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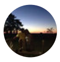

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ORIGINAL PAPER

Harpagophytum procumbens Prevents Oxidative Stress and Loss of Cell Viability In Vitro

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Abstract *Harpagophytum procumbens,* popularly known as devil's claw, is a plant commonly used in the treatment of diseases of inflammatory origin. The anti-inflammatory effects of *H. procumbens* have been studied; however, the mechanism of action is not elucidated. It is known that excess of reactive oxygen and nitrogen species may contribute to increasing tissue damage due to inflammation. In the present study, we examined the effects of *H. procumbens* infusion, crude extract and fractions on lipid peroxidation (brain homogenates) induced by different pro-oxidants (Fe^{2+} or sodium nitroprusside) and the effects of ethyl acetate fraction (rich in phenolic compounds) on antioxidant defenses (catalase activity and thiol levels) and cell damage (brain cortical slices) induced by different pro-oxidants. All tested extracts of *H. procumbens* inhibited lipid peroxidation in a concentration-dependent manner. Furthermore, the ethyl acetate fraction had the highest antioxidant effects either by decreasing lipid peroxidation and cellular damage or restoring thiols levels and catalase activity. Taken together, our results showed that *H. procumbens* acts either by preventing oxidative stress or loss of cell viability. Thus, the previously reported anti-inflammatory effect of *H. procumbens* could also be attributed to its antioxidant activity.

Keywords Antioxidant · Devil's claw · Cerebral cortex · Catalase · Thiol levels · Lipid peroxidation

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Introduction

Oxidative stress is a biological condition that occurs due to an imbalance between production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) and antioxidant defenses. This condition has been related to numerous pathologies where ROS can contribute to their worsening by causing alterations in the cell membrane (lipid peroxidation and protein oxidation) and DNA mutations [1–3].

It is known that inflammatory process may lead to an over production of ROS and RNS, which in turn can contribute to increase tissue damage [4, 5]. Literature data have shown the relationship between the inflammation process and oxidative stress in various physiological disorders [5–9]. Agents capable of interfering with free radical generation and/or blockage of their effects in biological tissues have therapeutic importance [10, 11]. In this context, alternative ways have been considered as adjuvant treatment of numerous diseases, mainly those associated with oxidative stress [12–15]. Thus, medicinal plants and other natural compounds have been largely studied as alternative or adjuvant treatment [16–21] with low side effects.

Nature has been a source of medical products for millennia, with many useful drugs developed from plant sources [22, 23]. According to the World Health Organization, developing countries still use traditional medicine as a primary care in treatment of many diseases [24]. Therefore, studies of medicinal plants concerning their therapeutic potential as well as possible side effects have high clinical relevance.

Harpagophytum procumbens, popularly known as devil's claw, is a perennial plant belonging to the family Pedaliceae and originating from Southern Africa [21, 25, 26]. *H. procumbens* extracts are mainly used because of their potent anti-inflammatory and analgesic effect demonstrated in numerous studies [20, 27–32]. Recently, the National Agency for Health Surveillance (ANVISA) [33], organ that oversees and regulates the sale of drugs in Brazil, approved the use of *H. procumbens* (MS: 1.1860.0035). Due to its therapeutic action, *H. procumbens* has been indicated for the treatment of arthritis, osteoarthritis, tendonitis, and as an adjunct treatment of gout in humans.

Besides the action in inflammation and pain, other effects have been attributed to *H. procumbens.* It has been demonstrated that *H. procumbens* showed anticonvulsant activity in mice [34], antidiabetic properties in rats [31], uterotonic effect on uterine musculature of mammals [35], cholinesterase inhibition in vitro [36] and in vitro antiplasmodial effects of some components extracted from the plant [37]. Many of the pharmacological actions of *H. procumbens* have been attributed to the presence of iridoid glycosides, Harpagoside. However, another studies show the effects of *H. procumbens* are not exclusively due to the presence of harpagoside and, instead of this, their effects have been associated with the presence of other compounds present in plants, such as flavonoids [38–40]. Besides, there are few studies investigating the mechanisms by which *H. procumbens* exerts its effects, despite the wide use of this plant in humans.

Therefore, the aim of this study was to evaluate the effects of the infusion, crude extract and fractions of *H. procumbens* on oxidative stress parameters and cell viability in vitro.

Materials and Methods

Animals

Male Wistar rats (±2 months old), weighing between 200 and 250 g, from our own breeding colony (Animal House, UFSM, Brazil) were kept in cages with free access to food and water in a room with controlled temperature (22 ± 2 °C) and in 12 h light/dark cycle with lights on at 7:00 am. All experiments were performed in accordance to the guidelines of the National Council of Control of Animal Experimentation (CONCEA).

Drugs

Tris–HCl, thiobarbituric acid, malonaldehydebis-(dimethyl acetal)(MDA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 5,5′-Dithiobis(2-nitrobenzoic acid L-Glutathione reduced, Folin and Ciocalteu's phenol reagent, catechin, quercetin and rutin were obtained from Sigma (St. Louis, MO, USA). Hydrogen peroxide and trichloroacetic acid (TCA), sodium nitroprusside, ferrous sulfate, and hydrochloric acid were obtained from Merck (Brazil). *H. procumbens* powder was obtained commercially from Quimer Comercial LTD (São Paulo, Brazil). Methanol, acetic acid, gallic acid (GA), rosmarinic acid and caffeic acid were purchased from Merck (Darmstadt, Germany). Ethyl alcohol, chloroform, *n*-butanol, ethyl acetate were acquired from Nuclear (São Paulo, Brazil).

Preparation of Infusion and Fractions

The powdered roots of *H. procumbens* were added to boiling distilled water (5 g/l), where they were packed in a closed flask protected from light. After 10 min, the infusion was filtered.

To obtain the different fractions, the powdered roots of *H. procumbens* were added to 70 % ethanol and allowed to stand at room temperature for a week with daily shaking. After filtration, the extract was evaporated under reduced pressure to remove the ethanol. The extract was then re-suspended in water and partitioned successively with chloroform, ethyl acetate and *n*-butanol (3 × 200 ml for each solvent) [41].

Quantification of Phenolic and Flavonoid Compounds by HPLC–DAD

Reverse-phase chromatographic analyses were carried out under gradient conditions using a C18 column (4.6 mm × 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2 % acetic acid (A) and methanol (B), and the composition of the gradient was: 5 % B until 2 min and changed to obtain 25, 40, 50, 60, 70 and 100 % B at 10, 20, 30, 40, 50 and 60 min, respectively, following the method described by Laghari [42] with slight modifications. Fractions and infusion were tested at concentrations of 5 mg/ml. The flow rate was 0.8 ml/min and injection volume 40 μl, and the detection wavelengths were 254 nm for GA, 280 nm for catechin, 325 nm for caffeic and rosmarinic acids, and 365 nm for quercetin and rutin. All samples and mobile phase were filtered through a 0.45-μm membrane filter (Millipore) and then degassed in anultrasonic bath prior to use. Stock solutions of reference standards were prepared in the HPLC mobile phase at a concentration range of 0.031–0.250 mg/ml for catechin, quercetin and rutin and 0.006–0.250 mg/ml for gallic, rosmarinic and caffeic acids. The chromatography peaks were confirmed by comparing their retention time with those of reference standards and by DAD spectra (200–500 nm). Calibration curves for the standards were: GA: Y = 48179x + 1236.5 (r = 0.9989); catechin: Y = 32741x + 1178.3 (r = 0.9995); caffeic acid: Y = 52055x + 1178.1 (r = 0.9997); rosmarinic acid: Y = 15534x + 1284.1 (r = 0.9993); rutin: Y = 55073x + 1327.4 (r = 0.9998); and quercetin: Y = 51704x + 1265.2 (r = 0.9996). All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by ICH (2005). LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve. High performance liquid chromatography (HPLC–DAD) was performed with an HPLC system (Shimadzu, Kyoto, Japan) and Prominence Auto Sampler (SIL-20A), equipped with Shimadzu LC-20AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV–VIS detector DAD (diode) SPD-M20A and Software LC solution 1.22 SP1.

Determination of Total Phenolic Compounds

The total phenolic content was determined by mixing the extracts with 1.25 ml of 10 % Folin-Ciocalteu's reagent (v/v), which was followed by the addition of 1.0 ml of 7.5 % sodium carbonate. The reaction mixture was incubated at 45 °C for 15 min, and the absorbance was measured at 765 nm. GA was used as standard for phenolic compounds [43].

Tissue Preparation

Rats were decapitated and cerebral (whole brain) tissue was rapidly dissected, placed on ice and weighed. Tissue was immediately homogenized in 10 Mm Tris–HCl, pH 7.4 (1:10, w:v). The homogenate was centrifuged for 10 min at 4,000×g to yield a pellet, which was discarded, and the low-speed supernatant (S1) was used for in vitro analysis.

TBARS Production

The potential to prevent lipid peroxidation in vitro by *H. procumbens* was determined pre-incubating a 200-μl aliquot of S1 for 1 h at 37 °C, with pro-oxidant agents (SNP 5 μM and Fe^{2+}100 μM) in the presence or absence of *H. procumbens* or GA as positive control. TBARS production was determined as described by Ohkawa [44].

Catalase

The activity of antioxidant enzyme Catalase was evaluated after pre-incubation of S1 at 37 °C for 1 h with pro-oxidant agents (Fe^{2+} or SNP) in the presence or absence of *H. procumbens* or GA. The reaction mixture was centrifuged at 3,000 rpm for 10 min and an aliquot of supernatant was used for measuring catalase activity by the method of Aebi [45], which monitores the disappearance of H_2O_2 in the presence of the brain homogenate (phosphate buffer pH 7.0 at 25 °C) at 240 nm. The enzymatic activity was expressed in μmol H_2O_2/min/g tissue.

Thiol Oxidation

In this experiment, an aliquot of S1 was pre-incubated at same experimental condition of catalase (describe above) and after 1 h, the protein and non-protein thiol levels were determined according to Ellman [46]. For non-protein thiol levels, 10 % TCA was added to an aliquot of the pre-incubation, centrifuged at 3,000 rpm for 10 min and the supernatans were then used. Ellman's reagent [5, 5′-dithiobis (2-nitrobenzoic acid), DTNB was added to the samples and the formed chromogen was measured spectrophotometrically at 412 nm. Results of protein and non-protein thiols levels were expressed as μmol protein thiol/g tissue and μmol non-protein thiol/g tissue, respectively.

Preparation of Cortex Slices

Rats were killed by decapitation, and the cortex was dissected on ice and placed in cold saline buffer medium containing (in mM) 120 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 25 HEPES, 1 KH_2PO_4 and 10 glucose, which was adjusted to pH 7.4 and previously aerated with O_2 [47]. Cross-sectional slices (0.4 mm thickness) were obtained using a McIlwain Tissue Chopper.

Cell Viability

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Five cortical slices were then transferred immediately to 24-well culture plates, each well containing saline medium and pre-incubated with different pro-oxidant agents, SNP (10 μM) or Fe^{2+} (200 μM), in the presence or absence of *H. procumbens* (100, 200 or 400 μg/ml) for 60 min at 37 °C in a water bath. GA was used as positive control (1, 5 or 10 µg/ml). Afterwards, the slices were washed three times with cold saline medium and maintained in the last. MTT (2 µg/ml) was added and the slices incubated for 30 min at 37 °C. MTT was converted into a purple formazan after cleavage of the tetrazolium ring by mitochondrial dehydrogenases. After the incubation period, formazan crystals were dissolved by the addition of 250 µl of DMSO, and the absorbance was measured at 570 and 630 nm [48].

The total protein content in slices was determined by the method of Lowry [49], using bovine serum albumin as standard. Protein content was used to normalize MTT score.

Statistical Analysis

Data were statistically analyzed by one-way ANOVA, followed by a post hoc test when appropriate. The results were considered statistically significant when $p < 0.05$.

Results

HPLC Fingerprint and Determination of Total Phenolic Compounds of *H. procumbens* Infusion, Crude Extract and Fractions

HPLC fingerprinting of *H. procumbens* infusion, crude extract and fractions revealed the presence of GA (retention time- tR = 14.19 min; peak 1), catechin (tR = 21.07 min; peak 2), caffeic acid (tR = 25.37 min; peak 3), rosmarinic acid (tR = 30.85 min; peak 4), phenol glycoside (tR = 33.97 min; peak 5), rutin (tR = 38.23 min; peak 6) and quercetin (tR = 50.11 min; peak 7), (Fig. 1; Table 1). The ethyl acetate fraction displayed the highest percentage of the main constituents identified in comparison to other fractions.

Corroborating the HPLC analysis, the quantification of phenolic compounds showed that the ethyl acetate fraction

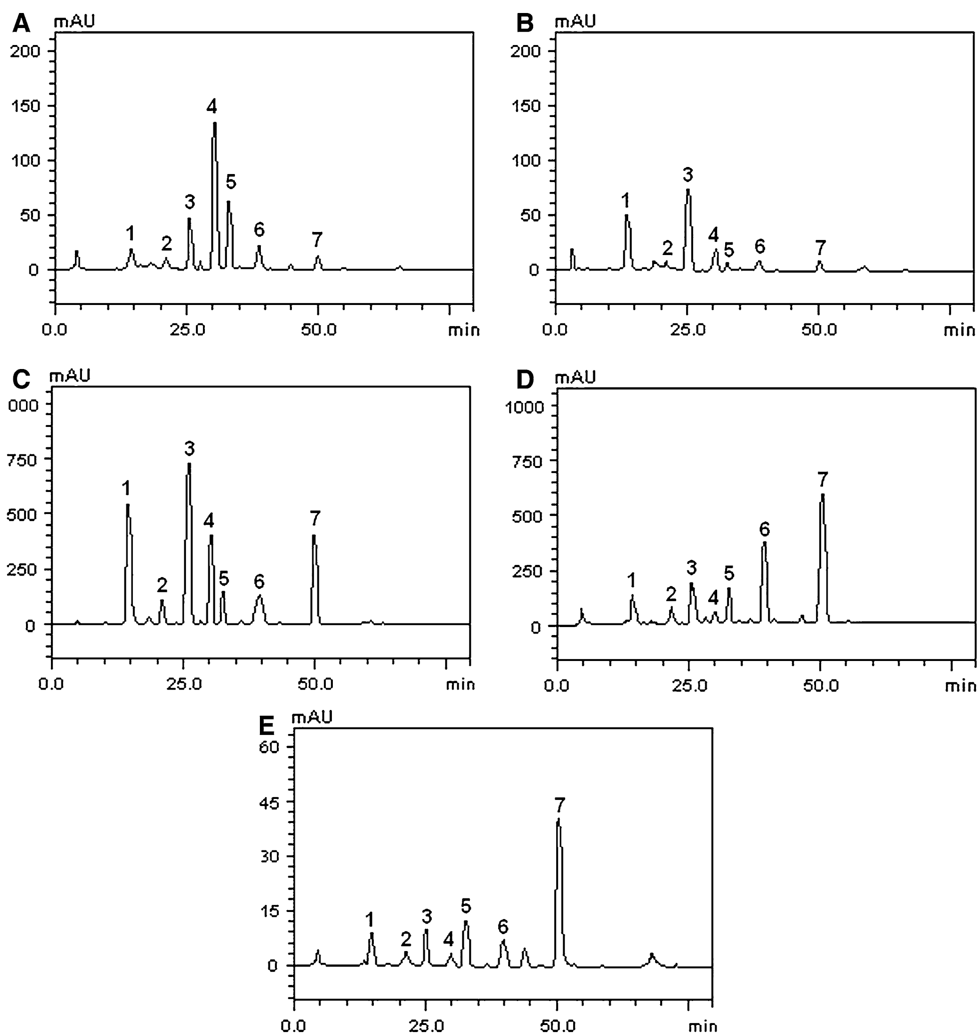

Fig. 1 Representative high performance liquid chromatography profile of *H. procumbens* **a** crude extract, **b** chloroform fraction, **c** ethyl acetate fraction, **d** butanolic fraction and **e** infusion. GA (peak 1), catechin (peak 2), caffeic acid (peak 3), rosmarinic acid (peak 4), phenol glycoside (peak 5), rutin (peak 6) and quercetin (peak 7)

Table 1 Composition of infusion, crude extract and fractions of *H. procumbens*

HP	Gallic acid (%)	Catechin (%)	Caffeic acid (%)	Rosmarinic acid (%)	Phenol glycoside (%)*	Rutin (%)	Quercetin (%)
CE	0.35 ± 0.03[a]	0.18 ± 0.01[a]	1.50 ± 0.06[a]	4.43 ± 0.05[a]	1.78 ± 0.01[a]	0.58 ± 0.01[a]	0.31 ± 0.02[a]
CF	1.62 ± 0.01[b]	0.07 ± 0.04[a]	2.76 ± 0.02[b]	0.56 ± 0.01[b]	0.10 ± 0.05[b]	0.19 ± 0.07[b]	0.18 ± 0.04[a]
EAF	10.63 ± 0.08[c]	2.59 ± 0.02[b]	17.51 ± 0.03[c]	8.23 ± 0.01[c]	3.62 ± 0.05[c]	4.94 ± 0.02[c]	8.70 ± 0.01[b]
BF	2.16 ± 0.09[d]	1.19 ± 0.02[c]	3.86 ± 0.01[d]	0.94 ± 0.03[d]	2.87 ± 0.06[d]	7.31 ± 0.04[d]	11.40 ± 0.01[c]
I	0.72 ± 0.04[e]	0.29 ± 0.01[a]	0.61 ± 0.05[e]	0.25 ± 0.04[b]	1.46 ± 0.07[a]	0.81 ± 0.02[a]	3.56 ± 0.07[d]
LOD	0.045	0.026	0.031	0.018	–	0.009	0.007
LOQ	0.148	0.085	0.102	0.060	–	0.030	0.023

Results are expressed as mean ± standard deviations (SD) of three determinations. Means followed by different letters, on each column, differ statistical by Tukey's test at $p < 0.05$

* Quantified was GA

CE crude extract, *CF* chloroform fraction, *EAF* ethyl acetate fraction, *BF* butanolic fraction, *I* infusion

Table 2 Determination of phenolic compounds in infusion, crude extract and fractions of *H. procumbens*

Method of extraction	phenol (μg GAE/mg plant) mean ± SEM
Infusion	2.37 ± 0.21[a]
Crude extract	5.13 ± 0.44[b]
Ethyl acetate	13.17 ± 0.50[c]
n-Butanol	5.62 ± 0.09[b]
Chloroform	4.95 ± 0.50[b]

Results are expressed as mean ± SEM from three to four independent experiments performed in duplicate. Means followed by different letters differ by Tukey's test at $p < 0.05$

of *H. procumbens* had the highest amount of total phenolic compounds, which was statistically different when compared to the other extracts tested. In contrast, *H. procumbens* infusion showed the least amount of these compounds in relation to the other extracts (Table 2).

Effects of *H. procumbens* on Oxidative Stress Parameters

Iron induced a significant increase in brain lipid peroxidation as demonstrated by the increase in TBARS formation ($p < 0.001$) when compared to basal level. Crude extract, fractions and infusion of *H. procumbens* significantly inhibited Fe^{2+}-induced TBARS formation in brain homogenate preparations in a concentration-dependent manner ($p < 0.001$). However, the inhibitory potency varied according to extract preparation. The potency order was ethyl acetate > chloroform > *n*-butanol > crude extract > infusion (Fig. 2; Table 3).

SNP induced an increase in TBARS production ($p < 0.001$) in brain preparations when compared to basal level, which was significantly inhibited by the crude extract, fractions and infusion of *H. procumbens* in a concentration dependent manner. The order of inhibitory potency between fractions was ethyl acetate > crude extract = *n*-butanol > chloroform > infusion (Fig. 3; Table 3). GA was able to protect against lipid peroxidation induced by both pro oxidants (Figs. 2, 3). In comparison, the IC50 values obtained with GA were similar to those obtained with the ethyl acetate fraction (Table 3).

Statistical analyses revealed that pro oxidant agents used were able to decrease catalase activity (Fig. 4a, $p < 0.001$ and Fig. 4b, $p < 0.05$) and both protein (Fig. 5a, $p < 0.001$ and Fig. 5b, $p < 0.05$) and non-protein thiol levels (Fig. 5c, $p < 0.001$ and Fig. 5d, $p < 0.01$) when compared to basal level. Ethyl acetate fraction prevented the consumption of catalase, induced by Fe^{2+} (Fig. 4a, $p < 0.01$) or SNP (Fig. 4b, $p < 0.05$). Similarly to catalase, ethyl acetate fraction was effective in preventing the oxidation of thiols induced by both pro-oxidant tested (Fig. 5a and 5c, $p < 0.00$; Fig. 5b and 5d, $p < 0.01$). GA has also been able to protect against the consumption of catalase and thiol content.

Effects of *H. procumbens* Ethyl Acetate Fraction on Cell Viability of Cortex Slices Submitted to Different Pro-oxidants

Since the ethyl acetate fraction had the highest phenolic content and antioxidant effects, we tested its effect on cell viability. The MTT assay showed that the pro-oxidants used caused a significant decrease in cell viability (Fig. 6). The ethyl acetate fraction significantly prevented cell damage induced by Fe^{2+} (Fig. 6a) and SNP (Fig. 6b) in slices of rat cerebral cortex. GA was also able to prevent cell damage induced by both pro-oxidants.

Discussion

Harpagophytum procumbens is a plant widely used by population due to its anti-inflammatory and analgesic

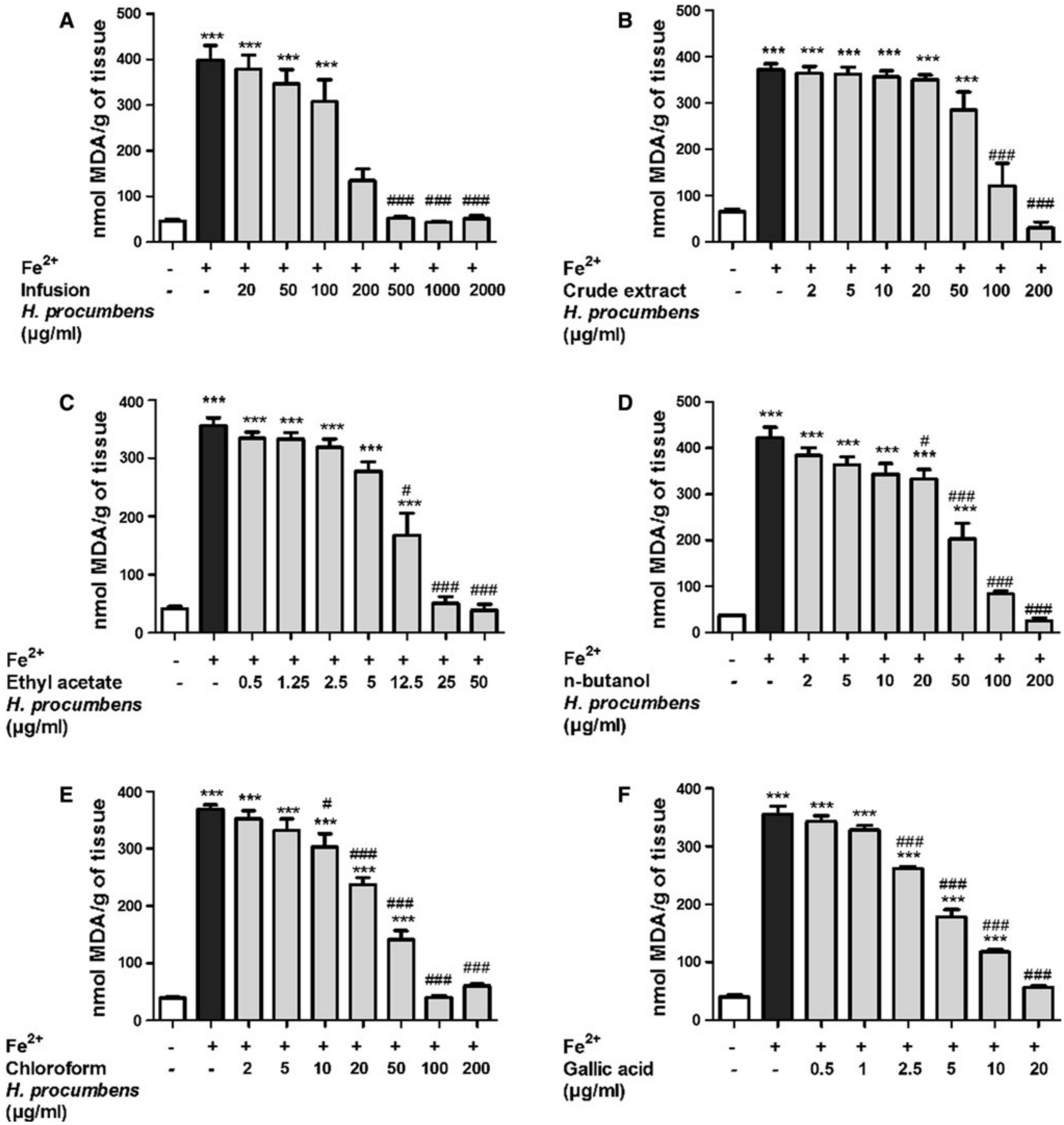

Fig. 2 Effects of infusion (**a**), crude extract (**b**) and fractions (**c** ethyl acetate, **d** *n*-butanol, **e** chloroform) of *H. procumbens* on basal (control) or Fe^{2+} (100 µM)-induced lipid peroxidation in rat brain homogenates. GA was used as a control antioxidant (**f**). Data show mean ± SEM from three to four independent experiments performed in duplicate. One-way ANOVA followed by Dunnett's test. *, **, *** Significant differences compared to basal and #, ##, ### Significant differences in relation to that induced by Fe^{2+}

action [50]. However, little is known about the mechanism of its pharmacological action. It is demonstrated that there is a strong relationship between inflammation and oxidative stress in the affected site [4, 5, 51]. It is known that brain is particularly vulnerable to oxidative stress due to its high content of polyunsaturated fatty acids and high oxygen consumption. Furthermore, the brain has low levels of antioxidant enzymes (e.g., catalase and glutathione peroxidase), which further facilitates the establishment of an oxidative state in brain cells [1, 52]. Here, GA was used as a positive control because it is a phytochemical compound well known in the literature, due to their antioxidant

Table 3 IC_{50} (μg/ml) values for inhibition by infusion, crude extract and fractions of *H. procumbens* of TBARS production induced by different pro-oxidants in rat brain preparations

Method of extraction	Pro-oxidants	
	Fe^{2+}	SNP
Infusion	158.40 ± 4.23[a]	37.20 ± 4.37[a]
Crude extract	87.33 ± 10.39[b]	5.15 ± 0.27[b]
Ethyl acetate	11.07 ± 1.32[c]	0.95 ± 0.005[c]
n-Butanol	49.92 ± 3.56[d]	6.41 ± 1.43[b]
Chroform	37.81 ± 4.44[d]	10.41 ± 0.40[b]
Gallic acid	5.02 ± 0.59[e]	0.61 ± 0.13[c]

Results are expressed as mean ± SEM from three to four independent experiments performed in duplicate. Means followed by different letters, on each column, differ statistically by Tukey's test at $p < 0.05$

characteristics. Furthermore, it is present in all of our extracts, being in greater quantity in the ethyl acetate fraction.

Thus, our first aim in the present study was to evaluate the effects of *H. procumbens* on brain lipid peroxidation induced by different pro-oxidants and compare its effects with GA. All *H. procumbens* extracts were able to prevent lipid peroxidation induced by SNP or iron in brain homogenates.

Lipid peroxidation caused by different pro-oxidant agents was easily prevented by *H. procumbens* crude extract, infusion or its fractions, indicating that the plant has a good antioxidant activity in vitro. In the present study, *H. procumbens* demonstrated a higher capacity to protect against lipid peroxidation induced by SNP than by iron, as showed by the IC_{50} results (Table 3). These result is in agreement with Wagner [53], that showed the highest effect of quercitrin to protect against lipid peroxidation induced by SNP than by other pro-oxidants.

Corroborating the findings on the TBARS test, the fingerprint of the extracts by HPLC showed different amounts of phenolic and flavonoid compounds in different extracts. Ethyl acetate fraction had the highest quantity of these compounds followed by butanolic fraction. This result has been demonstrated in other studies since these solvents can extract a greater amount of antioxidant compounds [41, 54, 55]. In addition, the ethyl acetate fraction showed a high index of total phenolic compounds, and its IC_{50} value was significantly lower than the other extracts. Also, the ethyl acetate fraction exhibited the highest activity against lipid peroxidation induced by both pro-oxidants used compared with other extracts. This relationship between phenolic compounds and antioxidant activity has already been described by several authors [12, 13, 53, 55].

Considering that the ethyl acetate fraction showed the highest capability against lipid peroxidation and the highest quantity and variety of antioxidants compounds, our second objective was to investigate whether it would be able to protect against possible damage in antioxidants defenses induced by Fe^{2+} or SNP, we decided to analyze the catalase activity and thiol levels (protein and non-protein).

The ethyl acetate fraction was also able to protect against a decrease of catalase activity and thiol levels. These markers are consumed in response to cellular oxidative stress. These results are in agreement with Bhattacharya and Bhattacharya [56], which demonstrated a dose-dependent increase in the activity of catalase and glutathione peroxidase in brain of rats treated with *H. procumbens* extract for 14 days. Another study showed that flavonoids such as quercetin and genistein were able to prevent the loss of reduced glutathione induced by copper and iron in U937 cells in vitro [57]. Furthermore, it is noteworthy that the results obtained from the ethyl acetate fraction were similar to those obtained with GA, demonstrating its high potential to protect against oxidative damage (Figs. 2, 3, 4, 5).

To check if the ethyl acetate fraction would act on a system with viable cells, we decided to investigate a possible protection against decrease in cell viability induced by iron or SNP in rat brain cortical slices.

In our study, the MTT assay showed that the *H. procumbens* ethyl acetate fraction had the ability to prevent the loss in cell viability induced by both pro-oxidants used (iron or SNP), which are able to generate reactive species, establishing the oxidative process and leading to cell death [2]. Therefore, *H. procumbens* acts either by preventing oxidative stress or loss of cell viability.

It is known that SNP causes cytotoxicity via either release of cyanide and/or nitric oxide (NO) [58–61]. Indeed, SNP is a good chemical inducer of lipid peroxidation [53], since it rapidly releases NO• in tissue preparations [58]. This radical easily produces peroxynitrite (ONOO−) and superoxide anion radical (O2•−), thus leading to lipid peroxidation and production of additional free radicals [62].

Similar to the SNP, iron can cause cytotoxic effects, such as by catalyzing the decomposition of H_2O_2 with the formation of hydroxyl radical ($\dot{OH}$) [63], which is normally considered the most reactive and damaging intermediate produced during cellular metabolism [63–66]. Besides, free iron is found in increased levels in some degenerative diseases [67, 68]. However, in a non-hazardous way, iron plays an important function in the respiratory chain, where its oxidation and reduction transports the electrons derived from food oxidation to molecular oxygen (O_2) [69].

There are literature data showing that flavonoids naturally present in plants have chelating capacity, thereby preventing oxidative cellular damage [53, 70, 71]. In this study the iron-induced oxidative damage on lipids and different extracts of *H. procumbens* were able to prevent

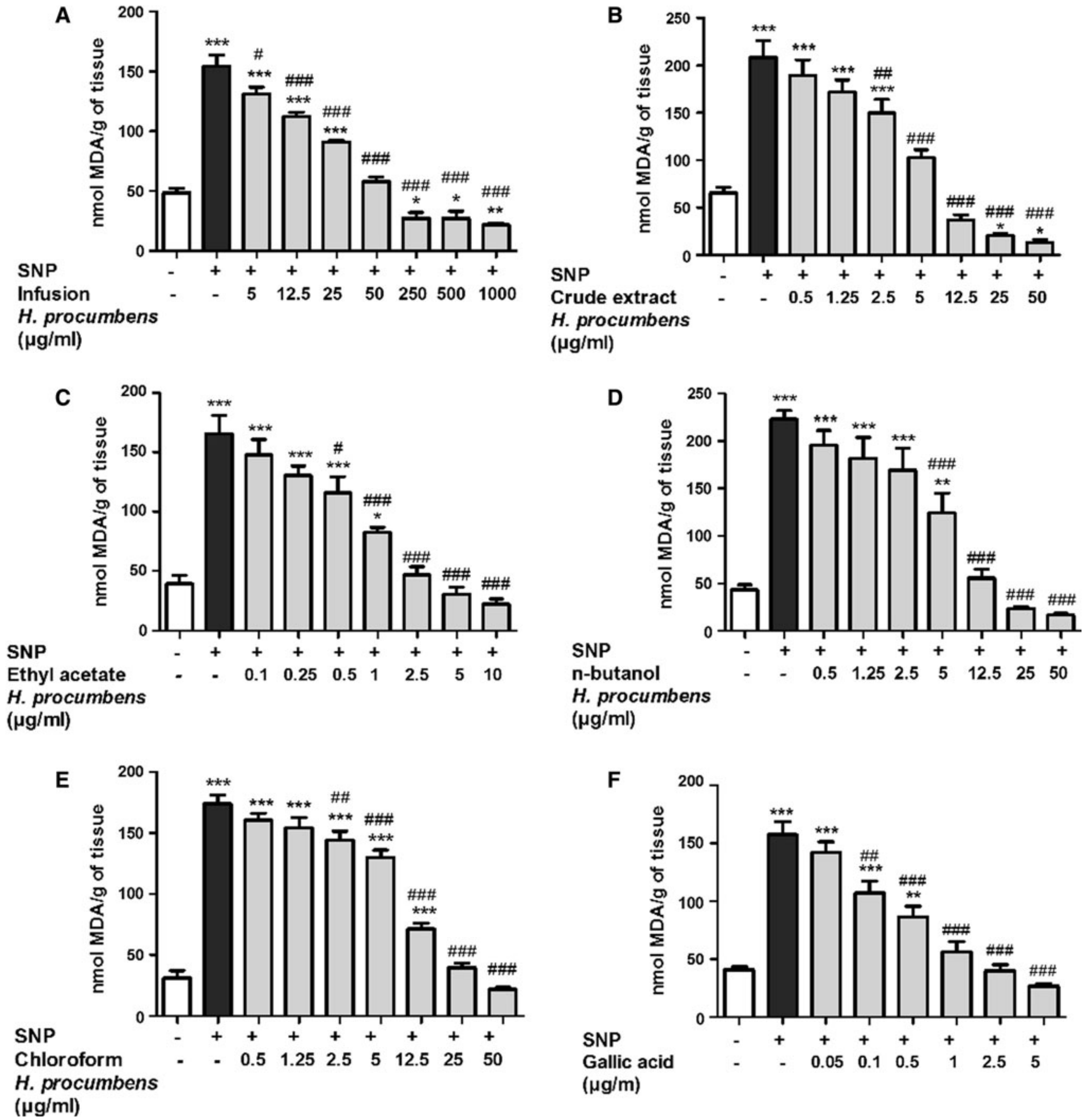

Fig. 3 Effects of infusion (**a**), crude extract (**b**) and fractions (**c** Ethyl acetate, **d** *n*-butanol, **e** Chloroform) of *H. procumbens* on basal (control) or SNP (5 µM)-induced lipid peroxidation in rat brain homogenates. GA was used as control antioxidant (**f**). Data show mean ± SEM from three to four independent experiments performed in duplicate. One way ANOVA followed by Dunnett's test. *, **, *** Significant differences compared to basal and #, ##, ### Significant differences in relation to induced by SNP

lipid peroxidation. In all extracts, flavonoids such as rutin and quercetin were found. A possible protection mechanism of *H. procumbens* could be through chelation of iron by flavonoids, preventing the formation of (OḢ) in Fenton reactions.

Previously published data have shown that the efficacy of *H. procumbens* is dependent on the mixture of a variety and amount of compounds present in plants and not just a single active component, such as harpagoside [39, 40, 72]. Kaszkin and collaborators [39], evaluated the anti-inflammatory and antioxidant activity by testing extracts containing high and low concentrations of harpagoside as well as harpagoside alone. As result, they showed that the best effect occurs in the presence of extracts with high

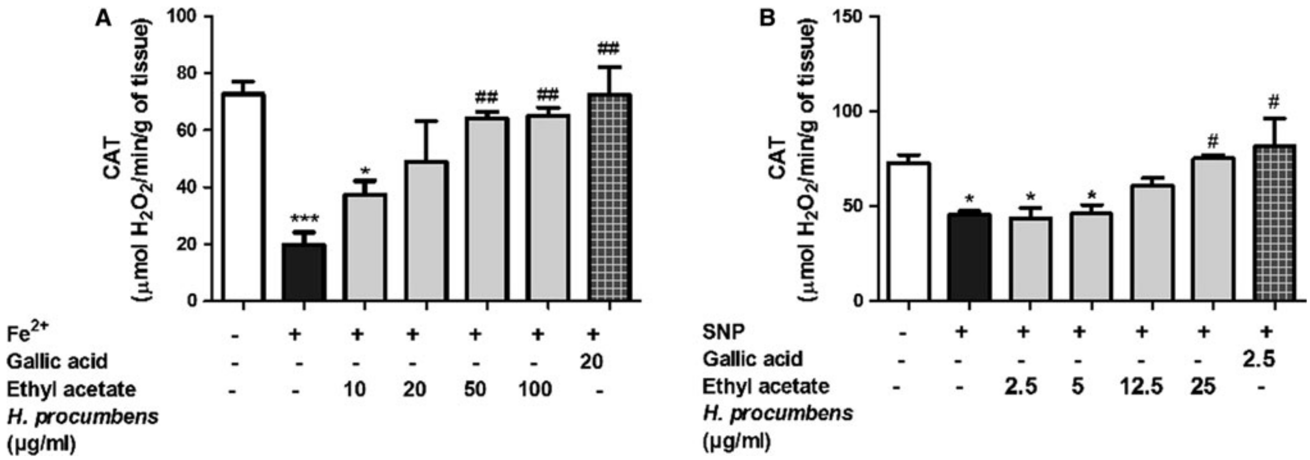

Fig. 4 Effects of *H. procumbens* ethyl acetate fraction on catalase levels of rat brain homogenates submitted to the action of different pro-oxidant agents: **a** Fe^{2+}(100 μM) and **b** SNP (5 μM). GA was used as control antioxidant. Data show mean ± SEM from three to four independent experiments performed in duplicate. One way ANOVA followed by Dunnett's test. *, **, *** Significant differences compared to basal and #, ##, ### Significant differences in relation to pro-oxidant agent

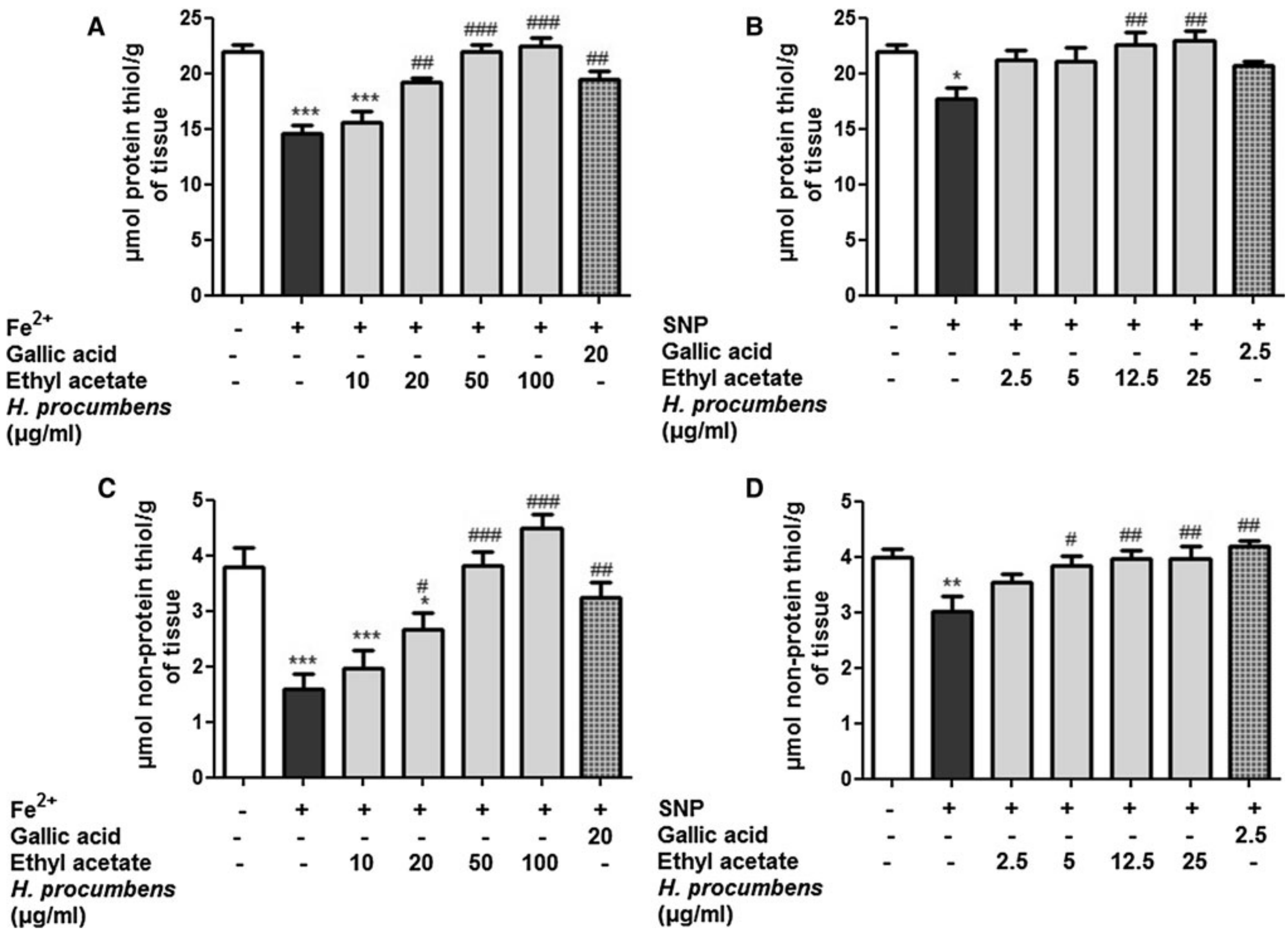

Fig. 5 Effects of *H. procumbens* ethyl acetate fraction on thiols content (protein and non-protein) of rat brain homogenates submitted to the action of different pro-oxidant agents. Protein thiol oxidation induced by Fe^{2+} (**a**) and induced by SNP(**b**). Non-protein thiol: Induced by Fe^{2+} (**c**) + and induced by SNP (**d**). GA was used as control antioxidant. Data show mean ± SEM from three to four independent experiments performed in duplicate. One way ANOVA followed by Dunnett's test. *, **, *** Significant differences compared to basal and #, ##, ### Significant differences in relation to pro-oxidant agent

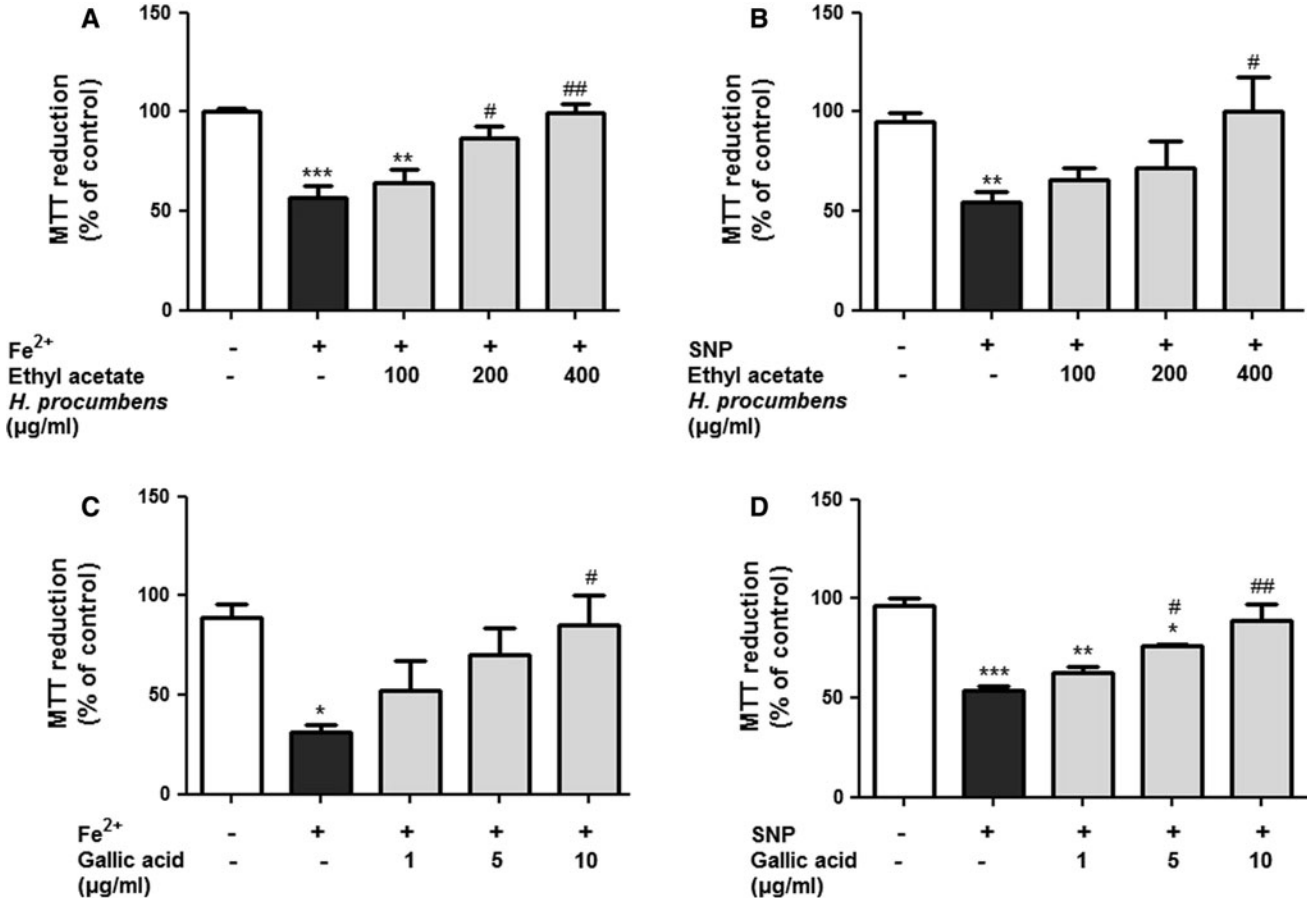

Fig. 6 Effects of *H. procumbens* ethyl acetate fraction on cell viability of rat brain cortical slices submitted to the action of different pro-oxidant agents: **a** Fe^{2+} (200 μM) and **b** SNP (10 μM). Cell viability was analyzed by quantification of MTT reduction. The data were corrected for the total amount of protein contained in the slices and were expressed as percentage of the control (considered as 100 %). GA was used as control antioxidant (c and d). Data show mean ± SEM from three to four independent experiments performed in duplicate. Statistical analysis was performed by one-way ANOVA followed by Dunnett's test. *, **, *** compared to basal and #, ##, ### represent differences in relation to that induced with pro-oxidant agents

concentrations of harpagoside. However, when harpagoside was tested alone, there was no effect, showing that the effectiveness of *H. procumbens* is due to the presence of several compounds.

Conclusion

In conclusion, all extracts of *H. procumbens* tested in this study were able to prevent either oxidative stress and loss of cell viability induced by well known pro-oxidant agents. These results are interesting, because this plant is widely used for the treatment of diseases related to inflammatory processes that generate painful stimuli, and the exact mechanism by which it exerts its therapeutic effect is not well elucidated. Our findings showed the high antioxidant capacity of *H. procumbens* through in vitro tests, and this effect can thus be related to the therapeutic effect of this plant. However, more studies must be performed to investigate its mechanism of action with the aim of exploring the whole therapeutic potential of *H. procumbens.*

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Conflict of interest The authors declare that they have no conflict of interest.

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