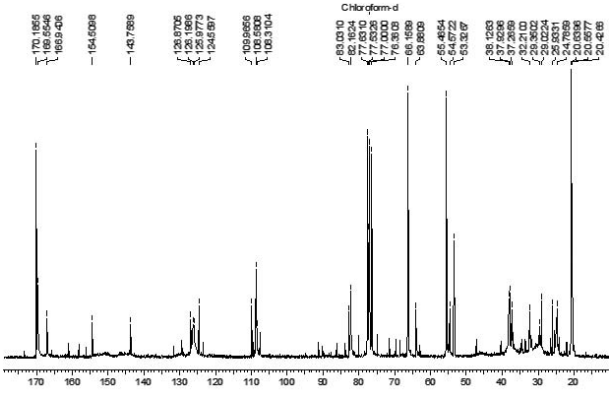

Figure 8. ^{13}C NMR spectrum of SF 2125 fraction. ($CDCl_3$, 75 MHz).

scorpioides has been studied in different biological tests and has shown cytotoxic effects in most of these. Previous studies show that dichloromethane and hexane fractions from the extract show fungicide and bactericide properties (Freire et al., 1996). Also, many *Vernonia* species are used as trypanocidals (Tchinda et al., 2002) and antihelmintics (Hordegen et al., 2003). We have now advanced our understanding of the antitumoral potential of *V. scorpioides* 2125 sub- fraction. Investigations into the mechanism of action for the tumor-reducing activity and also the compounds responsible for the cytotoxic activity are currently in progress.

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