RESEARCH ARTICLE

Brazilian *Bidens pilosa* Linné yields fraction containing quercetin-derived flavonoid with free radical scavenger activity and hepatoprotective effects

Maicon Roberto Kviecinski[1], Karina Bettega Felipe[1], João Francisco Gomes Correia[1], Eduardo Antonio Ferreira[1], Maria Helena Rossi[2], Fernando de Moura Gatti[2], Danilo Wilhelm Filho[3] and Rozangela Curi Pedrosa[1]*

[1]Departamento de Bioquímica, Universidade Federal de Santa Catarina, Florianópolis, Brazil; [2]Centro de Sanidade Animal, Instituto Biológico, São Paulo, Brazil; [3]Departamento de Ecologia e Zoologia, Universidade Federal de Santa Catarina, Florianópolis, Brazil

Bidens pilosa is a plant used by Amazonian and Asian folks for some hepatopathies. The hydroethanol crude extract and three fractions were assessed for antioxidant and hepatoprotective effects. Higher levels of scavenger activity on the 1,1-diphenyl-2-picrylhydrazyl radical, inhibition of deoxyribose oxidation and lipid peroxidation *in vitro* were detected for the ethyl acetate fraction (IC_{50} ~4.3–32.3 μg/ml) followed by the crude extract (IC_{50} ~14.2–98.0 μg/ml). The ethyl acetate fraction, again followed by the crude extract, showed high contents of total soluble polyphenols (3.6±0.2 and 2.1±0.2 GAE/mg, respectively) and presence of a quercetin-derived flavonoid identified as quercetin 3,3′-dimethyl ether 7-O-β-D-glycopyranoside. Both products were assayed for hepatoprotector effects against CCl_4-induced liver injury in mice. Markers of oxidative stress and hepatic injury were evaluated. The results showed that the 10-day pretreatments (15 mg/kg, p.o.) protected the livers against injury by blocking CCl_4-induced lipid peroxidation and protein carbonylation and the DNA fragmentation was decreased (~60%). The pretreatments avoided the loss of the plasma ferric reducing/antioxidant power and the elevation of serum transaminases and lactate dehydrogenase activities. The results suggest that the main constituents responsible for the hepatoprotective effects with free radical scavenger power associated are well extracted by performing fractionation with ethyl acetate. The findings support the Brazilian traditional use of this plant and justify further evaluations for the therapeutic efficacy and safety of the constituents of the ethyl acetate fraction to treat some liver diseases.

Keywords: *Bidens pilosa L.*; *hydroethanol maceration*; *ethyl acetate fractionation*; *free radical scavenger*; *hepatoprotection*; *CCl_4 toxicity*

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Free radicals may be causative factors in the etiology of some hepatopathies (1, 2). The oxidative stress can be induced by a variety of factors such as exposure to xenobiotics and including carbon tetrachloride (CCl_4). The CCl_4 has been used to study liver damage induced by free radicals in mice because the hepatic injury caused by this compound is quite analogous to its hepatotoxicity in humans (3).

In this sense, the free radical scavenger activity and the protective effects of several plants rich in antioxidants against CCl_4 hepatotoxicity have been reported (4, 5). *Bidens pilosa* Linné (Asteraceae) is a medicinal plant with a wide occurrence in the tropical regions and has a history of use by Amazonian Indians and Eastern medicine for several purposes including the treatment of liver diseases, malaria, and tumors (6–8). Previous studies have revealed a broad phytochemical constitution with little peculiarities according to the place where the plant is found. The major compounds already isolated from the plant are flavonoids and polyacetylenes (9).

Some promising results were already found when some isolated flavonoids were obtained from the Chinese

B. pilosa and assayed as a pool of total flavonoids for hepatoprotective effects (6). Here, the effects of *B. pilosa* cultivated in South America (Brazil) are examined. A crude hydroethanol extract was obtained and fractionated to yield three other fractions. The plant products were submitted to phytochemical evaluations monitored by assays *in vitro* and *in vivo* focusing on free radical scavenger power and hepatoprotective effects.

Material and methods

Plant

Bidens pilosa L. (Asteraceae) was collected at the Jureia Botanical Reserve (Atlantic Forest), São Paulo state, 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.

Extraction

The dried aerial parts of the plant were powdered and exhaustively macerated with ethanol-water solution (9:1) at room temperature for 3 days (three times). The solvent was eliminated under low pressure to obtain a dried hydroethanol crude extract (HCE), yielding 8.3% (w/w) in terms of starting material. After, by performing fast column silica gel 60 chromatography with vacuum and applying solvents in increasing polarity order, three fractions were obtained from the HCE. They were named chloroform fraction (f-$CHCl_3$), ethyl acetate fraction (f-EtOAc), and methanol fraction (f-MeOH) according to the respective solvent used. The fractionation with chloroform yielded 24.9%, while the fractionation with ethyl acetate yielded almost 36% and the methanolic one about 40% (w/w) in terms of the initial HCE. The phytoproducts were kept at room temperature in a dryer device protected against the light.

Determination of total polyphenol content (TPC)

The total polyphenol content (TPC) was determined in each plant sample according to the Folin-Ciocalteu method (10). The reaction mixture was composed of 0.1 ml of extracts, 7.9 ml of distilled water, 0.5 ml of Folin-Ciocalteu reagent, and 1.5 ml of 20% sodium carbonate. It was mixed and allowed to stand for 2 hours. The absorbance was measured at 765 nm and the results were expressed as gallic acid equivalents per milligram of extract (GAE/mg).

Chemical characterization on f-EtOAc

The f-EtOAc was submitted to reversed phase high performance liquid chromatography (HPLC; Shimadzu LC 6AD, J.T. Baker ODS C-18 column, 5 µm, 4.6 × 250 mm) using 5% aqueous acetic acid: methanol (65:35) as mobile phase at a flow rate of 1 ml/min and ultraviolet detector (Shimadzu, SPD 6AV). The mobile phase was prepared according to that employed by Felicio et al. (11). The spectra of nuclear magnetic resonance (NMR) were recorded on Brucker AC 200 in DMSO-d_6. The ultraviolet–visible (UV–vis) spectra were obtained in a Techcomp Model UV8500 II in MeOH, with a subsequent addition of the usual reagents: NaOAc, NaOH, $AlCl_3$, HCl, and H_3BO_3. The characterization was based on spectroscopic analyses and comparison with literature (12).

Free radical scavenging activity in vitro

The scavenging activity of *B. pilosa* (HCE, f-MeOH, f-EtOAc, and f-$CHCl_3$) was evaluated by using the 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenger method measured at 518 nm (13). The hydroxyl radical ($HO^{\cdot}$) was generated by the deoxyribose method, and its reaction with thiobarbituric acid (TBA) was indirectly detected by measuring the chromophore at 532 nm (14). Protection against induced lipid peroxidation (LPO) *in vitro* was assayed by using the TBA method as described by Chen and Tappel (15). The activities of the phytoproducts were evaluated in the range 0.5 to 500 µg/ml and the results were expressed by the half maximal inhibitory concentration (IC_{50}), which was the concentration (in µg/ml) of phytoproduct required to inhibit the generation rates of radicals or lipid peroxidation by 50%. Silymarin, a mixture of four flavonolignans that possesses a recognized free radical scavenger activity as well as a hepatoprotective effect, was used for the positive control (16).

Animals

Male Balb-c mice, weighing 20 ± 5 g, were housed under controlled conditions and had free access to laboratory chow and water. Animals were allowed to acclimatize for 5 days prior to the treatments. Animals were fasted for 8 hours before experiments but water was allowed *ad libitum*. All animal procedures were approved by the Ethics Committee of Universidade Federal de Santa Catarina, in accordance with the Principles of Laboratory Animal Care published by the National Institutes of Health (NIH Publication #86–23, revised 1996, US).

Hepatoprotective activity

Hepatotoxicity was induced in mice by an intraperitoneal administration of a single dose of CCl_4 (0.5 ml/kg) done on the day after following the 10-day pretreatments with HCE and f-EtOAc except in the normal control (NC), which received only the vehicle (4). For the experimental pretreatments, the dose was previously determined in a group of 12 animals allocated in four subgroups that received daily for 10 days HCE or f-EtOAc at 1.5, 15,

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Table 1. Groups of animals, experimental pretreatments, and challenge

Group	10-day pretreatment	Dose	Challenge on day 11
Normal control group (NC)	Vehicle	0.5 ml	None
Negative control group (NEG)	Vehicle	0.5 ml	CCl_4
Positive control group (SIL)	Silymarin	15 mg/kg	CCl_4
HCE group	HCE	15 mg/kg	CCl_4
f-EtOAc group	f-EtOAc	15 mg/kg	CCl_4

150, or 300 mg/kg, respectively. The safety of HCE and f-EtOAc was evaluated by observation of behavioral changes and monitoring of the weight of liver and body. After dose selection, mice were randomly divided into five groups ($n=12$), pretreated for 10 days, and then challenged according to Table 1.

Twenty-four hours after CCl_4 administration, blood was collected and the serum was separated for the evaluation of the biochemical markers of hepatic injury. After, the animals were sacrificed and the livers were immediately excised, weighed, and kept on ice for the analysis of biomarkers of oxidative stress and the endpoints of hepatotoxicity measured according to the following.

Biochemical markers of hepatic injury

The serum enzymatic activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were measured spectrophotometrically according to Reitman and Frankel (17) and Bergmeyer and Bernt (18) using a commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

Biomarkers of oxidative damage and antioxidant defenses

Malondialdehyde was used as a lipid peroxidation marker and measured in the livers by its capacity to react with thiobarbituric acid to produce thiobarbituric acid reactive substances (TBARS), a pigment that can be quantified spectrophotometrically (19). Results were expressed as contents of TBARS $\pm$ standard deviation (SD) (in nmol/mg of protein) using $\varepsilon=153$ mmol/L per cm. Oxidative damage to proteins was quantified as carbonyl protein contents. The method is based on the spectrophotometric detection of the end product of the reaction of 2,4-dinitrophenylhydrazine with carbonyl proteins to form protein hydrazones, and the results were expressed as nmoles of carbonyl group per milligram of protein using $\varepsilon=22$ mmol/L per cm (20).

The plasma ferric reducing/antioxidant power (FRAP) was determined in a reaction in which a Fe^{3+}/tripyridyltriazine complex is reduced by the plasma to produce a chromophore that is determined spectrophotometrically (21). Reduced glutathione (GSH) was measured by a spectrophotometric method through a reaction in solution done with hepatic extracts plus disodium hydrogen phosphate and 5, 5′-dithiobis-(2-nitrobenzoic acid; DTNB) to form a chromophore read at 412 nm using $\varepsilon=14.1$ mmol/L per cm. Results were expressed in µmol per g of tissue (22). The catalase activity (CAT) was determined by measuring its capacity to decompose hydrogen peroxide in solution under absorbance at 240 nm with time, at 37°C. The results were expressed in mmol/min per g of tissue and $\varepsilon=40$ mmol/L per cm (23).

Detection of DNA damage

DNA fragmentation was assessed using the comet assay (24). Samples (100 mg) of liver tissue were homogenized in 1 ml of phosphate-buffered saline (PBS; 1:9) and 10 µl of this solution was added to 70 µl of 0.75% low-melting point agarose. The cell/agarose mixture was added to a fully frosted microscope slide coated with a layer of 300 µl of normal-melting agarose (1%). The slides were immersed in a cold and freshly made lysis solution (2.5 mM NaCl, 100 mM ethylenediamine tetraacetic acid [EDTA], 1% Triton X-100, 10% dimethyl sulfoxide [DMSO], and 10 mM Tris, pH 10) for a minimum of 2 hours. Subsequently, the slides were incubated in freshly prepared alkaline buffer (300 mM NaOH and 1.0 mM EDTA, pH 13) for 30 minutes. The DNA was subjected to electrophoresis for 30 minutes at 280 mA and 25 V and then neutralized with 0.4 M Tris, pH 7.5. Finally, DNA was stained with ethidium bromide (2.0 µg/ml). Images of 100 randomly selected comets (50 from each of two replicate slides) were analyzed from each animal and their lengths (nuclear region + tail) were measured in arbitrary units. A DNA damage index (DI) was compiled according to tail size, from undamaged (0) to maximally damaged (400), resulting in a single DNA damage score for each animal and, consequently, for each group studied. Thus, the DI of each group ranged from 0 (completely undamaged) to 400 (maximum damage).

Statistical analysis

All the biochemical parameters were measured in triplicate. Data were expressed as mean $\pm$ SD. Comparisons and differences were denoted by means of Student's *t*-test and/or the ANOVA test complemented by the Tukey-Kramer multiple comparison test. The $P<0.05$ was regarded as significant.

Results and discussion

Bidens pilosa L. has a long history of use by Amazon Indigenous and Asian medicine as hepatoprotector. All parts of the plant are used in the medicinal preparations, which are mainly water–alcohol macerations in bottles or herbal teas used orally or topically (25). It is a constituent

of some liver elixirs commercially available in some countries. The HCE was prepared and evaluated here in an attempt to correspond to the popular use of the plant. Additionally, HCE was fractionated to yield three other products technically obtained. Then their antioxidant potential and hepatoprotective effects were evaluated.

Data depicted in Table 2 demonstrate that HCE and its three fractions presented a content of total polyphenolics. The results indicated that f-EtOAc followed by HCE have high contents of such compounds (Table 2). Further evaluations on f-EtOAc were done through HPLC and the chemical characterization was based on spectroscopic methods. Three major peaks were obtained after retention times of 3.10, 3.35, and 3.78 min at 258 nm. The majority of constituents of f-EtOAc ($t_R = 3.10$ min) were isolated by chromatography on a preparative scale and then submitted to spectroscopic analyses (^1H and ^{13}C NMR, UV–vis, and thin layer chromatography analysis). Spectral data obtained for this compound corresponded to those obtained for the flavonoid quercetin 3,3′-dimethyl ether 7-O-β-D-glycopyranoside described in the literature (12). These data are according with the strong antioxidant activity shown by f-EtOAc (Table 2 and Table 3).

The *in vitro* scavenging activity of the plant samples was first discerned by the DPPH radical assay, which primarily evaluates proton radical-scavenging ability. There were differences in terms of scavenging capacity on the DPPH radicals between the least effective fraction (f-$CHCl_3$) and the most effective one (f-EtOAc), which presented activity comparable to that of Silymarin (Table 2).

The HCE and the fractions were evaluated for their ability to inhibit hydroxyl radical generation using a deoxyribose reaction system. Other earlier studies that used this system to assess the effects of natural products reported that molecules able to inhibit the deoxyribose degradation are those that can chelate iron ions and render them poorly active in the Fenton reaction (26). The results showed that all the samples and Silymarin exhibited inhibitory activity against hydroxyl radical generation *in vitro*. Again, f-EtOAc and HCE were the most effective samples. With stronger action, f-EtOAc presented lower IC_{50} (Table 2).

The results demonstrate that some derivatives from *B. pilosa* were effective at protecting the rat liver homogenates against induced lipid peroxidation (LPO) *in vitro*. Most plant products assayed here showed a high lipid peroxidation inhibitory activity in a concentration-dependent manner *in vitro*, displaying $IC_{50} < 100$ μg/ml and again f-EtOAc exhibited the strongest activity among the examined samples. The higher contents of polyphenols in f-EtOAc were apparently associated with the higher antioxidant capacities *in vitro* (Table 2).

Previous studies have already demonstrated that the ethyl acetate fractions and the butanolic ones particularly, partitioned from the total crude extract of Chinese *B. pilosa*, could exhibit significant antioxidant activity *in vitro* (IC_{50} 14–17 μg/ml), comparable to that of α-tocopherol (27). Here, although sometimes superior, IC_{50}s were found for the Brazilian samples, data of f-EtOAc (IC_{50} 4.3–32.3 μg/ml) confirmed its expressive activity when compared to our positive control Silymarin (Table 2). Silymarin is an extract from *Silybum marianum* used in clinical practice in some countries for the treatment of toxic liver diseases. Its major active constituent is Silibinin, an antihepatotoxic flavolignan (28). Silymarin presented IC_{50} 7.6–13.9 μg/ml in the assays of the antioxidant power *in vitro*. It is interesting to note that data of f-EtOAc and HCE, related to the most important *in vitro* antioxidant activities, were associated with the higher contents of polyphenolics. As a consequence of these preliminary results, in the following, the activities *in vivo* were determined only employing these two samples.

The CCl_4 hepatotoxicity depends on the reductive dehalogenation of CCl_4 catalyzed by cytochrome P 450 in the hepatocyte leading to the generation of trichloromethyl peroxy radicals, an unstable complex reported to

Table 2. Activity of *Bidens pilosa* (hydroethanol crude extract and fractions) or Silymarin (0.5 to 500 μg/ml) on scavenging of 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH) and hydroxyl radicals ($^{\bullet}OH$), inhibition of lipid peroxidation *in vitro* (LPO), and total polyphenol content in the plant samples (TPC)

Sample	DPPH IC_{50} (μg/ml)	$^{\bullet}OH$ IC_{50} (μg/ml)	LPO IC_{50} (μg/ml)	TPC (GAE/mg)
HCE	98.0 ± 1.0[a]	14.2 ± 3.2[a]	35.8 ± 3.9[a]	2.1 ± 0.2
f-EtOAc	32.3 ± 6.7[a]	4.3 ± 1.5	25.1 ± 4.5	3.6 ± 0.2[b]
f-MeOH	102.9 ± 2.4[a]	18.5 ± 8.2[a]	90.4 ± 3.4[a]	1.0 ± 0.1
f-$CHCl_3$	248.0 ± 1.8[a]	33.0 ± 6.0[a]	166.3 ± 2.6[a]	0.8 ± 0.1
Silymarin	13.9 ± 0.7	7.6 ± 0.8	10.1 ± 4.5	–

Note: Hydroethanol crude extract (HCE), ethyl acetate fraction (f-EtOAc), methanol fraction (f-MeOH), and chloroform fraction (f-$CHCl_3$). Values are expressed as means ± SD ($n = 3$). IC_{50}: half maximal inhibitory concentration was obtained by linear regression.
[a]Denotes significant differences at $P < 0.05$ in comparison to the positive control (SIL).
[b]In comparison to HCE, f-MeOH, or f-$CHCl_3$.

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Table 3. Effects on the plasma ferric reducing/antioxidant power (FRAP), hepatic reduced glutathione content (GSH), and hepatic catalase activity (CAT) in mice pretreated with the hydroethanol crude extract (HCE) or the ethyl acetate fraction (f-EtOAc) from *Bidens pilosa* (15 mg/kg, p.o., 10 days), after CCl_4 on day 11 (0.5 ml/kg, i.p.), and controls

	Biomarkers of oxidative damage and antioxidant defenses		
Group	FRAP (mM)	GSH (μmol/g)	CAT (mmol/min.g)
NC	21.7 ± 0.7[a]	34.1 ± 3.1[a]	163.7 ± 29.6[a]
NEG	11.7 ± 0.8	8.1 ± 3.6	517.6 ± 67.9
f-EtOAc	20.7 ± 0.7[a]	30.1 ± 0.4[a]	366.4 ± 44.9[a,b]
HCE	18.9 ± 0.9[a]	25.8 ± 1.5[a,b]	447.1 ± 33.1[b]
Silymarin	24.5 ± 2.0[a]	29.8 ± 4.2[a]	441.6 ± 44.3[b]

Note: Values are expressed as means ± SD ($n = 6$).
[a]Denotes significant statistic differences at $P < 0.05$ in comparison to the negative control group (NEG).
[b]In comparison to the normal control group (NC).

be highly reactive. These radicals attack the membrane lipids causing their peroxidation, and may bind covalently to lipids and proteins causing damage to DNA and triggering processes leading to cell damage (3).

Fig. 1 shows some data about the toxicity of CCl_4. In the animals from the negative control, the levels of hepatic lipid peroxidation and carbonyl proteins were strongly increased. Data indicated that pretreatment with f-EtOAc and HCE is effective in preventing the increment of both lipid peroxidation and protein carbonylation levels in livers. Pretreatments with the *B. pilosa* products led normalization of the levels of these injury markers when compared to the normal control. Accordingly, the activities of f-EtOAc and HCE at 15 mg/kg were considered comparable to that of Silymarin (Fig. 1A/B). This dose was determined by previous observations for behavioral changes after oral administration of a range of doses of HCE or f-EtOAc repeatedly for 10 days. As we had observed *in vitro* a tendency for dose-dependent effects, the best dose in our case should reach maximal protective effects and, thus, in principle the dose should be the highest one that does not cause apparent toxicity. We found that none of the mice exhibited any abnormal behavioral responses at doses of 1.5 or 15 mg/kg, but mice that received 150 mg/kg or above showed slight toxic symptoms. These included inactiveness, loss of appetite, slow movement, dizziness, and erection of hairs. The administration of repeated daily doses of 15 mg/kg for 10 days did not influence the weight of the body or livers of the mice.

Sometimes a direct association between the antioxidant activity and the reducing power of certain plant extracts can be found. The reducing properties are generally associated with the presence of reducing agents, which have been shown to exert an antioxidant action by breaking the free radical chain by donating hydrogen atoms (29). Data in Table 3 demonstrate that plasma FRAP was found decreased in response to CCl_4 in

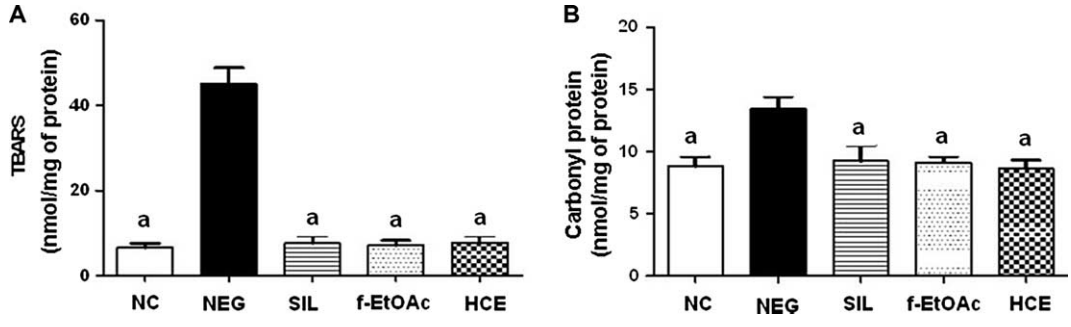

Fig. 1. CCl_4 led to increased levels of hepatic lipid peroxidation in treated mice from the negative control group (NEG) compared to the normal control group (NC). The ethyl acetate fraction (f-EtOAC), hydroethanol crude extract (HCE), and the positive control Silymarin (SIL) protected livers from pretreated mice against lipid peroxidation (A); animals from the negative control group (NEG) treated by CCl_4 presented increased levels of carbonyl proteins compared to the normal control group (NC). The f-EtOAC, HCE, and the positive control Silymarin (SIL) protected livers from pretreated mice against oxidative damage to proteins (B). All values are expressed as means ± SD, $n = 6$. [a]Denotes significant statistic difference compared to NEG ($P < 0.05$).

animals from the negative control. Once again, the data show that f-EtOAc and HCE prevented the CCl_4 toxic effects. The f-EtOAc caused total recovery in terms of the plasma FRAP to levels comparable to that of the normal control. The HCE was a little less active, while Silymarin exhibited the best activity in this respect (Table 3).

Pretreatment with f-EtOAc, HCE, or Silymarin (15 mg/kg, 10 days) was effective in avoiding the GSH depletion induced by CCl_4. The GSH levels in livers recovered in more than 85% when the pretreatments were done with f-EtOAc or Silymarin. Pretreatment with HCE caused about 75% recovery of GSH when compared to the negative control (Table 3). The CCl_4 led to increased CAT activity in livers of challenged mice. Only f-EtOAc was little effective in avoiding the elevation of CAT activity (Table 3). The results show a significant increase in the activities of serum AST, ALT, and LDH thus confirming the hepatocellular damage in mice challenged by CCl_4 (Table 4). After pretreatment with f-EtOAc or HCE, the activities of these enzymes decreased. Animals pretreated with f-EtOAc presented activities of transaminases and LDH closer to those of the normal group (Table 4). The favorable results of HCE and especially f-EtOAc from *B. pilosa* were confirmed by their prevention of DNA fragmentation induced by CCl_4 in hepatocytes of mice (Fig. 2). Fluorescence microscopy revealed a reduction ($\sim$30%) in terms of DNA fragmentation in samples of hepatic tissue from animals pretreated with Silymarin, and a near 50% reduction in samples from animals pretreated with HCE, when compared to the negative control group (NEG). Pretreatment with f-EtOAC was able to cause a decrease of around 60% in terms of DNA fragmentation when compared to the negative control group (Fig. 2).

Table 4. Effects of the hydroethanol crude extract (HCE) and the ethyl acetate fraction (f-EtOAc) from *B. pilosa* and Silymarin against the hepatotoxicity induced in mice by CCl_4

	Serum enzymes		
Group	AST (U/L)	ALT (U/L)	LDH (U/L)
NC	9.1 ± 1.1^{a}	29.2 ± 3.5^{a}	237.2 ± 38.3^{a}
NEG	68.4 ± 1.6	91.0 ± 1.5	541.4 ± 41.2
f-EtOAc	12.3 ± 1.4^{a}	33 ± 10^{a}	272.4 ± 33.7^{a}
HCE	$21.3 \pm 0.9^{a,b}$	$48.0 \pm 2.1^{a,b}$	295.1 ± 29.3^{a}
Silymarin	$24.4 \pm 1.4^{a,b}$	$53.0 \pm 2.5^{a,b}$	289.6 ± 30.5^{a}

[a]Denotes significant statistic differences at $P < 0.05$ in comparison to the negative control group (NEG).
[b]In comparison to the normal control group (NC).
Note: Values are expressed as means $\pm$ SD ($n = 6$).
Abbreviations: Alanine aminotransferase (ALT); aspartate aminotransferase (AST); lactate dehydrogenase (LDH).

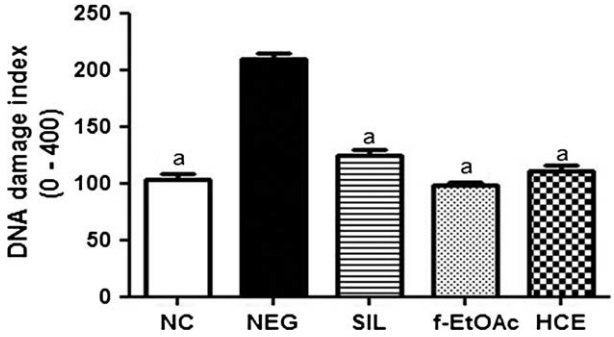

Fig. 2. DNA damage index (0–400) in hepatocytes from mice from the normal control group (NC), negative control group (NEG), positive control group pretreated with Silymarin at 15 mg/kg, p.o. (SIL), and from mice pretreated with the extracts from *Bidens pilosa*, the hydroethanol crude extract (HCE) or fraction ethyl acetate (f-EtOAc) both at 15 mg/kg (p.o) and on the 11th day treated with CCl_4 (0.5 ml/kg, i.pl). All values are expressed as means $\pm$ SD, $n = 6$. [a]Denotes significant difference compared to NEG ($P < 0.05$).

Although rather speculative, the *in vivo* findings might be due to the free radical scavenging properties of the polyphenol constituents and flavonoids of *B. pilosa*, but other mechanisms might also be involved. Oxidative stress is consistently present in liver intoxications (30). The present study provides important information on the therapeutic virtues of these plant products, which are apparently well in line with its properties as reported by traditional medicine.

Recently, a study by Yuan et al. (8) reported that a 10-day pretreatment with a brown powder (50 and 100 mg/kg) extracted from samples of *B. pilosa* purchased at a crude drug market (China) reduced the CCl_4-induced elevated liver index (liver weight/body weight), serum ALT and AST levels, and hepatic malonyl dialdehyde content and restored hepatic superoxide dismutase and glutathione peroxidase activities in acute liver injury in mice. Actually, the powder was a kind of a pool of purified flavonoids and glucosides in which they identified hyperoside, rutin, maritimetin, quercetin, okanin, *iso*-okanin, 7-*O*-(4,6-diacetyl)-d-glucopyranoside, (*Z*)-6-*O*-(3,6-di-*O*-acetyl-d-glucopyranosyl)-6,7,3,4-tetrahydroxyaurone, and 2,4,6-trimethoxy-4-*O*-dglucopyranosyl-dihydrochalcone. Interestingly, data obtained by administering the Brazilian samples under the same 10-day scheme of pretreatment is corroborative and comparable even considering that this work detected activity in pretreatments done with the HCE and especially f-EtOAc. Again, it is noteworthy that we did not observe apparent toxicity for HCE or f-EtOAc at the mentioned doses (1.5 or 15 mg/kg). Additionally, data obtained here indicate advantages in terms of bioactivity by applying fractionation with ethyl acetate. Some protective effects of some constituents of *B. pilosa* are reaffirmed here besides their free radical scavenging power. We recognized the value of some

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specific constituents of the plant, mainly quercetin-derived flavonoids.

From the overall results, we conclude that some constituents of *B. pilosa* do possess free radical scavenging activity, which exert a beneficial action in preventing liver damage induced by CCl_4. Since the preliminary analyses of the samples showed the presence of polyphenols by identifying quercetin-derived glycosylated flavonoids in the ethyl acetate fraction, the antioxidant and hepatoprotective activities of substances like these are known and, therefore, we speculate that these constituents might be the main components responsible for the observed effects. The results support the popular use of the plant as hydroethanol solutions, although the derived products enriched in flavonoids were even more effective in preclinical evaluations. These findings justify further evaluations of the efficacy and safety of the liver elixirs made with *B. pilosa* L. enriched in flavonoids.

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***Rozangela Curi Pedrosa**
Laboratório de Bioquímica Experimental – LABIOEX
Departamento de Bioquímica
Centro de Ciências Biológicas
Universidade Federal de Santa Catarina – CEP: 88040-900
Florianópolis, SC, Brazil
Tel: +55 483721 5048
Fax: +55 483721 9672
Email: roza@ccb.ufsc.br

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