

# Differential Molecular Targets for Neuroprotective Effect of Chlorogenic Acid and its Related Compounds Against Glutamate Induced Excitotoxicity and Oxidative Stress in Rat Cortical Neurons

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**Abstract** The present study has been designed to explore the molecular mechanism and signaling pathway targets of chlorogenic acid (CGA) and its main hydrolysates, caffeic (CA) and quinic acid in the protective effect against glutamate-excitotoxicity. For this purpose 8-DIV cortical neurons in primary culture were exposed to 50  $\mu\text{M}$  L-glutamic acid plus 10  $\mu\text{M}$  glycine, with or without 10–100  $\mu\text{M}$  tested compounds. Chlorogenic acid and caffeic acid via their antioxidant properties inhibited cell death induced by glutamate in dose depended manner. However, quinic acid slightly protects neurons at a higher dose. DCF, JC-1 and  $\text{Ca}^{2+}$  sensitive fluorescent dye fura-2, were used to measure intracellular ROS accumulation, mitochondrial membrane potential integration and intracellular calcium concentration  $[\text{Ca}^{2+}]_i$ . Results indicate that similarly, CGA acts as a protective agent against glutamate-induced cortical neurons injury by suppressing the accumulation of endogenous ROS and restore the mitochondrial membrane potential, activate the enzymatic antioxidant system by the increase levels of SOD activity and modulate the rise of intracellular calcium levels by increasing the rise of intracellular concentrations of  $\text{Ca}^{2+}$  caused by glutamate overstimulation. PKC signaling cascade appear to be engaged in this

protective mechanism. Interseling, CGA and CA also exhibit antiapoptotic properties against glutamate-induced cleaved activation of pro-caspases; caspase 1,8 and 9 and calpain (PD 150606, Calpeptin and MDL 28170). These data suggest that neuroprotective activity of CGA ester may occurs through its hydrolysate, the caffeic acid and its interaction with intracellular molecules suggesting that CGA exert its neuroprotection via its caffeoyl acid group that might potentially be used as a therapeutic agent in neurodegenerative disorders associated with glutamate excitotoxicity.

**Keywords** Glutamate · Neuroprotection · Chlorogenic acid · Antioxidant · Apoptosis · Molecular mechanisms

## Abbreviations

CNS	Central nervous system
DCF	Dichlorofluorescein
FDA	Fluorescein diacetate
$\text{H}_2\text{O}_2$	Hydrogen peroxide
LDH	Lactate dehydrogenase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
ROS	Reactive oxygen species
MAPK	Mitogen apoptotic protein kinase
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DCF	2', 7'-dichlorofluorescein
CGA	Chlorogenic acid
CA	Caffeic
QA	Quinic acid
PBS	Phosphate-buffered saline
LDH	Lactate dehydrogenase
CAT	Catalase
SOD	Superoxide dismutase
NMDA	N-methyl-D-aspartate

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DMSO Dimethyl sulfoxide  
HBSS Hank's Balanced Salt Solution

## Introduction

ROS generation is a normal physiological process, particularly for activation of numerous signal transduction pathways [1]. However, over production or dysregulation of ROS activity contributes to the development of some prevalent neurobiological diseases related to the central nervous system (CNS). Glutamate excitotoxicity play a key role in a number of neurodegenerative disorders, including Huntington's disease and multiple sclerosis, Alzheimer's disease, Parkinson's disease [2, 3] as well as cerebral ischemic and traumatic brain injury and other neurological disorders [4].

Many studies have focused on bioactive compounds, in particular phenolic compounds and their antioxidant activities against oxidative stress, which could be directly associated with neuronal protection. Hydroxycinnamic acids are one of the most common groups of phenolic acids, which are present in many consumed fruit, vegetables and beverages. Among the most common and well-known hydroxycinnamic acids is chlorogenic CGA (5-*O*-caffeoylquinic acid). Chlorogenic acid, a caffeic acid ester linked to quinic acid, it is generally present in berries, cherries, apples, kiwis, artichokes, eggplants, plums and coffee and it is known to have several biological functions, including anti-inflammatory, antibacterial, anticarcinogenic and antioxidant activities [5]. It has been also found that chlorogenic acid has the ability to attenuate oxidative stress in numerous neurological disorders; it has been demonstrated that it acts as a neuroprotective agent against scopolamine-induced amnesia and significantly improved the impairment of short-term or working memory induced by scopolamine [6]. In addition, CGA has been reported to inhibit H<sub>2</sub>O<sub>2</sub>-induced apoptotic neuronal death through the up-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> as well as the blockage of H<sub>2</sub>O<sub>2</sub>-induced pro-apoptotic cleavage of caspase-3 and pro-PARP in primary cortical neurons [7].

Chlorogenic acid is broken down into caffeic and quinic acids before absorption. The neuroprotective effects of caffeic acid (3, 4-dihydroxycinnamic acid), as a major metabolite of chlorogenic acid, abundant in carrots and various berries [8] have been investigated through in vitro studies. CA is already regarded as potent antioxidant, function dependent on its chemical structure [9]. It has been reported to have neuroprotective effects against A $\beta$ -induced neurotoxicity in vitro and to inhibit peroxynitrite-induced neuronal injury [10, 11]. CA also acts as a selective 5-LOX inhibitor, protected mice from the aluminum induced neuronal damage through the down-regulation of overexpression of APP and amyloid *beta* protein [12]. The antioxidant-like

effect of caffeic acid has been shown to be neuron-protective in vivo and ameliorates brain injuries after focal cerebral ischemia under pathological conditions [13] and attenuated the alterations induced by Quinolinic acid, an endogenous glutamate agonist with a relative selectivity toward *N*-methyl-D-aspartate (NMDA) receptor, induced oxidative damage, mitochondrial dysfunction and cellular alterations in rat striatum [14].

Quinic acid has been shown to exert neuroprotective and neurotrophic effect on A $\beta$ -induced toxicity and potentiating activity in the neurite outgrowth of PC 12 cells in in vitro studies [15]. However, little is known about its mechanisms in the central nervous system.

More recently, chlorogenic acid and its metabolites exhibited neuroprotective activity against mechanistically distinct cell death-inducing agents in cultured cerebellar granule neurons [16]. Indeed, the role of CGA in protecting against neuronal death *in vitro* study was reported and it has been shown that CGA inhibited glutamate-induced neuronal cell death and protects neurons against ischemia [17]. Chlorogenic acid is broken down into caffeic and quinic acids before gastrointestinal absorption and several data indicate their beneficial effects have been obtained under in vitro or in vivo conditions may be correlated with their parent compound [18] and its neuroprotective potential may result from its main metabolite(s). In support of this observations, in the present work, pharmacological approaches were used to investigate and compared the protective effects of chlorogenic acid and its major metabolites in the neuroprotective against L-glutamate induced excitotoxicity in cortical neuron cell culture. The underlying molecular mechanisms involved and signaling pathway transduction involved are investigated suggesting its potential role in the prevention in neurological disorders related to the glutamate excitotoxicity in the SNC.

## Materials and Methods

### Animals

Wistar rats were kept in a temperature-controlled room (21  $\pm$  1 °C) under an established photoperiod (lights on from 7:00 am to 7:00 pm) with free access to food and water. All experiments were performed according to the recommendations of the ethical committee in Tunis and Spanish University for care and use of animals in conformity with NIH guidelines.

### Chemicals

Neurobasal medium, B27 supplement, D(+)-glucose, L-glutamine, foetal bovine serum (FBS), trypsin-EDTA, and the antibiotic-antimycotic solution were obtained from Gibco

(Invitrogen, Grand Island NY, USA), sodium pyruvate, calcein, chelerythrine, H89, U73122, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and insulin were purchased from Sigma Aldrich (St. Louis, MO, USA). Caspases inhibitors (Ac-YVAD-H, Ac-IETD-H and Ac-LEHD-H) and Calpain inhibitors (PD150606, MDL28170, calpeptin) were purchased from international peptide. DCF: 2', 7'-dichlorofluorescein (DCF), fura-2 AM and JC-1 were purchased from Molecular Probes Eugene, OR. Glutamate (Sigma, St. Louis, MO) and was first dissolved in an equimolar solution of NaOH. Chlorogenic acid, caffeic acid and quinic acid were purchased from Sigma Aldrich Spain; were dissolved in DMSO at a concentration of 100 mM as a stock solution.

### Antioxidant Activity

Free radical scavenging activity was determined spectrophotometrically according to the method of DPPH assay. The DPPH radical is reduced when reacting with an antioxidant compound, which can donate hydrogen. Serial concentrations of Trolox or stock solution compounds were diluted and then (25  $\mu$ l) was placed in a cuvette with 975  $\mu$ l of 0.04 mM methanolic solution of DPPH radical. Absorbance was measured at 517 nm 30 min in the dark later. All determinations were performed in triplicate. The DPPH inhibition percentage was calculated as the absorbance decrease of the antioxidant samples relative to the control. The antiradical activity was expressed as the DPPH inhibition percentage as calculated using the following formula:

$$\% \text{ inhibition} = (\text{control OD} - \text{sample OD}) / \text{control OD} \times 100.$$

### Cortical Neurons

Cortical neurons were obtained from the cortical lobes of E18 Sprague-Dawley rat embryos, according to previously described procedures [19]. The cells were resuspended in B27 Neurobasal medium plus 10% FBS and then seeded onto poly-L-ornithine-coated glass coverslips (12 mm in diameter) or 6-well plates at  $5 \times 10^4$  cells per coverslip or  $2 \times 10^6$  cells per well. Culture dishes pre-coated with poly-D-lysine (50 mg/ml in sterile water) overnight. The cultures were essentially free of astrocytes and microglia. They were maintained at 37 °C and 5% CO<sub>2</sub>. Cultures were used 8–10 days after plating for excitotoxicity insult.

### Induction of Excitotoxicity and Cell Viability Determination

To induce excitotoxicity insult, neurons were exposed to L-glutamic acid (50  $\mu$ M) for 10 min in HBSS containing 2.6 mM CaCl<sub>2</sub>, 10 mM glucose, 10 mM glycine, pH 7.4, at 37 °C. Then, in order to evaluate the effects of tested compounds on excitotoxicity, cells were stimulated with

L-glutamic acid in the presence of different concentrations of each compound (10, 25, 50 and 100  $\mu$ M) and these compounds were maintained for 2 h after glutamate exposure. For cell survival experiments, neurons culture ( $5 \times 10^4$  cells/well) were incubated for 30 min with the fluorescent probe calcein acetoxymethyl ester (calcein-AM), then rinsed with phosphate-buffered saline (PBS). Fluorescence intensity was measured ( $\lambda$  excitation = 485 nm and  $\lambda$  emission = 530 nm) with a FL800TBI fluorescence microplate reader (Bio-Tek Instruments, Winooski, USA). The fluorescence in the control cells was taken as 100% viability. Data are mean percentages of viable cells versus the respective controls. Phenolic compounds stocks were dissolved in DMSO and were diluted in culture medium to the final desired concentration (maximal concentration of DMSO 0.001%). All experiments were performed in triplicate and the values provided here are the averages of at least three independent experiments.

### Measurement of Cell Cytotoxicity

Measurement of LDH activity in culture medium was determined using a colorimetric method, the CytoTox96<sup>®</sup> non-radioactive Cytotoxicity assay. Neurons were seeded onto 96-well plates ( $2 \times 10^4$  cells/well). The CytoTox96 assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Release LDH in culture supernatants is measured with a 30-minute coupled enzymatic assay that results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Visible wavelength absorbance at 490 nm data is collected using a standard 96-well plate reader. The results were expressed as a percentage of total LDH release from neurons. Data are expressed as the mean  $\pm$  SEM from three independent experiments performed in quadruplicate. The results were expressed as a percentage of total LDH release from neurons.

### Determination of Intracellular Reactive Oxygen Species (ROS)

ROS were detected by using a fluorescent probe, 2', 7'-dichlorofluorescein (DCF) resulting from the deacetylation and oxidation of the non-fluorescent compound DCFH<sub>2</sub>-DA. After treatment, cells were incubated with 30  $\mu$ M of cell permeant DCFH<sub>2</sub>-DA in Neurobasal serum-free loading medium at 37 °C for 30 min. At the end of the incubation, DCFH<sub>2</sub>-DA was removed and cells were washed twice with PBS. The intensity of fluorescence was measured ( $\lambda$  excitation = 485 nm and  $\lambda$  emission = 530 nm) with a FL800TBI fluorescence microplate reader (Bio-Tek Instruments, Winooski, USA). Results are expressed as the

mean  $\pm$  SEM from three independent experiments performed in quadruplicate.

### Measurement of Mitochondrial Membrane Activity

Mitochondrial membrane potential was quantified using the JC-1 fluorescent probe. Neuronal cultures ( $5 \times 10^4$  cells/well) were exposed to L-glutamate alone or with phenolic compounds (100  $\mu$ M) as described. At the end of treatments, neurons were incubated in the presence of the JC-1 probe (10  $\mu$ g/ml) for 15 min at 37 °C and then washed twice with PBS. In living cells, the lipophilic dye JC-1 enter into the mitochondria through membrane potential and form aggregates producing an intense orange signal. Whereas, in dead cells, where the mitochondrial membrane potential collapses the monomeric JC-1 remains cytosolic and stains the cell in green. Fluorescence intensity was measured with a fluorescence microplate reader (Bio-Tek Instruments, Winooski, USA) and expressed as a ratio of the fluorescence emission at 590 nm (orange, intact mitochondrial membrane potential) versus 530 nm (green, collapsed mitochondrial membrane potential). Results are expressed as the mean  $\pm$  SEM performed in quadruplicate from three independent experiments.

### Measurement of Antioxidant Enzyme Activities

We next analyzed if CGA and its metabolites can modify the equilibrium of the endogenous antioxidant system. Cortical neurons ( $2 \times 10^6$  cells per well) were subjected to the excitotoxic stimulus in the absence or presence of CGA, CA and QA and at the end of the treatment, culture medium was removed; cells were washed twice with PBS and then homogenized in the same solution at 4 °C. Cells were harvested by centrifugation (350 g, 4 °C, 10 min) and the cell pellet were resuspended in 50 ml of ice-cold lysing buffer containing 50 mM Tris-HCl (pH 8), 10 mM EDTA, 100 mM phenylmethyl-sulfonylfluoride and 1% Triton X-100. Samples were then centrifuged (16,000 $\times$ g, 20 min, 4 °C) and supernatants were finally stored at -20 °C until enzyme activity determinations.

The activity of SOD was measured using a spectrophotometric assay, which consists in measuring epinephrine autoxidation induced by superoxide anion. Samples were incubated for 3 min with a mixture containing bovine catalase (0.4 U/L), epinephrine (5 mg/mL) and Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> buffer (62.5 mM, pH 10.2). The oxidation of epinephrine was measured at 480 nm.

Catalase activity was of H<sub>2</sub>O<sub>2</sub>. Samples were mixed with 30 mM H<sub>2</sub>O<sub>2</sub> in PBS. The disappearance of H<sub>2</sub>O<sub>2</sub> was measured at 240 nm for 180 s at 30-s intervals. Catalase activity was calculated using the extinction coefficient of 40/mM/cm for H<sub>2</sub>O<sub>2</sub>.

### Determination of Calcium Release [Ca<sup>2+</sup>]<sub>i</sub>

Measurement of intracellular calcium of [Ca<sup>2+</sup>]<sub>i</sub> was determined by fluorescent probe fura-2 AM (Molecular Probes, Eugene, OR). Cells were incubated with 5  $\mu$ M fura-2-AM in culture medium for 30 min at 37C°, and then washed with HBSS containing 20 mM HEPES [pH 7.4], 10 mM glucose, and 2 mM CaCl<sub>2</sub>. Experiments were carried out in a coverslip chamber, continuously perfused with incubation buffer at 2 ml/min. At the end of the assay, in situ calibration was performed with the successive addition of 10 mM ionomycin and 2 M Tris-50 mM EGTA, pH 8.5. The [Ca<sup>2+</sup>]<sub>i</sub> concentration was estimated by the 340/380 ratio method, using a K<sub>d</sub> value of 224 nM. Data are expressed as the mean  $\pm$  SEM from three independent experiments performed in quadruplicate.

### Caspase Activity

The peptide caspase inhibitors are able to block caspase activity in vitro. We investigate the involvement of caspase 1, 8 and 9 in neuroprotective target of tested phenolic acid using synthetic caspase inhibitor peptides. For that purpose, neurons were seeded onto 96-well plates ( $2 \times 10^4$  cells/well), and incubated at day 8 in vitro with glutamate plus glycine in the presence or absence of polyphenols, as described above. Caspase inhibitors: Caspase 1 inhibitors (Ac-YVAD-H); Caspase 8 inhibitor (Ac-IETD-H) and Caspase 9 inhibitor (Ac-LEHD-H) were prepared in DMSO according to the instructions of the manufacturer (Peptides International, Louisville, KY) and added to cultures 30 min prior to glutamate induction. All experiments were performed in quadruplicate, and the values provided are the average of at least three independent experiments.

### Calpain Activity

To evaluate the Calpain activity on live cells, calpain peptide inhibitor (International peptide) was used according to the manufacturer's instructions. Neuronal cultures ( $5 \times 10^4$  cells/well) were exposed to L-glutamate alone or with phenolic acids (100  $\mu$ M) in the presence of the calpain inhibitors 3-(4-iodophenyl)-2-mercapto-(Z)-2-propeionic acid (PD150606) (100  $\mu$ M), calpeptin (20  $\mu$ M), or N-[[[(phenylmethoxy)carbonyl]-L-valyl]-phenylalaninal (MDL28170) (10  $\mu$ M). Calpain inhibitors were added for 30 min before exposure to L-glutamate. Calcein-AM was used here to quantify cell viability and Fluorescence was measured using a Synergy-HT fluorimeter (Bio-Tek Instruments Incl., Beverly, MA, USA). All experiments were

performed in quadruplicate, and the values provided are the average of at least three independent experiments.

### Statistical Analysis

Data are presented as the mean  $\pm$  SEM from three independent experiments performed in quadruplicate. Statistical analysis of the data was performed by using Student's t-test and ANOVA, followed by Bonferroni's test.  $**p < 0.01$ ;  $***p < 0.001$  and NS not statistically different from control;  $#p < 0.05$ ;  $##p < 0.01$ ;  $###p < 0.001$  versus glutamate-treated cells and ns not statistically different from glutamate alone.

## Results

### Antioxidant Potential: DPPH Assay

The antioxidant effects of chlorogenic acid (CGA) and its metabolites, caffeic acid (CA) and quinic acid (QA), were investigated. All three chemicals were separately used at a concentration range of 10–100  $\mu$ M and tested by DPPH assay. The scavenging DPPH radical assay results indicated that examined compounds here could scavenge DPPH free radical at a dose dependent manner. CA and CGA exhibited potent antioxidant activity against the DPPH radical formation. At 100  $\mu$ M, the highest DPPH activity was observed with CA followed by CGA; (101.10 and 97.5%, respectively) while QA revealed itself as a weak antioxidant activity (Fig. 1).

### Effect of Chlorogenic Acid and its Metabolites on Glutamate Mediated—Excitotoxicity

We evaluated the effectiveness of chlorogenic acid and its occurring metabolites against neuronal death following

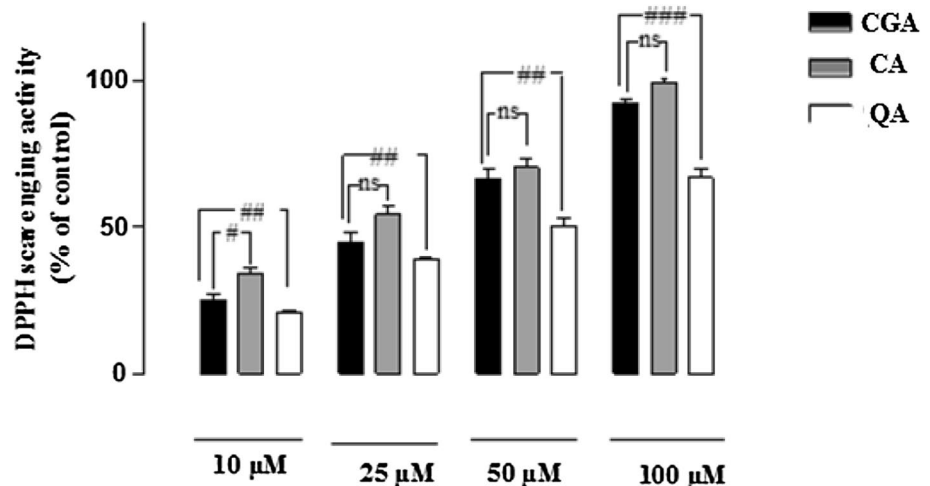
excitotoxic insults mediated by NMDA receptors (glutamate 50  $\mu$ M plus glycine 10  $\mu$ M; 10 min) as a model of neuronal excitotoxicity. As seen in Fig. 2, L-glutamate-induced decrease in cell viability by approximately 40% and was ameliorated dose-dependently by chlorogenic acid and caffeic acid; More there, both CGA and caffeic acid significantly enhanced cell survival to a similar degree and shows a complete reversal of the toxic effect glutamate at different concentrations. Although only a high concentration of quinic acid (100  $\mu$ M) exhibited a significant increase of cell viability but the results did not reach statistical significance compared with cells treated with caffeic or chlorogenic acid. We used a concentration 100  $\mu$ M in the following experiments since it had the maximal neuroprotection with the tested compounds.

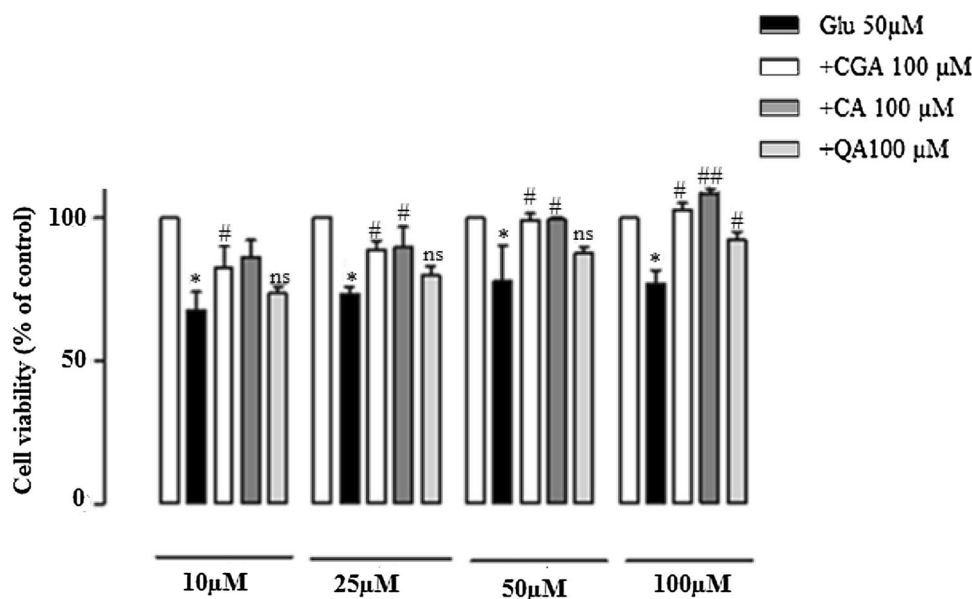
### ROS Scavenging Activity and Mitochondrial Membrane Potential

Glutamate excitotoxicity lead to excessive production of ROS causing oxidative damage and cell death. In order to evaluate the ability of tested phenolic compounds to directly scavenge free radicals and endogenous ROS generations, neurons were incubated with fluorescent probe CMH-2DCFDA which forms the fluorescent DCF compound upon oxidation with ROS. Results show that ROS generation as the consequences of glutamate excitotoxic insults, are similarly attenuated by CA and CGA and increase above control levels, but quinic acid did not alter the glutamate-induced increase in ROS production and failed to protect neurons against the overproduction of ROS after excitotoxicity insult (Fig. 3).

Considering the temporal pattern of free radical production after glutamate exposure (50  $\mu$ M; 10 min) ROS production rise up until reaching its maximum at 60 min after the excitotoxic insult by 2.41 fold compared with non

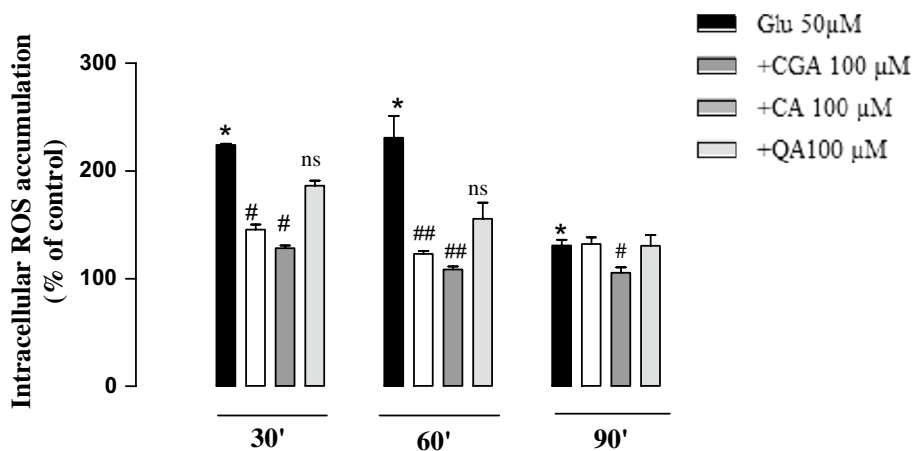
**Fig. 1** Antioxidant activities of chlorogenic, caffeic acid, and quinic acid determined by the DPPH assay. Values represent the mean  $\pm$  SEM of triplicates from three different experiments (n=3). ANOVA followed by the Bonferroni's test.  $#p < 0.05$ ;  $##p < 0.01$ ;  $###p < 0.001$  and ns, not statistically different when compounds were compared





**Fig. 2** Effects of graded concentrations (10–100 μM) of each compound chlorogenic acid caffeic acid and quinic acid on glutamate induced-excitotoxicity. Cells were incubated with L-glutamate/glycine (50/10 μM) for 10 min followed by tested compounds at the indicated concentrations were maintained for 2 h after glutamate exposure. After the treatments the medium was changed and cells were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> for oth-

ers 24 h. Each value is the mean (±SEM) calculated from at least six different wells from four independent cultures. ANOVA followed by the Bonferroni's test. \* < 0.05 ; \*\*p<0.01; \*\*\*p<0.001 and NS not statistically different from control; #p<0.05; ##p<0.01; ###p<0.001 versus glutamate-treated cells and ns not statistically different from glutamate alone



**Fig. 3** Effect of CGA and its related compounds on intracellular accumulation of ROS produced by activation of NMDA glutamate receptors in cultured cortical neurons. Cortical neurons were stimulated with glutamate (50 μM) in the presence of glycine (10 μM) and treated with CGA, CA and QA (100 μM) following stimulation at different times. Cellular ROS were detected by measuring the fluorescence of 2', 7'-dichlorofluorescein (DCF), and the results are

expressed as a percentage of fluorescence with respect to that of control cells. Each value represent the mean (±SEM) of triplicates from three different experiments (n=3). ANOVA followed by the Bonferroni's test. \*p<0.05 ; \*\*p<0.01; \*\*\*p<0.001 and NS not statistically different from control; #p<0.05; ##p<0.01; ###p<0.001 versus glutamate-treated cells and ns not statistically different from glutamate alone

treated cells. The reduction of ROS levels of caffeic acid was maintained at later stages until 90 min and had a statistically significant reduction levels, although chlorogenic acid attenuates oxidative stress caused by glutamate disappeared at 60 min after excitotoxic insult.

Since the generation of ROS in excitotoxic insults is associated with alterations and permeabilize the mitochondrial membrane, we next measured mitochondrial membrane potential using JC-1 as a fluorescent probe. Neurons exposed to L-glutamate /Glycine show decrease of the red signal



(590 nm); indicating the alteration of mitochondrial membrane integrity. Incubation of cells with CGA and caffeic acid totally suppressed the deleterious effects of L-glutamate on mitochondrial membrane potential and kept the mitochondrial membrane potential around the value of the resting potential only for 30 min ( $96.34 \pm 2.6$  and  $100.2 \pm 5.9\%$ , respectively). However, lower intensity was observed with quinic acid with green fluorescent stains indicating altered membrane in dead cells and failed to decrease the depolarization of the mitochondrial membrane potential after excitotoxic insult (Fig. 4).

### Modulation of the Antioxidant Enzyme System

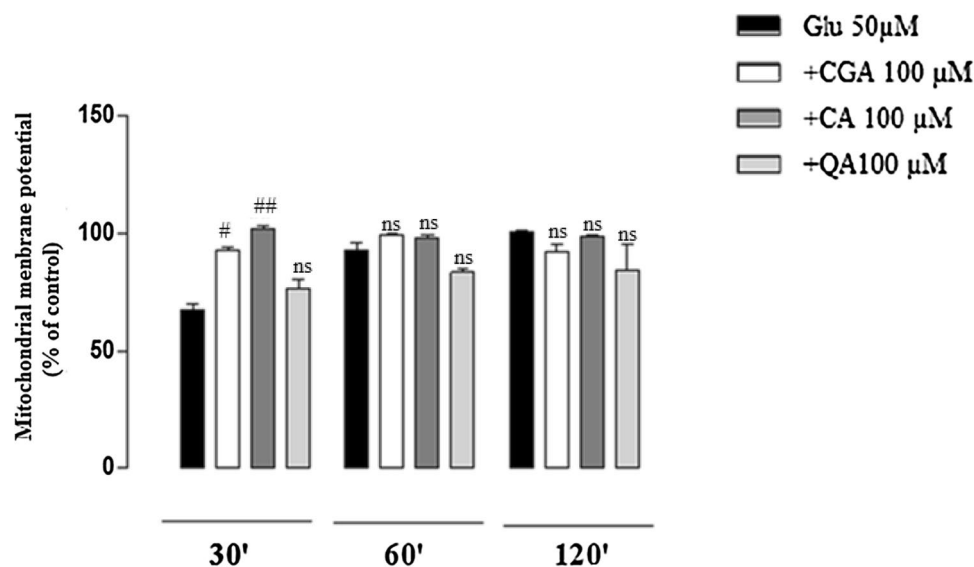
The antioxidant defense system has been developed by the organism as a protective mechanism against ROS formation. Among the most reported endogenous antioxidant systems are the activity of the enzymes SOD and CAT. In order to know if the protective effects of tested compounds against L-glutamate-induced oxidative stress are related to the antioxidant enzyme system. The results in Fig. 5 show that L-glutamate significantly decreased the levels of the antioxidant enzymes CAT, and SOD when compared with the levels in the control cells. More specifically, catalase activity was half of the respective control values. However,

levels of SOD was restored above control levels when neurons were treated with CGA and caffeic acid but not in the presence of quinic acid. Remarkably, QA has succeeded to restore catalase levels.

CGA and CA were almost identically effective; CGA increased by  $98.56 \pm 3.98$  and CA by  $105.6 \pm 4.7\%$ .

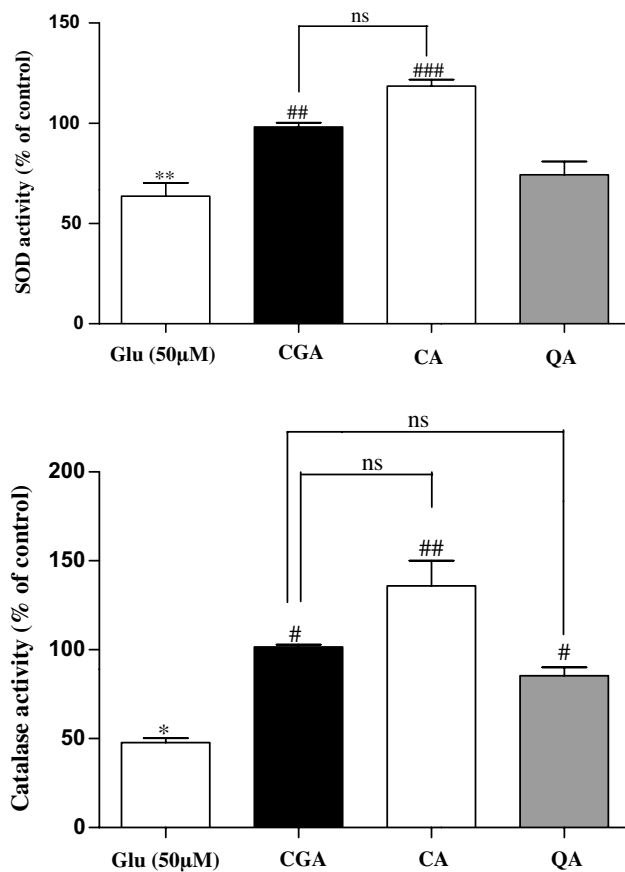
### Effect of CGA and its Metabolites on Intracellular Calcium

Excitotoxic insult mediates by glutamate receptors cause an elevation in the concentration of cytosolic  $\text{Ca}^{2+}$ . To further investigate the potential mechanisms underlying CGA and its related compounds-mediated cell death, the cytoplasmic free  $\text{Ca}^{2+}$  concentration was determined in cell culture with free-2 specific probe. Firstly, the time course of  $[\text{Ca}^{2+}]_i$  (mean  $\pm$  4 SEM) with respect to basal values before incubation with glutamate was determined (Fig. 6a). We observed previously that NMDA receptor activation in cortical neurons caused a significant  $[\text{Ca}^{2+}]_i$  increases which are reduced by CGA and Caffeic acid but not by quinic acid. Histogram in Fig. 6b depicts the changes in the peak amplitude of  $[\text{Ca}^{2+}]_i$ . The intracellular calcium concentration data in Fig. 6b represents the time to peak  $[\text{Ca}^{2+}]_i$  response increases when agonist as added.



**Fig. 4** Effect of CGA and its metabolites on glutamate-induced alteration of mitochondrial membrane potential in cultured cortical neurons at different time course. CGA and CA attenuate the depolarization of the mitochondrial membrane. The mitochondrial transmembrane potential was assessed by using the JC-1 probe, and the ratio of fluorescence emissions 590 versus 530 nm. The results are expressed as percentage of control. Each value is the mean ( $\pm$  SEM)

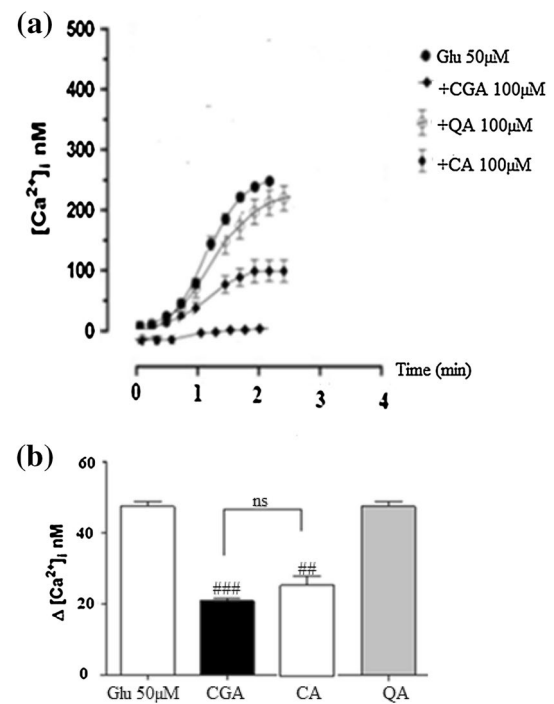
calculated from at least four different wells from three independent cultures ( $n=3$ ). ANOVA followed by the Bonferroni's test. ANOVA followed by the Bonferroni's test. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  and NS not statistically different from control; # $p < 0.05$ ; ## $p < 0.01$ ; ### $p < 0.001$  versus glutamate-treated cells and ns not statistically different from glutamate alone



**Fig. 5** Effect of CGA and its metabolites on SOD and catalase activities in cultured rat cortical neurons 1 h post-excitotoxic stimulus in the absence or presence of 100 µM CGA, CA and QA. SOD activity was determined by measurement of epinephrine autoxidation induced by superoxide anion, and catalase activity by the decrease of rate  $H_2O_2$ . Data were normalized with respect to non cells represented as a percentage of U/mg protein with respect to its control (100%). Each value is the mean ( $\pm$  SEM) of at least four different dishes from three independent cultures ( $n=3$ ). ANOVA followed by the Bonferroni's test. \* $p < 0.05$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and NS not statistically different from control. # $p < 0.05$ ; ## $p < 0.01$ ; ### $p < 0.001$  versus glutamate-treated cells. ns not statistically different when compounds were compared

### Signal Transduction Pathways Involved in the Neuroprotective Effect of Chlorogenic Acid and its Related Compounds

To investigate the signaling cascade involved in the neuroprotection action of each phenolic compounds, the whole ester, the chlorogenic acid as its main related metabolites via glutamate overstimulation, neurons were pre-incubated with the PKA inhibitor H89 ( $2 \times 10^{-5} M$ ), the PLC inhibitor U73122 ( $10^{-5} M$ ), the PKC inhibitor chelerythrine ( $10^{-6} M$ ) for 30 min, at 37 °C. The PLC inhibitor and the PKC inhibitor totally abrogated the neuroprotective action of CGA and CA against glutamate induced cell death. In



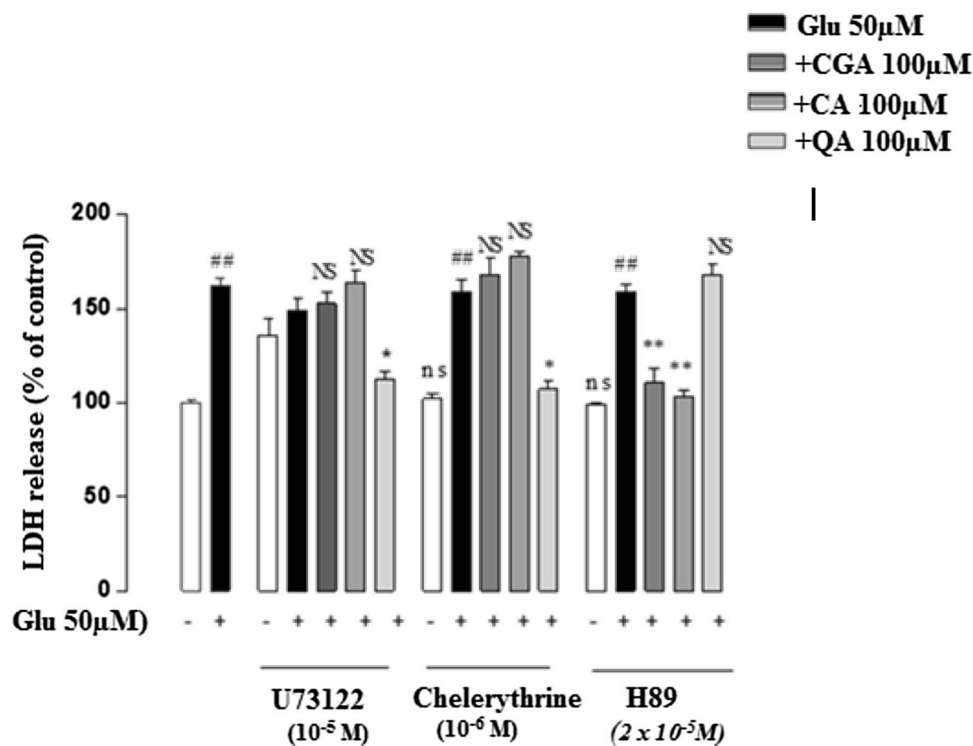
**Fig. 6** Effect of L-glutamate and phenolic compounds on intracellular calcium concentration. Cells were stimulated by glutamate/glycine (50/10 µM) and followed by 100 µM of each compounds.  $[Ca^{2+}]_i$  was determined using fluorescent probe fura 2-AM. **a** Curves in A illustrate the time course of the  $[Ca^{2+}]_i$  increase  $\pm$  SEM ( $n=3$ ) with respect to basal values. **b**  $[Ca^{2+}]_i$  increases induced by L-glutamate 50 µM are reduced by CGA and caffeic acid but not by quinic acid. ANOVA followed by the Bonferroni's test. \* $p < 0.05$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and NS not statistically different from control. # $p < 0.05$ ; ## $p < 0.01$ ; ### $p < 0.001$  versus L-glutamate-treated cells; ns not statistically different when compounds were compared

contrast, the selective PKA inhibitor H89 did not modify the effect of tested compounds on glutamate-induced cell death; suggesting that PKC signaling pathways' is involved in the neuroprotective mechanism of both CGA and CA. Meanwhile, for the quinic acid, its protective effects is totally abrogated on cell survival when neurons were pretreated with the PKA inhibitor suggesting that the neuroprotective activity of quinic acid can be accounted for by activation of the PKA (Fig. 7).

### Effects of Caspases and Calpain Inhibitor on Glutamate-Induced Cell Death

Caspases and calpain inhibitor were able to block caspases and calpain activity in neurons. In L-glutamate treated cells, cell survival was about 72.77% ( $P < 0.05$ ) compared with that of the control group. Glutamate-induced cell death was fully blocked by caspases specific inhibitors on cortical neurons cell culture. Pretreatment with specific caspase inhibitor Ac-YVAD-H, Ac-IETD-H and Ac-LEHD-H (100 µM)





**Fig. 7** Differential intracellular signaling pathways involved in the neuroprotective effect of CGA and its related compounds on cortical neuron cells. Cultured neurons at 8 DIV were pre-incubated for 30 min in the absence or presence of with the PKA inhibitor H89 ( $2 \times 10^{-5}$  M), the PLC inhibitor U73122 ( $10^{-5}$  M), the PKC inhibitor chelerythrine ( $10^{-6}$  M) and then stimulated for 10 min with L-glutamate 50  $\mu$ M without or with tested compounds at the same dose (100  $\mu$ M). Cell survival was quantified by measuring FDA fluo-

rescence intensity, and the results are expressed as percentages of the control. Each value is the mean ( $\pm$ SEM) of at least of six different wells from three independent cultures. ANOVA followed by the Bonferroni's test. \*  $< 0.05$  ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  and NS not statistically different from control; # $p < 0.05$ ; ## $p < 0.01$ ; ### $p < 0.001$  versus glutamate-treated cells and ns not statistically different from glutamate alone

30 min before glutamate exposure had no effect on the neuroprotective effect of CGA and CA which suggested that caspase-dependent apoptosis accounted for the neuroprotective effect of these phenolic compounds against glutamate excitotoxicity.

Calpain or  $Ca^{2+}$ -dependent proteases play an important role in the execution of apoptosis and activation of calpain has been implicated in neuronal apoptosis. Pretreatment with specific calpain inhibitor also totally abrogated the deleterious effect of glutamate induced cell death and preserve the neuroprotective effect of CGA and CA (Fig. 8).

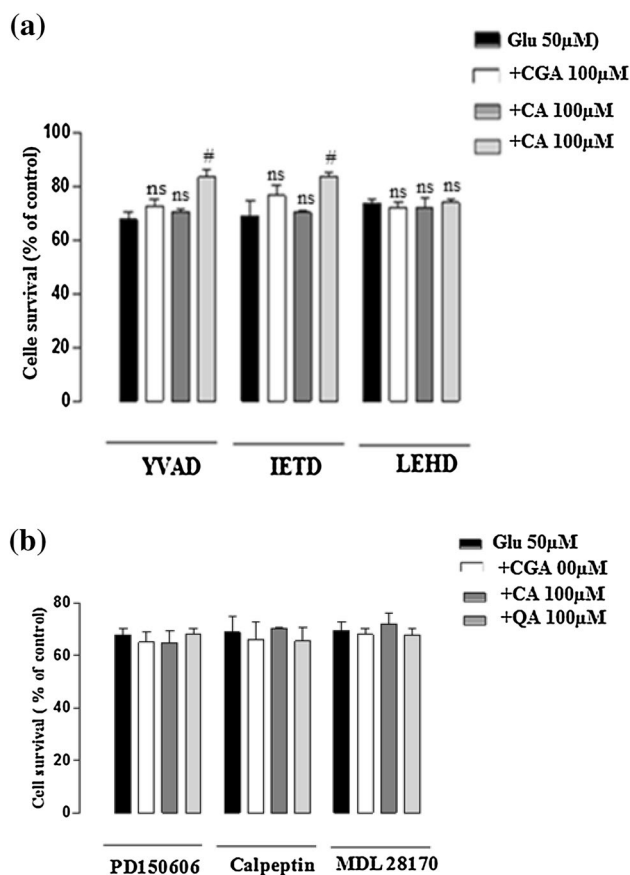
### Discussion

Generally, “excitotoxicity” was referred to as a neuronal degeneration triggered by the over- or prolonged activation of glutamate receptors in the central nervous system (CNS) by excitatory amino acids [20]. Glutamate excitotoxicity appears to play an important role in a number of

neurodegenerative diseases, including Alzheimer diseases, Parkinson diseases, Huntington disease and multiple sclerosis [2, 3].

Chlorogenic acid (CGA) is a cinnamic acid, which possesses different isomeric forms, is the predominant phenolic compounds in coffee and berries. Pharmacological effects of chlorogenic acid have been demonstrated on the CNS, and appear to exert many beneficial effects against oxidative stress to reduce the risk of developing neurodegenerative disorders [7, 16, 17]. CGA is capable of mediating oxidative stress and attenuating cell apoptosis due to different oxidative stressors and free radicals by modulating the accumulation of reactive oxygen species and by regulating the expression of key proteins and enzymes involved in cell apoptosis [21].

Since, chlorogenic acid the whole compounds is known to be poorly absorbed, extensively metabolized *in vivo*, and in the small intestine, it is hydrolyzed to caffeic acid and quinic acid which have been identified in the plasma and urine of rats fed [22]. The neuroprotective effect of CGA and its hydrolysates, caffeic acid and quinic acid against



**Fig. 8** Effect of caspases and calpain Inhibition activities in cultured neuron cortical rat at 30 min post stimulus. **a** Cells were pre incubated for 30 min with peptide caspase inhibitors at 100 µM: YVAD (casp1 inhibitor) ; IETD (casp-8 inhibitor) or LEHD (casp-9 inhibitor) in the absence or presence of tested compounds before NMDA receptors activation. **b** Neurons were incubated for 30 min with calpain inhibitor PD150606 (100 µM); Calpeptin (20 µM) or MDL 28,170 (10 µM) before addition of agonists, and cell survival was measured at 30 min post stimulus. In all data ,values are not statistically different versus glutamate-treated cells. Values are illustrated as a percentage with respect to the corresponding control, untreated cells (100%), and data are means ± SEM., n=3. In all instances, significance is given as \*\*p < 0.01; \*\*\*p < 0.001; NS not statistically different versus control. ##p < 0.01; ###p < 0.001 and ns not statistically different versus glutamate-treated cells

glutamate excitotoxicity were investigated with differences between observations [16, 17] but molecular mechanisms and signaling pathways transduction targeted accounting for neuroprotective effect of CGA, the whole ester and its hydrolysates, separately requires further research.

In a first investigation, we demonstrated that CGA did not differ statistically to caffeic acid to protect neurons against L-glutamate induced neuronal death. In contrast, quinic acid showed a lower range of neuroprotective activity and levels of cell viability reach of statistical significance only with a higher dose 100 µM.

Our results were in accordance with some previous studies, which have investigated the neuroprotective effects of CGA and its metabolites in vitro, indicating that CGA protects neurons from glutamate-induced neuronal cell death [17]. However, in the study of Taram et al. chlorogenic acid did not protect Cerebellar granule neurons CGNs and primary cultures of rat cortical neurons and only caffeic acid provided significant protection from excitotoxicity-induced cell death [16]. Differences between these observations could be due to several factors such as cell type, primary cell cultures or stable cell line cultures, tissue culture conditions, source of cells (fetal, middle age or adult brains), age of cultures, purity of cultures concentration and duration (minutes to hours) of glutamate exposure, nature of the neurotoxin used, and extent of Ca<sup>2+</sup> influx [19].

Excitotoxicity thought overstimulation of over activation of glutamate receptors has been reported as the most important trigger of neurotoxicity upon excitatory amino acids and neuronal death in some conditions mediated by the extracellular glutamate release and interaction with ionotropic receptors agonists receptors [23]. The ability of glutamic acid excitotoxicity is mediated, in most cases, by an interaction with NMDA receptors, leading to an uncontrollable rise of ROS and intracellular calcium concentrations. L-Glutamate also induces the partial depolarization of the mitochondrial membrane that triggers alterations in the membrane potential of mitochondria [20] and causes enhanced intracellular substrate oxidation and reactive oxygen species ROS production leading to apoptotic neuronal death. Measurement of mitochondria activity with the membrane potential-sensitive probe JC-1 revealed that treatment with glutamate resulted in a decrease of the proportion of active mitochondria, and that treatment with both caffeic and CGA prevented the deleterious effect of glutamate. Caffeic acid appears the most reactive compounds to neutralize the excess of ROS production. Its strongly suggest that the mechanism for antioxidant action of CA is associated with the formation of the iron complex, which inhibits the reaction between hydroxyl radical and the target molecule improving the capacity of CA to inhibit ROS formation.

In turn, the results obtained with chlorogenic acid and caffeic acid are consistent with previous reports showing that excitotoxic insults produce a rapid mitochondrial membrane depolarization which can be abolished by antioxidants [24]. CGA and CA may protect mitochondria through its direct free radicals scavenging ability and acting as a chain-breaking antioxidant though the the anti-radical properties of polyphenols lead to reduction of hydroxyl and superoxide radicals [25].

As for the antioxidant/scavenging activity of these compounds, was well documented [9, 26] and it was widely reported that chlorogenic acid and related compounds are well known to be antioxidants [27]. The scavenging of

the DPPH radical is a simple model reaction providing relevant information about the ability of acids to scavenge free hydroxyl radicals. Antioxidant activity of phenolic acids and their derivatives depends on the number and position of hydroxyl groups bound to the aromatic ring, the binding site and mutual position of hydroxyl groups in the aromatic ring, and the type of substituents [28].

CGA have been the subject of scientific research due to their antioxidant capacity to scavenge free radicals, which is attributed to double bonds and hydroxyl groups in its structure. In contrast to these observations, the binding of quinic acid to caffeic acid causes an increase in the antioxidant activity of caffeic acid while decreasing DPPH radical scavenging activities [29]; some other reports indicate that chlorogenic and caffeic acids are equally effective antioxidants activity [30].

In agreement with our results, caffeic acid has potent antioxidant properties, a function dependent on its chemical structure and the presence of a catechol moiety [28] suggesting that this antioxidant activity might contribute to its neuroprotective properties. The antioxidant activity of CA was previously studied by Nardini et al. and reported that CA inhibited, in a dose-dependent manner, human LDL lipoperoxidation induced by cupric ions [31]. CA is able to reduce lipoperoxyl radicals (ROO $\cdot$ )—by donating a hydrogen atom—to its corresponding hydroperoxide, which inhibits the lipid peroxidation chain reaction [25].

Another hypothesis to restore mitochondrial membrane dysfunction is chelating ability of phenolic compound. A previous study has suggested that CA has a chelating-antioxidant mechanism and it is strongly suggest that it works in vitro by efficiently chelating iron ions and, thus, minimizing the effects of Fenton-generated hydroxyl radicals. The antioxidant capacity of CA is due to the formation of complexes with metal ions, capable of preventing in vitro oxidative damage [32]. CA is able to complex Fe(II) ions and to inhibit in vitro hydroxyl radical formation and lipid peroxidation of rat liver membranes chain reaction which gives it the capability to act in the cell membrane microenvironment of different tissues preventing lipid peroxidation and other oxidative processes mediated by lipid peroxidation products [33].

Additionally, chlorogenic acid revealed the ability to form complexes with metal ions. This could be attributed to a more complex structure of others phenolic acid which is a cinnamate ester obtained by formal condensation of the carboxy group of *trans*-caffeic acid with the 3-hydroxy group of quinic acid. Thus, the presence of group carboxylic group in hydroxycinnamic acids family may have an influence on the chelation capacity, as well as on scavenging activity (iron-chelation properties of phenolic acids bearing catechol groups) [34].

All these reactions provoke inhibition or reduction in the formation of free radicals, they interrupt the propagation of free radical chain reactions, or they delay the start or reduce the reaction rate. Furthermore, metal chelation can result in prevention of metal redox cycling, occupation of all metal coordination sites, formation of insoluble metal complexes, steric hindrance of interactions between metals, and formation of lipid intermediates [35].

Scavenging or detoxification the excess ROS is achieved by an efficient antioxidative enzymatic system. Endogenous antioxidants play an important role in alleviating oxidative stress and its consequences [36]. Previous studies have suggested that anti-oxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) can function as a defense mechanism against ROS-mediated cellular damage, thereby proposing that these anti-oxidant enzymes may be useful as a therapeutic agent against ROS-related diseases [37]. Herein, we demonstrate that the CGA is able to stimulate both SOD and catalase activities in neurons. The mechanism involved in the polyphenols induced increase of SOD and catalase activity in a model of glutamate induced excitotoxicity is currently unknown, but it has been demonstrated that dephosphorylation of SOD and catalase in a calcium- and protein kinase C-dependent manner is associated with an increase in the activity of these two enzymes [38, 39]. Thus, the stimulatory effect of CGA and CA on SOD and catalase activities could be ascribed to the intracellular calcium increase inducing SOD and catalase activation through a dephosphorylation process. This finding supposes that chlorogenic acid via its caffeoyl groupement maintain the equilibrium of the endogenous enzymatic antioxidant to reinforce defense and remedy deleterious effects of excitotoxic damage.

Another hypothesis were suggested and support the fact that ROS generation during glutamate overstimulation is likely may due to the Ca $_2^+$  influx into the cytosol leading to neuronal damage via activation of enzymatic system in association with ROS generation and lipid peroxidation [40]. Depolarization of mitochondrial membrane also can activate plasma membrane voltage Ca $_2^+$  channels leading to additional Ca $_2^+$  influx. In support of this hypothesis, it has been shown it have been reported that CGA in coffee protects neurons from glutamate neurotoxicity during brain ischemia by regulating Ca $_2^+$  entry into neurons [17]. Caffeic acid also protects primary cultures of rat cortical neurons from the excitotoxic effects of L-glutamate by attenuated excessive calcium influx caused by glutamate [41]. Apparently, caffeic acid potently modulates the intracellular calcium concentration neuron cells through the control calcium influx or activation of calcium sensitive molecules. Thus, the inhibition of Ca $_2^+$  elevation by CGA through caffeic acid moiety and this effect might be attributed to a direct reduction in the amount of calcium entering through voltage-dependent

$\text{Ca}_2^+$  channels or to the secondary effects caused by other processes such as the alteration plasma membrane potential following modulation of  $\text{K}^+$  channels and support the finding of the capacity of some polyphenols to reduce calcium entry via glutamate receptors [42].

Collectively, these data suggest that the intrinsic radical scavenging activity of phenolic compounds has been linked to scavenge the excess of intracellular ROS generation and modulating mitochondrial membrane potential in a response to cytosolic  $\text{Ca}_2^+$  dysregulation followed by alteration of membrane potential and permeability transition, suggesting that these events may be considered as an initial line of defense against glutamate induced cell death.

Activation of MAPK kinase cascade signaling pathway is proposed to be involved in the neuroprotective effect of chlorogenic acid and its related hydrolysates against glutamate excitotoxicity using specific protein kinases inhibitor peptides: U73122 PLC inhibitor, Chelerytrine, PKC inhibitor or H89, PKA inhibitor. PLC inhibitor U73122 and the PKC inhibitor chelerythrine abrogated similarly the neuroprotective effect of chlorogenic acid and caffeic acid. These observations suggest that chlorogenic acid and its related compound caffeic acid and quinic acid compounds separately, exert their protective effects through different targeted transduction pathways. More there, the neuroprotective activity of chlorogenic acid the whole compounds can be contributed by the caffeoyl groupment and ascribed specifically to activation of the PLC/PKC signaling pathway. Since, activation of PKC by polyphenols is a key event in neuroprotection, and already known as a potent regulator of apoptotic pathways and was shown to be the target of many polyphenols for providing survival signaling. PKC signaling pathways regulate important molecular events involved in associative memory storage, and signaling deficits of PKC signaling pathways play an important role in the pathophysiology of neurodegenerative disorders like Alzheimer disease [43]. Protein kinase C is a calcium regulated kinase that modulate NMDA receptors function. PKC, enhance voltage-dependent  $\text{Ca}_2^+$  channel function and glutamate release [41]. However, quinic acid appears to exert its neuroprotective through the PKA signaling pathway.

It is widely accepted that permeabilization and mitochondrial dysfunction causes activation of caspases, the effectors of apoptotic cell death [42]. We have thus investigated the possible effect of phenolic tested compounds on L-glutamate-induced caspase activation in cultured cortical neurons. Therefore, active caspases can be controlled by of peptide inhibitors that directly interact with the protease; these peptide inhibitors are able to block caspases and inhibit their activities and per consequent inhibit cell death in vitro [44]. Caspases were divided into two important intracellular family group, upstream initiators and downstream effectors. The upstream initiators activate the effectors caspases

in response to un-upstream stimuli to leads to the apoptotic phase execution by cleavage of various substrates as a proenzyme [45]. Caspase 1, 8 and 9 were considered as caspases initiator of Caspase 3 that has already been identified as a key mediator of neuronal cell death [46]. Caspase 8 and caspases 9 have been implicated as principal procaspases and thus critical to distinguish cell death pathway: Caspases 8 activation is involved in the extrinsic or receptor-mediated pathways; the intrinsic or mitochondrial pathway involves activation of caspase 9 [47]. Herein, caspase 1, caspase 8 and caspase 9 inhibitor was used to block caspases activities. Our results showed that similarly CGA and caffeic acid inhibited L-glutamate-induced cleaved activation of caspase-8 and -9 which further confirmed that CGA and CA almost, similarly could inhibit L-glutamate-induced apoptosis in cortical neurons indicating that the protective effect of CGA and CA can be accounted for by an inhibition of caspase 8 and 9 that in turn inhibit caspase 3. Both caspases 8 and 9 then activate caspase 3, the major effector and the key mediator of neuronal cell death.

Moreover, an increase in the intracellular  $\text{Ca}^{2+}$  level is thought to trigger a cascade of biochemical processes, including calpain activation. It has been demonstrated that calpains is implicated in an early involvement in glutamate excitotoxicity induced cell death [48] and that caspases have the propriety to up-regulate calpain activities through modification of calpastatin (an endogenous calpain inhibitor) by proteolytic cleavage [49]. In addition, the rise levels of  $\text{Ca}^{2+}$  may be one of the causes of activations of proteolytic enzymes as calpains, which are considered mediators of both necrotic cell death and apoptosis [50]. Pretreatment of cortical neurons with calpain inhibitors, PD150606, calpeptin, and MDL28170 prior to tested compounds totally abrogated the protective effect of caffeic acid. Caffeic acid can inhibit glutamate induced calpain activity supporting its pronounced neuroprotective potential probably resulting from oxidative stress suppression.

Altogether, these data indicate that CGA acts as a protective agent against L-glutamate induced cortical neurons injury through maintain antioxidant enzymatic system and inhibiting apoptosis, as well as regulating neurons MAPK signaling cascade. These finding suggest that activation of PKC by caffeoyl group of chlorogenic acid appears to be a key event in its neuroprotective effect via proposed following mechanisms: maintain mitochondrial membrane potential, scavenging endogenous ROS production and increasing the levels of SOD activity.

## Conclusion

Recently, numerous bio-molecules such as phenolic compounds, having therapeutic abilities, have been developed as



new drug candidates. However, their large molecular sizes and poor hydrophilicities reduce their bioavailabilities or delivery to cells or tissues. This data demonstrate that the neuroprotective effects of chlorogenic acid a cinnamate ester obtained by formal condensation of the carboxy group of *trans*-caffeic acid with the 3-hydroxy group of quinic acid, on glutamate-induced excitotoxicity and brain damage might be attributed to the caffeoyl group, a relation structure function that is associated with anti oxidative ,anti apoptotic activities leading to the anti-excitatory potential of these active compounds and to have clinical benefits and might potentially be used as a therapeutic agent for neurodegenerative diseases. The neuroprotective effects of these compounds in vivo are currently under investigation.

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