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Neuroprotective Effects of *Castanea sativa* Mill. Bark Extract in Human Neuroblastoma Cells Subjected to Oxidative Stress

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ABSTRACT

One of the major features of neurodegenerative disease is the selective vulnerability of different neuronal populations that are affected in a progressive and often stereotyped manner. Despite the susceptible neuronal population varies between diseases, oxidative stress is implicated as the major pathogenic process in all of them. Natural Extract of *Castanea sativa Mill*. bark (ENC), recently characterized in its phenolic composition, acts as antioxidant and cardioprotective agent. Its neuroprotettive properties, however, have never been investigated. The aim of this study was to assess neuroprotection of ENC in in vitro models of oxidative-stress-mediate injury. Human neuroblastoma SH-SY5Y cells treated with glutamate (50 mM for 24 h) or hydrogen peroxide (25 μM for 1 h followed by 24 with medium) were used. The results showed that the addition of ENC (1–50 μg/ml) to cell medium before the neuronal damage provided neuroprotection in both experimental models used, while its addition after the injury was ineffective. In conclusion, the present results suggest that ENC could be a valuable support as dietary supplement, combining beneficial preventive neuroprotettive effects with a high antioxidant activity. J. Cell. Biochem. 117: 510–520, 2016. © 2015 Wiley Periodicals, Inc.

KEY WORDS: *Castanea sativa* Mill; NEUROPROTECTION; NEURODEGENERATIVE DISEASES; OXIDATIVE STRESS; EXCITOTOXICITY

Neuroprotection refers to the strategies and relative mechanisms which protect the central nervous system (CNS) against neuronal injury occurring in both chronic (e.g., Alzheimer's disease, AD, and Parkinson's disease, PD) and acute (e.g., stroke or trauma) neurodegenerative disorders [Dunkel et al., 2012]. In the context of the neurodegenerative pathologies, it is speculated that the administration of a drug able to interfere with multiple pathways involved in the onset and progression of the disease may slow and ultimately stop the evolution of the disease itself [Djaldetti et al., 2003]. The etiology of different neurodegenerative diseases, however, is a non-linear processes involving many common starting points of molecular networks, reciprocally and dynamically interacting to produce a common final, neurodegenerative outcome [Nieoullon, 2011]. Oxidative damage has been shown to be one of such starting point in acute as well as in chronic neurological disorders, damaging macromolecules, such as lipids, proteins, and nucleic acids, thus leading to cell injury and death [Li et al., 2012, Fischer and Maier, 2015]. In addition, recent studies have shown that reactive oxygen species (ROS) affect cell death/survival signaling pathways [Hensley and Harris-White, 2015]. For example, in AD Wnt signal transduction pathways, 5′-adenosine monophosphate activated protein kinase (AMPK), mechanistic target of rapamycin (mTOR) complexes, and activation of the Sirtuin 1 (silent mating-type information regulator 2 homolog 1)/peroxisome proliferator-activated receptor gamma co-activator 1-α (Sirt1/PGC-1α) axis are affected by oxidative stress [Godoy et al., 2014]. In PD, despite the mechanisms involved in selective degeneration of dopaminergic neurons in the nigrostriatal system are still unknown, some evidence suggests the involvement of ROS in such mechanisms. Oxidative stress may arise from the metabolism of dopamine with the production of potentially harmful free radical species [Cobb and Cole, 2015]. Compared to other brain areas, *subtantia nigra pars compacta* is exposed to a higher rate of ROS formation and to higher levels of oxidative stress. The reason for this is not clear yet, but it

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may be related to the energy metabolism of these cells or to their high content of dopamine [Cobb and Cole, 2015]. Finally, also the role of neuroinflammation in neurodegeneration has been demonstrated to be related to oxidative stress [Fischer and Maier, 2015]. Some Authors reported that this event may start from the periphery and that peripheral conditions strongly affects several brain diseases through blood-brain barrier alteration [Deretzi et al., 2011]. In particular, it has been postulated that changes in intestinal permeability might determine systemic and/or central nervous system inflammation [Galland, 2014]. Consequently, the administration of substances able to restore gastrointestinal function and to prevent neuronal damage may result in a huge clinical benefit.

Despite great advances in the knowledge about the molecular events triggering neurodegenerative diseases, therapies to efficiently inhibit their progression have not yet been developed. Most neurodegenerative diseases are late-onset and remain asymptomatic for most of the phases, and occur when the damage has already affected most neurons. Moreover, most treatments are symptomatic but no causative and for all these reasons current therapies show limited efficacy and limited value to patients. It may be possible to prevent or halt the disease progression to a great extent if therapies start at the initial stage of the disease. A rational approach aimed at exerting a disease-modifying activity and inhibiting the progression of neurodegenerative disorders may be focused on substances which at the same time modulate several molecular networks, thus restoring cells functionality. Several polyphenols possesses these properties, acting as neuroprotective agents by affecting many targets involved in the onset and progression of different chronic pathologies, including cardiovascular and cancer diseases [Khurana et al., 2013]. In particular, different tannins provide neuroprotection owing to their antioxidant- and anti-inflammatory activity [Rojanathammanee et al., 2013], the ability to inhibit β-secretase [Youn and Jun, 2013] as well as anomalous tau protein aggregation [Yao et al., 2013] and to restore gastrointestinal function in several inflammatory conditions [Rosillo et al., 2012]. Furthermore, several orally administered tannins and tannins-rich extracts have been shown to provide benefits at CNS level [Nakajima et al., 2013].

The extract obtained by the bark of *Castanea sativa* Mill (ENC) contains high amounts of hydrolizable tannins such as vescalagin, castalagin, vescalin, castalin, gallic acid, and ellagic acid, whose qualitative and quantitative chemical characterization has been recently performed [Chiarini et al., 2013]. ENC has been shown to modulate the entire gastrointestinal tract motility [Budriesi et al., 2010; Micucci et al., 2014], to act as antioxidant and cytoprotective agent in rat cultured cardiomyocytes [Chiarini et al., 2013], to possess antihelmintic, antimicrobial, and antiviral activities, to reduce oxidative stress as well as to prevent DNA damage when orally administered [Frankic and Salobir, 2011]. Its neuroprotective effects, however, have never been assessed.

The aim of the present study was to assess neuroprotection of ENC by using in vitro model of excitotoxic- or oxidative stress-mediated injury, that is human neuroblastoma SH-SY5Y cells treated with glutamate or hydrogen peroxide. Furthermore, in order to evaluate preventive and curative potential of ENC, the extract was added before (pre- treatment) or after/during (post-treatment) cell injury, respectively. Results showed that the addition of ENC to cell medium before the neuronal damage provided neuroprotection in both experimental models, while its addition after the injury was ineffective.

MATERIALS AND METHODS

MATERIALS

ENC® was supplied by Alchemistry (Alchemistry SRL, Cesena, Italy) and was obtained as previously reported [Budriesi et al., 2010]. The fine brown powder (92–95% dry matter) contains 77% of pure tannin on a dry matter basis. The chemical composition of the batch used in the experiments was as follows: water, 2.9%; tannin, 77.8%; non-tannin, 17.7% (oligosaccharides, salts, vegetable resins, and gums coming from the hydrolysis process of chestnut wood); insoluble, 1.6%; crude fibers, 0.24%; ash, 1.7%. The major components of tannins were vescalagin, castalagin, and ellagic acid. Other minor compounds, detected in trace levels, 5-*o*-galloylhamamelose, (3, 5-dimethoxy-4-hydroxyphenol)-1-*o*-*β*-D-(6′-*o*-galloyl)-glucoside isomer, m-digallic acid, kurigalin isomer, and chestanin [Chiarini et al., 2013]. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), dimethylsulfoxide, propidium iodide and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Deionized water was obtained from an Elix 10 water purification system from Millipore (Bedford, MA).

CELL CULTURES

Human SH-SY5Y neuroblastoma cells were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutammine (2 mM), in a humidified atmosphere of 95% air and 5% CO_2 at 37C. Cells were sub-cultured when they were 70–80% confluent, fed twice each week and used for assays during exponential phase of growth.

GLUTAMATE- OR HYDROGEN PEROXIDE-INDUCED INJURY IN SH-SY5Y CELLS

SH-SY5Y cells were seeded into 96-well plates (8×10^4 cells/ml, final volume 200 μl). After 24 h, the cell culture medium was aspirated and the wells were washed three times with phosphate-buffered saline (PBS). For concentration response curves, cells were treated with l-glutamate (GLU) (1–100 mM for 24 h) or hydrogen peroxide (1–200 μM for 1 h and after 24 h with medium) and then cell viability assay performed as described below. GLU was prepared as 500 mM stock solution in 1 M HCl which was re-equilibrated at pH 7.5 before dilution to working concentrations [Croce et al., 2013]. Hydrogen peroxide was freshly prepared from 30% stock solution prior to each experiment. 50 mM GLU or 25 μM H_2O_2 were selected for further studies as they produced a significant toxicity of around 50% cell death.

ENC TREATMENTS

ENC was prepared immediately before use as a stock solution (10 mg/ml in PBS) and pH was adjusted to 7.5 before dilution to the desired final concentration. To evaluate preventive and curative potential of ENC, the extract was added before (pre-treatment) or after/during

(post-treatment) cell injury, respectively. In particular, SH-SY5Y cells were pretreated for 2 h with ENC (1–50 μg/ml) before adding GLU or H_2O_2 to the medium, while the extract was co-present with GLU or H_2O_2 to assess its curative potential (post-treatment).

CELL VIABILITY ASSAY

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay was used to determine cell viability. Briefly, after treatment with glutamate or hydrogen peroxide, 20 μl of MTT solution (5 mg/ml in PBS) were added to each well and incubation continued at 37°C for 4 h. The medium was then removed, replaced with 200 μl DMSO and plates were shaken for 20 min. Absorbance was measured at 540 nm wavelength using a Multiskan Go™ microplate reader (Thermo Scientific, Waltham, MA).

CELLS MORPHOLOGICAL ASSAYS

Apoptotic cells experiencing damage in the nuclei are featured by cell shrinkage, membrane blebbing, and presence of apoptotic bodies. In order to register such changes caused by GLU or H_2O_2, SH-SY5Y cells were examined by a phase-contrast light microscope. The results were evaluated using the grade scale, described in USP 28 (United States Pharmacopeia edition 2005) (grades 0–4) for assessment of the cytotoxic potential of tested materials, as follows: grade 0-none reactivity (discrete intracytoplasmic granules, no cell lysis); grade 1- slight reactivity (no more than 20% of the cells are round, loosely attached and without intracytoplasmic granules; occasional lysed cells are present); grade 2-mild reactivity (no more than 50% of the cells are round and devoid of intracytoplasmic granules, no extensive cell lysis and empty areas between cells); grade 3- moderate (up to 70% of cells are rounded or lysed); grade 4-severe (nearly complete destruction of the cells).

APOPTOSIS ASSAYS

Cell cycle and sub-G0/G1 population analysis was used to check for cell apoptosis by using flow cytometry [Riccardi and Nicoletti, 2006]. SH-SY5Y (2.5×10^5 cells/ml, final volume of 2 ml) were treated with ENC and then subjected to injury as detailed before. Afterward, they were treated with trypsin-EDTA, collected by centrifugation at 1500 rpm for 10 min and thoroughly rinsed with cold PBS. Pellets were resuspended in 300 μl of cold PBS and then 700 μl of ice-cold ethanol was added drop by drop under gently mixing and by keeping tubes on ice. Cells were then fixed at −20°C for 24 h. Ethanol was removed by washing thoroughly with PBS and the pellets were then resuspended in 300 μl of PBS added with 3 μl of RNase (10 mg/ml). Tubes were incubated for 30 min at room temperature and washed again with PBS. The final pellet was resuspended in 1 ml of PBS, added with 10 μl of PI (2 mg/ml), and incubated for 30 min at room temperature in the dark. Red fluorescence (DNA) was detected through a 563–607 nm band-pass filter using a FACScan flow cytometer (BD Biosciences, San Jose, CA). In flow cytometric histograms, apoptotic cells have a signal in the sub-diploid regions and are well-separated from the normal G0/G1 cells peak. A minimum of 10^4 cells per sample were collected and the percentage of apoptotic cell accumulated in the sub-G0/G1 peak was calculated by using Cell Quest software (BD Biosciences, San Jose, CA).

INTRACELLULAR ROS CONTENT

The intracellular ROS level was detected using the oxidation-sensitive fluorescence probe 2′, 7′-dichlorofluorescein diacetate (DCFH-DA). Before any treatment, cells were incubated with 5 μM DCFH-DA in the dark for 30 min and then gently washed in PBS. Fluorescence reading was taken at 1 h or 24 h with fluorescence microplate reader using excitation and emission wavelengths of 485 nm and 535 nm, respectivelly. Intracellular antioxidant activity was expressed as the percent of inhibition of intracellular ROS produced by glutamate- or H_2O_2-exposure [Chiarini et al., 2013].

DAPI STAINING

Assessment of apoptosis based on nuclear morphology was performed using the 4′,6-diamidino-2-phenylindole (DAPI) staining kit (Life Tecnologies Italia, Monza, Italy) according to the manufacturer′s protocol, with slight modifications. At the end of the experiment, cells were rinsed with ice-cold PBS buffer and immediately fixed with 4% paraformaldehyde (30 min, 25°C) followed by three washings with PBS. PBS/Triton (0.2% v/v) was then added (10 min, 25°C) and afterward cells were stained with DAPI. After washing again with distilled water, cells were placed on a slide for fluorescence microscopy with the anti-fade mounting medium 1, 4-diazabicyclo octane in glycerol (DABCO) and examined by a reverse-phase microscope (Leica Microsystems, Wetzlar, Germany). Nuclei with fragmented and condensed DNA were scored as apoptotic cells. For quantification, three independent experiments were carried out and five fields were randomly selected by operator which was blind to the condition of the assay.

ANALYSIS OF DATA

Data were collected as quadruplicate from at least three independent experiments. The results were expressed as mean ± error standard. Cell viability was expressed as percent of untreated cells (controls) or as percent of recovery from injury which varied from 0 (no damage recovered) to 100% (totally recovered damage). Statistical significance was assessed by using unpaired Student′s *t*-test or ANOVA (ordinary or repeated measures followed by Dunnett *post test*) (GraphPad Prism version 5.04, GraphPad Software Inc., San Diego, CA), as appropriate. In all comparisons, the level of statistical significance (*P*) was set at 0.05.

RESULTS

EFFECTS OF ENC ON SH-SY5Y CELL VIABILITY

To evaluate the effects of ENC *per se* on SH-SY5Y cells viability, cells were incubated with increasing ENC concentrations (0–100 μg/ml) for 24, 48, and 72 h. ENC treatment proved to be generally devoid of cytotoxic effects and only a slight reduction in cell viability (~20%) was in fact encountered at the highest concentration tested at all scheduled times (Fig. 1, panel A). Flow cytometric analysis of cellular DNA, used to determine the effect of ENC on cell cycle distribution, showed that the extract did not affect the percent of cell in the different phase of cell cycle (Fig. 1, panel B). Finally, the morphological analysis performed by using contrast phase microscopy and DAPI staining after 24 h of treatment with the highest

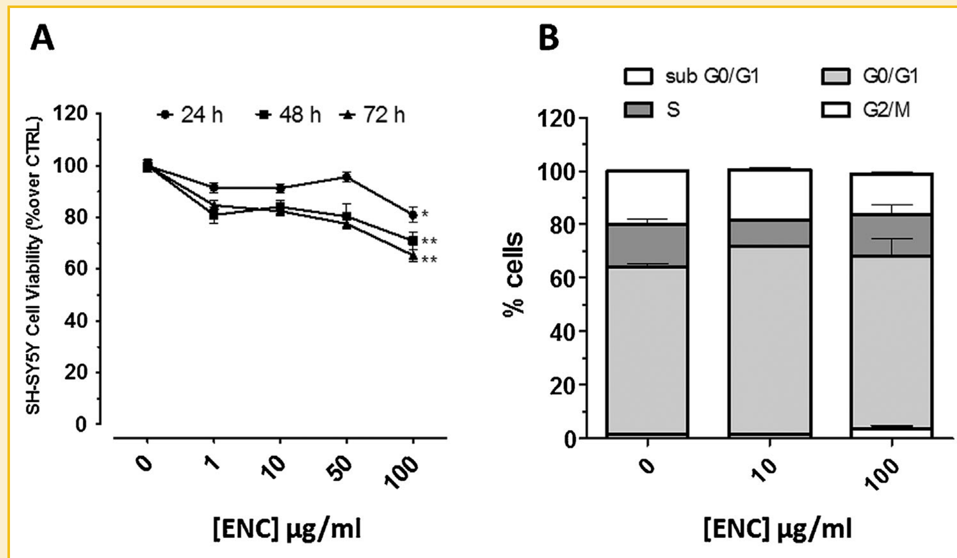

Fig. 1. Effects of Natural Extract Chestnut bark (ENC) on SH-SY5Y cell viability and cell cycle distribution. (Panel A). The cells were incubated with increasing concentrations of ENC (0–100 µg/ml) and MTT test performed at 24, 48 and 72 h time-points. (Panel B). Percentage of cells in the subG0/G1 (apoptotic), G0/G1, S or G2/M phase determined by flow cytometry after propidium iodide staining. Cells were treated with 10 or 100 µg/ml of ENC for 24 h ore left untreated (Controls, CTRL). For both Panel A and B, data are reported as means ± e.s.m. of four independent experiments (triplicate or quadruplicate were performed in each experiment). Statistical evaluation was performed by using ANOVA followed by Dunnet *post test*. MTT assay: $^{*}P < 0.05$, $^{**}P < 0.01$ vs CTRL. Cell cycle: no significant changes were observed after ENC treatments.

concentration of ENC (100 µg/ml) confirmed previous data. The extract evoked grade 1 signs of cytotoxicity in neuroblastoma cells, being ~20% of cells round, loosely attached and without intracytoplasmic granules (Fig. 2, panel A). In the DAPI assay, which specifically stains the nuclei, the presence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies upon ENC treatment at a concentration of 100 µg/ml was generally observed in few cells (Fig. 2, panel A). This was confirmed also by quantitative

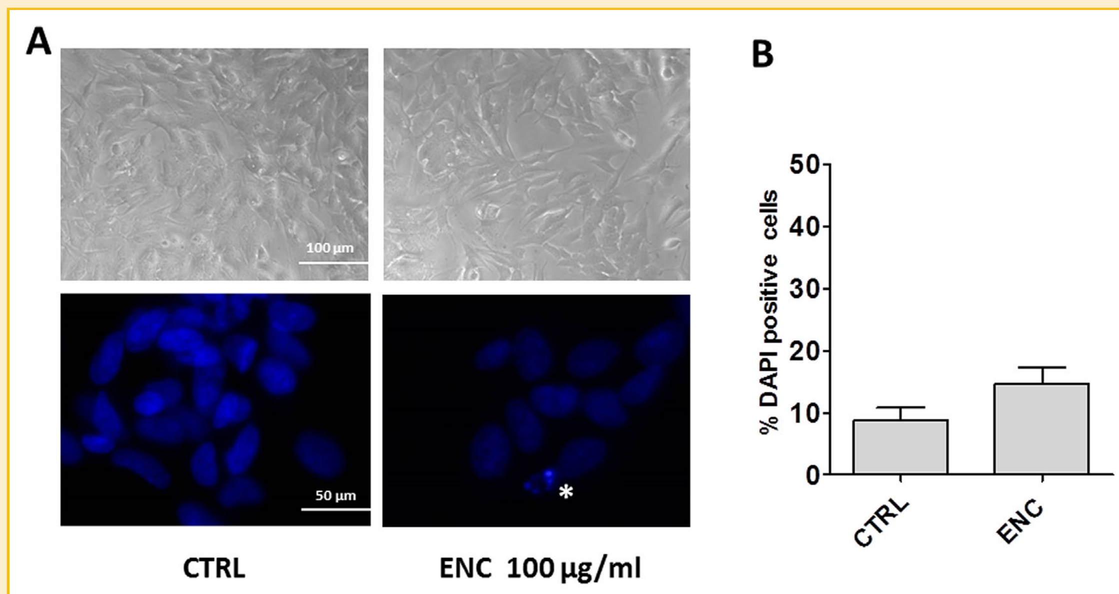

Fig. 2. Effects of Natural Extract Chestnut bark (ENC) on SH-SY5Y cell morphology. (Panel A). Morphological comparison performed at contrast phase microscopy (scale bar 100 µm OM 100X) or by using fluorescence microscopy after DAPI staining (scale bar 50 µm, OM 400X) between untreated (CTRL) and ENC -treated (100 µg/ml for 24 h) SH-SY5Y cells. Asterisk indicate cells with fragmented nuclei and condensed DNA and considered as apoptotic. Each photograph was representative of three independent observations. (Panel B). DAPI staining quantitative analysis shown as mean ± e.s.m. of three independent experiments.

analysis (Fig. 2, panel B) which showed a slight increase in DAPI positive cells. Consequently, in consideration of the light toxicity observed at the highest concentration of ENC, its neuroprotective effects were deeply investigated in the range 0–50 μg/ml.

EFFECTS OF ENC ON GLUTAMATE-INDUCED SH-SY5Y TOXICITY

In order to determine a concentration of GLU which caused 50% reduction in cell viability accompanied by apoptotic-mediated cell death to be used to assess ENC protection, SH-S5Y5 cells were incubated at increasing GLU concentrations (0–100 mM) for 24 h and cell viability measured using the MTT assay. Results demonstrated that cell viability decreased with increasing concentrations of GLU, with an IC_{50} of 50.1 mM and a maximum effect of 84.7 ± 0.9% at the highest concentration used (Fig. 3 panel A). Furthermore, cell cycle analysis showed significant induction of cell apotosis as indicated by sub-G0/G1 increase upon exposure to GLU (from 16.9 ± 3.1% at 20 mM up to 41.4 ± 4.3% at 80 mM) in comparison to control cells (Fig. 3, panel B). Observations under the phase-contrast microscope confirmed that SH-SY5Y cells following treatment with GLU decreased growth and committed varying degrees of apoptotic death with shrinkage and deformation of cell bodies (Fig. 3, panel C). In the light of these results, treatment with 50 mM GLU for 24 h was then chosen for further experiments.

To evaluate the ability of ENC to attenuate GLU-induced toxicity, neuroblastoma cells were incubated with different concentrations of ENC for 2 h before excitotoxic injury. Results demonstrated that the extract had a concentration-dependent neuroprotective effect against GLU-induced injury as compared with the control group (CTRL) (Fig. 4 panel A); 50 μg/ml ENC was the most effective concentration in exerting neuroprotection, since cells recovery was 47.4 ± 5.8% ($P < 0.01$ vs GLU).

Flow cytometric analysis of cellular DNA was used to study changes in SH-SY5Y cell cycle. As reported in Figure 3, panel B, GLU induced a significant increase in proportion of sub-G0/G1 hypodiploid cells (+31.8 ± 3.0%, $P < 0.001$ vs CTRL) which was accompanied by a reduction in the percentage of cells in the G0/G1 and s phase (Fig. 4, panel B). Interestingly, flow cytometry data indicate that cells pretreated with ENC at 10 and 50 μg/ml concentration may have re-entered cell cycle since the percentage of cells in sub G0/G1, G0/G1, and s phase gradually attained control values when increasing ENC concentration. Since during GLU-induced toxicity generation of ROS strongly contribute to cell death, DCFH-DA assay was used to quantify intracellular ROS formation. As reported in Figure 4, panel C, a concentration- and time-dependent decrease in ROS production was observed in ENC-pretreated SH-SY5Y cells following exposure to GLU. The inhibitory maximal effect, was observed at 10 μg/ml (24 h) and

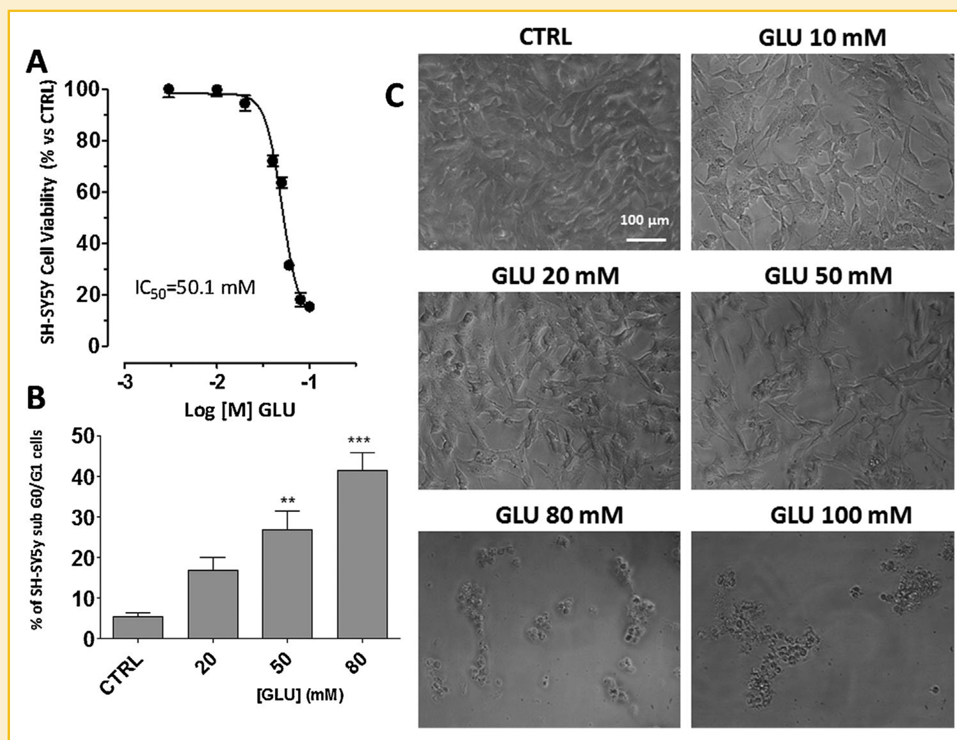

Fig. 3. Effects of Glutamate (GLU) on SH-SY5y cell viability. (Panel A). Glutamate concentration–cell death response curves (by MTT assay). (Panel B). Percentage of SH-SY5Y cells in subG0/G1 phase (flow cytometry) after treatment with increasing concentrations of GLU for 24 h. For both Panel A and B, data are reported as means ± e.s.m. of four independent experiments (triplicate or quadruplicate were performed in each experiment). Statistical evaluation was performed by using ANOVA followed by Dunnet *post test*. $^{**}P < 0.01$, $^{***}P < 0.001$ vs CTRL. (Panel C). Morphological comparison performed at contrast phase microscopy (scale bar 100 μm OM 100x) between untreated (CTRL) and GLU-treated (10–100 mM for 24 h) SH-SY5Y cells. Each photograph was representative of three independent observations.

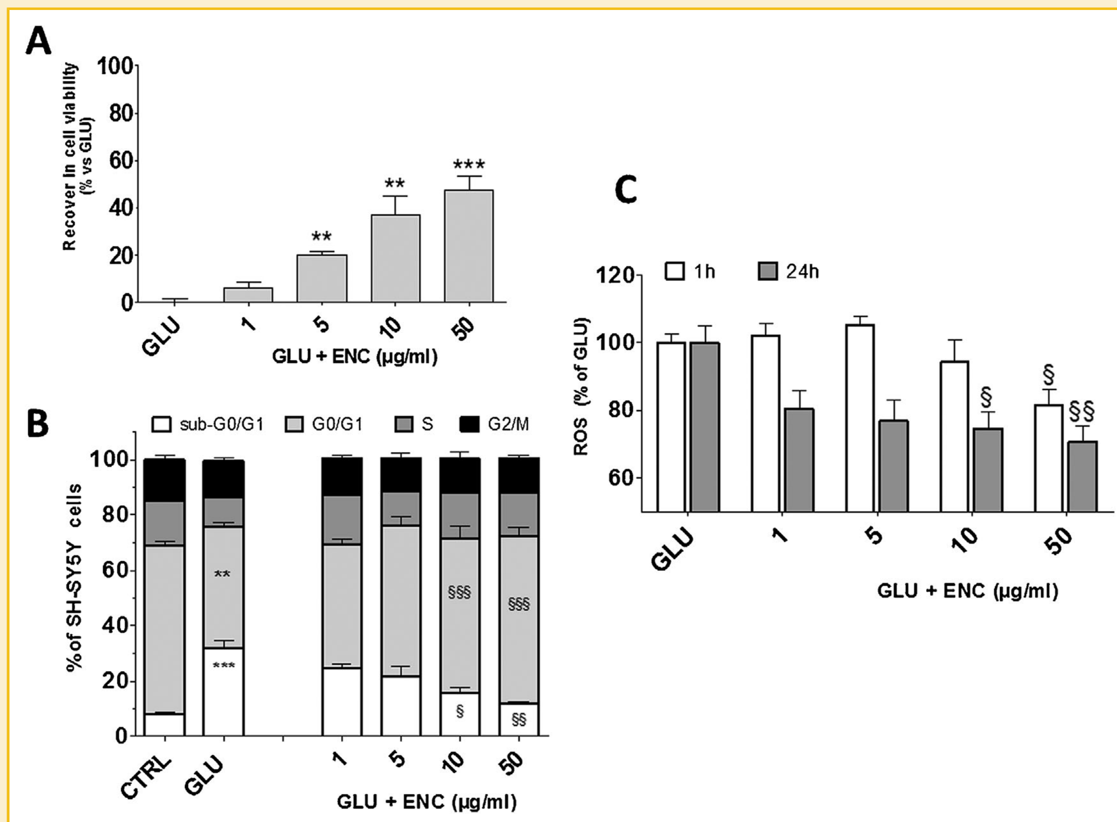

Fig. 4. Effects of 2 h pretreatment with Natural Extract of Chestnut bark (ENC)(1–50 μg/ml) on Glutamate (GLU)-induced cytotoxicity (50 mM for 24 h) and ROS production in SH-SY5Y cells. (Panel A). cell viability assessed by MTT assay. Data are reported as percent of recovery from injury which varied from 0 (no damage recovered) to 100% (totally recovered damage). (Panel B). Percentage of cells in the subG0/G1 (apoptotic), G0/G1, S or G2/M phase determined by flow cytometry after propidium iodide staining. (Panel C). Intracellular ROS production determined by using the peroxide-sensitive fluorescent probe DCFH-DA and expressed as the percent of inhibition of intracellular ROS produced by GLU. Data are reported as means ± e.s.m. of four independent experiments (triplicate or quadruplicate were performed in each experiment). Statistical evaluation was performed by using ANOVA followed by Dunnet *post test.* $^{**}P < 0.01$, $^{***}P < 0.001$ vs CTRL. §$P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$ vs GLU.

50 μg/ml (1 h and 24 h) ENC concentrations, at which a reduction of about 30% in ROS formation was measured.

Morphology analysis of cells performed by using phase contrast microscopy confirmed previous data, revealing a reversion in the characteristic morphological features of apoptosis induced by GLU and exerted by ENC. In particular, GLU-induced toxicity was characterized by shrunken cell bodies with broken neuries, the tendency to round-up and detach from the culture plate (grade 2–3 toxicity). These apoptotic changes were markedly prevented by pretreatment of ENC especially at the highest concentration (50 μg/ml) (Fig. 5, panel A). These morphological observations were further substantiated by those obtained by using DAPI staining, which highlight changes of the chromatin in apoptotic cells. As reported in Figure 4, after GLU treatment the number of fluorescent cells which contained condensed and fragmented nuclear material of varying sizes and showing the features of apoptotic bodies, which usually appear during the final phase of apoptosis (indicated by asterisk) was dramatically increased. In contrast, the number of floating cells displaying the fluorescence of the dye, when cells were pretreated with ENC was significantly lower, particularly at 50 μg/ml ENC concentration (Fig. 5, Panel B). Finally, the treatment of SH-SY5Y cells with ENC (0–50 μg/ml) during glutamate- induced injury (post-treatment) was completely ineffective (data not shown).

EFFECTS OF ENC ON HYDROGEN PEROXIDE-INDUCED SH-SY5Y TOXICITY

The excessive generation of intracellular ROS is a common feature of many neurodegenerative pathologies. Thus, to reproduce ROS cytotoxicity underlying neuronal loss a model of direct oxidative stress was used by exposing the human neuroblastoma cell line SH-SY5Y to H_2O_2. SH-SY5Y cells were thus incubated with increasing concentrations of H_2O_2 (0–800 μM) for 1 h. Afterward hydrogen peroxide was replaced by normal medium, cells incubated for further 24 h and cell viability finally assessed. The aim was to determine a concentration of H_2O_2 which caused 50% reduction in cell viability accompanied by apoptotic-mediated cell death and to be used to assess ENC protection in further experiments. As expected, a decrease in cell viability was observed following H_2O_2 treatment ($IC_{50} = 21.6$ μM) (Fig. 6, panel A) and a concentration of H_2O_2 that resulted in approximately 50% viability (25 μM) was then chosen. Afterward, the possibility that ENC could revert toxic effects induced

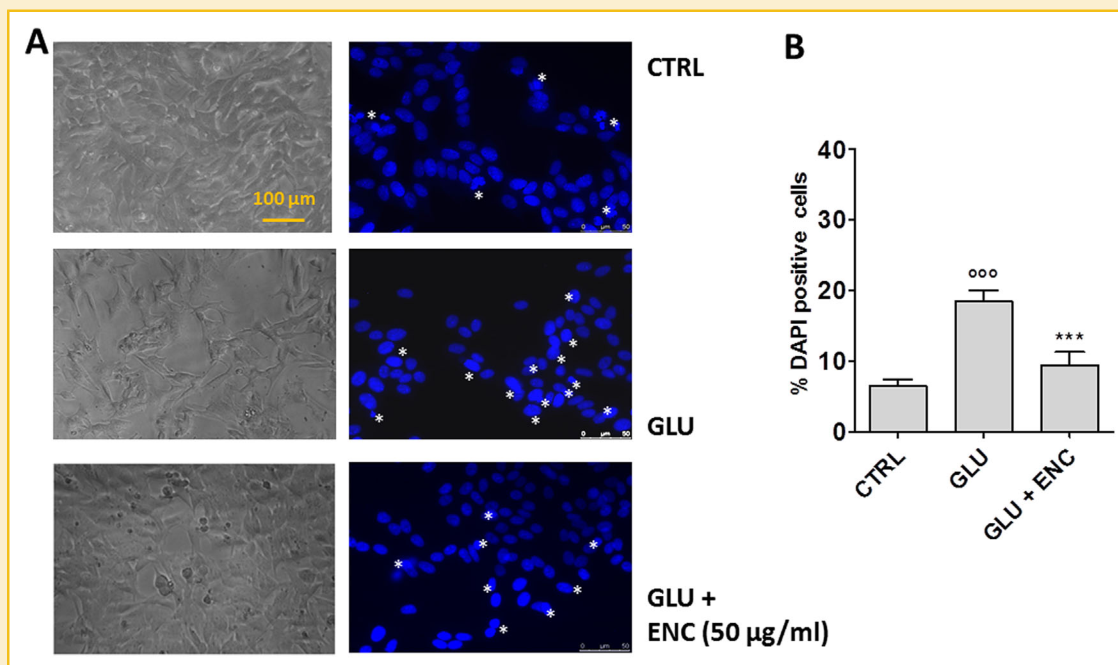

Fig. 5. (Panel A). Morphological comparison performed at contrast phase microscopy (scale bar 100 µm OM 100X) or by using fluorescence microscopy after DAPI staining (scale bar 50 µm, OM 400X) between SH-SY5Y untreated cells (CTRL), cells treated with glutamate 50 mM for 24 h (GLU) or cells pretreated for 2 h with 50 µg/ml Natural Extract of Chestnut bark (ENC) before Glutamate. Asterisks indicate cells with fragmented nuclei and condensed DNA and considered as apoptotic. Each photograph was representative of three independent observations. (Panel B). DAPI staining quantitative analysis shown as mean ± e.s.m. of three independent experiments. Statistical evaluation was performed by using ANOVA followed by Dunnet *post test*. °°°$P < 0.01$ vs CTRL; ***$P < 0.001$ vs GLU.

by H_2O_2 was deeply investigated. As reported in Figure 6, (panel B), pretreatment with ENC 2 h before and during the noxious stimulus, afforded concentration-dependent protection. This was observed starting from ENC 5 µg/ml (cell recovery 21.6 ± 5.5%, $P < 0.01$ vs H_2O_2), while maximal effect was achieved at the highest concentrations of 50 µg/ml (cell recovery 32.1 ± 2.7%, $P < 0.01$ vs H_2O_2), suggesting a cytoprotective effect of ENC against oxidative-stress-induced injury.

To examine whether ENC could also hamper H_2O_2-mediated ROS formation in neuroblastoma cells, DCFH-DA assay was performed. In the presence of ENC a concentration- and time-dependent reduction of intracellular ROS formation caused by hydrogen peroxide was observed (Fig. 6, panel C). Pretreatment of the cells with ENC, in fact, afforded significant protection already at 1 µg/ml after 24 h, while maximum effects was achieved at 50 µg/ml at both tested times.

ENC neuroprotection was evident also by analyzing phase contrast images. Figure 7 (panel A, left), in fact, reports cell damage caused by H_2O_2, where the number of cells decreased and their outlines were irregular (grade 2–3 toxicity). On the contrary, cells treated with ENC gradually recovered their initial density and exhibited a healthier appearance as ENC concentration increased. ENC also reduced apoptotic cell death induced by H_2O_2 as determined by DAPI staining (Fig. 7, panel A, right). A huge amount of the cells were DAPI-positive after treatment with H_2O_2, supporting the involvement of an apoptotic program in oxidative stress-induced SH-SY5Y neuronal death. On the contrary, ENC reduced in a concentration-dependent fashion the number of condensed or fragmented nuclei upon H_2O_2 treatment, the maximum effect being achieved at the highest concentration used (Fig. 7, panel B).

Finally, the treatment of SH-SY5Y cells with ENC (0–50 µg/ml) after H_2O_2- induced cell death or ROS formation was completely ineffective (data not shown).

DISCUSSION

The incidence of neurodegenerative disorders is expected to rise in the next years and the identification of effective treatments represents a significant challenge for medicine. Preventive strategies may reduce both the risk of developing neurodegenerative diseases and of symptom progression. Therefore, more research is needed to develop compounds with neuroprotective properties which could have therapeutic applications, especially when considering the relationship between gut alterations and neurodegenerative diseases [Forsyth et al., 2011].

Many vegetal extracts and natural compounds exert neuroprotective activities affecting different molecular networks in vitro and in vivo [Wang et al., 2010; Wei et al., 2013], produce a prebiotic activity [Campos et al., 2012] and restore intestinal permeability [Wei et al., 2013]. Chestnut (*Castanea sativa* Mill.) trees bark and wood are important sources of tannins and other phenolic compounds and the extract used in this study contains high amounts of vescalagin, castalagin, vescalin, castalin, gallic acid, and

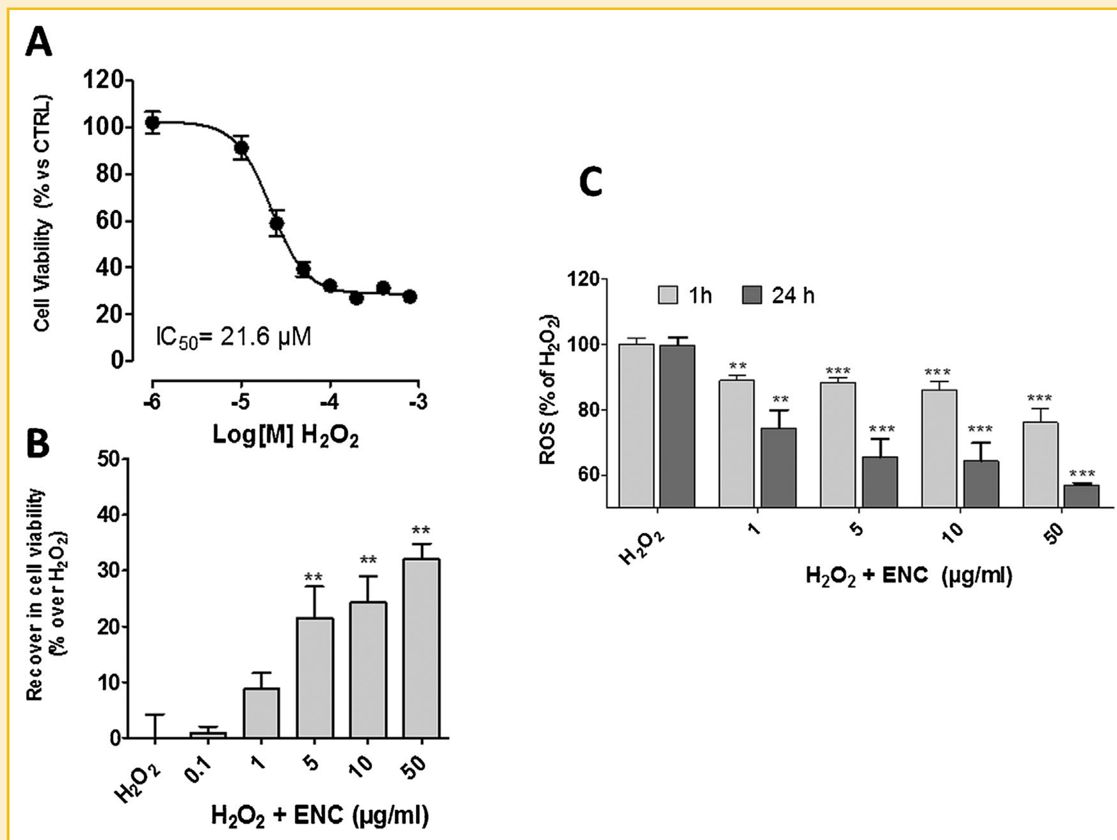

Fig. 6. Effects of H_2O_2 on SH-SY5Y cell viability and ROS production. (Panel A). Hydrogen peroxide concentration–cell death response curves. Cells were incubated with increasing concentrations of H_2O_2 (0–800 μM) for 1 h. Afterward hydrogen peroxide was replaced by normal medium, cells incubated for further 24 h and cell viability assessed by MTT assay. (Panel B). Effects of 2 h pretreatment with Natural Extract of Chestnut bark (ENC) (1–50 μg/ml) on H_2O_2-induced cytotoxicity (25 μM for 1 h followed by 24 h with medium). Data are reported as percent of recovery from injury which varied from 0 (no damage recovered) to 100% (totally recovered damage). For both Panel A and B, data are reported as means ± e.s.m. of four independent experiments (triplicate or quadruplicate were performed in each experiment). Statistical evaluation was performed by using ANOVA followed by Dunnet *post test.* $^{**}P < 0.01$ vs H_2O_2. (Panel C). Effects of 2 h pretreatment with ENC (1–50 μg/ml) on H_2O_2-mediated ROS production determined by using the peroxide-sensitive fluorescent probe DCFH-DA. Data were expressed as the percent of inhibition of intracellular ROS produced by H_2O_2 and reported as means ± e.s.m. of four independent experiments (triplicate or quadruplicate were performed in each experiment). Statistical evaluation was performed by using ANOVA followed by Dunnet *post test.* $^{**}P < 0.01$ vs H_2O_2.

ellagic acid. Many ellagitannins, including castalagin, and vescalagin, have potent antitumor, antioxidant, antimicrobial, and antimalaric properties [Khennouf et al., 2003; Seeram et al., 2005; Reddy et al., 2007; Buzzini et al., 2008; Auzanneau et al., 2012]. In addition, tannins may improve CNS functionality by affecting intestinal permeability [van Ampting et al., 2010; Ren et al., 2012]. No scientific data about ENC potential neuroprotective effects, however, are reported and for this reason these have been here investigated.

A common feature of neurodegenerative diseases is extensive evidence of oxidative stress, which might contribute to the dysfunction and death of specific cells, favoring disease pathogenesis [Li et al., 2013; Fischer and Maier, 2015; Hensley and Harris-White, 2015]. In the present study human brain neuroblastoma SH-SY5Y cells treated with two stressors (GLU and hydrogen peroxide) were used as an experimental models of oxidative stress pertaining to neuronal death. GLU-induced cell death model was selected because it relies on the endogenous production of ROS. High levels of extracellular glutamate, in fact, have been shown to be toxic to nerve cells in culture by two distinct processes: excitotoxicity, which occurs through the activation of ionotropic glutamate receptors, and a programmed cell death pathways called oxidative glutamate toxicity, or oxytosis [Tan et al., 2001], mediated by CySS/glutamate antiporter, depletion of GSH, down regulation of SOD activity, leading finally to apoptosis [Kritis et al., 2015].

The results demonstrated that ENC may have potential neuronal diseases preventive capabilities since it can protect SH-SY5Y cells when added to the medium before the injury. In particular, ENC is neuroprotective against glutamate toxicity, at least in part by the reduction of ROS formation. One mechanism of ROS-induced cell death is DNA damage [Azqueta and Collins, 2012]. Pretreatment with ENC, however, reduced DNA damage induced by glutamate stress, as shown by flow cytometry- and DAPI staining-assays. This suggests that ENC-mediated ROS reduction might protect against apoptosis resulting from DNA damage. Future studies, however, should be undertaken in order

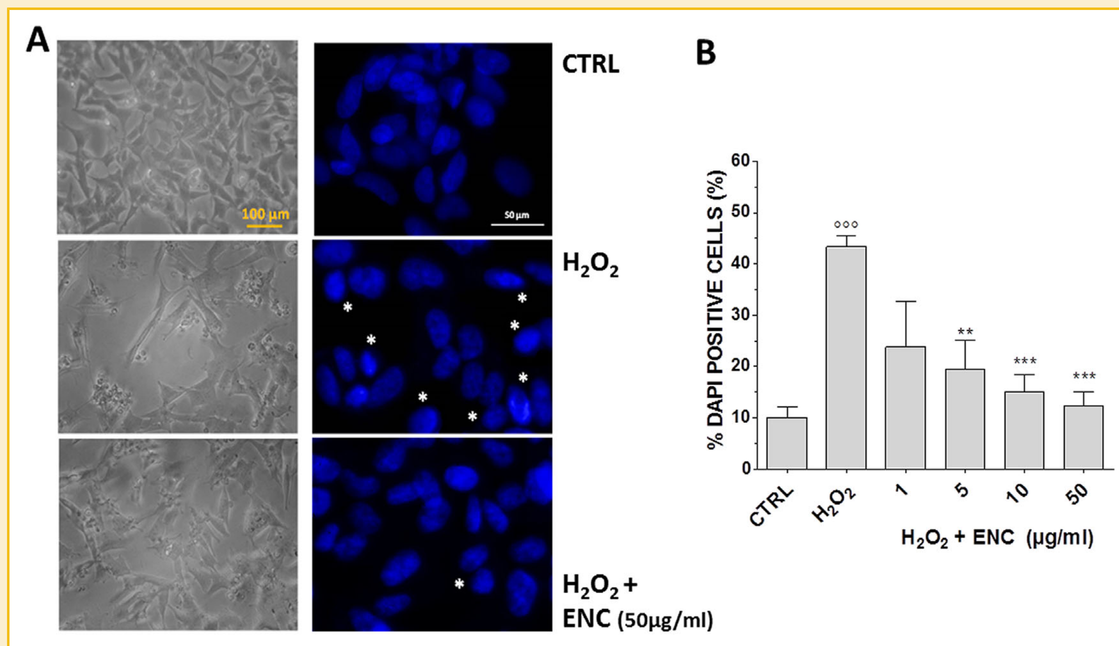

Fig. 7. Morphological comparison performed at contrast phase microscopy (scale bar 100 μm OM 100x) or by using fluorescence microscopy after DAPI staining (scale bar 50 μm, OM 400X) between SH-SY5Y untreated cells (CTRL), cells treated with H_2O_2 (25 μM for 1 h followed by 24 h with medium) or cells pretreated for 2 h with 50 μg/ml Natural Extract of Chestnut bark (ENC) before H_2O_2. Asterisks indicate cells with fragmented nuclei and condensed DNA and considered as apoptotic. Each photograph was representative of three independent observations. (Panel B). DAPI staining quantitative analysis shown as mean ± e.s.m. of three independent experiments. Statistical evaluation was performed by using ANOVA followed by Dunnet *post test*. °°°$P < 0.01$ vs CTRL; **$P < 0.01$, ***$P < 0.001$ vs H_2O_2.

to further elucidate the mechanisms underlying DNA repair due to ENC.

H_2O_2-induced apoptotic cell death depends on the concentration of H_2O_2 and on the exposure time to this agent [Chen et al., 2009]. Moderate concentrations of H_2O_2 induce DNA cleavage and morphologic changes leading to apoptosis [Gardner et al., 1997]. In the present study H_2O_2 caused a dose-dependent loss of SH-SY5Y cell viability. Pre-treatment with ENC significantly protected cells, suggesting that the extract prevented SH-SY5Y cells from undergoing H_2O_2-induced apoptotic cell death. Staining the apoptotic nuclei with DAPI, in fact, revealed that ENC attenuated also the induction of apoptotic features, such as cell shrinkage, nuclear condensation, and DNA fragmentation, compared to cells treated with H_2O_2 alone.

Interestingly, ENC concentrations exerting neuroprotective effects towards GLU and H_2O_2 damage (mostly at 10–50 μg/ml) were in the same range of those producing protective effects in rat cardiomyocites exposed to H_2O_2 [Chiarini et al., 2013]. Tannins, in fact, are able to inhibit lipid peroxidation and lipoxygenases in vitro and to scavenge free radicals such as hydroxyl, superoxide, and peroxyl [Gyamfi and Aniya, 2002]. While antioxidant effects of condensed tannins have been reported by several studies, there is little information on the antioxidant activity of water soluble tannins [Yoshida et al., 2010; Ito, 2011]. Hydrolysable tannins, for their high degree of hydroxylated aromatic functions, show high antioxidant activity [Koleckar et al., 2008]. Moreover, extracts from *Castanea Sativa* leaves have been shown to exert an antioxidant effect and to be useful in the prevention of photoaging and oxidative-stress-mediated skin diseases [Calliste et al., 2005; Almeida et al., 2008; Almeida et al., 2015]. The present results confirm antioxidant properties of ellagitannins and outlines their ability to prevent oxidative-mediate injury in human neuroblastoma cells. ENC, however, could elicit neuroprotection also by other mechanism (s). Recent findings, in fact, report that castalagin interferes with the Akt-dependent, Erk-dependent, JNK-dependent, and p38 MAPK-dependent pathways in osteoclasts [Iwatake et al., 2015]. All these pathways are impaired during oxidative stress which occurs in neurodegerative diseases, giving rise to cellular injury through apoptosis, and autophagy processes [Maiese et al., 2012]. Despite the possibility that castalagin could interfere with these closely integrated pathways also in the brain needs to be demonstrated, the possibility that a single compound could simultaneously interfere with different steps of the kinase cascade offers an exciting scenarios for the development of new treatment strategies.

Finally, the prospects to target the prevention and/or the onset of neurodegenerative disorders through ENC administration might also occur in vivo, as several studies demonstrated that oral administration of tannins produces brain CNS benefits [Nakajima et al., 2013; Farbood et al., 2015]. In addition, over the last decade, development of the so-called drug carriers such as liposomes- and nanoparticle-mediated drug delivery could represents one promising strategy to successfully increase the

CNS penetration of several therapeutic moieties, included nutraceuticals and natural compounds [Sahni et al., 2011].

In conclusion, taken together the present findings outline for the first time that ENC affords neuroprotection when administered before oxidative stress mediated-damage (preventive effect), while when added to the medium during the injury its curative properties were not observed. Greater understanding of how ENC as a whole, as well as its single components could affect the pathways which led to oxidative stress-mediated consequences in the CNS especially during aging is worth to be studied since it can foster novel and hopefully safe clinical treatment avenues for the prevention of CNS chronic disorders.

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