

Neuroprotective Effects of Naturally Occurring Polyphenols on Quinolinic Acid-Induced Excitotoxicity in Human Neurons

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Key Words

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ABSTRACT

Quinolinic acid (QUIN) excitotoxicity is mediated by elevated intracellular Ca²⁺ levels, and nitric oxide (NO•) mediated oxidative stress resulting in DNA damage, PARP activation, NAD⁺ depletion and cell death. We evaluated the effect of a series of polyphenolic compounds (i.e. epigallocatechin gallate (EPCG), catechin hydrate, curcumin, apigenin, naringenin and gallotannin) with antioxidant properties on QUIN induced excitotoxicity on primary cultures of human neurons. We showed that the polyphenols, EPCG, catechin hydrate and curcumin can attenuate QUIN-induced excitotoxicity to a greater extent than apigenin, naringenin and gallotannin. Both EPCG and curcumin were able to attenuate QUIN-induced Ca²⁺ influx and nNOS activity to a greater extent compared to apigenin, naringenin and gallotannin. While Ca²⁺ influx was not attenuated by catechin hydrate, nNOS activity was reduced most likely through direct inhibition of the enzyme. All polyphenols reduced the oxidative effects of increased NO• production thereby reducing the formation of 3-nitrotyrosine and PARP activity, and hence preventing NAD⁺ depletion and cell death. In addition to the well-known antioxidant properties of these natural phytochemicals, the inhibitory effect of some of these compounds on specific excitotoxic processes such as calcium influx provides additional evidence for the beneficial health effects of polyphenols in excitable tissue, particularly within the CNS.

INTRODUCTION

Quinolinic acid (QUIN) cytotoxicity is known to be involved in the pathogenesis of several CNS disorders, including Alzheimer's disease (AD) [1-3], Amyotrophic Lateral Sclerosis (ALS) [4], Huntington's disease [5], and the AIDS dementia complex (ADC) [6]. We have previously shown that the NMDA receptor can be activated by pathophysiological concentrations of QUIN in both human astrocytes and neurons, rendering these cells susceptible to injury via an excitotoxic process [7]. Excitotoxicity can occur through over-activation of the NMDA receptor with subsequent influx of Ca^{2+} , activation of both nNOS and iNOS, and excess generation of $NO\bullet$ [8].

$NO\bullet$ is a potent vasodilator and an important neurotransmitter, that is not considered toxic at physiological concentrations [9]. However, the $NO\bullet$ radical is largely unstable in the cellular system, and can react via complex pathways to yield tertiary reactive nitrogen species (RNS) such as the NO_2^- and the peroxynitrite free radical [10]. These molecules can cause DNA damage leading to activation of the nuclear DNA nick sensing enzyme poly(ADP-ribose) polymerase (PARP-1) [11]. Activated PARP-1 synthesises ADP-ribose (ADPR) polymers from NAD^+ [11]. Over activation of PARP-1 can lead to depletion of intracellular NAD^+ and ATP stores leading to a number of deleterious processes including mitochondrial permeability [12], overproduction of superoxide [12], and the release of cell death mediators, [11]. Therefore strategies directed at reducing QUIN induced $NO\bullet$ production and free radical damage may prove beneficial in treatments of neurodegenerative disease.

Extensive investigation have been undertaken to determine the neuroprotective effect of polyphenolic rich

beverages such as teas and red wine [13-16]. Several neuroprotective mechanisms of action have been proposed, including antioxidant and/or antiinflammatory properties [17]. Studies have shown that frequent consumption of fruit and vegetable juices, which are high in polyphenols, are associated with a substantially decreased risk of AD [18]. The "Kame Project" found that subjects who reported drinking juices three or more times per week were 76 per cent less likely to develop signs of AD than those who drank less than one serving per week. Even drinking juices once or twice a week was found to reduce the risk by 16 per cent [18]. Numerous studies have shown that green tea polyphenols can protect against excitotoxicity in neuronal cells although the exact mechanism remains unclear [19]. Tea consumption *ad libitum* by rodents was shown to afford neuroprotection against oxidative damage in normal aging [20], and through combination with the NMDA channel blocker memantine against brain excitotoxicity [21]. Some studies have shown that tea and wine derived catechins, in parallel with the individual flavonol quercetin can reduce the concentrations of increased reactive oxygen and reactive nitrogen species (ROS/RNS) [22-25] and intracellular calcium levels in the synapse [26]. Other studies have indicated a significant inhibitory effect of catechins and apigenin upon iNOS activity [27, 28]. However, no study to our knowledge, has reported the potential inhibitory effect of naturally occurring polyphenolic compounds on nNOS activity and intracellular calcium influx in human neurons following exposure to pathophysiological concentrations of QUIN.

In the present study, we evaluated the potential inhibitory effect of several polyphenolic compounds present in green

tea, namely epigallocatechin gallate (EPCG), catechin hydrate, curcumin, apigenin, naringenin, and gallotannin (Table 1) on QUIN-mediated elevations in nNOS activity in cultured human neurons using the citrulline assay. nNOS activity was verified by nitrite determination in culture supernatant using the fluorometric Griess diazotisation assay. Intracellular Ca^{2+} influx was measured using a fluorometric assay. The potential neuroprotective effects of these polyphenols on QUIN mediated NAD^+ depletion and PARP-1 activation was also investigated using well-established spectrophotometric assays. Immunohistochemistry was used to detect the formation of poly(ADP-ribose) (PAR) polymers. PAR formation is directly correlated to DNA strand breaks [11]. Our data show that EPCG, catechin hydrate and curcumin can significantly reduce QUIN-induced excitotoxicity compared to apigenin, naringenin and gallotannin. Likewise, the neuroprotective effects of these polyphenols on QUIN-mediated NAD^+ depletion, PARP-1 activation and DNA damage correlate with reduced nNOS activity and/or decreased intracellular Ca^{2+} influx.

MATERIALS AND METHODS

Reagents and Chemicals

Dulbecco's phosphate buffer solution (DBPS), Fura-2-AM fluorophore, and all other cell culture media and supplements were from Invitrogen (Melbourne, Australia) unless otherwise stated. Nicotinamide, bicine, β -nicotinamide adenine dinucleotide reduced form (β -NADH), 3-[-4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), HEPES, D-glucose, alcohol dehydrogenase (ADH), sodium pyruvate, TRIS, γ -globulins, quinolinic acid (QUIN),

DAPI, epigallocatechin gallate, catechin hydrate, curcumin, apigenin, naringenin, and gallotannin were obtained from Sigma-Aldrich (Castle-Hill, Australia). Phenazine methosulfate (PMS) was obtained from ICN Biochemicals (Ohio, USA). Bradford reagent was obtained from BioRad, Hercules (CA, USA). Rabbit anti-MAP2 was obtained from Millipore (Melbourne, Australia). Mouse anti-poly(ADP-ribose) (10H) was obtained from Alexis Corporation (Pastlach, Switzerland). Mouse anti-3-nitrotyrosine (3-NT), secondary anti-mouse IgG and anti-rabbit Alexa 488 (green) or Alexa 594 (red)-conjugated antibodies were obtained from Molecular Probes (Eugene, OR). All commercial antibodies were used at the same concentrations specified by the manufacturer.

Cell Cultures

Human foetal brains were obtained from 16-19 week old fetuses collected following therapeutic termination with informed consent. Mixed brain cultures were prepared and maintained using a protocol previously described by Guillemain et al. (2005) [2]. Neurons were prepared from the same mixed brain cell cultures as previously described [29]. Briefly, cells were plated in 24-well culture plates coated with Matrigel (1/20 in Neurobasal) and maintained in Neurobasal medium supplemented with 1% B-27 supplement, 1% Glutamax, 1% antibiotic/antifungal, 0.5% HEPES buffer, and 0.5% glucose. Cells were maintained at 37°C in a humidified atmosphere containing 95% air/5% CO_2 .

Measurement of Neuronal Nitric Oxide Synthase Activity Using the Citrulline Assay

Neuronal nitric oxide synthase activity was assayed by monitoring the conversion of L-[³H]arginine to L-[³H]citrulline as previously described [30]. Cells were treated with 50-1200 nM QUIN for 30 minutes. After incubation, the reaction was terminated by adding 0.3 M HClO₄ (pH. 5.5) containing EDTA (4mM). Radiolabelled citrulline is neutral at a pH of 5.5, and is separated from the positively charged arginine using a column containing analytical grade cation-exchange resin (AG Dowex 50W-X8). L-[³H]citrulline amount was measured using a Beckman LS6500 scintillation counter. Results were expressed as ng L-citrulline/500 µg protein/30 minutes. In another set of experiments, neuronal cells were pre-incubated for 15 minutes with 1-100 µM EPCG, catechin hydrate, curcumin, apigenin, naringenin, and gallotannin respectively. Afterwards, nNOS activity in the presence of 550 nM QUIN was quantified as described above.

Nitrite Determination by Fluorometric Griess Diazotisation Assay

Nitrite production in culture supernatant was measured using the fluorometric Griess diazotisation assay as previously described. In the Griess assay, NO₂ is allowed to react with an aromatic amine in acidic medium to yield a fluorescent azo derivative. Briefly, neurons were treated with 50-1200 nM QUIN for 30 minutes and 100 µl of culture supernatant was placed in a 96-well microplate. Diaminonaphthalene (DAN) was diluted to 10 mM in deionised water from the original 100 mM DMSO stock solution,

and 1% HCl was added to the aqueous mixture to generate a working stock of DAN. Afterwards, 100 µl of DAN was added to each sample and incubated for 10 minutes at room temperature. An additional 100 µl of 2M NaOH was added and the fluorescence intensity was then recorded at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. In another set of experiments, neuronal cells were pre-incubated for 15 minutes with 1-100 µM EPCG, catechin hydrate, curcumin, apigenin, naringenin, and gallotannin respectively. Afterwards, the amount of nitrite produced in the presence of 550 nM QUIN was quantified as described above.

Calcium Influx Studies Using Fluorometry

To measure intracellular Ca²⁺, human neurons were loaded (~1 hour, room temperature) with 3.5 µg/ml Fura-2-AM in loading solution containing (in mM): 135 NaCl, 5KCl, 1 MgCl₂, 1 CaCl₂, 5 glucose, and 10 HEPES (pH 7.4). Probenicid dissolved in 1M NaOH was added to the loading solution at a final concentration of 4 mM to reduce dye leakage. Following the recommended 1 hr incubation period, the loading solution was removed and replaced with HBSS containing 50 mM glycine. Addition of selected polyphenols (EPCG, catechin hydrate, curcumin, apigenin, naringenin, and gallotannin) was undertaken 15 minutes prior to the addition of QUIN to ensure adequate diffusion time was provided to attain equilibrium. The calcium influx experiments were subsequently performed using Fluostar Optima Fluorometer (NY, USA). Filter excitation and emission was set at 485 nm and 520 wavelengths respectively. For each well, fluorescence was measured via orbital scanning of 10 locations at a 3 mm radius every 0.5

seconds, and the average of these readings was recorded. Baseline fluorescence was measured during the first 10 seconds of the experiment, followed by injection of QUIN (in HBSS). Fluorescent readings were subsequently taken for an additional 90 seconds. Negative controls included injection of only HBSS solution without an agonist.

NAD(H) Microcycling Assay for the Measurement of Intracellular NAD⁺ concentrations

Intracellular NAD⁺ concentration was measured spectrophotometrically using the thiazolyl blue microcycling assay established by Bernofsky and Swan [31] adapted for 96 well plate format by Grant and Kapoor [32]. Human neurons were pre-incubated for 15 minutes with 30 and 50 μ M EPCG, catechin hydrate, curcumin, apigenin, naringenin, and gallotannin respectively. Afterwards cells were treated with QUIN (550 nM) and intracellular NAD⁺ levels were measured 24 hours later.

Extracellular LDH Activity as a Measurement for Cytotoxicity

The release of lactate dehydrogenase (LDH) into culture supernatant correlates with the amount of cell death and membrane damage, providing an accurate measure of cellular toxicity. LDH activity was assayed using a standard spectrophotometric technique described by Koh and Choi [33]. After neuronal cells pre-incubation with 1-100 μ M EPCG, catechin hydrate, curcumin, apigenin, naringenin, and gallotannin respectively, neuronal cells were treated with QUIN (550 nM) and extracellular LDH activity was assessed in culture supernatant after 24 hours.

PARP Assay for the Measurement of Intracellular PARP Activity

PARP activity was measured using a new operational protocol relying on the chemical quantification of NAD⁺ modified from Putt et al, [34] and adapted for 24 well format by Braidy et al. [7]. After a 15-minute pre-incubation with the selected polyphenolic compounds, neurons were then treated with QUIN (550 nM) and incubated for 15 minutes. DPBS solution was then aspirated and PARP lysing buffer (200 μ l) was added to the cell plate. The buffer solution contained MgCl₂ (10 mM), Triton X-100 (1%), and NAD⁺ (20 μ M) in Tris buffer (50mM, pH 8.1). The plate was then incubated for 1 hour and PARP activity was assayed as previously described (Braidly et al. submitted) [7].

Bradford Protein Assay for the Quantification of Total Protein

NAD⁺ concentration, PARP and extracellular LDH activities were adjusted for variations in cell number using the Bradford protein assay described by Bradford [35].

Immunocytochemistry for the Detection of Poly(ADP-ribose) and 3-Nitrotyrosine Formations

The method for immunocytochemistry has been previously described (Guillemin et al. 2005b). Cells were incubated with mAb Poly(ADP-ribose) (PAR) and mAb 3-Nitrotyrosinase (3-NT) together with the phenotypic marker (MAP-2). Selected secondary antibodies (goat anti-mouse IgG or goat anti-rabbit coupled with Alexa 488 or Alexa 594) were used. The following controls were performed for each labelled experiment: (1) isotypic antibody controls; and (2) incubation with only the secondary labelled antibody. Cell counting was performed in a blinded manner. The whole

controls and untreated chamber slides were counted. Enumeration of each slide was classified according to the following scheme: DAPI staining for total cell number, MAP-2 immunoreactivity for neurons, and 3-NT and PAR for 3-nitrotyrosine and poly(ADP-ribose) staining respectively.

Data Analysis

Results obtained are presented as the means \pm the standard error of measurement (SEM). One way analysis of variance (ANOVA) and post hoc Tukey's multiple comparison tests were used to determine statistical significance between treatment groups. Differences between treatment groups were considered significant if p was less than 0.05 ($p < 0.05$).

RESULTS

Effect of EPCG, Catechin Hydrate, Curcumin, Apigenin, Naringenin, and Gallotannin on QUIN-Induced nNOS Activity and Extracellular Nitrite Production in Human Neurons

We investigated the effect of QUIN on nNOS activity in cultured human neurons. Primary human neurons were treated with QUIN for 30 minutes at increasing concentrations. A dose dependent increase in nNOS activity was observed with increased concentrations of QUIN (Fig 1A). As expected the increase in nNOS activity correlated well with increasing release of nitrite into the extracellular medium (Fig 1B).

To determine if polyphenols can influence QUIN-induced nNOS activity due to QUIN in human neurons, we tested the effect of selected polyphenolic compounds on nNOS activity in cultures pre-treated with selected polyphenols for 15 minutes. All polyphenols tested

produced a dose-dependent decrease in nNOS activity in human neurons, with EPCG, catechin hydrate, and curcumin showing higher potency than apigenin, naringenin and gallotannin. These results correlate well with reduced extracellular nitrite release from those same neuronal cell cultures (Fig 1D).

Effect of EPCG, Catechin Hydrate, Curcumin, Apigenin, Naringenin, and Gallotannin on Intracellular NAD⁺ levels, Extracellular LDH and PARP Activation in Human Neurons

To determine the effect of polyphenols on intracellular NAD⁺ levels, endogenous PARP activation and cell viability, we measured intracellular NAD⁺ levels, PARP and extracellular LDH activities in human neurons after 24-hour treatment. Treatment with EPCG and curcumin significantly increased intracellular NAD⁺ levels in a dose dependent manner (Fig 2A), but no significant difference was observed for PARP (Fig 2B) and LDH activities (Fig 2C). On the other hand, Gallotannin induced a dose-dependent decrease in intracellular NAD⁺ levels (Fig 2A), and a dose-dependent increase in extracellular LDH activity (Fig 2C). No significant difference was observed for PARP activity (Fig 2B). Similarly, no significant differences were observed in intracellular NAD⁺ levels (Fig 2A), PARP (Fig 2B) and extracellular LDH activities (Fig 2C) for apigenin and naringenin.

Effect of EPCG, Catechin Hydrate, Curcumin, Apigenin, Naringenin, and Gallotannin on QUIN-mediated NAD⁺ depletion, Extracellular LDH and PARP Activation in Human Neurons

To assess the effects of polyphenols on QUIN-mediated NAD⁺ depletion, PARP activation and extracellular LDH release (cell death), we measured intracellular

NAD⁺ levels, PARP and extracellular LDH activities in human neurons after 24-hours of treatment. Addition of EPCG, catechin hydrate and curcumin (50 μM) significantly attenuated QUIN-mediated NAD⁺ depletion after 24 hours (Fig 3A). Apigenin, naringenin and gallotannin also prevented NAD⁺ depletion at the same concentrations (50 μM), but to a lesser extent (Fig 3A). As previously shown, neurons treated with QUIN at 550 nM for 1 hour had a PARP activity significantly increased compared to the control (Fig 3B). Concomitant treatment of these cells with EPCG, catechin hydrate and curcumin (50 μM) significantly reduced PARP activity compared to QUIN treatment alone. Treatment with apigenin and naringenin and gallotannin (50 μM) also reduced PARP activity, but to a significantly lower degree than EPCG, catechin hydrate or curcumin (Fig 3B). These results closely correlate with results presented for NAD⁺ (Fig 3A). Neurons treated with QUIN (550 nM) in the presence of selected polyphenols (50 μM) showed significantly reduced evidence of cell death as measured by extracellular LDH activity in culture supernatants after 24 hours (Fig 3C). Extracellular LDH activity was significantly reduced in the presence of either, EPCG, catechin hydrate and curcumin compared to apigenin, naringenin and gallotannin (Fig 3C). These results again directly correlate with data shown for NAD⁺ depletion and PARP activity (Fig 3A and 3B).

QUIN Induces Intracellular Ca²⁺ Levels in Cultured Human Neurons

Human foetal neurons were incubated with QUIN and a significant dose dependent increase in intracellular Ca²⁺ influx was observed (Fig 4). As, reactive nitrogen species were increased with increasing concentrations of QUIN (Fig 1), it is

reasonable to conclude that the formation of NO• is a downstream event in the QUIN-induced excitotoxic cascade mediated by Ca⁺⁺ influx.

Effect of EPCG, Catechin Hydrate, Curcumin, Apigenin, Naringenin, and Gallotannin on QUIN-Induced [Ca²⁺]_i in Cultured Human Neurons

As mentioned above, QUIN stimulation induced a significant increase in intracellular Ca²⁺. Each of the polyphenols, EPCG, curcumin, apigenin, naringenin, and gallotannin, significantly reduced intracellular Ca²⁺ influx (Fig 4). Attenuation of increased Ca²⁺ influx was greatest with EPCG and curcumin compared to apigenin and naringenin respectively (Fig 5). Interestingly, catechin hydrate did not ameliorate QUIN-induced increase in intracellular Ca²⁺ (Fig 5).

Detection of 3-Nitrotyrosine Formation in Cultured Human Neurons

Immunocytochemistry was used to visualise protein nitration due to increased NO• production in cultured human neurons. Increased protein nitration in the form of increased 3-NT was observed in 20% of QUIN-treated cells compared to non-treated cells (Fig 6A and 6B). Likewise, staining for 3-NT was less detectable in QUIN treated neurons pre-incubated with EPCG (0%), catechin hydrate (0%) and curcumin (0%) compared to cells treated with apigenin (7%), naringenin (9%) and gallotannin (12%) (Fig 6A and 6B).

Detection of Poly(ADP-ribose) Expression in Cultured Human Neurons

Immunocytochemistry studies were used to detect poly(ADP-ribose) (PAR) formation following treatment with QUIN and selected polyphenols. The amount of PAR formed in living cells gives direct

indication of the extent of DNA damage. Higher immunoreactivity for PAR staining (25%) was detected in human neurons in the presence of QUIN (550 nM) compared to untreated cultures, and cells co-treated with 50 μ M of EPCG (4%), Catechin hydrate (5%), Curcumin (4%), Apigenin (10%), Naringenin (11%), and Gallotannin (12%) for 1 hour respectively (Fig 7A and 7B). The presence of EPCG, catechin hydrate and curcumin in QUIN exposed neurons resulted in the lowest PAR formation compared to cells treated with the other polyphenols (Fig 7A and 7B). This indicates that the latter compounds exhibit a poorer neuroprotective effect against DNA damage compared to EPCG, catechin hydrate and curcumin.

DISCUSSION

The excitotoxin QUIN is one of the major end products of tryptophan catabolism in the central nervous system (CNS). Increased QUIN production by activated microglia / infiltrating macrophages has been reported in the brain in aging, and in neuroinflammatory diseases [1]. For example, QUIN is found at high concentrations in immuno-active amyloid plaques in the AD brain [1, 2, 29]. Given the complex aetiology and mechanisms of AD, QUIN is likely to play a pivotal role in the neurodegenerative changes occurring in the brain [1, 29, 36, 37].

We and others have previously demonstrated the involvement of NOS in QUIN toxicity on human astrocytes and neurons [7, 38, 39]. This neurotoxic involvement of NOS has been confirmed by the use of the NOS inhibitor, L-NAME that can protect human primary neurons and astrocytes *in vitro* against QUIN toxicity [7, 40]. NOS inhibitors have also been found effective to protect mice and

monkey models from the development of AD pathophysiology [41].

Another way to attenuate increased NO• production and consequent energy depletion due to QUIN is to block the NMDA receptor. We have previously shown that the NMDA ion channel blocker MK-801 can protect human neurons from QUIN-induced excitotoxicity [7]. However, long-term NMDA receptor inhibition by MK-801 as been previously shown to be toxic to cultures of rat cortical neurons [42]. Alternatively, polyphenols with their ROS/RNS scavenging, metal chelating and anti-inflammatory properties represent a promising additional option for the modulation of excitotoxic cell death that may potentially be effective in conditions such as AD treatment. The neuroprotective effects of green tea polyphenols and their potential in the treatment of AD have been extensively reviewed [43-45].

In this study, we evaluated the effects of several polyphenolic compounds on QUIN-mediated elevations in nNOS activity and nitrite production. The activity of nNOS was determined by the stoichiometric conversion of L-[³H]arginine to L-[³H]citrulline [30]. The activity of nNOS was considerably enhanced in a dose dependent manner with increasing concentrations of QUIN within 30 minutes with a subsequent increase in nitrite production (Fig. 1). These results are consistent with previous reports showing increased NO• production in the striatum within 2 hours following QUIN injection [38] [46].

Conversely, a dose dependent decrease in nNOS activity and nitrite production was observed in QUIN-treated neuronal cells pre-incubated with selected polyphenolic compounds (Fig 1). EPCG, catechin hydrate and curcumin showed greater inhibitory effect on nNOS activity

and subsequent nitrite production compared to apigenin, naringenin, and gallotannin (Fig. 1). The modulatory effect of polyphenolic compounds on the nitric oxide synthase family has been previously reviewed in Youdim et al. (2002) [45]. EPCG, catechin hydrate, and curcumin can suppress NO• production in cultures of RAW 264.7 macrophages and human peripheral blood mononuclear cells following a 24 hour stimulation with lipopolysaccharide (LPS) [47]. Moreover, apigenin has been shown to down-regulate iNOS expression and NO• production in RAW 264.7 macrophages [48]. Taken together, these results suggest that polyphenols can inhibit NO• production by significantly reducing iNOS expression and activity. However, this present study is the first to examine the inhibitory effects of polyphenolic compounds on nNOS activity in primary cultures of human neurons. Consistent with the above results, EPCG, catechin hydrate, and curcumin showed a significant reduction in 3-nitrotyrosine (3-NT) formation compared to QUIN-treated cells alone (Fig 6). Apigenin, naringenin and gallotannin also exerted a protective effect against 3-NT formation but to a lesser extent than the other polyphenols (Fig 6).

We have previously shown that QUIN can induce PARP-1 activity and subsequent NAD⁺ depletion in primary cultures of human astrocytes and neurons at pathophysiological concentrations [7]. In that earlier study, NOS inhibition using L-NAME significantly reduced NAD⁺ depletion and PARP-1 activation in cultured human neurons exposed to cytotoxic concentrations of QUIN [7]. The present study showed that the polyphenols, EPCG, catechin hydrate and curcumin, which have a greater inhibitory effect on nNOS activity and nitrite production, can prevent DNA damage (indicated by

reduced PAR formation (Fig 7) and PARP-1 activation (Fig 3)) and block the subsequent depletion of NAD⁺ stores, thereby preserving the cells energy dependent functions (Fig 3). Apigenin, naringenin and gallotannin also showed a neuroprotective effect against PARP-1 activation and NAD⁺ depletion, but to a lesser extent than the previously mentioned polyphenols most likely due to their lower inhibitory effect on nNOS activity (Fig 3).

While treatment with catechin hydrate, apigenin and naringenin alone showed no significant difference in intracellular NAD⁺ levels, and PARP and LDH activities across the range of concentrations tested, increased intracellular NAD⁺ levels were observed following treatment with EPCG and curcumin alone (Fig 2). This is consistent with the observation that PARP activity (and therefore NAD⁺ turnover) was also lowest following treatment with both EPCG and curcumin at 50 and 100 μM (Fig 2B). On the other hand, gallotannin showed a dose dependent decrease in intracellular NAD⁺ levels (Fig 2A), with a corresponding decrease in cell viability (Fig 2C). This may be explained by the observation by others that Gallotannin strongly inhibits NMNAT-1 activity, with no detectable activity observed at 100 μM [49].

The results of the present study show that QUIN can induce intracellular Ca²⁺ influx in a dose-dependent manner (Fig 4), and that this reduces the viability of cultured human neurons. To determine whether the neuroprotective effect of these polyphenols was due to a direct nNOS inhibition or via intracellular Ca²⁺ modulation, we examined the effect of these polyphenols on intracellular Ca²⁺ influx in human neurons following QUIN stimulation. We found that EPCG and

curcumin were able to attenuate QUIN-induced Ca^{2+} influx to a greater extent than apigenin, naringenin and gallotannin (Fig 5). Catechin hydrate however did not attenuate the observed increase in Ca^{2+} in QUIN-treated neuronal cultures (Fig 5). EPCG has been previously shown to attenuate glutamate-induced cytotoxicity via intracellular ionotropic Ca^{2+} modulation in PC12 cells, although the exact mechanism remains unclear [50]. Curcumin has been shown to exert a potent antioxidant effect on $\text{NO}\bullet$ related radical generation [51]. Curcumin has also been shown to antagonise several important pathways involved in NOS mediated neurotoxicity, including activation of nuclear factor kappa B (NF- κ B), Jun N-terminal Kinase (JNK) pathway, and protein kinase C (PKC) [26, 52, 53]. PKC partly phosphorylates the core NMDA receptor subunit NR1, which potentiates increased Ca^{2+} influx following NMDA receptor activation [26]. A decreased phosphorylation of NR1 may protect against QUIN-induced excitotoxicity when the levels of QUIN are significantly elevated. We found that catechin hydrate did not reduce QUIN induced Ca^{2+} influx in human neurons. This is consistent with another study, where catechin hydrate only slightly inhibited the phosphorylation of PKC [26]. However, catechin hydrate significantly reduced QUIN-induced nNOS activity and $\text{NO}\bullet$ production. It is possible that inhibition of nNOS activity by catechin hydrate may be mediated through a direct action on the enzyme itself. For example, nitrite and peroxynitrite inhibition by catechins has been attributed to the 3'4'-catechol group on the B-ring [26].

Apigenin and naringenin are known to protect against excitotoxic insults in human neurons independent of NOS activity. Silva et al. (2008) showed

that the apigenin derivative biapigenin prevented kainate excitotoxicity by protecting cultured neurons from delayed calcium deregulation due to excessive NMDA receptor activation [54]. Further studies have focussed on the binding of naringenin to GABA_A receptors as a potential neuroprotective mechanism of action in the CNS [55, 56].

Our results show that gallotannin is less active against nNOS activity and demonstrated poor nitrite scavenging properties (Fig 1). However, gallotannin was able to attenuate QUIN-induced Ca^{2+} influx in human primary neurons to a similar extent as apigenin. Other studies have shown that gallotannin can significantly reduce Ca^{2+} influx only when administered simultaneously with glutamate [26]. This suggests a possible competitive inhibitory process.

Importantly the concentrations used in these experiments are within the achievable range of serum levels following oral consumption of these polyphenols. For example, one human study reported that the serum concentration of curcumin was $1.77 \pm 1.87 \mu\text{M}$ [57]. In another rat study, daily oral consumption of a glyconated form of catechin resulted in a serum concentration of $34.8 \pm 6.0 \mu\text{M}$ [58]. The amount of EPCG in a single cup of green tea is approximately $300 \mu\text{M}$ [59]. Therefore, the calculated maximum serum concentration of EGCG may reach $60 \mu\text{M}$ in a 60 kg human after oral consumption of a single cup of tea. In the present study, the polyphenols were tested at a standardised concentration of $50 \mu\text{M}$. While this concentration is relevant to serum levels in humans it is relevant to notes that lower concentrations of these polyphenols may also be neuroprotective if administered over a longer period of time.

Several epidemiological studies have predicted neurodegenerative diseases

to be a major public health problem in the twenty-first century [60]. In Australia, it has been projected that while the total ageing population will increase by 40% in 2042, the population with Alzheimer's disease will increase by three and a half times due to ageing population demographics [61]. The neuroprotective effects of these green tea polyphenols were obtained in an experimental pre-treatment model. The efficacy of these polyphenols *in-vivo* is dependent on the ability of these polyphenols to cross the blood brain barrier (BBB). Curcumin, EPCG and catechin have been reported to pass through the BBB [62, 63]. The permeability of apigenin, naringenin, and gallotannin remains unknown.

In a recent meta-analysis of 187 retrospective studies, EPCG, curcumin, catechin hydrate, melatonin, resveratrol, vitamin C and vitamin E were identified as naturally occurring compounds that show efficiency in slowing down the spectre of AD symptoms [64]. Results from our study and others add support to this observation and may encourage individuals to select foods that contain these beneficial compounds (e.g. red grapes, blue berries, peanuts, etc.). This will be important to improve population health in general, and in ageing populations in particular.

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Table 1. Structure of selected green tea polyphenols used in this study.

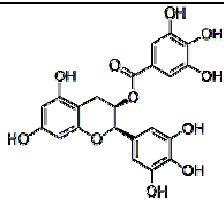
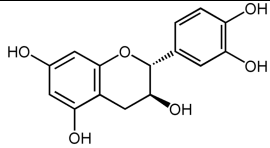
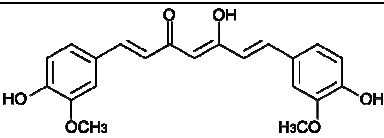
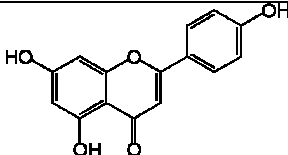
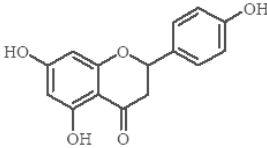
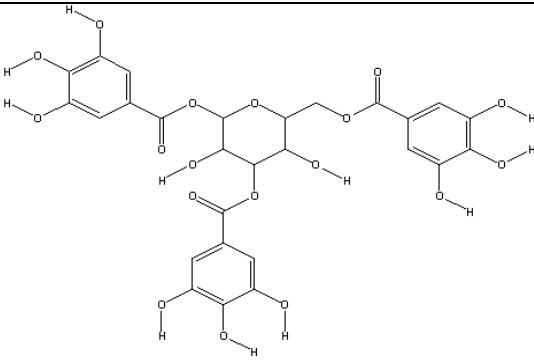
Polyphenol	Chemical Structure
EPCG	
Catechin Hydrate	
Curcumin	
Apigenin	
Naringenin	
Gallotannin	

Figure 1. Effect of polyphenols on QUIN induced nNOS activity and nitrite production in human neurons. Effect of : (A) QUIN on nNOS activity for 30 minutes. *p<0.05 compared to previous dose (B) QUIN on extracellular nitrite production. *p<0.05 compared to previous dose (C) EPCG, Catechin Hydrate, Curcumin, Apigenin, Naringenin, and Gallotannin on nNOS activity in the presence of QUIN (550 nM) for 30 minutes. *p<0.05 compared to 550 nM QUIN alone; (D) EPCG, Catechin Hydrate, Curcumin, Apigenin, Naringenin, and Gallotannin on extracellular nitrite production in the presence of QUIN (550 nM). *p<0.05 compared to 550 nM QUIN alone; (n=4 for each treatment group).

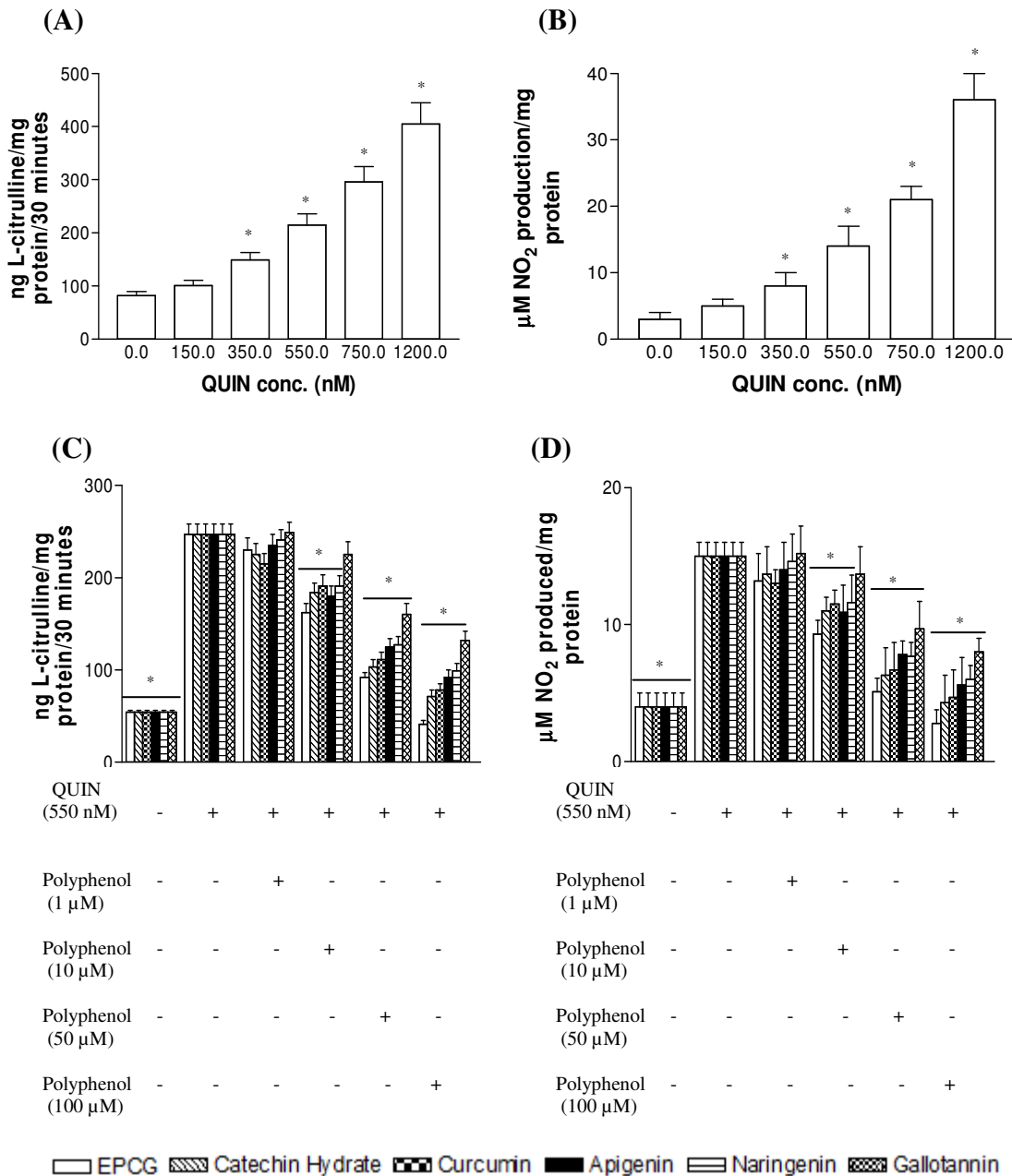


Figure 2. Effect of polyphenols on intracellular NAD⁺ levels, PARP activation and cell death in human neurons. Effect of: (A) EPCG, Catechin Hydrate, Curcumin, Apigenin, Naringenin, and Gallotannin on intracellular NAD⁺ levels for 24 hours. *p<0.05 compared to medium alone; (B) EPCG, Catechin Hydrate, Curcumin, Apigenin, Naringenin, and Gallotannin on PARP activity for 1 hour. *p<0.05 compared to medium alone; (C) EPCG, Catechin Hydrate, Curcumin, Apigenin, Naringenin, and Gallotannin on extracellular LDH activity. *p<0.05 compared to medium alone; (n=3 for each treatment group).

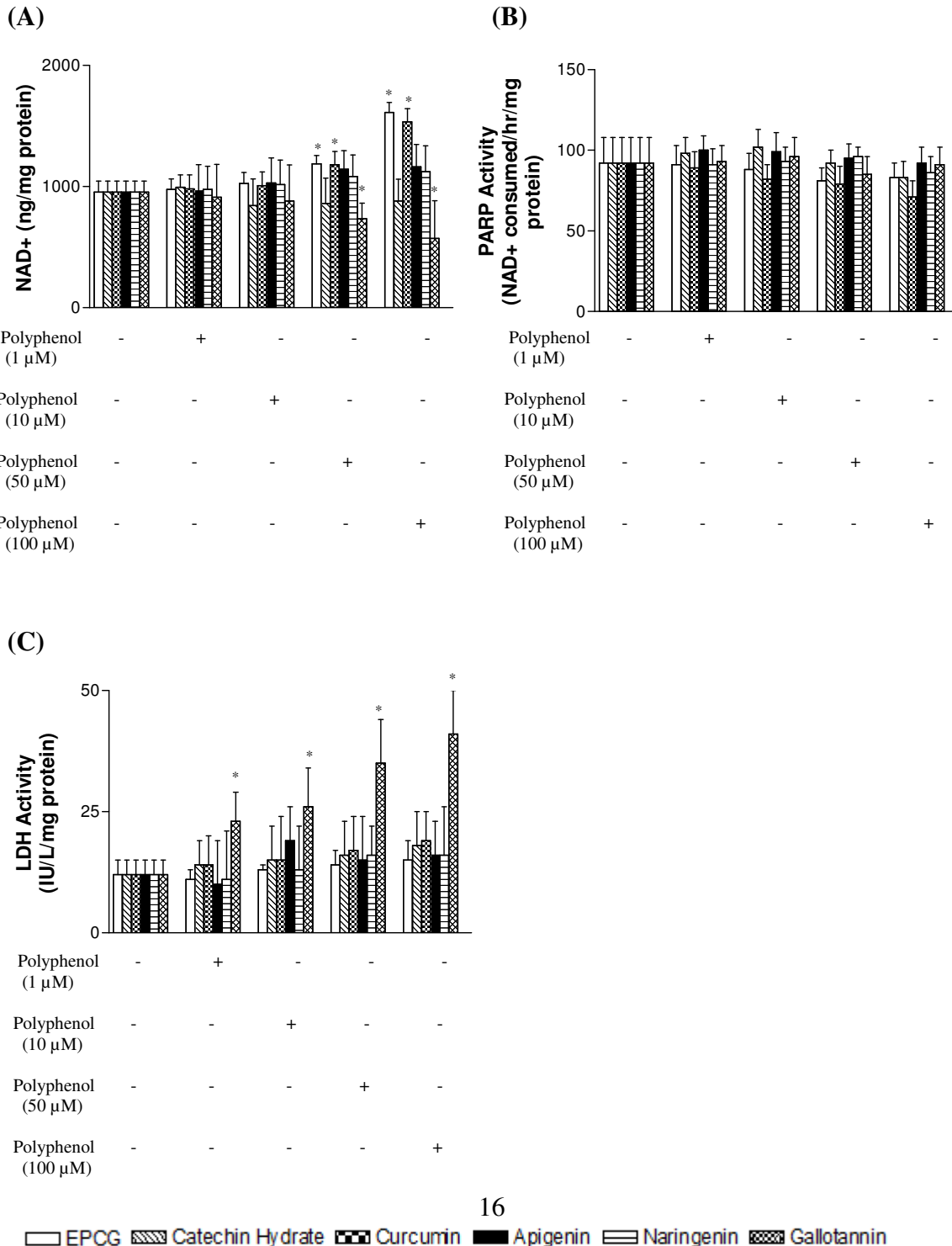
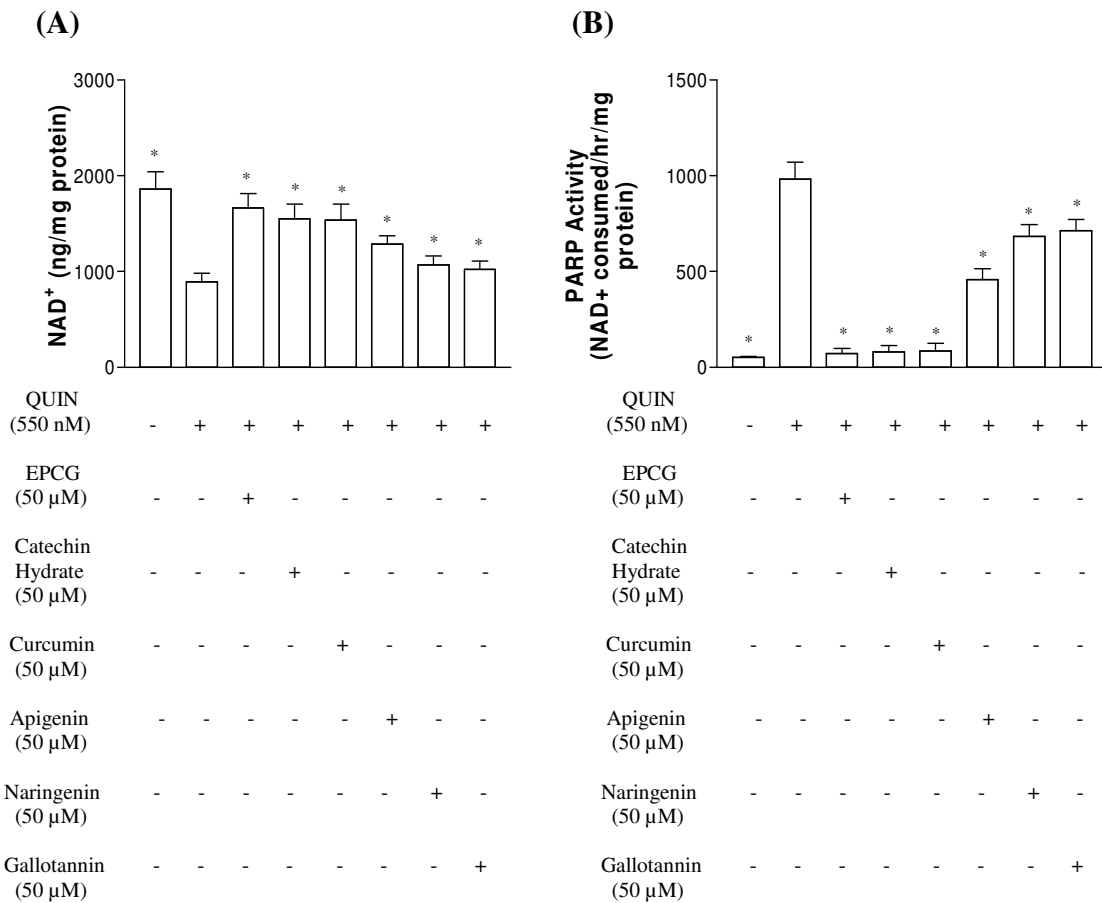


Figure 3. Effect of polyphenols on QUIN induced NAD depletion, PARP activation and cell death in human neurons. Effect of: (A) EPCG (50 μ M), Catechin Hydrate (50 μ M), Curcumin (50 μ M), Apigenin (50 μ M), Naringenin (50 μ M), and Gallotannin (50 μ M) on intracellular NAD⁺ levels in the presence of QUIN (550 nM) for 24 hours. *p<0.05 compared to 550 nM QUIN alone; (B) EPCG (50 μ M), Catechin Hydrate (50 μ M), Curcumin (50 μ M), Apigenin (50 μ M), Naringenin (50 μ M), and Gallotannin (50 μ M) on PARP activity in the presence of QUIN (550 nM) for 1 hour. *p<0.05 compared to 550 nM QUIN alone; (C) EPCG (50 μ M), Catechin Hydrate (50 μ M), Curcumin (50 μ M), Apigenin (50 μ M), Naringenin (50 μ M), and Gallotannin (50 μ M) on extracellular LDH activity in the presence of QUIN (550 nM). *p<0.05 compared to 550 nM QUIN alone; (n=4 for each treatment group).



(C)

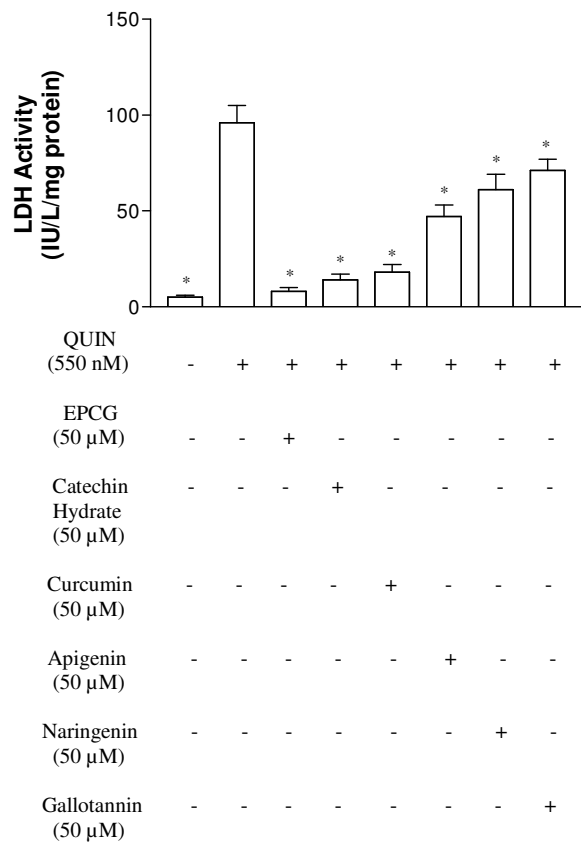


Figure 4. QUIN induces Ca²⁺ influx in human neurons.

(A) Representative trace of [Ca²⁺]_i induced by QUIN (150 nM, 550nM and 1200 nM).

(B) Quantified amplitude of neuronal response to QUIN at the aforementioned concentrations. * p<0.05 compared to no QUIN; (n=4 for each treatment group).

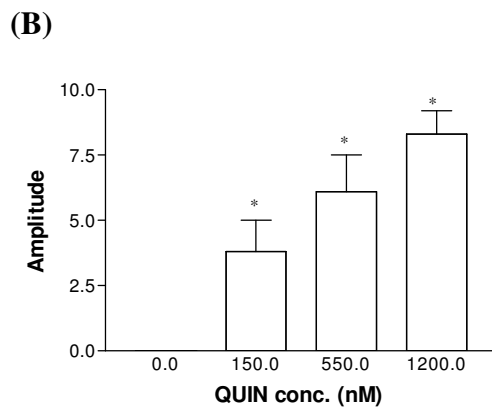
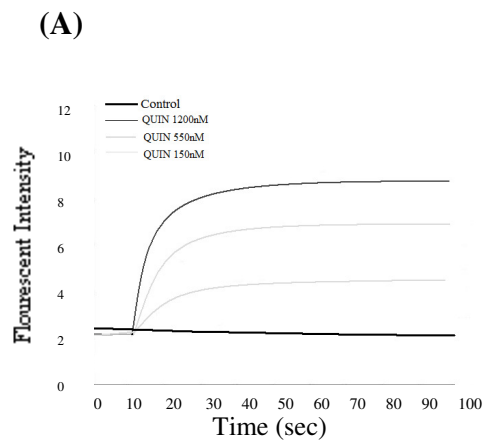
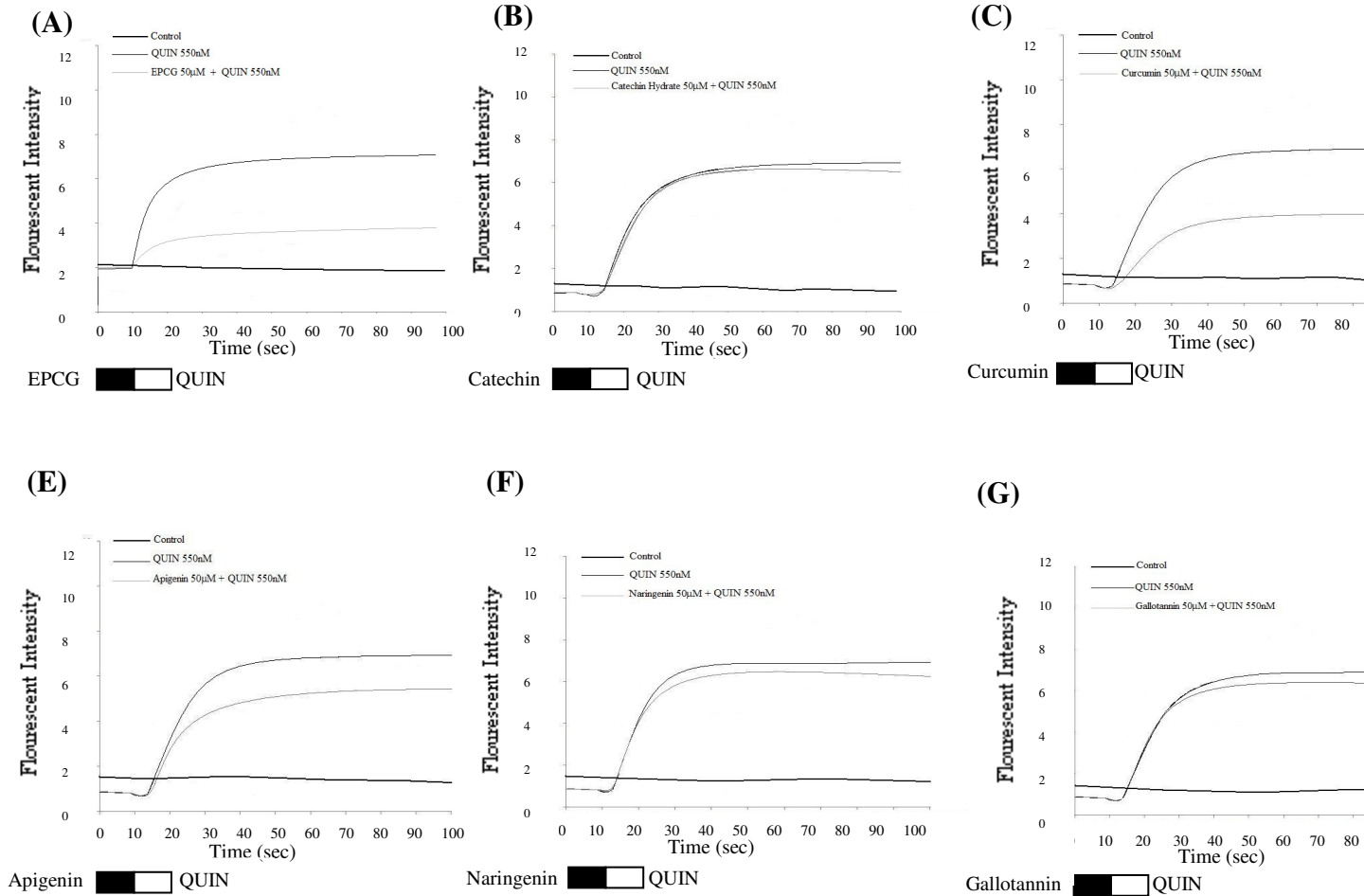


Figure 5. Effect of polyphenols on QUIN induced Ca²⁺ influx in human neurons. Representative trace of [Ca²⁺]_i induced by 550 nM QUIN in the presence of (A) EPCG, (B) Catechin Hydrate, (C) Curcumin, (D) Apigenin, (E) Naringenin, (F) Gallotannin. (G) Quantified amplitude of neuronal response to QUIN and EPCG, Catechin Hydrate, Curcumin, Apigenin, Naringenin, and Gallotannin. The polyphenols were washed out during QUIN administration, since the polyphenols may influence its fluorescence. *p<0.05 compared to 550 nM QUIN; (n=4 for each treatment group).



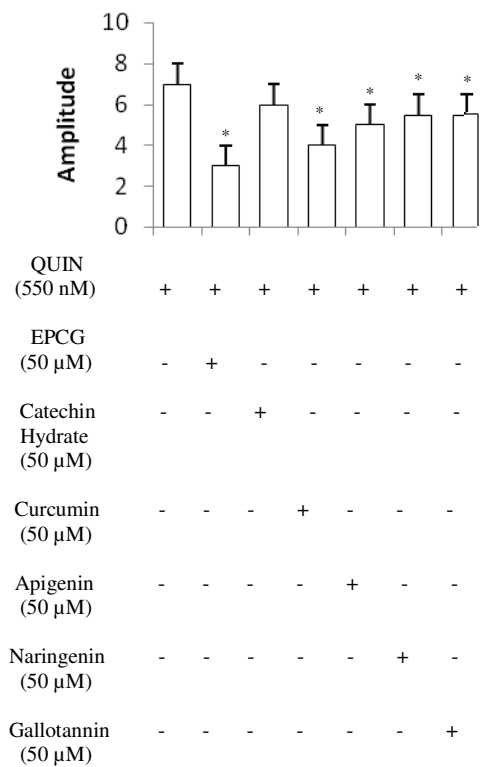
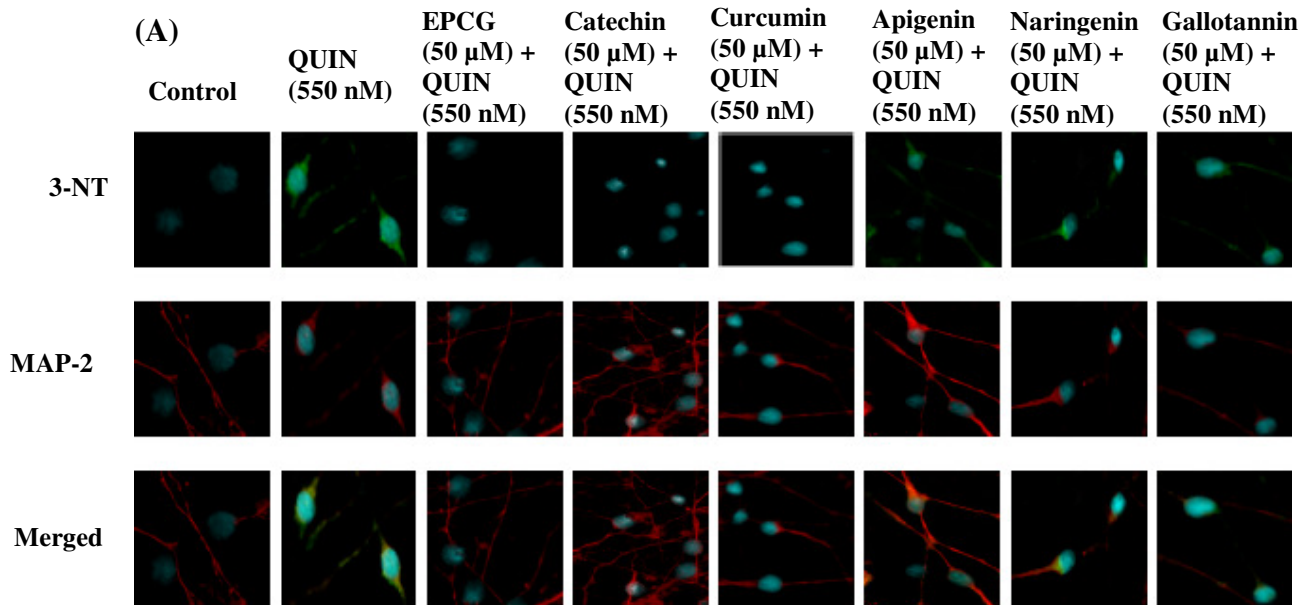


Figure 6. Immunocytochemical detection of 3-NT in purified primary human neurons after QUIN (550 nM) stimulation.

Staining for 3-NT in human neurons: Top row, double staining for 3-NT/green and DAPI/blue; Centre, double staining for MAP-2/red and DAPI/blue; Bottom row, Merged 3-NT/green, MAP-2/red and DAPI/blue. **(B)** Numeration of fluorescence intensity of 3-NT in human neurons using immunocytochemistry. Histogram shows the percentage of human neurons expressing 3-NT relative to the total number of neuronal cells after 24 hours of treatment. * $p < 0.05$ compared to 550 nM QUIN alone; (n=4 for each treatment group).



(B)

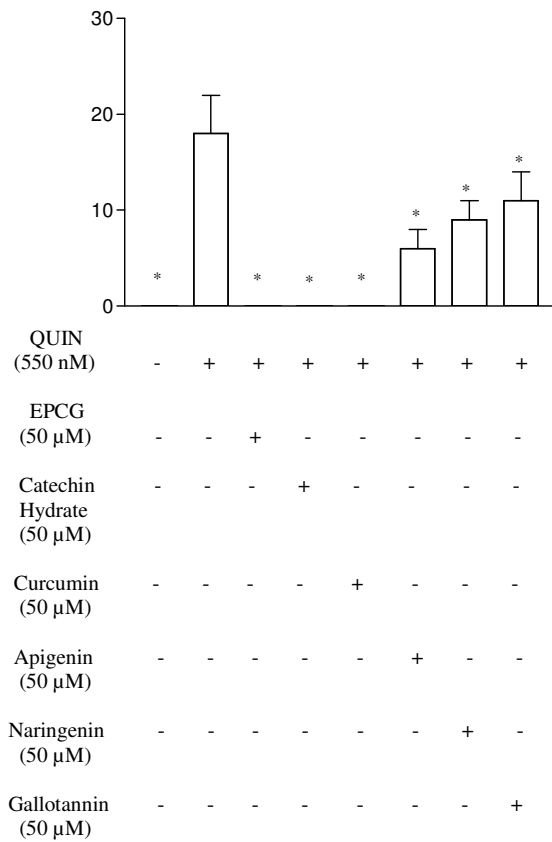
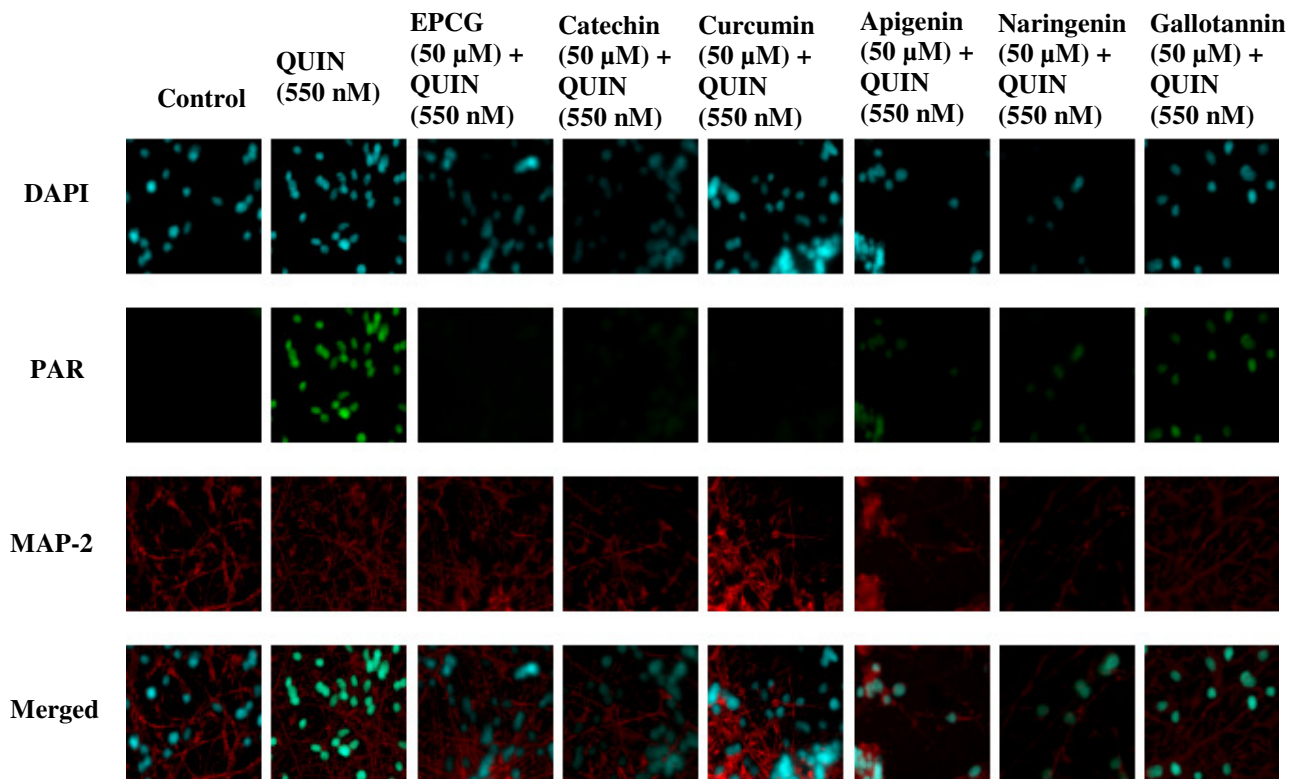


Figure 7. Immunocytochemical detection of PAR in purified primary human neurons after QUIN (550 nM) stimulation.

Staining for PAR in human neurons: Top row, nuclear staining for DAPI/blue; 2nd row, staining for PAR/green; 3rd row, double staining for DAPI/blue and MAP-2/red; 4th row, Merged PAR/green, MAP-2/red and DAPI/blue. **(B)** Numeration of fluorescence intensity of PAR in human neurons using immunocytochemistry. Histogram shows the percentage of human neurons expressing PAR relative to the total number of neuronal cells after 1 hour of treatment. **p*<0.05 compared to 550 nM QUIN alone; (n=4 for each treatment group).

(A)



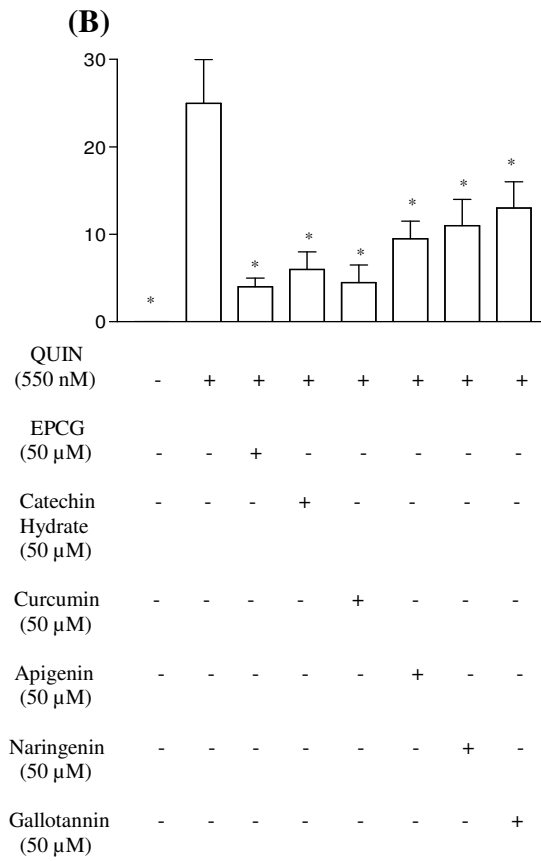


Figure 8. Schematic representation of the protective effects of EPCG, curcumin, catechin hydrate, apigenin, naringenin and gallotannin

The excitatory neurotoxin QUIN leads to over-activation of NMDA receptors followed by sustained Ca^{2+} influx. The calcium influx leads to the formation of $\text{NO}\cdot$ by the activation of nNOS. Highly reactive free radicals are formed which can cause oxidative damage to DNA leading to over-activation of PARP-1 and subsequent NAD^+ depletion and cell death due to energy restriction. Polyphenols can inhibit QUIN-induced excitotoxicity. However, each polyphenolic compound exerts its neuroprotective effect through distinct mechanism.

