



## Original Article

 Aqueous extract of *Baccharis trimera* improves redox status and decreases the severity of alcoholic hepatotoxicity

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## ABSTRACT

The metabolism of ethanol occurs mainly in the liver, promoting increase of reactive oxygen species and nitrogen, leading to redox imbalance. Therefore, antioxidants can be seen as an alternative to reestablish the oxidizing/reducing equilibrium. The aim of this study was to evaluate the antioxidant and hepatoprotective effect of aqueous extract of *Baccharis trimera* (Less.) DC., Asteraceae, in a model of hepatotoxicity induced by ethanol. The extract was characterized and *in vitro* tests were conducted in HepG2 cells. It was evaluated the cells viability exposed to aqueous extract for 24 h, ability to scavenging the radical DPPH, besides the production of reactive oxygen species and nitric oxide, and the influence on the transcriptional activity of transcription factor Nrf2 (12 and 24 h) after exposure to 200 mM ethanol. The results showed that aqueous extract was non-cytotoxic in any concentration tested; moreover, it was observed a decrease in ROS and NO production, also promoting the transcriptional activity of Nrf2. *In vivo*, we pre-treatment male rats Fisher with 600 mg/kg of aqueous extract and 1 h later 5 ml/kg of absolute ethanol was administrated. After two days of treatment, the animals were euthanized and lipid profile, hepatic and renal functions, antioxidant status and oxidative damage were evaluated. The treatment with extract improved liver function and lipid profile, reflecting the reduction of lipid microvesicles in the liver. It also promoted an increase of glutathione peroxidase activity, decrease of oxidative damage and MMP-2 activity. These results, analyzed together, suggest the hepatoprotective effect of *B. trimera* aqueous extract.

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## Introduction

Ethanol is the most used alcohol in alcoholic beverages and its abusive consumption is associated with various health problems worldwide (Lívero and Acco, 2016). The metabolism of ethanol occurs mainly in the liver, where it is converted to acetaldehyde by alcohol dehydrogenase and subsequently oxidized to acetate by acetaldehyde dehydrogenase (Cederbaum, 2012). These enzymes use NAD<sup>+</sup> as cofactor and generate NADH, thus decreasing the

NAD<sup>+</sup>/NADH ratio, affecting several metabolic pathways (Smith et al., 2007), besides promoting the increase of acetaldehyde adducts and reactive oxygen species (ROS), leading to oxidative stress (Lu et al., 2012; Han et al., 2016). In some circumstances, the microsomal oxidation system of ethanol (CYP2E1) can be activated, which contributes even more to the ROS formation (Smith et al., 2007; Ceni et al., 2014; Hernández et al., 2015).

Alcoholic fatty liver disease (AFLD) is the first response of the liver to ethanol use and is characterized by accumulation of lipids in hepatocytes (Ceni et al., 2014). An optimal pharmacological treatment for AFLD would reduce inflammatory parameters, oxidative stress and lipid accumulation, and avoid fibrotic events. However, the development of a drug that is capable of acting on so many different pathways is extremely difficult. For this reason, a single

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drug therapy has not been developed, but combined therapies in an attempt to reverse hepatocyte injury (Lívero and Acco, 2016).

Due to the great importance of oxidative stress in the pathogenesis of AFLD, several studies have focused on the use of antioxidants to prevent oxidative damage and improve liver function (Ceni et al., 2014; Hernández et al., 2015; Lívero and Acco, 2016). ROS can be neutralized by enzymatic antioxidants, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) and nonenzymatic as glutathione, vitamins and dietary antioxidants (Chen et al., 2015; Han et al., 2016). The antioxidant enzymes are mainly regulated by the factor-2 nuclear-erythroid factor (Nrf-2) (Lushchak, 2014; Chen et al., 2015; González et al., 2015). This factor is usually found in the cell cytoplasm associated with the ECH-associated calcite-like protein (Keap-1), which allows its labeling and degradation Nrf2 via proteasome (Chen et al., 2015). However, in the presence of oxidative stress the factor migrates to the nucleus, where it binds to the antioxidant response element (ARE), promoting the antioxidant enzymes expression (Lushchak, 2014; Chen et al., 2015; González et al., 2015; Kim and Keum, 2016).

In addition to the enzymatic antioxidants, the inclusion of antioxidants in the diet is of great importance and the consumption is related to the reduction of the risk of the development of diseases associated with the accumulation of free radicals, since in these compounds substances that act in synergism in the protection of cells and tissues can be found (Bianchi and Antunes, 1999). In fact, some studies have demonstrated the beneficial effect of natural dietary antioxidants (Al-Sayed et al., 2014; Al-Sayed et al., 2015; Pádua et al., 2010; Lívero et al., 2016a,b; Fahmy et al., 2017). Thereby, medicinal plants have attracted the attention of researchers as potential agents against alcoholic liver injury because of their antioxidant potential and the few side effects (Ding et al., 2012). However, most plant species are only empirically used, and there are few studies that prove their therapeutic efficacy (Foglio et al., 2006).

In this sense, *Baccharis trimera* (Less.) DC., Asteraceae, is a medicinal plant used in popular culture and widely distributed in South America (Bona et al., 2005). In Brazil, this plant is popularly known as carqueja and used as gastric protector (Lívero et al., 2016a,b), hypoglycemic (Oliveira et al., 2011), anti-inflammatory (Karam et al., 2013) and antioxidant (Pádua et al., 2013; de Araújo et al., 2016). Some studies are focused in the use of medicinal plants in ethanol-induced intoxication, however most studies use only alcoholic/hydroethanolic extracts and it is known that the solvent used for the extraction of the secondary metabolites is involved in the biological activity of these plants (Rates, 2001). Based on these evidences and on the fact that there are no studies with aqueous extract of *B. trimera* in ethanol-induced hepatotoxicity available, our goal was to characterize this extract and verify its effect on protection against alcoholic hepatotoxicity *in vitro* and *in vivo* model.

## Materials and methods

### Plant material

The aerial parts of *Baccharis trimera* (Less.) DC., Asteraceae, were collected in Ouro Preto city, in Minas Gerais state, Brazil. The specimens were authenticated and deposited at the Herbarium José Badini (UFOP), OUPR 22.127. After identification, the aerial parts were dried in a ventilated oven (30 °C), pulverized and stored in plastic bottles. To obtain the aqueous extract, approximately 100 g of the plant was extracted with 1 l of water for 24 h, by maceration. The solids were removed by vacuum filtration and the solvent was removed by a rotary evaporator at 40 °C (Pádua et al., 2010).

### RP-UPLC-DAD-ESI-MS analyses

The aqueous extract was analyzed in the Ultra Performance Liquid Chromatography coupled to diode arrangement detector and mass spectrometry. In this assay, 20 mg of the sample was applied and diluted with 4 ml of MeOH/H<sub>2</sub>O (9:1). The eluate was dried and resuspended in a solution of methanol and then filtered on Chromafil® PDVF syringe filters (polyvinylidene difluoride, 0.20 mm) in a volume sufficient to obtain 2 mg/ml concentration of the sample. For the analysis in HPLC-DAD-EM, 20 µl of the sample were injected into the liquid chromatograph, in the same conditions described by de Araújo et al. (2016).

### In vitro tests

#### DPPH radical-scavenging activity

The percentage of antioxidant activity of each substance was assessed by DPPH free radical assay, according to Araújo et al. (2015). In brief, aqueous extract was diluted in methanol 80% and dilutions were performed to obtain the concentrations (25–500 µg/ml). The standard curve was performed with the reference antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchrome-2-carboxylic acid). As blank methanol (80%) was used and the antioxidant activity was determined by the decrease in the DPPH absorbance and the percent inhibition was calculated using the following equation: % antioxidant activity = (1 – A<sub>Sample</sub> 515/A<sub>Control</sub> 515) × 100.

#### Cell culture

Hepatocellular carcinoma cell line (HepG2) was acquired from the Cell Bank from the Federal University of Rio de Janeiro. The cells were placed in sterile 75 cm<sup>2</sup> growth vials containing the DMEM culture medium and supplemented with antibiotic (Penicillin-Streptomycin) and 10% (v/v) fetal bovine serum. The bottles were incubated in an oven at 37 °C humidified with 5% carbon dioxide (CO<sub>2</sub>). Cells were used for assays when the confluence reached about 80%, so the medium was aspirated and the monolayer washed with buffered saline (PBS). After this, 2 ml of trypsin and EDTA solution (0.20% and 0.02%, respectively) were used. Subsequently, the cells were centrifuged and the supernatant was discarded and the cell pellet was resuspended in 1 ml of DMEM medium. The cells were then counted with Trypan Blue 0.3% in the Neubauer chamber. For each experiment triplicate was used, with biological duplicate (totaling *n* = 6 per group).

#### Cell viability assay

Cell viability was determined using colorimetric MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay as described previously (Fotakis and Timbrell, 2005). In brief, HepG2 cells (1 × 10<sup>5</sup>) were cultured in 96 well-plates with or without different aqueous extract concentrations of *B. trimera* (5–600 µg/ml), which was diluted in DMEM medium, and ethanol (5–800 mM) for 24 h. After incubation, medium was removed and MTT solution (5 mg/ml) was added and incubated for further 1 h at 37 °C. Subsequently, dimethyl sulfoxide was added to dissolve formazan crystals and the absorbance was measured at 570 nm. The cell viability percentage was calculated based in the formula below, where the control was assigned 100% viability.

$$\% \text{ of cell viability} = \frac{\text{absorbance of treated cells}}{\text{absorbance control}} \times 100$$

#### ROS and NO production

For the determination of reactive oxygen species and nitric oxide 2.5 × 10<sup>4</sup> cells were cultured in white 96 well-plates with

two different aqueous extract of *B. trimera* concentrations (10 and 50 µg/ml), diluted in DMEM medium. After an incubation of 3 h, the medium was removed and 200 mM of ethanol was added with 50 µM of carboxi-H<sub>2</sub>DCFDA (for ROS production) or 10 µM of DAF-FM (for NO production). The plate was incubated for 24 h and, then, HANKS was added. The reading was obtained in microplate reader, using 485 nm for excitation and 535 nm for emission microwave.

#### Luciferase reporter assay

To evaluate the effect of aqueous extract of *B. trimera* on transcriptional activity of Nrf2, Luciferase assay System was used, according to Silva et al. (2011), with some modifications. For this, a kit Dual Luciferase assay System was used. Briefly,  $1.5 \times 10^5$  HepG2 cells were plated in 24-well plates and incubated for 24 h. Then, medium without SFB was added and incubated for further 24 h. After this time, transfection was performed with 100 µl per well of mix (500 ng of lipofectamine, 100 ng of pRL-TK, 400 ng of pPGL37 and medium HG to complete the volume). Six-hour incubation was carried out and, then, 10 and 50 µg/ml of aqueous extract were added. After 3-hour incubation, 200 mM of ethanol was added and a new incubation was performed for 12 or 24 h. After this, 100 µl of lysis buffer (provided by the kit) was added and centrifuged for 4 min at 10,000 rpm. Then, 15 µl of supernatant and 35 µl of Luciferase II reagent (LAR-II) were read in luminometer (580 nm), providing the “Net A” value. After, 50 µl of Stop and Glo<sup>®</sup> were added, providing the “Net B” value. For calculations the NetA/NetB ratio was used.

#### In vivo tests

##### Animals

Male Fisher rats (220–250 g), obtained from the Laboratory of Experimental Nutrition from the Federal University of Ouro Preto, were kept on collective cages, in a 12 h light/dark cycle at room temperature and were fasted 12 h with water *ad libitum* before the experiment. All animals were used according to the Committee guidelines on Care and Use of Animal from Federal University of Ouro Preto, Brazil (No. 2016/01).

##### The experimental protocol

The animals were divided into three groups:

- Control group (C) ( $n = 7$ ): received 1 ml of water;
- Ethanol group (E) ( $n = 5$ ): received 1 ml of water and 1 h later 5 ml/kg of absolute ethanol (El-Naga, 2015).
- Aqueous extract of *B. trimera* (Aq) ( $n = 7$ ): received 600 mg/kg of extract and 1 h later 5 ml/kg of absolute ethanol (Pádua et al., 2010, 2013, 2014).

All treatments were administrated by gavage, totaling a volume of 1 ml of solution. The animals were treated for two consecutive days and 24 h after the last ethanol dose, they were euthanized by deep anesthesia induced by isoflurane. They were maintained on a 12 h fasting.

##### Analysis of biochemical serum parameters

The serum was used for determining the urea, creatinine, ALT, AST, protein total, glucose, total cholesterol, HDL, fraction non-HDL, triacylglycerides. All measurements were performed by commercial laboratories kits Labtest<sup>®</sup> (Lagoa Santa, MG, Brazil) and Bioclin<sup>®</sup> (Belo Horizonte, MG, Brazil).

##### Determination of antioxidant system

The antioxidant system was evaluated by SOD, catalase, glutathione peroxidase and reductase activity, beyond glutathione

total (oxidized and reduced). The assay for determination of indirect SOD-activity is based on SOD competition with superoxide radical, formed by self-oxidation of pyrogallol, which is responsible for MTT reduction and formation of formazan crystals (Marklund and Marklund, 1974). Catalase activity was determined based on its ability to convert hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) into water and molecular oxygen (Aebi, 1984). Glutathione system was determined by kit (Sigma–Aldrich, St. Louis, MO, USA). To determine catalase and superoxide dismutase activity, 100 mg of liver tissue was homogenized in phosphate buffer (pH 7.4). Total glutathione and reduced/oxidized glutathione concentrations were determined by the homogenization of 100 mg of tissue in 5% sulfosalicylic buffer. For correction of the dosages, the protein was measured by the Lowry method (Lowry et al., 1951). After homogenization, the samples were centrifuged at 10,000 × g for 10 min, at 4 °C. The supernatant was collected and used as the sample and all dosages were according to Bandeira et al. (2017).

##### Determination of oxidative stress markers

In order to evaluate oxidative damage thiobarbituric acid reactive substances (TBARS) and carbonyl protein such as markers were used. The TBARS concentration was determined based on thiobarbituric acid (TBA) binding to oxidized lipids, according to Buege and Aust (1978). In the method for determining carbonylated protein, it was used 2,4-dinitrophenylhydrazine (DNPH), which reacts with carbonyl groups to generate the corresponding hydrazone that can be analyzed spectrophotometrically, as described by Levine et al. (1994). For correction of the dosages, the protein was measured by the Lowry method (Lowry et al., 1951).

##### Gelatin zymography

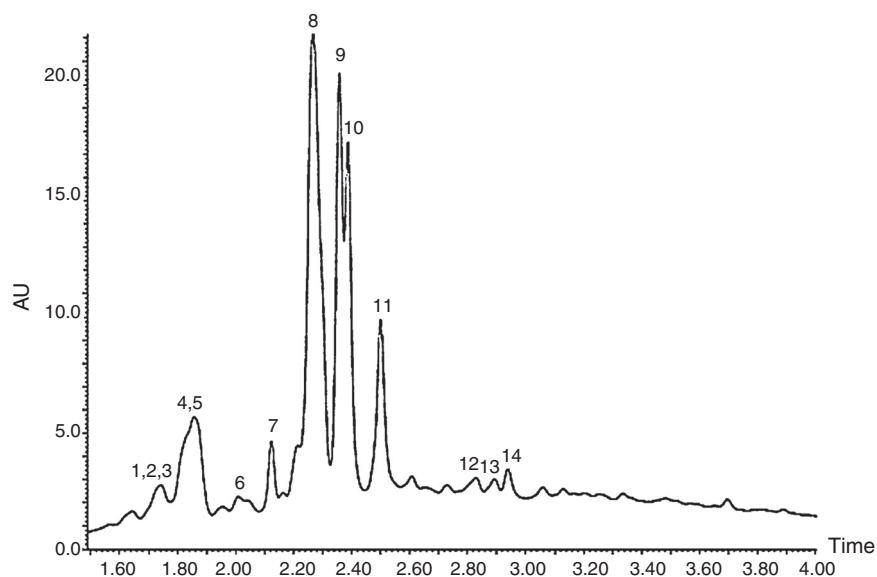
MMP-2 activity was detected using gelatin zymography. Briefly, 50 mg of tissue were homogenized in 200 of RIPA buffer (150 mM NaCl, 50 mM Tris, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 1 µl/ml protease inhibitor at pH 8.0). After homogenization, the samples were centrifuged at 10,000 × g for 10 min, at 4 °C and the supernatant was collected and used as the sample. The activity was measured according to Araújo et al. (2015).

##### Histological evaluation

For microscopic analysis, a portion of the liver from each animal of experimental groups was fixed in 10% formalin and immersed in paraffin. Sections of 4 µm were obtained and the slides were stained with hematoxylin and eosin (H&E). The photomicrographs were obtained at 40× magnification (Leica Application Suite, Germany). Liver histology was examined using eleven images obtained at random from the tissue and classified for the degree of microvesicular steatosis. The images were examined semi quantitatively, considering that the degree of lipid infiltration was graded reflecting the percentage of hepatocytes containing lipid droplets. It was given the values 0–3 according to the steatosis, where 0: none; 1: 1–33%; 2: 33–66%; 3: >66%, as described by Brunt et al. (1999).

##### Statistical analysis

The data were analyzed by Kolmogorov–Smirnov test for normality, and all data showed a normal distribution. All values are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA), with Bonferroni posttest. Prism 5.0 (GraphPad, La Jolla, CA, USA) was used to perform the analysis. Differences were considered significant when  $p < 0.05$ .



**Fig. 1.** RP-UPLC-DAD fingerprint section of aqueous extract of *Baccharis trimera*. 1: 3-*O*-feruloylquinic acid; 2: 4-*O*-caffeoylquinic acid; 3: 5-*O*-caffeoylquinic acid; 4: 3-*O*-caffeoylquinic acid; 5: 4-*O*-feruloylquinic acid; 6: 5-*O*-feruloylquinic acid; 7: apigenin-6,8-di-*C*-glucopyranosidum; 8: 3-*O*-isoferuloylquinic acid; 9: 5-*O*-isoferuloylquinic acid; 10: 6(8)-*C*-furanosyl-8(6)-*C*-hexosyl flavone; 11: 6(8)-*C*-hexosyl-8(6)-*C*-furanosyl flavone; 12: 3,4-di-*O*-caffeoylquinic acid; 13: 3,5-di-*O*-caffeoylquinic acid; 14: 4,5-di-*O*-caffeoylquinic acid; 15: 4-*O*-isoferuloylquinic acid.

**Table 1**  
Substances identified in the aqueous extract of *Baccharis trimera* by LC-DAD-ESI-MS.

| Peak | Compound  | RT (min) | UV (nm)      | LC-MS [M-H] <sup>-</sup> (m/z)<br>(fragmentation m/m) |
|------|---|----------|--------------|---|
| 1    | 3- <i>O</i> -feruloylquinic acid                          | 1.73     | 321.1        | 367.29<br>(193.0; 155.2; 148.9; 134.0)                |
| 2    | 4- <i>O</i> -caffeoylquinic acid                          | 1.74     | 320.1        | 353.38<br>(191.0; 178.9; 135.1)                       |
| 3    | 5- <i>O</i> -caffeoylquinic acid                          | 1.75     | 323.1        | 353.38<br>(191.2; 179.1; 135.1)                       |
| 4    | 3- <i>O</i> -caffeoylquinic acid                          | 1.86     | 323.1        | 353.38<br>(190.8; 179.0; 137.0)                       |
| 5    | 4- <i>O</i> -feruloylquinic acid                          | 1.88     | 323.1        | 367.36<br>(193.2; 172.8; 149.1; 134.0)                |
| 6    | 5- <i>O</i> -feruloylquinic acid                          | 1.96     | 320.1        | 367.38<br>(192.7; 172.7; 149.2; 134.2)                |
| 7    | Apigenin-6,8-di- <i>C</i> -glucopyranosidum               | 2.12     | 269.1; 321.0 | 593.42<br>(547.1; 503.0; 472.9; 431.1)                |
| 8    | 3- <i>O</i> -isoferuloylquinic acid                       | 2.25     | 318.1        | 367.59<br>(193.1; 172.8; 149.1; 133.8)                |
| 9    | 5- <i>O</i> -isoferuloylquinic acid                       | 2.47     | 323.1        | 367.17<br>(191.2; 172.8; 149.1; 134.1)                |
| 10   | 6(8)- <i>C</i> -furanosyl-8(6)- <i>C</i> -hexosyl flavone | 2.36     | 271.1; 331.1 | 563.48<br>(515.1; 473.1; 443.0; 383.1)                |
| 11   | 6(8)- <i>C</i> -hexosyl-8(6)- <i>C</i> -furanosyl flavone | 2.50     | 271.3; 333.2 | 563.20<br>(515.1; 472.9; 442.9; 383.2)                |
| 12   | 3,4-di- <i>O</i> -caffeoylquinic acid                     | 2.81     | 282.1; 320.5 | 515.20<br>(190.0; 162.9; 134.7)                       |
| 13   | 3,5-di- <i>O</i> -caffeoylquinic acid                     | 2.93     | 283.1; 321.2 | 515.17<br>(191.0; 163.0; 135.0)                       |
| 14   | 4,5-di- <i>O</i> -caffeoylquinic acid                     | 2.95     | 286.1; 320.3 | 515.40<br>(190.7; 163.0; 143.2; 127.0)                |
| 15   | 4- <i>O</i> -isoferuloylquinic acid                       | 3.46     | 322.1        | 367.22<br>(190.6; 163.0; 148.0)                       |

## Results

### RP-UPLC-DAD-ESI-MS analysis of aqueous extract

Twelve phenolic acids and three flavonoids were identified in the aqueous extract of *B. trimera*, by RP-UPLC-DAD-ESI-MS. The RP-UPLC-DAD fingerprint is shown in Fig. 1 (Table 1).

### Antioxidant activity in vitro of *Baccharis trimera*

To evaluate the ability of *B. trimera* to neutralize the DPPH radical, five concentrations of extract were used. It was observed that the highest concentration of extract (500 µg/ml) was able to inhibit DPPH by approximately 68%, similar to the 150,174–125,145 µg/ml of trolox standard concentration. This means that the extract at a

**Table 2**

Evaluation of *Baccharis trimera* ability to scavenging DPPH radical. The highest concentration of aqueous extract of *Baccharis trimera* was able to inhibit DPPH by approximately 68%, while the reference antioxidant (Trolox) required a concentration of 300 times greater to inhibit the same percentage. DPPH: 2,2-diphenyl-1-picrylhydrazyl; Trolox: 6-hydroxy-2,5,7,8-tetra-trihylchroman-2-carboxylic acid. The values are expressed as the mean  $\pm$  SD.

| DPPH radical scavenging activity                                 | Inhibition %    |
|--|-----------------|
| <i>Aqueous extract of Baccharis trimera</i> ( $\mu\text{g/ml}$ ) |                 |
| 500  | 68.1 $\pm$ 11.1 |
| 250  | 30.5 $\pm$ 1.38 |
| 100  | 8.10 $\pm$ 0.90 |
| 50   | 3.30 $\pm$ 0.88 |
| 25   | 0.59 $\pm$ 0.27 |
| <i>Trolox</i> ( $\mu\text{g/ml}$ )                               |                 |
| 200232   | 95.9 $\pm$ 0.19 |
| 175203   | 83.3 $\pm$ 3.05 |
| 150174   | 72.0 $\pm$ 2.82 |
| 125145   | 60.4 $\pm$ 4.79 |
| 100116   | 47.3 $\pm$ 1.24 |
| 75087  | 32.9 $\pm$ 1.53 |
| 50058  | 22.0 $\pm$ 0.77 |
| 25029  | 10.5 $\pm$ 1.06 |

concentration of approximately 300 times less than trolox is able to inhibit the same percentage of DPPH (Table 2).

#### *Baccharis trimera* aqueous extract does not show cytotoxicity in HepG2 cells

The results of Fig. 2 (panel A) showed that there was no significant difference in the HepG2 cells viability at concentrations of 5–25  $\mu\text{g/ml}$  of aqueous extract of *B. trimera*, with viability maintained above 85%. In the concentrations of 50–600  $\mu\text{g/ml}$  there was a significant reduction in viability in relation to the control, but maintained above 75%. In panel B a significant reduction was observed in cell viability from the concentration of 200  $\mu\text{M}$  of ethanol.

#### *Baccharis trimera* aqueous extract decreased a reactive species production in HepG2 cells incubated with ethanol

It can be observed in Fig. 2 that ethanol promoted the increase of ROS (panel C) and NO (panel D) in HepG2 cells. There was a reduction in these parameters when the cells were pretreated with aqueous extract in both concentrations (10 and 50  $\mu\text{g/ml}$ ), reaching similar levels to negative control (cells not incubated with ethanol).

#### *Baccharis trimera* aqueous extract modulates Nrf2 transcriptional activity in HepG2 cells

It can be observed in Fig. 2 that the ethanol is able to induce the Nrf2 transcriptional activity in 12 h (panel E) and 24 h (panel F) of incubation, when compared to the control. The Nrf2 transcriptional activity was increased when cells were pretreated with 10  $\mu\text{g/ml}$  of aqueous extract of *B. trimera*, in 12 h, when compared to the ethanol group without treatment. Whereas regarding the 24 h, the concentration of 50  $\mu\text{g/ml}$  of aqueous extract was able to induce the increase the Nrf2 transcriptional activity.

#### Evaluation of the effect of *Baccharis trimera* on biochemical parameters in alcoholic hepatotoxicity

Renal function was evaluated based on the parameters creatinine and urea. In order to evaluate liver function, the parameters ALT, AST and total protein were used. Total cholesterol, HDL, fraction non-HDL and triacylglycerol levels were determined for the lipid profile. Table 3 showed the increase in creatinine levels, total

**Table 3**

Biochemical marker levels in serum and plasma of rats. Animals received absolute ethanol (E); pretreatment with aqueous extract (Aq) and 1 h after received absolute ethanol. Control (C) received water. Different letters (a, b, c, d) indicate significant difference from each other at  $p < 0.05$ , while same letters indicate no significant difference ( $p > 0.05$ ).

| Biochemical parameters    | Treated groups                |                                 |                                |
|---------------------------|-------------------------------|---------------------------------|--------------------------------|
|                           | C                             | E                               | Aq                             |
| Urea (mg/dl)              | 59.6 $\pm$ 1.7                | 71.81 $\pm$ 5.5                 | 70.92 $\pm$ 6.1                |
| Creatinine (mg/dl)        | 0.375 $\pm$ 0.09 <sup>b</sup> | 0.7361 $\pm$ 0.087 <sup>a</sup> | 0.57 $\pm$ 0.079 <sup>a</sup>  |
| ALT                       | 18.99 $\pm$ 1.4 <sup>a</sup>  | 21.83 $\pm$ 2.02 <sup>a</sup>   | 12.0 $\pm$ 0.65 <sup>b</sup>   |
| AST                       | 37.02 $\pm$ 3.8               | 37.76 $\pm$ 3.5                 | 27.5 $\pm$ 2.69                |
| Total protein (mg/dl)     | 5.97 $\pm$ 0.65 <sup>a</sup>  | 4.12 $\pm$ 0.17 <sup>b</sup>    | 4.62 $\pm$ 0.4 <sup>b</sup>    |
| Glucose (mg/dl)           | 100.6 $\pm$ 5.8               | 108.5 $\pm$ 6.7                 | 103.1 $\pm$ 10.4               |
| Total cholesterol (mg/dl) | 97.15 $\pm$ 2.6 <sup>c</sup>  | 230.0 $\pm$ 28.24 <sup>a</sup>  | 144.0 $\pm$ 19.11 <sup>b</sup> |
| HDL (mg/dl)               | 43.8 $\pm$ 4.05               | 35.82 $\pm$ 0.31                | 42.92 $\pm$ 1.18               |
| Fraction non HDL (mg/dl)  | 58.8 $\pm$ 10.61 <sup>c</sup> | 189.1 $\pm$ 20.95 <sup>a</sup>  | 99.98 $\pm$ 25.82 <sup>b</sup> |
| Triacylglycerides (mg/dl) | 63.95 $\pm$ 12.72             | 53.86 $\pm$ 6.49                | 53.22 $\pm$ 12.78              |

cholesterol and non-HDL fraction, besides a decrease in total protein in the group of animals that received ethanol. Pretreatment with aqueous extract of *B. trimera* promoted a decrease in ALT activity, total cholesterol and non-HDL fraction and an increase in total protein, compared with ethanol group. It was not observed significant differences in urea levels, AST activity, glucose, HDL and triacylglycerides levels in any of the experimental groups.

#### Effect of *Baccharis trimera* on the antioxidant system in alcoholic hepatotoxicity

It could be observed in Fig. 3 that the ethanol consumption did not alter the activities of SOD (panel A) and catalase (panel B) enzymes, compared with control group. The aqueous extract did not alter these parameters either. Regarding glutathione system, the results showed a significant decrease in glutathione total (panel C) and glutathione peroxidase (GPx) activity (panel E), together with an increase in reduced/oxidize glutathione ratio (panel D) in ethanol group when compared with control group. *B. trimera* aqueous extract promotes an increase in GPx activity and a decrease in reduced/oxidize glutathione ratio. Glutathione reductase activity (panel F) did not alter in any of the experimental groups.

#### Evaluation of the effect of *Baccharis trimera* on markers of oxidative stress in alcoholic hepatotoxicity

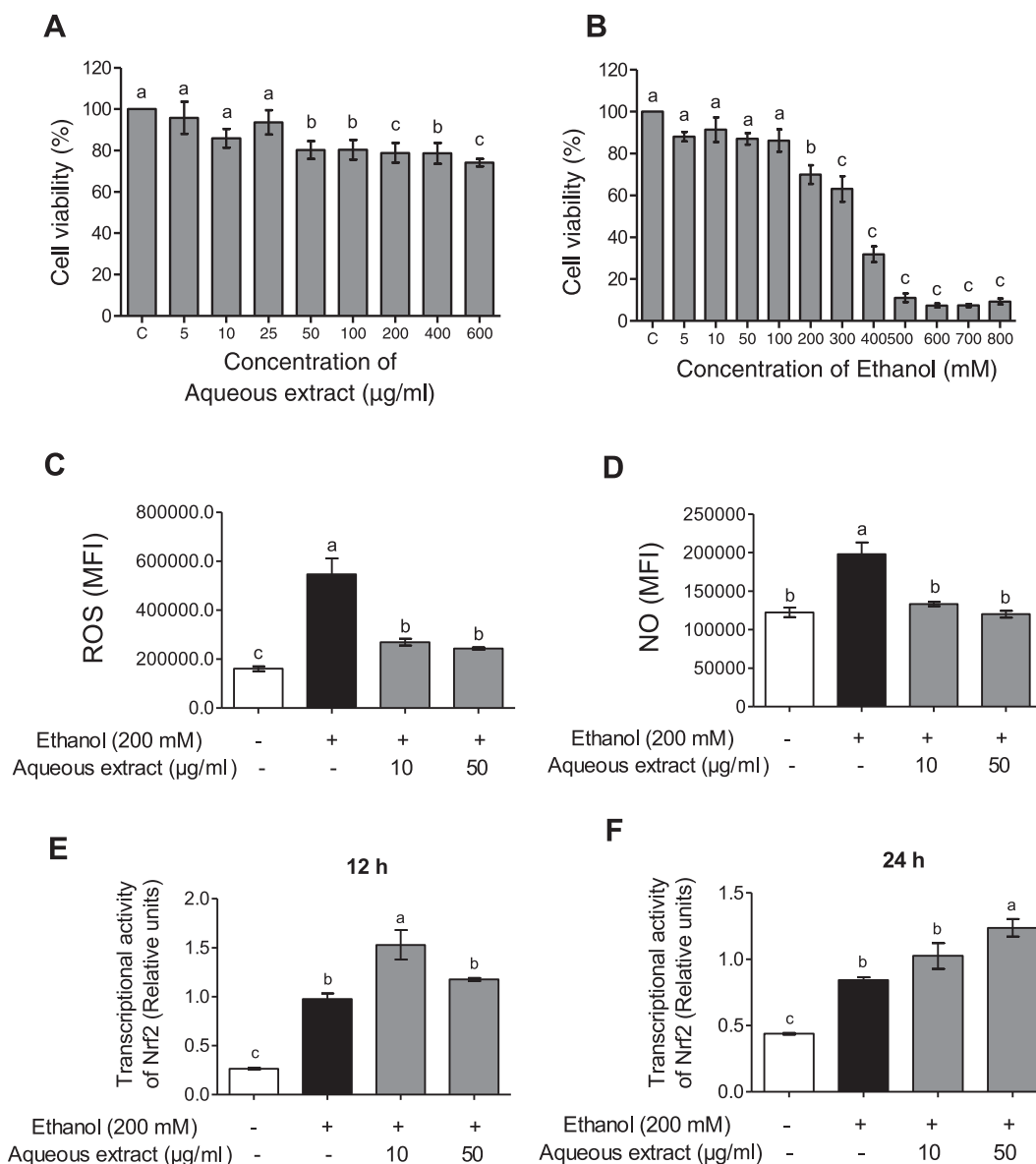
In ethanol group there was an increase in TBARS (Fig. 4 – panel A) and carbonylated protein (panel B) levels, compared to control group. The extract of *B. trimera* was able to reduce only the levels of TBARS, no alterations were observed in the levels of carbonylated protein.

#### *Baccharis trimera* decreases the MMP-2 activity in alcoholic hepatotoxicity

To evaluate the MMP-2 activity the zymography technic was used. Fig. 5A represents qualitative images of gel and 5B represents the quantitative activity (band density). Then, it was observed that ethanol promoted an increase in MMP-2 activity, when compared to the control. The treatment with *B. trimera* promotes the reduction of MMP-2 activity, in relation of ethanol group.

#### *Baccharis trimera* aqueous extract reduces micro-steatosis in alcoholic hepatotoxicity

It could be observed that the animals from ethanol group show microvesicular steatosis, mainly grade 3 and 1 (Fig. 6), whereas animals from control group exhibit mainly grade 0 and 1. Treatment



**Fig. 2.** Cell viability of *Baccharis trimer* aqueous extract (5–600 µg/ml) (A) and ethanol (5–800 mM) (B) for 24 h measured by MTT assay. Effect of *Baccharis trimer* aqueous extract on ethanol-induced ROS (C) and NO (D) production, expression MFI (Medium intensity of fluorescence). We also evaluated the effect of *Baccharis trimer* on ethanol-induced Nuclear factor E2-related factor 2 (Nrf2), via luciferase assay, for 12 h (E) and 24 h (F). The results were expressed as mean ± S.D. (n = 6). Different letters (a, b, c) indicate significant difference from each other at  $p < 0.05$ , while same letters indicate no significant difference ( $p > 0.05$ ).

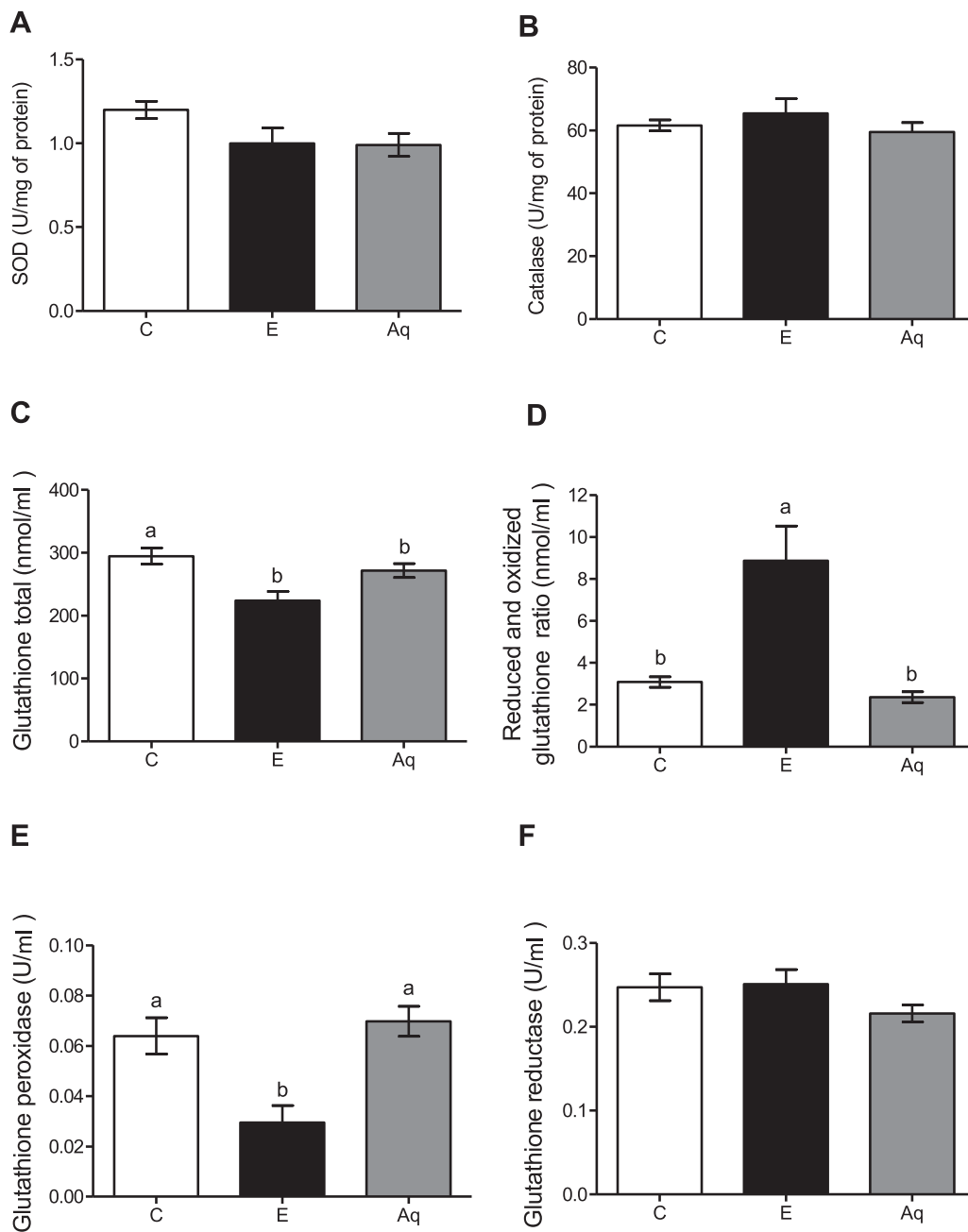
with *B. trimer* was able to reduce the degree of severity of the microvesicular steatosis, mainly grade 1.

## Discussion

The present study investigated the potential protective effects of *B. trimer* aqueous extract against hepatotoxicity induced by ethanol, as well the compounds present in this extract. *In vitro*, ability of the extract to scavenge free radicals and the effect of *B. trimer* on ethanol mediated ROS, NO and transcriptional activity of Nrf2 in HepG2 cells were examined. It was provided for the first time that *B. trimer* aqueous extract stimulates the transcriptional activity of Nrf2. *In vivo*, using a rat model of acute intoxication by ethanol, it was demonstrated that *B. trimer* alleviated the oxidative damages, improving the antioxidant defense and attenuating hepatic steatosis (Graphic abstract). This encourages the advancement of research indicating *B. trimer* as therapeutic agent for hepatoprotection.

Flavonoids, caffeic acid derivatives and diterpenes have been isolated from different extracts of *B. trimer* (Abad and Bermejo, 2007; Verdi et al., 2005; Lívero et al., 2016a,b). In our study, using LC-DAD-ESI-MS three flavonoids were detected in aqueous extract (apigenin-6,8-di-C-glucopyranosidium (7); 6(8)-C-furanosyl-8(6)-C-hexosyl flavone (10); 6(8)-C-hexosyl-8(6)-C-furanosyl flavone (11)) and twelve phenolic acids (3-O-feruloylquinic acid (1); 4-O-caffeoylquinic acid (2); 5-O-caffeoylquinic acid (3); 3-O-caffeoylquinic acid (4); 4-O-feruloylquinic acid (5); 5-O-feruloylquinic acid (6); 3-O-isoferuloylquinic acid (8); 5-O-isoferuloylquinic acid (9); 3,4-di-O-caffeoylquinic acid (12); 3,5-di-O-caffeoylquinic acid (13); 4,5-di-O-caffeoylquinic acid (14); 4-O-isoferuloylquinic acid (15)).

The ability of aqueous extract of *B. trimer* to sequester radicals was evaluated and the results showed that all tested concentrations showed an antioxidant capacity in a dose-dependent manner. For the purpose of determining the concentrations that would be used

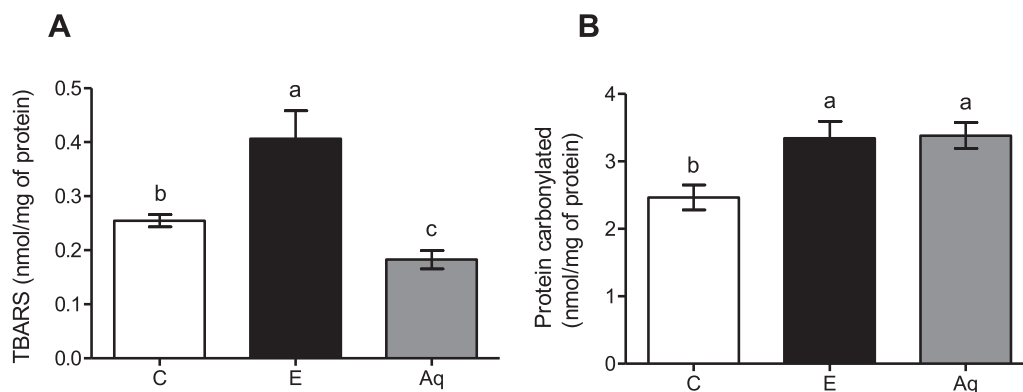


**Fig. 3.** Effect of *Baccharis trimera* aqueous extract on the level of SOD (A), catalase (B), glutathione total (C), reduced and oxidized glutathione ratio (D), activity of glutathione peroxidase (E) and glutathione reductase (F), in the livers of rats. Control (C) received water. Animals received absolute ethanol (E); pretreatment with aqueous extract (Aq) and 1 h after received absolute ethanol. Different letters (a, b) indicate significant difference from each other at  $p < 0.05$ , while same letters indicate no significant difference ( $p < 0.05$ ).

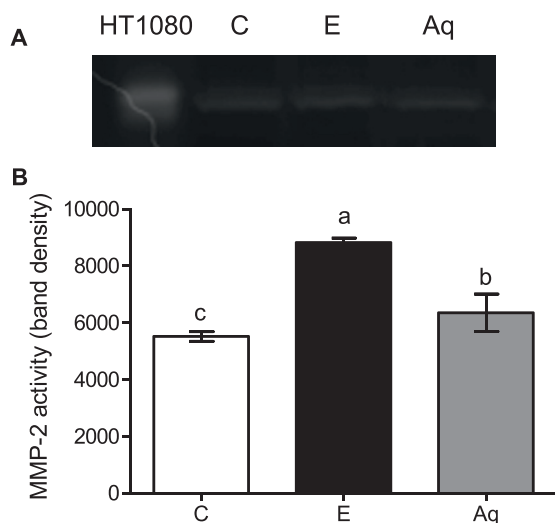
in the other *in vitro* assays, HepG2 cells were incubated with different concentrations of the aqueous extract and ethanol. The results showed that *B. trimera* aqueous extract was not cytotoxic at any concentration evaluated. Rodrigues et al. (2009) demonstrated that the aqueous *B. trimera* extract was not cytotoxic to bone marrow cells at any of the concentrations tested (500–2000  $\mu\text{g/ml}$ ). However, Nogueira et al. (2011) demonstrated the aqueous extract was cytotoxic in 500  $\mu\text{g/ml}$  in HTC and HEK cells (rat hepatoma cells and human embryo kidney epithelial cells, respectively) indicating that the toxicity may be tissue-specific. In relation to ethanol, there was a reduction in viability from 200 mM. Kumar et al. (2012) observed a significant decrease in HepG2 cells viability exposed to ethanol from 100 mM. Based on this, non-cytotoxic concentrations

of *B. trimera* were selected (10–50  $\mu\text{g/ml}$ ) and the ethanol cytotoxic concentration (200 mM) were used in the subsequent experiments.

When HepG2 cells were incubated with ethanol the production of ROS and NO was increased. Haorah et al. (2011) also found increased ROS and NO in endothelial cells treated with ethanol. This increase can be explained by the fact that the main metabolite of ethanol, acetaldehyde, activates NADPH oxidase and inducible nitric oxide synthase (iNOS), which leads to an increase in the production of ERO and nitric oxide (NO), causing oxidative damages (Haorah et al., 2008; Rump et al., 2010; Alikunju et al., 2011). Gong and Cederbaum (2006) observed that in hepatocytes isolated from rats there was an increase of Nrf2, probably due to the induction of CYP2E1 promoted by ethanol, which leads to the increase in



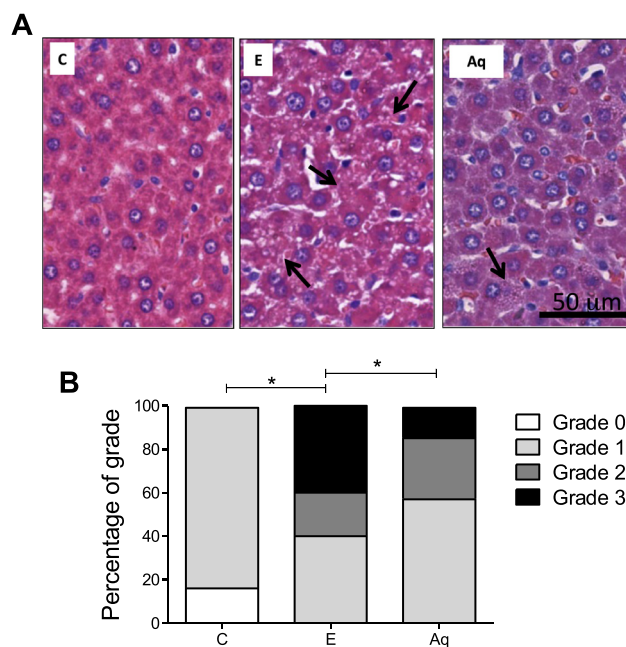
**Fig. 4.** Effect of *Baccharis trimera* aqueous extract on the level of TBARS (A) and carbonylated protein (B) in the livers of rats. Control (C) received water. Animals received absolute ethanol (E); pretreatment with aqueous extract (Aq) and 1 h after received absolute ethanol. Different letters (a, b) indicate significant difference from each other at  $p < 0.05$ , while same letters indicate no significant difference ( $p < 0.05$ ).



**Fig. 5.** Effect of *Baccharis trimera* aqueous extract on the MMP-2 activity, via gelatin zymography, in the livers of rats. A: Gel bands; B: band density. HT1080 fibrosarcoma cells were used as positive control. Control (C) received water. Animals received absolute ethanol (E); pretreatment with aqueous extract (Aq) and 1 h after received absolute ethanol. Different letters (a, b) indicate significant difference from each other at  $p < 0.05$ , while same letters indicate no significant difference ( $p < 0.05$ ).

ROS production, with consequent activation of Nrf2. These findings are in agreement with our results that found an increase in Nrf2 transcriptional activity in cells that received only ethanol. Ethanol induced Nrf2 transcriptional activity has also been demonstrated by others authors (Dong et al., 2011; Lu et al., 2012).

The pretreatment with *B. trimera* promoted the reduction of ROS and NO, returning to values similar to the control. Antioxidants can act directly through the elimination of ERO and ERN, or indirectly, through the modulation of signaling pathways (Paiva et al., 2015). Thus, this study and others showed that *B. trimera* has the ability to sequester radicals, inferring that the decrease in these species can be attributed, at least, to the plant's direct action de Oliveira et al., 2012; Pádua et al., 2013). In addition, it was also inferred that this decrease can be attributed to the indirect action, since *B. trimera* aqueous extract promoted an increase in Nrf2 transcriptional activity, promoting antioxidant protection against the stress by the ethanol. Lívero et al. (2016a,b) have already demonstrated that the hydroethanolic extract of *B. trimera* promotes an increase expression of Nrf2, but no other study has shown the effect of *B. trimera* over the Nrf2 activity. Thus, these data in agreement with the decrease of ROS and NO show that *B. trimera* may be effective in preventing stress induced by ethanol.



**Fig. 6.** Representative hematoxylin and eosin-stained histological sections of livers from rats (A). Semi-quantification was demonstrated in B. Severe microvesicular steatosis was observed in the ethanol group (E), but not in the control group (C). *Baccharis trimera* aqueous extract (Aq) attenuated fat accumulation in hepatocytes. The images were photographed at 400x magnification. Scale bar = 50  $\mu\text{m}$ . Asterisk (\*) indicates significant difference between two groups.

To confirm the effects an *in vivo* experiment was carried out, where the animals received only absolute ethanol or were pretreated with *B. trimera* aqueous extract. The results showed a worsening kidney function, decrease in total protein, but ALT and AST transaminases did not change in the ethanol group. There are several situations in which there is loss of correlation between serum levels of liver enzymes and a tissue injury, so that an increase of serum activities of liver enzyme markers does not necessarily reflect on liver cell death (Contreras-Zentella and Hernández-Muñoz, 2016). However, acetaldehyde formed during the metabolism of ethanol may form adducts with amino acids, reflecting the general decrease in protein synthesis and the decrease of plasma protein secretion (Smith et al., 2007). Pretreatment with *B. trimera* significantly decreased ALT activity. Lívero et al. (2016a,b) also found a decreased ALT activity when mice received hydroethanolic extract of *B. trimera*. The acute administration of alcohol may lead to a reduction or no change in



glucose concentration, this difference can be explained by the nutritional status at the time alcohol is administered (Steiner et al., 2015). Probable because our animals received balanced commercial feed no changes were found in glucose in any experimental group.

The results also showed an increase in total cholesterol, non-HDL fraction, beyond hepatic micro-steatosis, but TAG did not change in ethanol group. After acute consumption of high doses of ethanol the serum levels of TAG may increase, decrease or remain normal, however, the total flow that is absorbed by the liver is increased due to the stimulatory effects of ethanol on liver blood flow (Baraona and Lieber, 1979), promoting the accumulation of micro and/or macrovesicles lipids in hepatocytes (Baraona and Lieber, 1979; Lívero and Acco, 2016). *B. trimera* improved the lipid profile and decreased hepatic micro-steatosis, protecting against ethanol damage. Lívero et al. (2016a,b) showed that hydroethanolic extract of *B. trimera* decreases the expression of the *Scd1* gene, which is responsible for encoding the stearoyl-CoA desaturase-1, an important enzyme in the biosynthesis of the main fatty acids found in TAG. Maybe part of this mechanism contributes to the protection mechanism by the extract.

The ability of ethanol to induce oxidative stress and antioxidant depletion, such as glutathione, is well recognized (Lu and Cederbaum, 2008; Han et al., 2016). Our results showed that ethanol alterations in glutathione metabolism were more significant than alterations in SOD and CAT activities, since ethanol-treated rats exhibited a decrease in GPx activity and decrease in oxidized glutathione, reflecting an increase in the GSH/GSSG glutathione ratio. The decrease in GPx activity in ethanol-induced intoxication has also been demonstrated in other studies (Park et al., 2013; Li et al., 2014; Yan et al., 2014). GPx is one of the responsible for the reduction of H<sub>2</sub>O<sub>2</sub> to water, since a decrease in the activity of this enzyme was observed, it is possible to infer that in these animals there was probably accumulation of H<sub>2</sub>O<sub>2</sub>, contributing to oxidative stress. This inefficiency in the antioxidant response may justify the increase in the TBARS and carbonylated protein levels observed in our study. In carbon tetrachloride-induced hepatotoxicity models it has been shown that the decrease of glutathione is related to the increase of MDA (Azab et al., 2013; Al-Sayed and Abdel-Daim, 2014). *B. trimera* promoted increased GPx activity, contributing to an adaptive response with a consequent decrease in TBARS. In fact, the mechanisms involved in the antioxidant capacity of polyphenols include the suppression of ROS formation by inhibition of the enzymes involved in their production; direct elimination of ROS; or positive regulation in antioxidant defense (Hussain et al., 2016).

Hepatic fibrosis is an important histological feature associated with the progression of alcoholic liver disease, characterized by increased deposition of extracellular matrix components (ECM). The key event in liver fibrogenesis is the activation of hepatic stellate cells (HSC), which are a major source of ECM in the liver (Ceni et al., 2014; Lasek, 2016). Acetaldehyde is one of the main mediators of alcohol-induced fibrogenesis, as it may stimulate the synthesis of fibrillar collagens and structural glycoproteins of ECM. Acetaldehyde may further promote ECM remodeling by upregulation of metalloproteinase-2 (MMP-2) (Ceni et al., 2014). In addition, H<sub>2</sub>O<sub>2</sub> due to oxidative stress can activate MMP-2 (Hopps et al., 2015). This fact can explain the increase in MMP-2 activity found in the ethanol treated animals in our experiment. However, the pretreatment with *B. trimera* decreased the MMP-2 activity. It has already been shown that the caffeoylquinic acids have MMP-2 inhibitory activity (Benedek et al., 2007). Since that in the characterization of our extract it was demonstrated the presence of caffeoylquinic acids, it is possible to infer that this mechanism is involved with the observed protection.

Thus, when our results were analyzed all together suggest that *B. trimera* aqueous extract appears to be promising as therapeutic agent for hepatoprotection.

#### Author contributions

ACSR elaborated and performed all the work. GRA, GHBS GCB contributed in collecting plant sample and identification, confection of herbarium, chromatographic analysis. KPL and PHAM assisted in the practical laboratory part. CMA contributed with the zymography technique. BMS, ACAC and EMCR contributed with luciferase assay. WGL contributed with histological analyses. DCC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

#### Conflicts of interest

The authors declare no conflicts of interest.

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