Neurochem Res (2015) 40:1197–1210
DOI 10.1007/s11064-015-1582-5

ORIGINAL PAPER

Centella asiatica and Its Fractions Reduces Lipid Peroxidation Induced by Quinolinic Acid and Sodium Nitroprusside in Rat Brain Regions

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Received: 25 September 2014 / Revised: 9 April 2015 / Accepted: 12 April 2015 / Published online: 23 April 2015

Abstract Oxidative stress has been implicated in several pathologies including neurological disorders. *Centella asiatica* is a popular medicinal plant which has long been used to treat neurological disturbances in Ayurvedic medicine. In the present study, we quantified of compounds by high performance liquid chromatography (HPLC) and examined the phenolic content of infusion, ethyl acetate, *n*-butanolic and dichloromethane fractions. Furthermore, we analyzed the ability of the extracts from *C. asiatica* to *scavenge* the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) radical as well as total antioxidant activity through the reduction of molybdenum (VI) (Mo^{6+}) to molybdenum (V) (Mo^{5+}). Finally, we examined the antioxidant effect of extracts against oxidant agents, quinolinic acid (QA) and sodium nitroprusside (SNP), on homogenates of different brain regions (cerebral cortex, striatum and hippocampus). The HPLC analysis revealed that flavonoids, triterpene glycoside, tannins, phenolic acids were present in the extracts of *C. asiatica* and also the phenolic content assay demonstrated that ethyl acetate fraction is rich in these compounds. Besides, the ethyl acetate fraction presented the highest antioxidant effect by decreasing the lipid peroxidation in brain regions induced by QA. On the other hand, when the pro-oxidant agent was SNP, the potency of infusion, ethyl acetate and dichloromethane fractions was equivalent. Ethyl acetate fraction from *C. asiatica* also protected against thiol oxidation induced by SNP and QA. Thus, the therapeutic potential of *C. asiatica* in neurological diseases could be associated to its antioxidant activity.

Keywords *Centella asiatica* · Antioxidant · Quinolinic acid · Sodium nitroprusside · Lipid peroxidation

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Introduction

Reactive species are formed in cellular conditions by several mechanisms including autoxidation of unstable biomolecules such as dopamine and activation of neutrophils or nitric oxide synthases whose produce nitric oxide [1]. Oxygen metabolism also leads to the production of small quantities of reactive oxygen species (ROS), such as superoxide radical (O_2^{-}), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) [2]. Under physiological conditions, the production of free radicals and other reactive species are kept in an equilibrium state by antioxidant defense system [3]. These species are maintained at low, but measurable, concentrations in the cells, through a balance between their rates of production and removal by antioxidants [4].

Oxidative stress occurs when cellular antioxidant defense mechanisms fail to counterbalance and control the endogenous production of ROS and reactive nitrogen species (RNS) [5, 6]. Therefore, it has been related to numerous pathologies where ROS production can contribute

to worse the symptoms by causing alterations in the cell membrane (lipid peroxidation and protein oxidation) and DNA mutations [4, 7]. In the mid-1950s, Denham Harman articulated a 'free-radical theory' of ageing, speculating that endogenous oxygen radicals were generated in cells and resulted in a pattern of cumulative damage. Particularly, oxidative stress has been implicated as a major cause of cellular injuries in the central nervous system [8], as Alzheimer's disease [9], cognitive deficits [10], Parkinson's disease [11], and Huntigton's disease [12].

In this context, different neurotoxic agents have been used to induce oxidative stress in vitro [13–17] and in vivo [18–20] models, such as quinolinic acid (QA) and sodium nitroprusside (SNP). QA is a major metabolite of the kynurenine pathway of tryptophan metabolism [21] and is an endogenous glutamate agonist with relative selectivity to *N*-methyl-D-aspartate (NMDA) receptor [22] and is involved in neurotoxic events including epilepsy [23]. The activation of NMDA receptor leads to an increase in intracellular Ca^{2+} concentration, leading to ATP depletion, mitochondrial dysfunction, oxidative stress and cell damage [24]. SNP is a nitric oxide donor, which in turn is a reactive nitrogen radical that reacts with oxygen to form other reactive species in aqueous medium [25] contributing to oxidative stress.

Medicinal plants and other natural compounds have been largely studied as alternative or adjuvant in the treatment of pathologies involving oxidative damage. *Centella asiatica* is a psychoactive therapeutic plant belonging to the family Apiaceae that has been used for several years in Indian Ayurvedic medicine with antioxidant and antiinflammatory properties [26]. Previous reports demonstrated that *C. asiatica* significantly protected the brain from neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [27] and ameliorated memory deficits against D-galactose-induced senescence in mice [28]. In addition, different bioactive components of *C. asiatica* such as asiatic acid, asiaticoside, madecassoside and madecassic acid improves neurological damages. All these components are triterpenes which have effective activity against free radical generation. Several studies have showed that the components of *C. asiatica* present biological activity against different injuries. For example: asiatic acid ameliorates ischemic damage [29] and H_2O_2-induced injury in SH-SY5Y cells [26]; standardized extract containing madecassoside and asiaticoside has anxiolytic effects against chronic imobilization stress [30]; asiaticoside has been found to have therapeutic value against β-amyloid neurotoxicity [31]; madecassoside has neuroprotective effect on focal reperfusion ischemic injury and early stage of Parkinson's disease induced by MPTP in rats [32, 33]. However, the mechanisms involved in pharmacological properties of *C. asiatica* extracts as well as the relation of biological activity with the characterization of the components present in each extract are not well understood.

Thus, the aim of the present study was compare different extracts of *C. asiatica* against oxidative damage induced by QA and SNP in brain regions (cerebral cortex, striatum and hippocampus) verifying the importance of the composition of the extracts to *C. asiatica* action in specific brain structures.

Materials and Methods

Animals

Male adult rats (±2 months old), weighing 280–320 g, were obtained from a local breeding colony (Animal House, UFSM, Brazil). The animals were kept in a room, with free access to food and water, on a 12 h light/dark cycle, controlled temperature (22 ± 2 °C). The animals were used according to the guidelines of the National Council to Control of Animal Experimentation, Brazil.

Chemicals Reagents

Thiobarbituric acid (TBA) was purchased from Merck (Brazil). QA (2,3-pyridine dicarboxylic acid), Tris–HCl, malonaldehyde bis-(dimethyl acetal) (MDA), Folin and Ciocalteu's phenol reagent, sodium nitroprusside, madecassoside, quercetin, quercitrin, rutin, catechin, epicatechin and kaempferol were obtained from Sigma (St. Louis, MO, USA). Acetonitrile, formic acid, gallic acid, chlorogenic acid, rosmarinic acid and caffeic acid purchased from Merck (Darmstadt, Germany). The powder of *C. asiatica* was obtained from Pharma Nostra Comercial (Anápolis, GO, Brazil).

Preparation of Infusion and Fractions

The infusion of *C. asiatica* was prepared by dissolving 2 mg/mL of powder in boiling distilled water which was filtered after 10 min. To obtain the different fractions, the aqueous extract was evaporated under reduced pressure to remove the water. Aqueous extract was then re-suspended in water and partitioned successively with dichloromethane, ethyl acetate and *n*-butanol (3 × 200 mL for each solvent) [34]. The concentrations used were defined considering its in vitro effects of previous experiments from our group (data not shown) and data from literature [35].

Quantification of Compounds by HPLC–DAD

Chromatographic separation was performed with a reversed phase using C_{18} column (4.6 mm × 250 mm) packed with

5 μm diameter particles. Mobile phase was water containing 1 % formic acid (A) and acetonitrile (B), and the composition gradient was: 20 % of B until 0 min and changed to obtain 35, 45, 65, 80, 90 and 20 % of B at 15, 30, 35, 40, 45 and 60 min, respectively [36] with slight modifications. *C. asiatica* infusion, dichloromethane fraction, ethyl acetate fraction and butanolic fraction were analyzed dissolved in water at a concentration of 10 mg/mL. The presence of the following compounds was investigated, gallic acid, chlorogenic acid, caffeic acid, rosmarinic acid, madecassoside, catechin, epicatechin, quercetin, quercitrin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.7 mL/min, injection volume 50 μL and the wavelength were 206 nm for madecassoside, 254 nm for gallic acid, 280 nm for catechin and epicatechin, 327 nm for caffeic, rosmarinic and chlorogenic acids, and 366 nm for quercetin, quercitrin, rutin and kaempferol. All the samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030–0.250 mg/mL for kaempferol, quercetin, quercitrin, rutin, catechin, epicatechin and madecassoside; and 0.030–0.250 mg/mL for gallic, caffeic, rosmarinic and chlorogenic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–600 nm). Calibration curve for gallic acid: $Y = 13629x + 1195.8$ ($r = 0.9993$); catechin: $Y = 12407x + 1259.6$ ($r = 0.9997$); epicatechin: $Y = 12547x + 1193.4$ ($r = 0.9991$); caffeic acid: $Y = 11758x + 1359.2$ ($r = 0.9996$); chlorogenic acid: $Y = 14061x + 1325.3$ ($r = 0.9995$); rosmarinic acid: $Y = 12658x + 1195.3$ ($r = 0.9998$); madecassoside: $Y = 13628x + 1273.8$ ($r = 0.9995$); rutin: $Y = 12845x + 1065.7$ ($r = 0.9999$); quercetin: $Y = 13560x + 1192.6$ ($r = 0.9991$), quercitrin: $Y = 13719x + 1256.7$ ($r = 0.9993$) and kaempferol: $Y = 14253x + 1238.9$ ($r = 0.9997$). 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. 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 [37].

Determination of Total Phenolic Compounds

For the total phenolic determination, the extracts were mixed 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 ($NaCO_3$) as previously described [38]. The reaction mixture was incubated at 45 °C for 15 min, and the absorbance was spectrophotometrically measured at 765 nm. Galic acid (GA) was used as standard for phenolic compounds and results are showed as GA equivalent (GAE).

DPPH$^{\cdot}$ Radical Scavenging Method

The radical scavenging activity of the compounds was determined as previously described [39]. Each extract was tested at concentrations of 10, 20, 50, 100 and 150 μg/mL. Gallic acid was used as a control. DPPH$^{\cdot}$ (diluted in ethanol) was added to final concentration of 0.15 mM and allowed to react at room temperature during 30 min in dark conditions. The absorbance was spectrophotometrically measured at 518 nm.

Spectrophotometric Quantitation of Antioxidant Capacity

A sample solution aliquot of *C. asiatica* infusion and fractions in water was combined in a vial with reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The assay is based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH [40]. The extracts were tested at concentrations of 10, 20, 50, 100 and 150 μg/mL. Thus, they were capped and incubated in a water bath at 95 °C for 90 min and the absorbance was measured at 695 nm against a blank control. GA was used as a standard and the antioxidant capacity of the extracts were compared to GA.

Preparation of Brain Homogenates

Animals were anesthetized with ketamine:xylazine (90:12 mg/kg) and killed by decapitation on the day of the experiments. The encephalic tissue (whole brain) was quickly removed and placed on ice. Striatum, hippocampus and cortex were removed, weighed and immediately homogenized in Tris–HCl 10 mM (1:10), pH 7.4. The homogenate was centrifuged for 10 min at $3000 \times g$ to yield a pellet, which was discarded, and the low speed supernatant (S1) was used for in vitro analysis.

Prepare Solutions

QA or SNP solutions were prepared to obtain final concentrations of 1 mM [14] and 5 μM [38, 41] respectively. QA (Sigma) was dissolved in 0.1 M phosphate buffer (pH 7.4) and neutralized with 1 N NaOH for the solutions stock. SNP was dissolved in distilled water just before the experiment. Oxidizing agents were added to the reaction just before the pre-incubation.

Lipid Peroxidation Induced by QA or SNP

The potential to prevent lipid peroxidation in vitro by *C. asiatica* was determined by thiobarbituric acid reactive substances (TBARS) production according to the method previously described by Okawa [42]. Analyses were performed in brain structures by mixing 200 µL of S1 for 1 h at 37 °C, with pro-oxidant agents (QA 1 mM or SNP 5 µM) in the presence or absence of different concentrations of *C. asiatica* extracts. TBARS formation was determined spectrophotometrically at 532 nm, using malondialdehyde (MDA) as standard.

Protein and Non-protein Thiol Oxidation Induced by QA or SNP

As the ethyl acetate fraction from *C. asiatica* presented the best antioxidant activity in the majority of the experiments, we tested this fraction against protein and non-protein thiol oxidation induced by QA or SNP. In this experiment, an aliquot of S1 was incubated at 37 °C for 1 h in the presence of *C. asiatica* with pro-oxidant agents (QA or SNP) and after, the protein and non-protein content thiol were determined in the present study [41]. For non-protein thiol content, trichloroacetic acid (TCA) was added to an aliquot of the pre-incubation, centrifuged at 3000 rpm for 10 min and the supernatant was 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 were expressed as µmol protein thiol/g tissue and µmol non-protein thiol/g tissue, respectively.

Statistical Analysis

The results were statistically analyzed by one-way ANOVA followed by a post hoc test when appropriate. The results were considered statistically significant when $p < 0.05$. The fraction concentration that causes 50 % inhibition (IC_{50}) was determined by linear regression analysis from 4 individual experiments by statistical software.

Results

HPLC Analysis

HPLC fingerprinting of *C. asiatica* infusion and fractions revealed the presence of the gallic acid ($t_R = 6.61$ min; peak 1), catechin ($t_R = 9.18$ min; peak 2); madecassoside ($t_R = 11.45$ min; peak 3), chlorogenic acid ($t_R = 14.86$ min; peak 4), caffeic acid ($t_R = 18.03$ min; peak 5), rosmarinic acid ($t_R = 21.34$ min; peak 6), epicatechin ($t_R = 24.57$ min; peak 7), rutin ($t_R = 32.15$ min; peak 8), quercitrin ($t_R = 36.20$ min; peak 9), quercetin ($t_R = 37.12$ min; peak 10) and kaempferol ($t_R = 43.79$ min; peak 11) (Fig. 1; Table 1). The HPLC analysis revealed that flavonoids (quercetin, quercitrin, rutin and kaempferol), triterpene glycoside (madecassoside), tannins (catechin and epicatechin) and phenolics acids (gallic, chlorogenic, rosmarinic and caffeic acids) are present in the extract of *C. asiatica.*

The ethyl acetate fraction displayed the highest percentage of the main constituents identified in comparison to other fractions. *N*-butanolic fraction presented rutin, quercitrin and kaempferol as components with higher amount and infusion showed as main components chlorogenic acid and quercetin respectively. The highest percentage of madecassoside was found in dichloromethane fraction while in butanolic fraction it was not found (Table 1).

Total Phenolic Compounds of *C. asiatica* Infusion and Fractions

The quantification of phenolic compounds showed that ethyl acetate (EA) fraction of *C. asiatica* presented the highest quantity of GAE/mg of extract followed by dichloromethane fraction > *n*-butanol fraction > infusion (Table 2). Phenolic compounds are usually presented in higher polar fractions [43] as ethyl acetate fraction. These results corroborate with HPLC analysis of the ethyl acetate fraction.

Effects of *C. asiatica* and Its Fractions on $DPPH^{\cdot}$ Radical

The ability of *C. asiatica* extracts in quenching the stable free radical $DPPH^{\cdot}$ was showed in comparison with GA as positive control at concentrations of 10, 50, 100 and 150 µg/mL (Fig. 2). The inhibitory concentration (IC_{50}) of $DPPH^{\cdot}$ radical by different extracts of *C. asiatica* was in the following order: ethyl acetate > dichloromethane > infusion = *n*-butanolic fraction (Table 3, $p < 0.05$).

Total Antioxidant Activity of *C. asiatica* and Its Fractions

The infusion presented the lowest total antioxidant activity compared to GA (Fig. 3, $p < 0.05$). *N*-butanolic and dichloromethane fraction at concentrations of 50–150 µg/mL showed total antioxidant activity similar to GA at 5 µg/mL. Ethyl acetate fraction presented the highest total antioxidant activity compared with other extracts being 100 and 150 µg/mL similar to GA at 20 and 50 µg/mL, respectively (Fig. 3, $p < 0.05$).

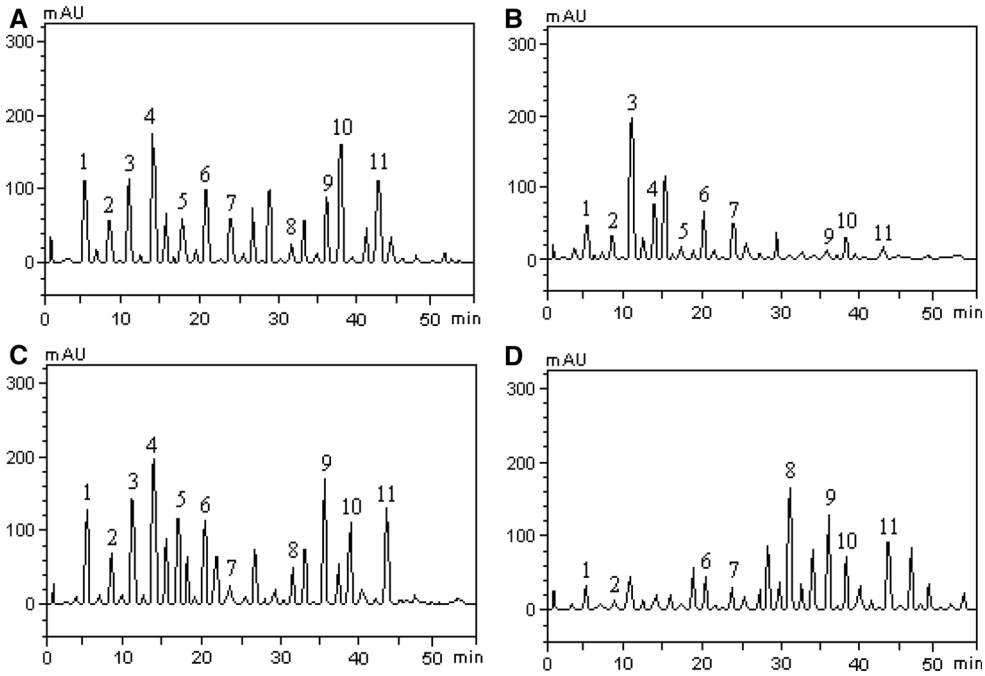

Fig. 1 Representative high performance liquid chromatography profile of *Centella asiatica* infusion (**a**), dichloromethane fraction (**b**), ethyl acetate fraction (**c**) and butanolic fraction (**d**). Gallic acid (peak 1), catechin (peak 2), madecassoside (peak 3), chlorogenic acid (peak 4), caffeic acid (peak 5), rosmarinic acid (peak 6), epicatechin (peak 7), rutin (peak 8), quercitrin (peak 9), quercetin (peak 10) and kaempferol (peak 11)

Table 1 Composition of *C. asiatica* infusion, dichloromethane, ethyl acetate fraction and butanolic fractions

Compounds	Infusion (mg/g)	Dichloromethane (mg/g)	Ethyl acetate (mg/g)	*n*-butanolic (mg/g)	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid	6.91 ± 0.02^{a}	2.89 ± 0.01^{a}	7.39 ± 0.01^{a}	1.46 ± 0.03^{a}	0.024	0.079
Catechin	4.07 ± 0.03^{b}	1.76 ± 0.02^{b}	4.16 ± 0.01^{b}	0.61 ± 0.01^{b}	0.007	0.023
Madecassoside	7.15 ± 0.03^{a}	14.07 ± 0.01^{c}	9.03 ± 0.03^{c}	–	0.032	0.105
Chlorogenic acid	11.83 ± 0.01^{c}	6.51 ± 0.02^{d}	15.14 ± 0.01^{d}	–	0.013	0.042
Caffeic acid	4.13 ± 0.02^{b}	0.81 ± 0.01^{e}	7.27 ± 0.03^{a}	–	0.035	0.115
Rosmarinic acid	6.28 ± 0.01^{ad}	4.19 ± 0.01^{f}	7.08 ± 0.01^{a}	2.79 ± 0.02^{c}	0.026	0.083
Epicatechin	4.27 ± 0.01^{b}	2.90 ± 0.03^{a}	1.39 ± 0.01^{e}	1.52 ± 0.01^{a}	0.021	0.070
Rutin	1.64 ± 0.03^{e}	–	2.83 ± 0.02^{f}	12.30 ± 0.03^{d}	0.042	0.138
Quercitrin	6.13 ± 0.01^{d}	0.72 ± 0.01^{e}	13.48 ± 0.01^{g}	8.69 ± 0.01^{e}	0.019	0.063
Quercetin	10.26 ± 0.02^{c}	1.68 ± 0.02^{b}	7.12 ± 0.01^{a}	5.42 ± 0.02^{f}	0.028	0.091
Kaempferol	7.29 ± 0.01^{a}	0.89 ± 0.03^{e}	8.16 ± 0.01^{c}	7.11 ± 0.02^{g}	0.015	0.049

Results are expressed as mean ± SEM from three to four independent experiments performed in duplicate. Means followed by different letters, on each column test and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated $p < 0.05$

Effect of *C. asiatica* and Its Fractions on Lipid Peroxidation Induced by QA or SNP

QA increased lipid peroxidation when compared with basal conditions ($p < 0.0001$), as well as SNP ($p < 0.0001$) in brain regions. Infusion (Fig. 4) of *C. asiatica* or its fractions (butanolic, Fig. 5; ethyl acetate, Fig. 6 or dichloromethane, Fig. 7) significantly inhibited QA (A, B, C)—or SNP (D, E, F)-induced TBARS formation in cortex, striatum and hippocampus homogenates. However, the inhibitory potency varied according with brain region, extract preparation and oxidant agent used which are demonstrated in Table 4.

The inhibitory potency (Table 4) obtained according with the extract from *C. asiatica* and brain region used was: in the cerebral cortex, striatum and hippocampus to QA-induced TBARS: ethyl acetate > *n*-butanolic = dichloromethane > infusion; for SNP-induced in the cerebral cortex was ethyl acetate > dichloromethane > infusion > *n*-butanolic, in the striatum was dichloromethane > ethyl acetate > infusion > *n*-butanolic and in the hippocampus was infusion > dichloromethane > ethyl acetate > *n*-butanolic.

Table 2 Total phenolic compounds of *C. asiatica* infusion and its fractions

Phenolic compounds (μg GAE/mg of extract)	Mean ± SEM
Infusion	1.82 ± 0.26[a]
Ethyl acetate	21.95 ± 0.44[b]
n-butanol	2.54 ± 0.01[a]
Dichloromethane	11.71 ± 0.49[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 Bonferroni's test at $p < 0.05$

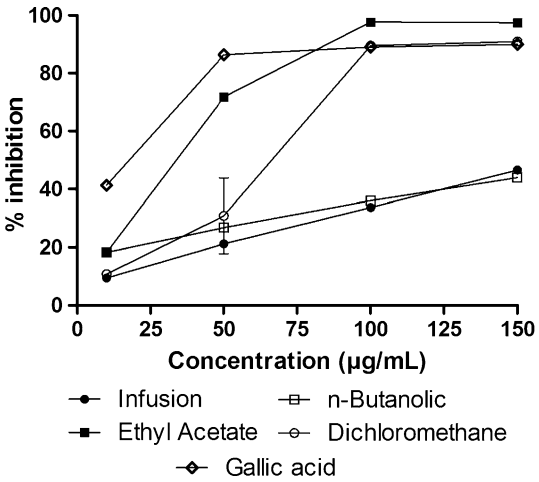

Fig. 2 Effects of different concentrations of infusion, ethyl acetate, *n*-butanolic and dichloromethane fractions from *Centella asiatica* on DPPH· assay. The results are expressed as percentage of inhibition and GA was used as a positive control. Data show mean ± SEM values averages from 3 independent experiments performed in duplicate

Table 3 IC_{50} (μg/mL) values of extracts from *C. asiatica* obtained on DPPH· assay

Extracts	IC_{50} (μg/mL)
Gallic acid (control)	13.33 ± 1.13[a]
Infusion	164.3 ± 3.34[b]
Ethyl acetate	38.86 ± 0.26[c]
n-butanolic	175.4 ± 3.72[b]
Dichloromethane	58.98 ± 2.34[c]

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

Effect of Ethyl Acetate Fraction from *C. asiatica* on Protein and Non-protein Thiol Oxidation Induced by QA or SNP

Statistical analyses revealed that pro-oxidant agents used were able to decrease the levels of both protein ($p < 0.05$, Fig. 8) and non-protein thiol content ($p < 0.05$, Fig. 9) as compared to basal level. Ethyl acetate fraction was effective in preventing the oxidation of thiols induced by both pro oxidant tested (Figs. 8, 9, $p < 0.05$). Similarly to lipid peroxidation, the protective effect of *C. asiatica* ethyl acetate fraction varied in according with brain region and pro-oxidant agent used.

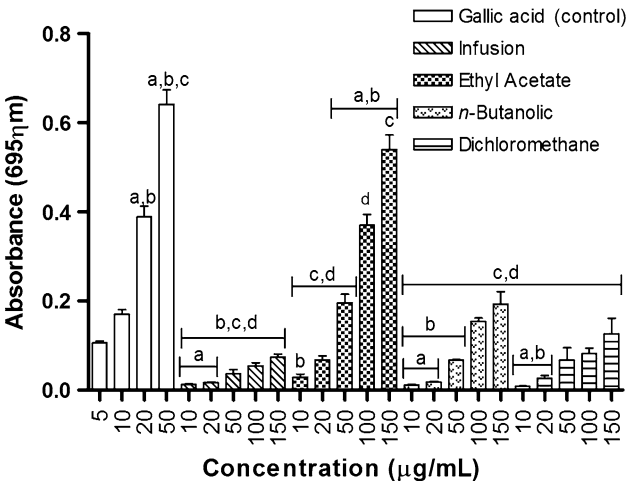

Fig. 3 Total antioxidant activity of the extracts from *C. asiatica* was measured by the phosphomolybdenum assay. Data are expressed as absorbance mean ± SEM (n = 3) and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant when $p < 0.05$. Significant difference compared to ([a]) 5 μg/mL GA; ([b]) 10 μg/mL GA; ([c]) 20 μg/mL GA and ([d]) 50 μg/mL GA

Discussion

The brain tissue is a target to oxidative damage due to its high Ca^{+2} trafficking across neuronal membranes [44, 45], high oxygen demand, high content of unsaturated fatty acids, rapid oxidative metabolic activity, and little endogenous antioxidant potential and insufficient neuronal cell repair capacity comparatively to its necessity [46]. In the present study, we show a significant increase in TBARS production and a decrease in thiol content in cerebral cortex, striatum and hippocampus when exposed to QA and SNP in vitro. It was also demonstrated that infusion and different fractions of *C. asiatica* have antioxidant action and ameliorated lipid peroxidation induced by QA and SNP. Also, ethyl acetate fraction from *C. asiatica* protected against the decreasing in thiol content induced by both pro-oxidants tested. However, the antioxidant potency of the fractions was dependent on the pro-oxidant used and of the tissue showing the presence of different constituents in each extract acts in a specific way depending on action mechanism of pro-oxidant used.

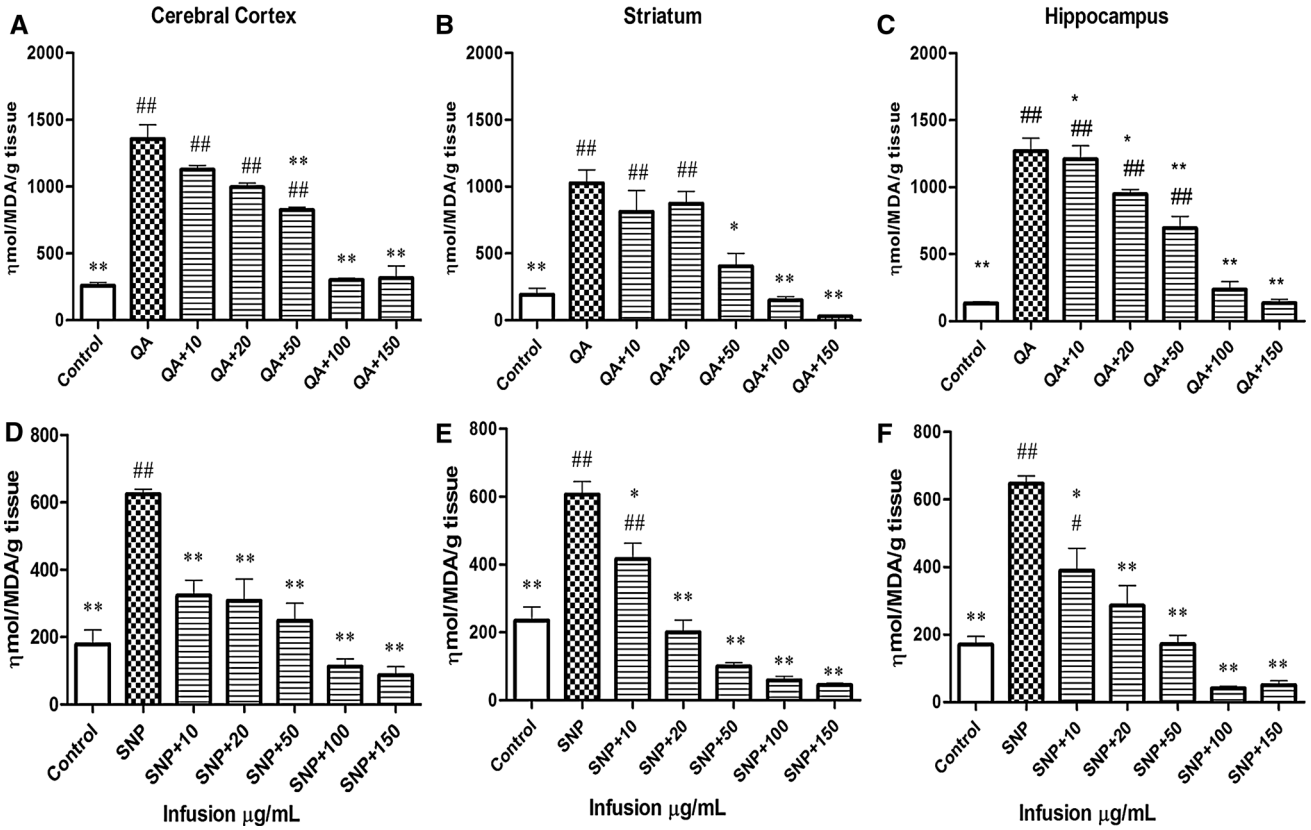

Fig. 4 Effect of infusion from *C. asiatica* on lipid peroxidation induced by QA (1 mM) (**a**–**c**) or SNP (5 μM) (**d**–**f**) in cerebral cortex (**a**, **d**), striatum (**b**, **e**) and hippocampus (**c**, **f**) of rats. Data are expressed as mean ± SEM (n = 3) and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant when $p < 0.05$. Significant differences are marked as (*) $p < 0.05$ or (**) $p < 0.0001$ when compared to QA or SNP group and marked as ($^{\#}$) $p < 0.05$ or ($^{\#\#}$) $p < 0.0001$ when compared to control group

Our study was drawn because there are no studies in vitro with *C. asiatica* fractions investigating its effects against neurotoxic agents and relating in vitro effects with the constituents present in each extract. Thus, we showed for the first time the in vitro effects of different extracts relating these effects with extracts composition. QA is a neurotoxic agent that can produce excitotoxicity and decrease cellular viability through free radical formation [47, 48] promoting an increase in extracellular glutamate levels that triggers oxidative stress via over-stimulation of NMDA receptors [49]. Furthermore, QA inhibits glutamate uptake in astrocytes [50]. We observed that QA was more potent in inducing lipid peroxidation than SNP, which could be attributed, at least in part, to multiple mechanisms by which QA produce its toxic effects [23]. However, in in vitro studies, the ability to induce lipid peroxidation depends on the formation of complexes with Fe(II) [51]. In homogenates treated with potent chelators such as deferoxamine at a concentration of 10 μM, the quinolinate is unable to produce lipid peroxidation. Recent studies showed the improvement of lipid peroxidation induced by QA with *V. officinalis* extract [52], red and white Ginger extracts [53] emphasizing beneficial effects of medicinal plants on neurotoxicity. These data are in agreement with our results since we show that *C. asiatica* extracts can reduce the increase in MDA levels in different brain structures induced by QA in vitro. Nevertheless, this protection is dependent of the oxidant agent used, antioxidant extract and brain structure since we observed that ethyl acetate fraction had the highest inhibitory potentials in all brain regions tested when it was used QA to induce lipid peroxidation. This effect is probably due to ethyl acetate constituents since this fraction has the highest quantity of phenolic content (Table 2) in comparison to the other fractions which was confirmed by HPLC analysis that demonstrated higher quantities of flavonoids, tannins and phenolics acids when compared with infusion, *n*-butanolic and dichloromethane fraction (Table 1). This result is in agreement with previous studies which have been demonstrating that the solvents used to obtain ethyl acetate fraction can extract a greater amount of antioxidant compounds [34, 54] particularly phenolic acids which are

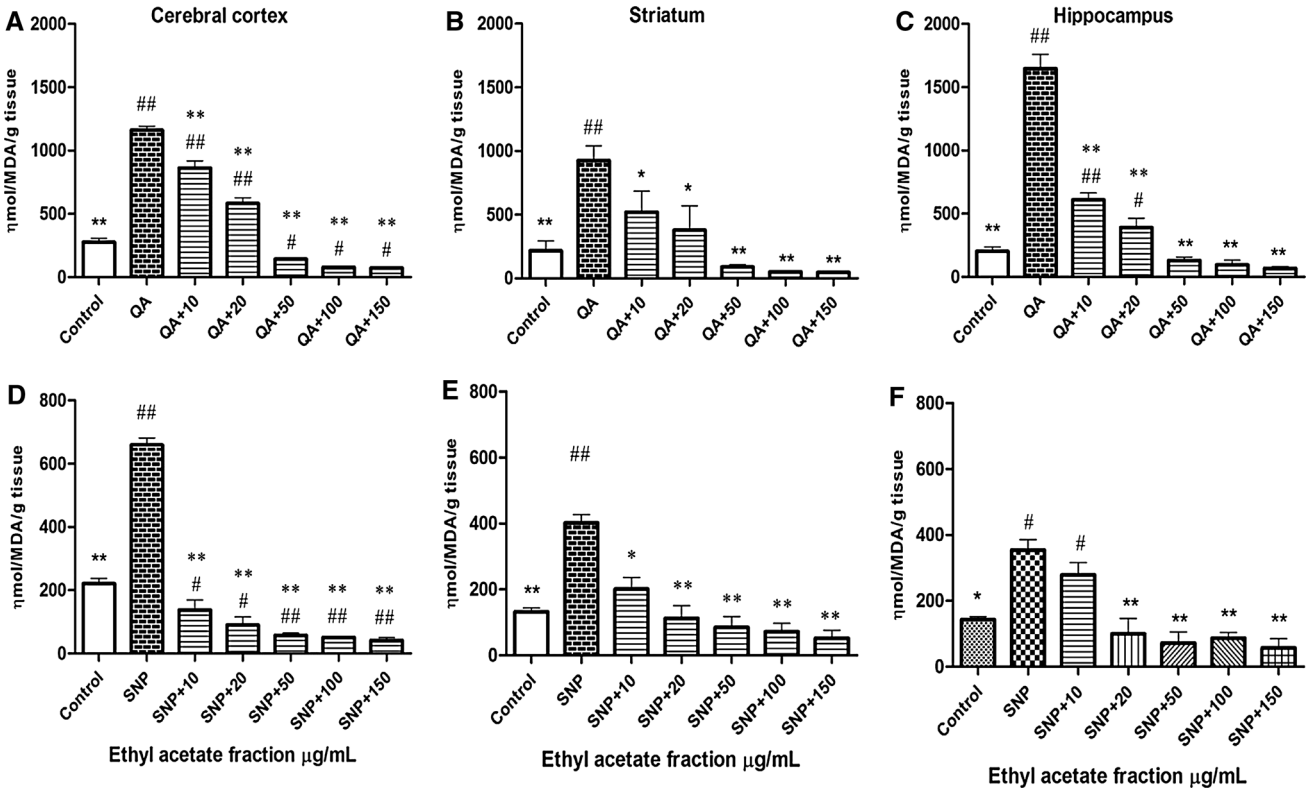

Fig. 5 Effect of ethyl acetate fraction from *C. asiatica* on lipid peroxidation induced by QA (1 mM) (**a**–**c**) or SNP (5 μM) (**d**–**f**) lipid peroxidation in cerebral cortex (**a**, **d**), striatum (**b**, **e**) and hippocampus (**c**, **f**) of rats. Data are expressed as mean ± SEM (n = 3) and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant when $p < 0.05$. Significant differences are marked as (*) $p < 0.05$ or (**) $p < 0.0001$ when compared to QA or SNP group and marked as ($^{\#}$) $p < 0.05$ or ($^{\#\#}$) $p < 0.0001$ when compared to control group

prominently present in the ethyl acetate fraction. Besides, literature data show that phenolic acids are able to decrease MDA levels and ROS production [55–58].

About the mechanism of its pharmacological action, it is known that phenol and triterpenes presents in the extract have high antioxidant potentials due to its capacity in chelate iron, preventing the formation of ($OH^{\cdot}$) in Fenton reaction [59]. In addition, the antioxidant activities of flavonoids are believed to be associated with their chemical structure and the two hydroxyl groups in the catechol B-ring [60], these groups can act by allowing donation of hydrogen stabilizing radical species [61, 62]. The triterpenes of *C. asiatica*, as madecassoside and asiaticoside, also have decrease MDA levels production in substantia nigra pars compacta in a model of Parkinson's disease induced by MPTP [33, 63]. Wanasuntronwong et al. [30] showed the importance of synergism between madecassoside and asiaticoside in anxiolytic effects and stimulation of glutamic acid decarboxylase (GAD), an enzyme involved in the synthesis of GABA and demonstrate similar activity of diazepam, an agonist of GABA A receptor [64]. These findings are corroborated by our study and suggest possible mechanism for the action of *C. asiatica* extracts in lipid peroxidation induced by QA in brain besides the effect of its reaction with iron, this effect could be observed in future in vivo studies. In previous studies we found that agonists of GABAergic receptors (such as muscimol) reverts neuronal damage caused by activation of NMDA and cell death in hippocampal cells [65, 66].

SNP exposure causes cytotoxicity via either release of cyanide and/or nitric oxide ($NO^{\cdot}$) and rapidly releases $NO^{\cdot}$ in tissue preparations, which in turn produces peroxynitrite (ONOO-) and superoxide anion radical ($O_2^{\cdot-}$), thus leading to lipid peroxidation [18, 67–69]. In cerebral cortex, ethyl acetate fraction had a highest potency in inhibiting TBARS induced by SNP. However, in striatum we observed that dichloromethane and *n*-butanolic fraction had better ability to protect from TBARS formation while in hippocampus, the infusion of *C. asiatica* had the better activity. These differences of fractions effects in homogenates of brain regions may be attributed to the differences in their iron content. Brain regions like striatum and hippocampus are largely enriched with non-heme iron which could promote ROS production through fenton reaction [70]. The

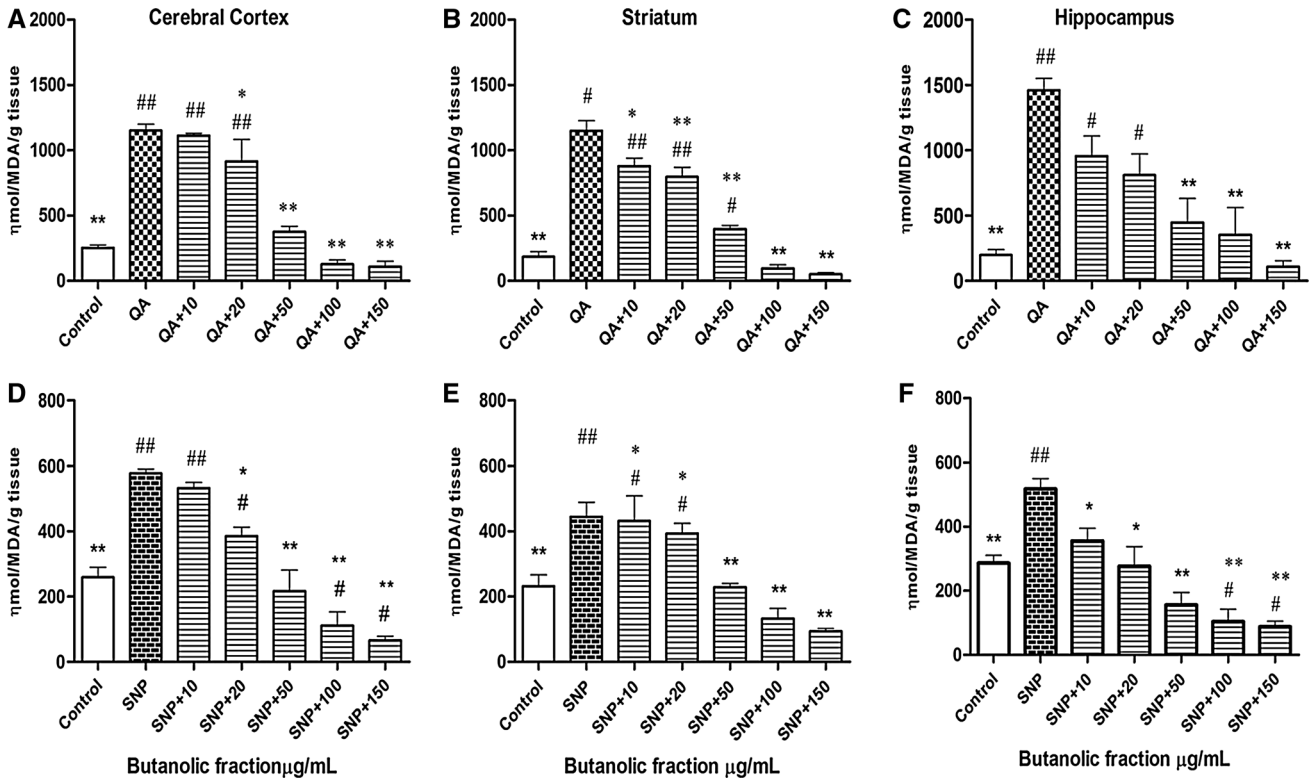

Fig. 6 Effect of *n*-butanolic fraction from *C. asiatica* on lipid peroxidation induced by QA (1 mM) (**a**–**c**) and SNP (5 μM) (**d**–**f**) lipid peroxidation in cerebral cortex (**a**, **d**), striatum (**b**, **e**) and hippocampus (**c**, **f**) of rats. Data are expressed as mean ± SEM (n = 3) and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant when $p < 0.05$. Significant differences are marked as (*) $p < 0.05$ or (**) $p < 0.0001$ when compared to QA or SNP group and marked as ($^{\#}$) $p < 0.05$ or ($^{\#\#}$) $p < 0.0001$ when compared to control group

constituent presents in each fraction probably are involved in their different effects since the compounds which are described in the literature by having potency to chelating iron and/or donating electrons [60–62] were present in highest quantity in infusion and ethyl acetate fraction and produced the highest effects in striatum and hippocampus. Interestingly, it was previously demonstrated that *C. asiatica* aqueous extract also presented neuroprotective effects in in vivo models [10, 71] showing that the compounds are able to cross the blood brain barrier. Besides literature data suggesting madecassoside as a determining factor to *C. asiatica* action in vivo [72] we were unable to demonstrate it since, ethyl acetate fraction presented the better antioxidant activity in the majority of our experiments and the highest concentration of madecassoside was in dichloromethane extract.

As the ethyl acetate fraction from *C. asiatica* presented the best antioxidant activity in the majority of the experiments, we tested this fraction against protein and non-protein thiol oxidation induced by QA or SNP. It is known that thiol groups are present in active site of the enzymes and its oxidation leads to reduction in the activity and the oxidation is caused also by ROS [73]. Non-protein thiol is mainly represented by glutathione which is an endogenous antioxidant and has been involved in neuroprotective activity due to thiol group present in its structure [74]. The ethyl acetate fraction was also able to protect against decrease in content of thiols. As in lipid peroxidation, the effect of ethyl acetate fraction from *C. asiatica* varied according with brain region and pro-oxidant used. However, we also observed that the concentrations of ethyl acetate fraction from *C. asiatica* necessary to prevent thiol oxidation were higher than in lipid peroxidation.

Additionally, using a total antioxidant activity assay, we demonstrated that the ethyl acetate fraction presented a greater antioxidant activity when compared with other fractions, which is probably due to the presence of phenolic content (Table 2). Similarly, the effect of antioxidant on $DPPH^{\cdot}$ radical scavenging is involved with their capacity to donate a hydrogen atom, and in the present study, we also demonstrated that the ethyl acetate fraction has the higher capacity into remove $DPPH^{\cdot}$ radical in comparison with the other fractions (Fig. 2; Table 3).

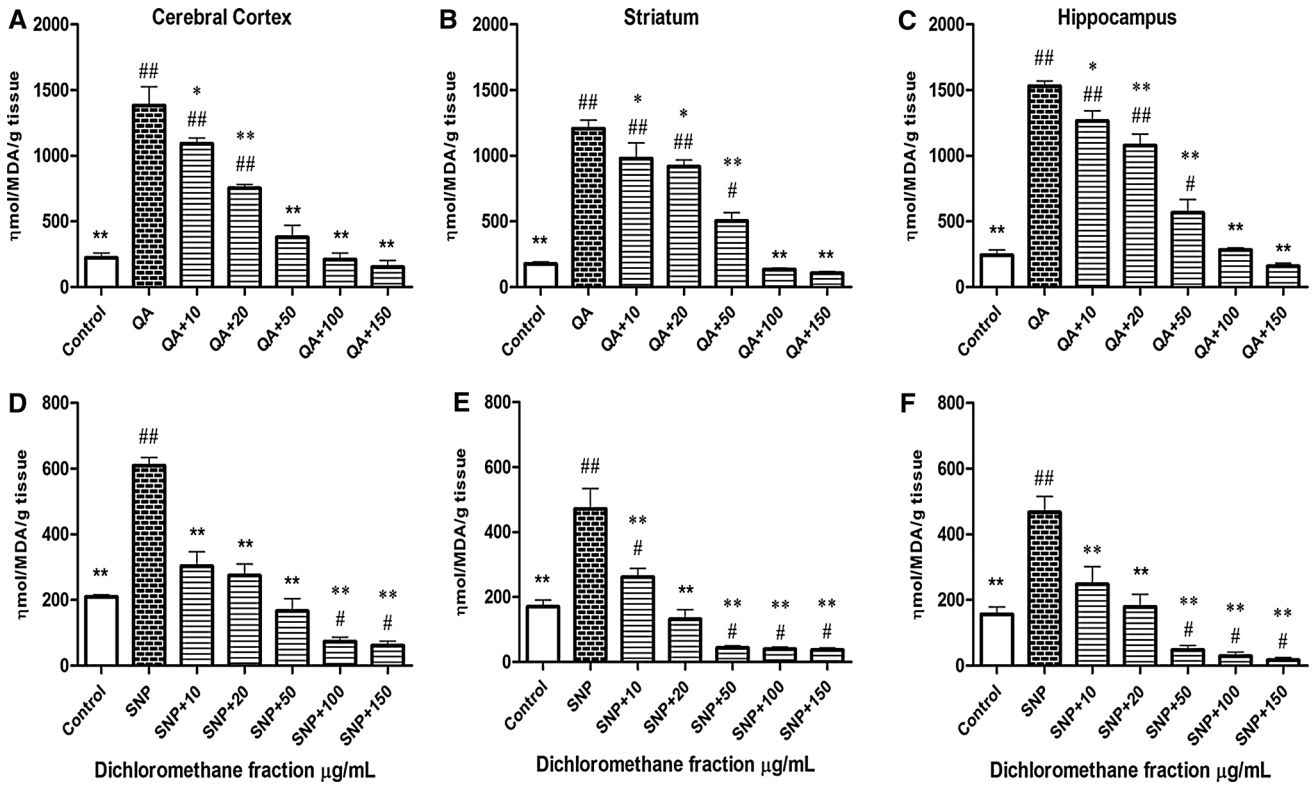

Fig. 7 Effect of dichloromethane fraction from *C. asiatica* on lipid peroxidation induced by QA (1 mM) (**a**–**c**) or SNP (5 μM) (**d**–**f**) lipid peroxidation in cerebral cortex (**a**, **d**), striatum (**b**, **e**) and hippocampus (**c**, **f**) of rats. Data are expressed as mean ± SEM (n = 3) and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant when $p < 0.05$. Significant differences are marked as (*) $p < 0.05$ or (**) $p < 0.0001$ when compared to QA or SNP group and marked as ($^{\#}$) $p < 0.05$ or ($^{\#\#}$) $p < 0.0001$ when compared to control group

Table 4 IC_{50} (μg/mL) values for infusion, ethyl acetate, *n*-butanolic and dichloromethane fractions from *C. asiatica* of TBARS production induced by QA (1 mM) and SNP (5 μM) in rat brain regions homogenate

Extracts	Pro-oxidants					
	QA			SNP		
	Cortex	Striatum	Hippocampus	Cortex	Striatum	Hippocampus
Infusion	$72.85 \pm 7.47^{a,*}$	$46.90 \pm 10.4^{a,*}$	52.64 ± 7.42^{a}	21.67 ± 2.20^{a}	$16.12 \pm 4.64^{a,b}$	17.15 ± 2.03^{a}
Ethyl acetate	11.82 ± 2.54^{b}	13.91 ± 2.90^{b}	13.55 ± 6.33^{b}	10.82 ± 0.74^{a}	$13.57 \pm 4.32^{b,e}$	21.83 ± 7.91^{a}
n-butanolic	25.36 ± 5.955^{b}	35.89 ± 1.42^{ab}	29.96 ± 2.45^{ab}	31.98 ± 0.57^{a}	$47.94 \pm 2.69^{b,c}$	22.97 ± 5.53^{a}
Dichloromethane	28.19 ± 0.60^{b}	42.84 ± 4.88^{ab}	37.45 ± 5.76^{ab}	14.62 ± 0.10^{a}	$11.05 \pm 3.72^{b,d,e}$	19.22 ± 2.59^{a}

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 Bonferroni's test at $p < 0.05$. While the lines, the values marked with (*) differ

Conclusions

In conclusion, all extracts of *C. asiatica* tested in this study were able to prevent lipid peroxidation and thiol oxidation in brain induced by two well-known pro-oxidant agents, QA and SNP. Also, *C. asiatica* presented DPPH scavenger activity and reduced of molybdenum (VI) to molybdenum (V). In part, these effects can be related to their phenolic content, including the presence of flavonoids since ethyl acetate fraction presented the best antioxidant activities and the highest content of flavonoids, tannins and phenolics acids, when compared to infusion, *n*-butanolic and dichloromethane fractions. These results are interesting because this plant could be used as a potential agent for the prevention of various neurological diseases associated with oxidative damage. However, additional studies are

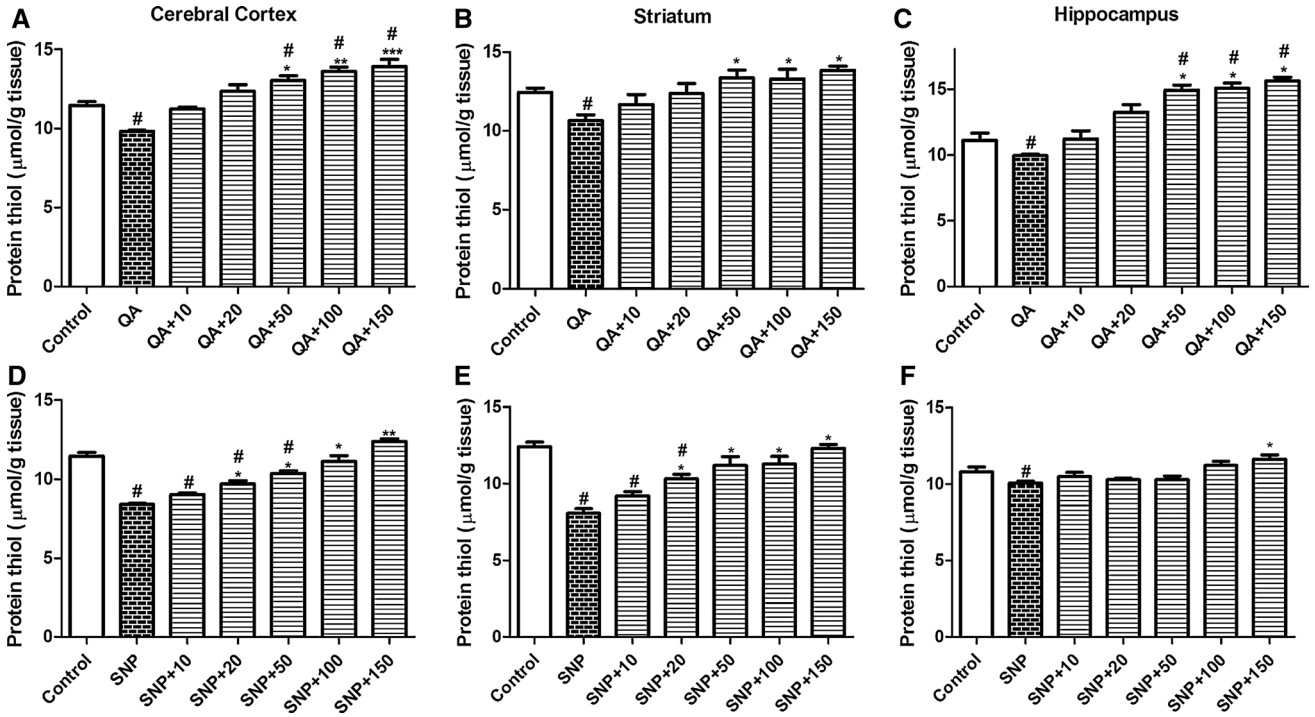

Fig. 8 Effect of ethyl acetate fraction from *C. asiatica* on protein thiol content of rat cerebral cortex (**a**, **d**), striatum (**b**, **e**) and hippocampus (**c**, **f**) incubated with QA (1 mM) (**a**–**c**) or SNP (5 μM) (**d**–**f**). Data are expressed as mean ± SEM (n = 3) and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant when $p < 0.05$. Significant differences are marked as (*, **, *** different symbols are expressing differences between the concentration of ethyl acetate fraction from *C. asiatica*) when compared to QA or SNP group and marked as (#) when compared to control group

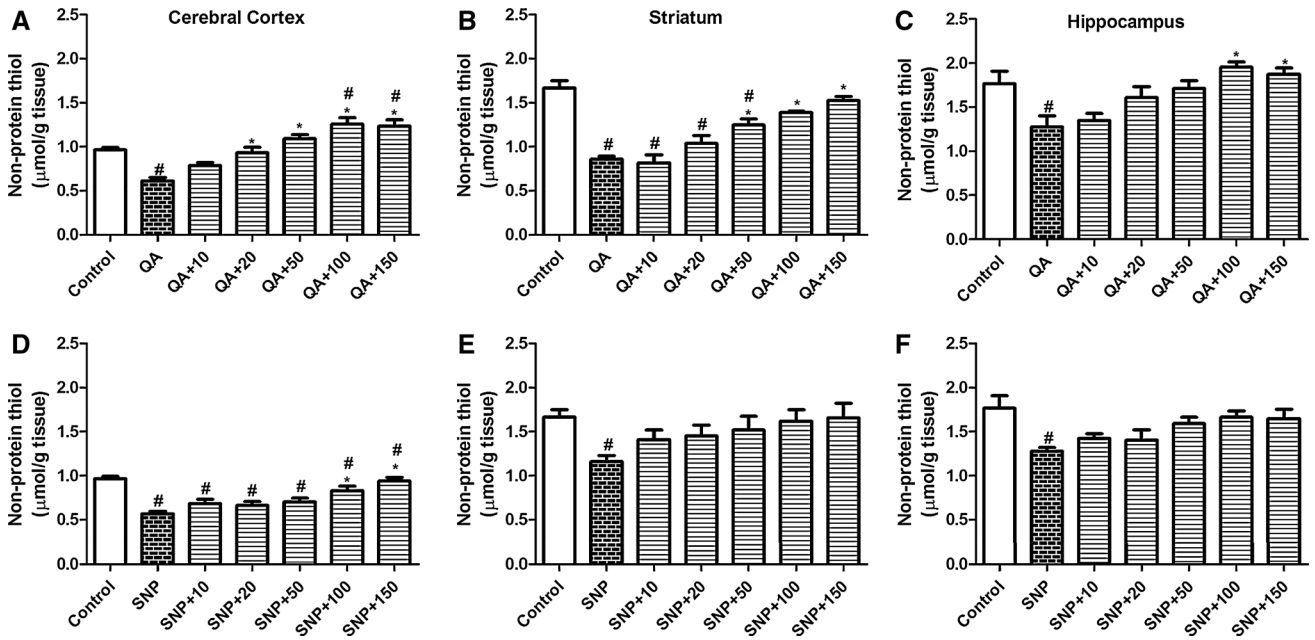

Fig. 9 Effect of ethyl acetate fraction from *C. asiatica* on non-protein thiol content of rat cerebral cortex (**a**, **d**), striatum (**b**, **e**) and hippocampus (**c**, **f**) incubated with QA (1 mM) (**a**–**c**) or SNP (5 μM) (**d**–**f**). Data are expressed as mean ± SEM (n = 3) and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant when $p < 0.05$. Significant differences are marked as (*) when compared to QA or SNP group and marked as (#) when compared to control group

necessary to investigate the exact mechanism responsible for protective effect of *C. asiatica*, and, its effects on in vivo models of oxidative stress.

Acknowledgments Financial support by FAPERGS (2080-2551/13-5-PqG-001/2013) and PRONEM FAPERGS(11/2029-1). CAPES and CNPq (475210/2013-1) is gratefully acknowledged. F.A.A.S, M.L.A. and R.F. are recipient of CNPq fellowship. A.B. is recipient of CAPES fellowship.

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

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