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Study of the antitumor potential of *Bidens pilosa* (Asteraceae) used in Brazilian folk medicine

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Abstract

Aim of the study: *Bidens pilosa* (L.) (Asteraceae) is a medicinal plant traditionally used in Brazil for treating conditions that can be related to cancer. Therefore the present study was carried out to evaluate the antitumor activity of extracts obtained from the aerial parts of this plant species.
Materials and Methods: The crude hydroalcoholic extract (HAE) (water:alcohol, 6:4) and solvent fractions (chloroform = $CHCl_3$,ethyl acetate = EtOAc, methanol = MeOH) were assessed for cytotoxicity assay by the brine shrimp and hemolytic, MTT and NRU assays. The antiproliferative potential of the crude extract and fractions was investigated in vivo using the Ehrlich ascites carcinoma (EAC) in isogenic Balb/c mice that were administered intraperitoneally 150 and 300 mg/kg body weight per day for nine days beginning 24 h after tumor inoculation.
Results: In *in vitro* cytotoxicity using Ehrlich ascites carcinoma cell line assay $CHCl_3$ extract proved to be more toxic than the crude HAE with an IC_{50} of 97 ± 7.2 and 83 ± 5.2 μg/mL to NRU and MTT, respectively. Histomorphological evaluations indicated that the treatment with $CHCl_3$ and HAE extracts significantly reduced ($P < 0.05$) body weight, abdominal circumference, tumor volume, packed cell volume and viable cell count, when compared to EAC control group. Furthermore, nonviable tumor cell count increased significantly ($P < 0.01$) only under treatment with $CHCl_3$ or HAE, and this was accompanied by a marked percentage increase in life span (54.2 and 41.7%, respectively). Biochemical assays revealed that $CHCl_3$ and HAE extracts were also able to decrease serum LDH activity (39.5 and 30.6%) and GSH concentration (94.6 and 50.7%) in ascitic fluid, respectively.
Conclusion: The chloroform fraction showed the best and methanolic the worst antitumor activity.

Keywords: Medicinal plants; *Bidens pilosa*; Cytotoxicity; Antitumor

1. Introduction

Brazil is a rich source of medicinal plants and a number of plant extracts are used against diseases in folk medicine, but only a few of these have been scientifically investigated. Plant-derived natural products such as flavonoids, terpenes, alkaloids, and so on have received considerable attention in recent years due to their diverse pharmacological properties, including cytotoxic and cancer chemopreventive effects (Di Carlo et al., 1999).

According to Cragg and Newman (2000), over 50% of the drugs in clinical trials for antitumor activity were isolated from natural sources or are related to them. Several plant products have been tested for antitumor activity and some of these, such as vincristine and taxol are now available as drugs of choice. The rich and diverse plant resources of the Amazon region are likely to provide effective antitumor agents. One of the best approaches in the search for antitumor agents from plant resources is the selection of plant based on ethnomedical leads, and testing the selected plants' efficacy and safety through mod-

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ern scientific methods. The study of the traditional indigenous medical practices in Amazonia and Southern Brazil brought to light ethnomedicinal use of *Bidens pilosa* Linné (Asteraceae) to treat certain tumors (Franco and Fontana, 2004). This plant is also known as an antitumor agent in Cuba and The Bahamas (Valdés and Rego, 2001).

Bidens pilosa is a widely occurring plant species in tropical regions and it has several popular names according to the place where it is found. In Brazil it is generally known as Picão-preto and it is widely used as folk medicine by indigenous people to treat a variety of illnesses including pain, fever, angina, diabetes, edema (water retention), infections and inflammation (Vasquez, 1990; Duke and Vasquez, 1994; Valdés and Rego, 2001). In addition, in the Amazon and South Brazil regions, infusion and hydroalcoholic solutions of *Bidens pilosa* are also regarded as useful in the treatment of malaria (Brandão et al., 1997) and even tumors (Alvarez et al., 1996; Kviecinski et al., 2008). Also, *Bidens pilosa* is a popular ingredient in herb teas in China (Chiang et al., 2004).

Phytochemical analyses of *Bidens pilosa* revealed a broad chemical constitution, and it was previously believed that this fact could explain its wide use. The main compounds already isolated from the plant are polyacetylenic and flavonoid substances (Brandão et al., 1997). Polyacetylenes are hydrocarbons that strongly absorb long-wave UV radiation, and their medicinal activity is altered upon exposure to light (photoactivation) (Wat et al., 1980). They have been reported to possess cytotoxic effects on parasites (Brandão et al., 1997) and modified cells in culture (Alvarez et al., 1996). The specific flavonoids present in *Bidens* have not been fully elucidated, although luteolin, quercetin and some others have been isolated so far (Chiang et al., 2004). Interestingly, quercetin has been shown to suppress tumors in mice (Devipriya et al., 2006).

Taking these findings into account, the present work was carried out to evaluate the antitumor activity of extracts of the aerial parts of *Bidens pilosa* against the Ehrlich ascites carcinoma in cell culture and in isogenic Balb/c mice.

2. Materials and methods

2.1. Plant samples and extract preparation

Bidens pilosa L. was collected at the Jureia Botanical Reserve (Atlantic Forest), state of São Paulo, Brazil. The plant was identified by Inês Cordeiro, and a voucher specimen (M.H. Rossi SP384167) was deposited at the Herbário do Estado Eneida P.K. Fidalgo, of the Botanical Institute, São Paulo, Brazil. This study followed the international, Brazilian and institutional rules concerning the biodiversity rights.

Dried aerial part of this plant was powdered and macerated in water–ethanol solution (6:4) at room temperature for 10 days and was reserved as crude hydroalcoholic extract (HAE). Another part of the dried whole plant was also powdered and exhaustively extracted with ethanol–water solution (9:1) at room temperature for 3 days (3 times), and the solvent was eliminated under low pressure to obtain a dried ethanol extract, and 8.3% (w/w) yield of extracts in terms of starting crude material was partitioned for fractions assays. Three fractions were obtained from dried ethanol extract by performing fast silica gel 60 column chromatography with vacuum and applying solvents with different polarities. The fractions were named chloroform ($CHCl_3$), ethyl acetate (EtOAc) and methanol (MeOH) fraction according to the respective solvent. The extracts were kept at room temperature in a dryer device protected against light.

2.2. Toxicity study

2.2.1. Brine shrimp assay

Brine shrimp (*Artemia salina* Leach) eggs were hatched in a beaker filled with seawater under constant aeration. After 48 h the nauplii were collected by pipette against a lighted background. Ten nauplii were transferred to each well of 24-well plates containing the samples. The extract concentration ranged from 10 to 1000 μg/mL and positive control and substance reference were $K_2Cr_2O_7$ and quinidin sulphate, respectively. The plates were maintained under illumination. Survivors were counted after 24 h of incubation and the percentage of deaths at each dose and in the control (seawater plus vehicle) was determined according to Meyer et al. (1982).

2.2.2. Hemolytic assay

The hemolytic activity of *Bidens pilosa* extracts was investigated following the method proposed by Costa-Lotufo et al. (2002). Each well of 96-well plates received 100 μL of 0.85% NaCl solution containing 10 mM $CaCl_2$. The first well was the negative control and contained only the vehicle (DMSO 10%), while the second well contained 100 μL of test extracts diluted by 50%. The extracts were tested at concentrations ranging from 10 to 2500 μg/mL. The serial dilution continued until the 11th well. The last well received 20 μL of 0.1% Triton X-100 (in 0.85% saline) to obtain 100% hemolysis (positive control). Then, each well received 100 μL of a 2% suspension of mouse erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the released hemoglobin was measured spectrophotometrically by absorbance at 540 nm.

2.3. Endpoints of cytotoxicity

The cytotoxic effect of *Bidens pilosa* extracts and doxorubicin, expressed as cell viability, was assessed using the murine-derived Ehrlich ascites carcinoma (EAC) cell line. The EAC cells were grown in suspension in RPMI medium, supplemented with 10% heat-inactivated fetal calf serum, 100 IU penicillin/mL and 100 μg streptomycin/mL in a humidified incubator with a 5% CO_2 atmosphere at 37 °C. The cells were plated onto 96-well plates at a density of 3×10^4/mL. The medium was removed 48 h after cell seeding and replaced with one containing test extract (50–1000 μg/mL) or doxorubicin (0.1–25 μg/mL), initially dissolved in methanol and then diluted in RPMI. The final concentration of methanol in the test and control media was 1%. The cells were exposed for 24 h to the test medium with or without *Bidens pilosa* extracts. Each extract concentration was tested in eight replicates and in three exper-

iments. At the end of incubation, two independent endpoints of cytotoxicity, neutral red uptake and MTT reduction, were evaluated and each one is presented in the form of IC_{50}.

2.3.1. Neutral red uptake (NRU)

Neutral red has been shown to produce changes in the structure of lysosomes as well as bringing about lysosomal enlargement and vacuolation when the cell dies. Consequently, the neutral red uptake assay was proposed by Borefreund and Puerner (1984) for the evaluation of cellular toxicity. Briefly, after 4 h of incubation with serum-free medium containing 50 μg of neutral red/mL, the cells were washed quickly with PBS and then 0.1 mL of an aqueous solution of 1% (v/v) acetic acid:50% (v/v) ethanol was added to each well to extract the dye. After rapid shaking in a microtitre plate shaker, absorbance was read at 540 nm.

2.3.2. Tetrazolium salt assay (MTT)

The MTT assay is a test of metabolic competence based upon assessment of mitochondrial performance, relying on the conversion of a yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). The cells were incubated with RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/mL streptomycin and 100 U/mL penicillin at 37 °C with 5% CO_2. For experiments, cells were plated in 96-well plates (10^6 cells/well in 100 μL of medium). After 24 h, the extracts (50–1000 μM) were added, dissolved in DMSO. Doxorubicin (0.01–0.58 μg/mL) was used as a positive control. At the end of 72 h incubation, the medium in each well was replaced by fresh medium (200 μL) containing 0.5 mg/mL of MTT. Three hours later, the formazan product of MTT reduction was dissolved in DMSO, and absorbance was measured using a multi-plate reader. The effect of extracts was determined as the percentage of reduced dye in the control samples at 550 nm.

2.4. Antitumor activity in vivo

2.4.1. Animals

Male isogenic Balb/c mice, weighing 20 ± 5 g, were housed under controlled conditions (12 h light–dark cycle, 22 ± 2 °C, 60% air humidity), and had free access to standard laboratory feed and water. All animals were allowed to acclimatize for at least 5 days prior to the first treatment. Animals fasted for 12 h before experiments, but water was allowed *ad libitum*. All animal procedures were conducted in accordance with legal requirements appropriate to the species (NIH publication #85.23, revised in 1985) and with the authorization of the local Ethics Committee (365/CEUA and 23080.016010/2005-32/UFSC).

2.4.2. Experimental protocol

The animals were divided into seven groups ($n = 12$). The normal group was not inoculated with tumor cells, while six groups were injected with EAC cells (0.2 mL of 5×10^6 cells/mice) intraperitoneally. This was taken as day 0 and the experimental treatment started 24 h later. From the 1st day, 100 μL/mouse per day of sterile saline was administered intraperitoneally to the negative control group (EAC-bearing mice). MeOH, EtOAc or $CHCl_3$ extracts at doses of 150 mg/kg and HAE at doses of 300 mg/kg were administered each day to the treated groups and the standard drug doxorubicin (DOX, 1.2 mg/(kg day)) was administered to each animal from the positive control group. The pharmacological treatment lasted 9 days. Twenty-four hours after the last dose, six mice from each group were sacrificed for the study of the antitumor activity. The rest of the animal groups were kept to check the survival time of EAC tumor-bearing hosts.

2.4.3. Tumor growth response

The antitumor effects of the extracts were determined by the change in body weight and abdominal circumference, mean survival time (MST) and percentage increased life span (% ILS). The MST of each group containing six mice was identified by recording the mortality on a daily basis for 30 days, and the % ILS was calculated using the following equations (Mazumder et al., 1997): MST = day of the first death + day oh the last death/2; ILS (%) = [(mean survival time of treated group/mean survival time of control group) − 1] × 100.

The effects of *Bidens pilosa* extracts were also assessed by the determination of the body weight, tumor volume, packed cell volume and viable and nonviable tumor cell count of EAC-bearing mice by the Trypan blue incorporation method (Freshney, 1999).

2.4.4. Assay for lactate dehydrogenase release

The release of lactate dehydrogenase (LDH EC 1.1.1.27) enzyme is a well-known method for the quantification of cell damage. The enzymatic activity was estimated in serum by the method of Kornberg (1995). The reaction mixture consisted of 0.1 mL of nicotinamide adenine dinucleotide reduced disodium salt (NADH, 0.02 M), 0.1 mL of sodium pyruvate (0.01 M), plus 0.1 mL of serum, and this was made up to 3 mL with sodium phosphate buffer (0.1 M; pH 7.4). The change in the absorbance at 340 nm was recorded at 30 s intervals over 3 min and the enzyme activity was calculated using a molar extinction coefficient of 6.220 M^{-1} cm^{-1} before being expressed as IU/L.

2.4.5. Reduced glutathione assay (GSH)

The ascitic GSH was measured according to Anderson (1985). Immediately after ascitic sample collection, acid homogenates were obtained by the addition of 12% trichloroacetic acid (1:4, v:v), and then centrifuged at 15,000 × *g* for 5 min. Supernatants from the acid extracts were added to 0.25 mM 5,5′-dithio-bis (2-nitrobenzoic acid) in 0.1 M potassium phosphate buffer pH 8.0, and the formation of thiolate anion was immediately determined.

2.5. Statistical analysis

All the biochemical parameters mentioned above were measured in triplicate. Data are presented as mean ± S.D. and were analyzed by ANOVA complemented with the Tukey–Kramer

Table 1
The toxicity of *Bidens pilosa* extracts and doxorubicin measured by Brine shrimp toxicity (LC_{50}, μg/mL), hemolytic activity (IC_{50}, μg/mL) and *in vitro* cytotoxicity to EAC cells measured by MTT (IC_{50}, μg/mL) and NRU (IC_{50}, μg/mL) assays

Bidens pilosa extracts	Brine shrimp toxicity	Hemolytic activity	NRU	MTT
HAE	>1000	>1000	701.7 ± 5.3	944.2 ± 8.7
MeOH	>1000	>1000	>1000	>1000
EtOAc	>1000	>1000	234.0 ± 8.7	467.3 ± 6.9
$CHCl_3$	>1000	>1000	83.0 ± 5.2	97.0 ± 7.2
DOX	>1000	>1000	0.6 ± 0.1	0.4 ± 0.1

multiple comparison test. *P*-values less than 5% ($p < 0.05$) were considered to be statistically significant.

3. Results

3.1. Toxicity study

In order to verify whether the toxicity of *Bidens pilosa* is related to membrane disruption or unspecific mechanisms, different *Bidens pilosa* extracts and doxorubicin were tested for their ability to induce the lysis of mouse erythrocytes and *Artemia saline* death. The results obtained from the hemolytic and microcrustaceous assays showed that neither the examined extracts nor doxorubicin exhibited important unspecific toxicity (Table 1).

3.2. Endpoints of cytotoxicity

The cytotoxicity of *Bidens pilosa* extracts and doxorubicin on the Ehrlich tumor cell line was evaluated using the specific mitochondrial and lysosomal toxicity measured by MTT and NRU assays, whose results are presented in Table 1. The $CHCl_3$ extract was the most active in these assays, exhibiting IC_{50} values between fourfold and ninefold lower than other fractions. However, doxorubicin showed the lowest IC_{50} in the NRU and MTT assays. The EtOAc and HAE extracts also possess cytotoxic activity, although only extracts with an IC_{50} value lower than 200 μg/mL were considered active (Suffness and Pezzuto, 1991). The MeOH extract examined was considered inactive by these assays.

3.3. Antitumor activity in vivo

The $CHCl_3$ extract showed significant antitumor activity in EAC-bearing mice. The effects of *Bidens pilosa* extracts (at a dose of 150 mg/kg) on body weight, abdominal circumference, mean survival time, tumor volume, packed cell volume, tumor cell count (viable and nonviable cells) are shown in Table 2. Treatment with $CHCl_3$ and EtOAc extracts reduced significantly ($P < 0.05$) the tumor body weight, abdominal circumference, tumor volume, packed cell volume and viable cell count when compared to the EAC control group. Furthermore, nonviable tumor cell count increased significantly ($P < 0.01$) when compared to the EAC-bearing mice control group, but only when the treatment was done with $CHCl_3$ or HAE.

The mean survival time showed an important increase with $CHCl_3$ and HAE (69.6 and 41,7%, respectively), whereas the standard drug doxorubicin-treated group had a mean survival time of 100%.

The effect of the treatments with *Bidens pilosa* $CHCl_3$, EtOAc, HAE and MeOH extracts and DOX on percentage life span of EAC-bearing mice is shown in Table 3. ILS% increased significantly ($P < 0.001$) with $CHCl_3$, HAE and EtOAc when compared to the EAC control group, whereas DOX and normal groups had ILS = 100%. There was no response in the EAC-bearing group treated with MeOH extract.

Table 2
Effect of *Bidens pilosa* crude extract in EAC-bearing mice treated with 150 mg/kg (MeOH, EtOAc, $CHCl_3$), 300 mg/kg (HAE) and doxorubicin (DOX, 1.2 mg/kg) on body weight increase, abdominal circumference, mean survival time (MST), tumor volume, packed cell volume, and viable and nonviable tumor cell count of EAC-bearing mice and normal mice

Parameter	Normal mice	EAC control (5×10^6 cells)	DOX	$CHCl_3$	EtOAc	HAE	MeOH
Increase in body weight (g)	1.7 ± 0.5***	8.1 ± 1.7	2.2 ± 0.8***	6.09 ± 1.3*	6.1 ± 1.1*	6.9 ± 2.2	7.5 ± 2.2
Increase in abdominal circumference (cm)	0.2 ± 0.1***	2.7 ± 0.4	0.3 ± 0.1***	1.2 ± 0.4*	1.8 ± 0.3*	1.4 ± 0.4*	2.4 ± 0.4
Tumor volume (mL)	–	10.4 ± 2.1	–	5.8 ± 2.7**	7.1 ± 1.9*	7.3 ± 0.7*	10.1 ± 0.9
Packed cell volume (mL)	–	3.4 ± 0.5	–	2.4 ± 0.4	2.1 ± 0.3	2.01 ± 0.4	3.1 ± 0.5
Viable tumor cell count (10^7 cells/mL)	–	15.9 ± 3.4	–	8.6 ± 2.4*	11.2 ± 2.5*	9.7 ± 2.0*	13.0 ± 2.0
Nonviable tumor cell count (10^7 cells/mL)	–	1.1 ± 0.8	–	5.0 ± 0.3**	2.8 ± 0.5	3.5 ± 0.2**	1.8 ± 0.2
MST (days)	30.0***	11.5 ± 0.1	30.0***	19.5 ± 0.1**	15.0 ± 0.1*	16.3 ± 0.3*	12.0 ± 0.4

Note: Data are expressed as the mean of six mice ± S.E.M. (***), (**) and (*) significantly different in extracts-treated groups compared with the EAC-bearing mice ($P < 0.001$, $P < 0.01$ and $P < 0.05$).

Table 3
Effect of *Bidens pilosa* extracts on increased life span (ILS %), serum LDH activity and ascitic GSH contents in EAC-bearing mice treated with 150 mg/kg ($CHCl_3$, EtOAc, MeOH), 300 mg/kg (HAE) or doxorubicin (DOX, 1.2 mg/kg)

Bidens pilosa extracts	LDH activity	Ascitic GSH content	%ILS
Normal mice	578.5 ± 50***	NE	100
EAC-bearing mice	2607.7 ± 71	15.0 ± 0.8	0
DOX	803.9 ± 58***	NE	100***
$CHCl_3$	1025.4 ± 60**	5.1 ± 0.5***	54.2 ± 3.6***
EtOAc	1354.0 ± 68	8.5 ± 0.6***	34.8 ± 4.7***
HAE	1229.7 ± 35*	7.0 ± 0.6***	41.74 ± 3.5***
MeOH	1541.3 ± 40	15.0 ± 0.4	0

Note: Data are expressed as the mean of six mice ± S.E.M. (***), (**) and (*) means statistically differences compared to EAC-bearing mice ($P < 0.001$, $P < 0.01$ and $P < 0.05$), respectively.

Ehrlich ascites carcinoma injection alone in mice led to a marked increase in serum LDH activity (273.3%) when compared to the normal group. The treatment with $CHCl_3$, HAE and EtOAc extracts from *Bidens pilosa* resulted in a significant ($P < 0.01$ and $P < 0.05$) decrease in the activity of this enzyme, at 39.5, 30.6 and 27.2%, respectively, when compared to EAC-bearing mice (Table 3). Remarkably, DOX administration protected against the LDH rise as indicated by the serum enzyme activity with a reduction of 54.5%. There was no change in the LDH activity in the EAC-bearing group treated with MeOH extract.

Table 3 shows the levels of GSH detected in the ascitic tumor fluid of EAC-bearing mice with different treatments. The administration of *Bidens pilosa* extracts ($CHCl_3$, HAE and EtOAc) lead to a 94.6, 50.7 and 50.1% depletion of GSH in the tumor fluid of treated animals when compared to the negative control group ($P < 0.001$). The results obtained when the animals were treated with MeOH did not differ significantly from those of the EAC-bearing mice control group. Since the standard drug doxorubicin caused total inhibition of tumor development in inoculated mice it was not possible to evaluate the GSH ascitic level in this group.

4. Discussion and conclusions

The $CHCl_3$ extract showed important activity measured by endpoints of cytotoxicity MTT and NRU assays, indicating the presence of some cytotoxic compounds in this extract, which and these could be responsible for the strong *in vitro* activity. Interestingly, the EtOAc extract was two to three times less active compared to the $CHCl_3$ and HAE extracts, which better represent its traditional medicine use. Previous studies have demonstrated the cytotoxic activity of *Bidens pilosa* extracts against HeLa and KB carcinoma cell lines (Sundararajan et al., 2006).

Despite the results obtained from MTT and NRU assays, none of the tested *Bidens pilosa* extracts possessed any activity against *Artemia salina* nauplii or against mouse erythrocytes. These data suggest that the cytotoxic activity was not related to lytic properties or membrane instability induced by the extracts (Costa-Lotufo et al., 2002).

In addition, this study revealed that the crude hydroalcoholic extract and the three fractions of *Bidens pilosa*, a plant used in Brazilian folk medicine for the treatment of tumors, presented some cytotoxic activity. Further studies will be necessary for an improved characterization of this property in more extensive biological evaluations. It is possible that this activity is related to another mechanism of toxicity such as mitochondrial or lysosomal interactions since the results were obtained by the MTT and NRU assays. In this sense, the present study was carried out also to evaluate the antitumor effect of the crude hydroalcoholic extract and fractions of *Bidens pilosa* in mice inoculated with the same kind of tumor, the Ehrlich ascites carcinoma (EAC).

In EAC-bearing mice, a regular rapid increase in the ascites tumor volume was noted. Ascites fluid is the direct nutritional source for the tumor cells and a rapid increase in the ascitic fluid with tumor growth would be a means to meet the nutritional requirement for these cells (Prasad and Giri, 1994). The treatment performed with the $CHCl_3$ fraction decreased the tumor volume and tumor cell count, and increased the percentage of Trypan blue-positive stained dead tumor cells. The most reliable criterion for judging the value of any antitumor drug is the prolongation of the life span of animals (Clarkson and Burchenal, 1965). The $CHCl_3$ fraction increased the percentage of life span (%ILS) as shown in Table 3. It was found that this extract increases the life span of EAC-bearing mice by decreasing the nutritional fluid volume and combating tumor growth. The crude hydroalcoholic extract also reduced tumor volume and increased the %ILS more efficiently compared to $CHCl_3$. It is important to note that doxorubicin, the drug employed as the standard, caused total inhibition of tumor development.

The treatment of tumor-inoculated mice with doxorubicin also caused a strong reduction in plasma LDH activity when compared to the negative control (Table 3). Cancer cells produce and retain LDH to maintain growth of the tumor and the increased serum LDH level is well known as a common characteristic in humans and animals with malignant tumors (Wolf and Williams, 1973), and it is believed that this elevation is caused by enzyme leakage from dead cancer cells occurring during high tumoral cell turnover. In fact, data obtained through evaluating the LDH activity in EAC-inoculated mice confirmed the ability of this kind of tumor to induce a strong increase in the enzyme activity, since the levels were increased about fourfold when compared to the normal control group (Table 3). It was particularly interesting to observe that the tumor retraction caused by doxorubicin was associated with a 54.7% decrease in serum LDH activity compared to the EAC-group, suggesting that these levels became normalized. In this experimental evaluation doxorubicin appeared to present itself as a cure for Ehrlich carcinoma when compared to the EAC-group, suggesting that these levels became normalized. When the LDH activity was measured in animals treated with the $CHCl_3$ extract, a significant difference in this parameter was observed when compared to the EAC-group. The HAE extract also showed a down-regulation effect in LDH activity but this effect was more important than that observed with the $CHCl_3$ treatment. Since the $CHCl_3$ and HAE extracts were able to decrease LDH, it appears that they both have an antitumor effect.

Several researchers have already tried to correlate GSH levels with tumors. It has been reported, for instance, that many tumors can be considered GSH-dependent and it is suspected that cancer cells use GSH for protection against oxidative damage and increased levels of GSH in tumors are related to anti-chemotherapeutic effects and multidrug resistance (Shimura et al., 2000). Also, some studies have shown an increase in the sensitivity of these cells to cytotoxic agents through GSH depletion (Mitchell et al., 1989). It is possible to predict the efficacy of chemotherapy if the content of tumoral GSH is monitored by any method (Shimura et al., 2000). Data obtained on the fluid of EAC-inoculated mice revealed that the administration of *Bidens pilosa* MeOH extract failed to decrease the GSH levels in tumoral tissue in treated mice compared to non-treated mice (negative control). On the other hand, the administration of the $CHCl_3$ extract, which is supposed to possess a greater concentration of polyacetylene compounds with cytotoxic activity (Brandão et al., 1997) did cause a marked decrease in GSH levels compared to the EAC-group. In addition, the hydroalcoholic extract was able to significantly decrease GSH levels, and this response was more effective than that obtained with $CHCl_3$ extract. If we accept that the efficacy of chemotherapy is associated with a reduction of ascitic GSH contents, it is reasonable to suppose that the $CHCl_3$ extract has an antiproliferative effect.

The results of the Ehrlich ascites carcinoma assay, an experimental model with an antitumoral response strongly related to immunomodulation, suggest that this effect if due to a different mechanism than cytotoxicity. A recent *in vitro* study with *Bidens pilosa* infusion (Abajo et al., 2004) showed that this plant enhance cytokine production as interleukin-1 and TNFα, which are essential mediators of host inflammatory responses to natural immunity. It is important to note that the immune response has a decisive role in the death mechanism of tumoral cells. Therefore, these findings may shed some light on the possible mechanism of antiturmoral effect of *Bidens pilosa* fractions related to the regulation of immune parameters.

Finally, it is important to note that the crude water:ethanol extract that represent the traditional use of this plant, showed an antitumor activity only at a high dose (300 mg/kg), while $CHCl_3$ showed significant antitumor activity with half the administered dose.

In conclusion, our study suggests that the $CHCl_3$ extract, which do not correspond to the popular medicinal formulation of *Bidens pilosa*, showed the best antitumor activity, since it displayed important cytotoxicity *in vitro* (MTT and NRU assays), increased the life span of EAC-tumor bearing mice, and decreased both, serum LDH activity and the GSH content of the tumor fluid. However, the intraperitoneal way used in the present work do not correspond to the traditional oral administration but it represents a first approach of the experimental evaluation of an antitumoral activity of natural products. In addition, it is important to note that Mirvish et al. (1985) reported a weak esophageal cocarcinogen effect of elevated quantity of dried leaves from *Bidens pilosa* (50 g/kg in the diet) in rats exposed to methyl-*n*-amylnitrosamine. Other experimental models might be able to evaluate a cocarcinogenic risk.

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