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

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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 (IC₅₀ ~ 4.3–32.3 µg/ml) followed by the crude extract (IC₅₀ ~ 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 CCl₄-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 CCl₄-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; CCl₄ toxicity

Received: 19 September 2010; Revised: 8 December 2010; Accepted: 12 December 2010; Published: 18 January 2011

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 (CCl₄). The CCl₄ 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 CCl₄ 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 polycyctenes (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 the initial HCE. The ethyl acetate yielded almost 36% and the methanolic one chloroform yielded 24.9%, while the fractionation with (f-EtOAc), and methanol fraction (f-MeOH) according to the respective solvent used. The fractionation with chloroform fraction (f-CHCl₃), 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 Bruker AC 200 in DMSO-d₆. 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, AlCl₃, HCl, and H₂BO₃. 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-CHCl₃) was evaluated by using the 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenger method measured at 518 nm (13). The hydroxyl radical (HO·) 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 (IC₅₀), 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 CCl₄ (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,
Scavenging of free radicals and hepatoprotection by \textit{B. pilosa}

A triazine 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 $\varepsilon = 14.1 \text{ mmol/L per cm}$. Results were expressed in $\mu$mol 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 $\varepsilon = 40 \text{ mmol/L per cm}$ (23).

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

<table>
<thead>
<tr>
<th>Group</th>
<th>10-day pretreatment</th>
<th>Dose</th>
<th>Challenge on day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control group (NC)</td>
<td>Vehicle</td>
<td>0.5 ml</td>
<td>None</td>
</tr>
<tr>
<td>Negative control group (NEG)</td>
<td>Vehicle</td>
<td>0.5 ml</td>
<td>CCl$_4$</td>
</tr>
<tr>
<td>Positive control group (SIL)</td>
<td>Silymarin</td>
<td>15 mg/kg</td>
<td>CCl$_4$</td>
</tr>
<tr>
<td>HCE group</td>
<td>HCE</td>
<td>15 mg/kg</td>
<td>CCl$_4$</td>
</tr>
<tr>
<td>f-EtOAc group</td>
<td>f-EtOAc</td>
<td>15 mg/kg</td>
<td>CCl$_4$</td>
</tr>
</tbody>
</table>

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 $\varepsilon = 153 \text{ mmol/L per cm}$. 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 nmols of carbonyl group per milligram of protein using $\varepsilon = 22 \text{ mmol/L per cm}$ (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).

#### 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 $\mu$l of this solution was added to 70 $\mu$l 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 $\mu$l 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 $\mu$g/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

\textit{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 ($^1$H 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 Silibinin (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 exhibit 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 Silicbum 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).

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 (·OH), inhibition of lipid peroxidation in vitro (LPO), and total polyphenol content in the plant samples (TPC).

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH $IC_{50}$ (µg/ml)</th>
<th>·OH $IC_{50}$ (µg/ml)</th>
<th>LPO $IC_{50}$ (µg/ml)</th>
<th>TPC (GAE/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCE</td>
<td>98.0 ± 1.0$^a$</td>
<td>14.2 ± 3.2$^a$</td>
<td>35.8 ± 3.9$^a$</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>f-EtOAc</td>
<td>32.3 ± 6.7$^a$</td>
<td>4.3 ± 1.5</td>
<td>25.1 ± 4.5</td>
<td>3.6 ± 0.2$^b$</td>
</tr>
<tr>
<td>f-MeOH</td>
<td>102.9 ± 2.4$^a$</td>
<td>18.5 ± 8.2$^a$</td>
<td>90.4 ± 3.4$^a$</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>f-CHCl$_3$</td>
<td>248.0 ± 1.8$^a$</td>
<td>33.0 ± 6.0$^a$</td>
<td>166.3 ± 2.6$^a$</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Silymarin</td>
<td>13.9 ± 0.7</td>
<td>7.6 ± 0.8</td>
<td>10.1 ± 4.5</td>
<td>–</td>
</tr>
</tbody>
</table>

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$_3$. 

Citation: Libyan J Med 2011, 6: 5651 - DOI: 10.3402/lj.m.v6i60.5651
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 CCl₄. 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 CCl₄ in

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 CCl₄ on day 11 (0.5 ml/kg, i.p.), and controls

<table>
<thead>
<tr>
<th>Group</th>
<th>FRAP (mM)</th>
<th>GSH (µmol/g)</th>
<th>CAT (mmol/min.g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>21.7 ± 0.7²</td>
<td>34.1 ± 3.1³a</td>
<td>163.7 ± 29.6³a</td>
</tr>
<tr>
<td>NEG</td>
<td>11.7 ± 0.8</td>
<td>8.1 ± 3.6</td>
<td>517.6 ± 67.9</td>
</tr>
<tr>
<td>f-EtOAc</td>
<td>20.7 ± 0.7²</td>
<td>30.1 ± 0.4²a</td>
<td>366.4 ± 44.9³a</td>
</tr>
<tr>
<td>HCE</td>
<td>18.9 ± 0.9²</td>
<td>25.8 ± 1.5³a,³b</td>
<td>447.1 ± 33.1³b</td>
</tr>
<tr>
<td>Silymarin</td>
<td>24.5 ± 2.0²</td>
<td>29.8 ± 4.2³a</td>
<td>441.6 ± 44.3³b</td>
</tr>
</tbody>
</table>

Note: Values are expressed as means ± SD (n = 6).

²Denotes significant statistic differences at P < 0.05 in comparison to the negative control group (NEG).
³In comparison to the normal control group (NC).

Fig. 1. CCl₄ 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 CCl₄ 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 CCl₄ 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 CCl₄. 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 CCl₄ 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 CCl₄ (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 CCl₄ in hepatocytes of mice (Fig. 2). Fluorescence microscopy revealed a reduction (~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 CCl₄

<table>
<thead>
<tr>
<th>Group</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>LDH (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>9.1 ± 1.1a</td>
<td>29.2 ± 3.5a</td>
<td>237.2 ± 38.3a</td>
</tr>
<tr>
<td>NEG</td>
<td>68.4 ± 1.6</td>
<td>91.0 ± 1.5</td>
<td>541.4 ± 41.2</td>
</tr>
<tr>
<td>f-EtOAc</td>
<td>12.3 ± 1.4a</td>
<td>33 ± 10a</td>
<td>272.4 ± 33.7a</td>
</tr>
<tr>
<td>HCE</td>
<td>21.3 ± 0.9a,b</td>
<td>48.0 ± 2.1a,b</td>
<td>295.1 ± 29.3a</td>
</tr>
<tr>
<td>Silymarin</td>
<td>24.4 ± 1.4a,b</td>
<td>53.0 ± 2.5a,b</td>
<td>289.6 ± 30.5a</td>
</tr>
</tbody>
</table>

aDenotes significant statistic differences at P < 0.05 in comparison to the negative control group (NEG).

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 CCl₄

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 CCl₄-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
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 CCl₄. 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.

**Acknowledgements**

R.C. Pedrosa (Proc. 300718/2003-9) and D. Wilhelm Filho (Proc. 309438/2008-0) are recipients of research grants from the Conselho Nacional de Pesquisa (CNPq-MCT, Brazil) Additionally, the authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) for providing financial assistance in the form of research scholarships.

**Conflict of interest and funding**

The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

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