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Natural compounds attenuate heavy metal-induced PC12 cell damage

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Abstract

Objectives: To investigate the neuroprotective effects of six natural compounds (caffeine, gallic acid, resveratrol, epigallocatechin gallate [EGCG], L-ascorbic acid and alpha tocopherol [Vitamin E] on heavy metal-induced cell damage in rat PC12 cells.

Methods: In this *in vitro* experiment, rat PC12 cells were exposed to four heavy metals ($CdCl_2$, $HgCl_2$, $CoCl_2$ and $PbCl_2$) at different concentrations and cell apoptosis, necrosis and oxidative stress were assessed with and without the addition of the six natural compounds.

Results: The metals decreased cell viability but the natural compounds attenuated their effects on apoptosis, necrosis and reactive oxygen species (ROS) levels. Mitochondrial protein changes were involved in the regulation.

Conclusion: Overall, the natural compounds did provide protection against the metal-induced PC12 cell damage. These data suggest that natural compounds may have therapeutic potential against metal-induced neurodegenerative disease.

Keywords

Metal salts, apoptosis, necrosis, ROS, natural compounds, polyphenols, PCA, PC12 cells

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Introduction

Metals have an important role in the functioning of all living organisms in a variety of ecosystems. They act as cofactors for essential enzymes to maintain homeostasis. While biometals are required for normal body functioning, excess accumulation of metal ions, especially heavy metals in the central nervous system (CNS), can result in severe problems and have been linked to neurodegeneration.[1–6]

One of the mechanisms by which metals might induce neurodegeneration is through oxidative stress.[2] Once the imbalance between the production of free radicals (reactive oxygen species, ROS) and

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antioxidant defences occurs, oxidative damage will develop. Cell apoptosis is often induced in these processes.[7,8] Natural compounds, such as plant polyphenols, have been suggested to have high antioxidant activity and they can chelate metal ions, so may have protective effects in cardiovascular diseases, diabetes, cancers and neurodegenerative diseases.[9–12]

In the present *in vitro* study, we investigated the neuroprotective effects of six natural compounds (caffeine, gallic acid, resveratrol, epigallocatechin gallate [EGCG], L-ascorbic acid and alpha tocopherol [Vitamin E] on heavy metal-induced cell damage in rat PC12 cells.

Methods

Materials

PC12 cells (rat adrenal pheochromocytoma cells) were obtained from American Type Culture Collection (ATCC, USA). Roswell Park Memorial Institute 1640 (RPMI-1640) medium and foetal bovine serum (FBS) were also purchased from ATCC and AlamarBlue cell viability assay reagent (DAL1100) was obtained from Thermo Fisher Scientific, USA. (H2DCFDA). 2′,7′-Dichlorofluorescin diacetate (H_2DCFDA), a chemically reduced form of fluorescein used as an indicator for ROS production, was purchased from Sigma-Aldrich, USA. The Annexin V-FITC apoptosis staining/detection kit with propidium iodide staining solution, was supplied by BD Biosciences, USA.

Antibodies against the mitochondrial proteins, Bax, Bcl-2, Cytochrome C, Caspase-3 and β-actin, were obtained from Santa Cruz Biotechnology, Santa Cruz, USA. ABTS (2,2'-azino-bis [3-ethylbenzthiazoline-6-sulphonic acid]), metal salts and small molecules were purchased from Sigma-Aldrich, USA. All other chemicals used in this study were all analytical grade.

Cell culture, viability and test substances

PC12 cells were cultured in RPMI-1640 medium supplemented with 5% (v/v) FBS in a humidified incubator 5% CO_2 atmosphere at 37°C. The culture medium was changed after cell density had reached 3×10^6 cells/ml. The cell viability of the PC12 cells was evaluated using AlamarBlue cell viability assay methods according to the manufacturer's instructions.

For sub-culturing, approximately 1×10^4 cells were placed into 96-well plates. Cells were incubated with 100 µl metal salts or natural compounds for 24 hours. The four metal salts, at concentrations of 10, 25, 50, 100, 200, 400, 600, 1000 and 1500 µM, were: cadmium chloride ($CdCl_2$), mercuric chloride ($HgCl_2$), cobalt chloride ($CoCl_2$) and lead chloride ($PbCl_2$). The six natural compounds, at concentrations of 5, 20, 80, and 320 µg/ml, were: caffeine, gallic acid, resveratrol, epigallocatechin gallate (EGCG), L-ascorbic acid and alpha tocopherol (Vitamin E).

Following incubation with the test substances, the cells were incubated for three hours with 10 µl AlamarBlue reagent. The absorbance was detected at 570nm by a microplate reader. PC12 cells without chemicals were used as the control group and cell viability results were expressed as percentage of control. Half-maximal inhibitory concentration (IC_{50}) for each metal salt was obtained by fitting the cell viability curves to the Hill equation.[13]

Cell apoptosis, necrosis and ROS production

PC12 cells were incubated with relative IC_{50} concentrations of metal salts to determine cell damage. $CdCl_2$, $HgCl_2$, $CoCl_2$ and

$PbCl_2$ at 500, 300, 100 and 130 μM, respectively, were incubated with PC12 cells for 24 hours. Thereafter, PC12 cells were washed twice using FBS and the samples were dyed with propidium iodide. The apoptotic and necrotic rates were measured using Annexin V-FITC apoptosis detection kit. The cells were assessed by fluorescence-activated cell sorting (FACS) using the Cell Quest software (BD, Pharmingen).

ROS production was evaluated using H_2DCFDA fluorescence dye as described previously.[14] PC12 cells were sub-cultured into 6-well plates. Cells were incubated with test substances for 24 hours after which time μM H_2DCFDA was added for 30 minutes in the dark. All samples passed through a 40 μm cell strainer before being loaded into FACS flow cytometry (Calibar; BectonDickinson) quantified at least 1×10^4 cells for each sample.

Antioxidant activity assay

The ABTS method, which is a spectrophotometric technique, was used to evaluate the free radical scavenging abilities of the six natural compounds.[15] ABTS stock solution was prepared by reacting equal volumes of 7 mM ABTS solution with 2.45 mM potassium persulfate solution. The mixture was mixed and kept in dark for 16 h at room temperature. Before use, the stock solution was diluted with ethanol to give an absorbance of 0.70 ± 0.02 at 734 nm. The test samples (10 μl) at different concentrations were added to 1ml ABTS working solution. The control was the ABTS solution without any test sample. After mixing the samples for 5 minutes, the absorbance (A) of the resulting solution at 734 nm was measured. Inhibition of ABTS radical was calculated using the following equation:[15]

$$\text{ABTS scavenging effect } (\%) = [(A_{\text{control}} - A_{\text{sample}}) \times 100/A_{\text{control}}]$$

Western blot assay

PC12 cells were incubated with 130 μM lead chloride and 80 μg/ml EGCG for 24 hours. The cells were collected and western blot performed as previously described.[16] RIPA buffer containing protease inhibitors was used to collect cells. Samples with equal amounts of proteins were separated on 12% SDSPAGE then the separated proteins were blotted onto PVDF membrane, and probed with selective antibodies to mitochondrial proteins (i.e., Bax, Bcl-2, Cytochrome C, Caspase-3 and β-actin) and incubated for one hour. The intensity of the optical bands was quantified using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, USA).

Statistical analysis. All assays were repeated at least three times and data were expressed as mean ± standard deviation (SD). Data were analysed using the Statistical Package for Social Sciences (SPSS®) for Windows® release 22.0 (IBM Corp., Armonk, NY, USA). Student's *t*-test was used to evaluate significance compared with controls and a *P*-value <0.05 was considered to indicate statistical significance.

Using ClustVis software, Principal Component Analysis (PCA) was used to model the multivariate data sets. For PCA, data from all metal salts were used as control and the difference between co-culture natural compounds and metal salts were plotted.

Results

Metal salts

Cell viability. Metal salts ($CdCl_2$, $HgCl_2$, $CoCl_2$ and $PbCl_2$) were added to PC12 cells in concentrations up to 1500 μM and dose-dependent decreases in cell viability

were observed in all test groups (Figure 1A–1D). Compared with the other metals, the steepest cell viability curve was observed for $CoCl_2$ and the flattest for $CdCl_2$. Likewise, the IC_{50} for $CoCl_2$ was 97 µM and for $CdCl_2$ was 525 µM (Figure 1E).

Cell apoptosis, necrosis and ROS production. PC12 cell apoptosis and necrosis were mea sured by Annexin V and propidium iodide double straining techniques. Significant cell apoptosis and necrosis were observed in the metal test groups whereas these two events were rarely seen in the control group (Figure 2A).

PC12 cells exposed to $CdCl_2$ and $PbCl_2$ were the most sensitive to cell apoptosis compared with the other metals. The $CdCl_2$ and $PbCl_2$ groups generated approximately 30% apoptotic cells which was approximately two-fold higher than cells incubated with $HgCl_2$ or $CoCl_2$ (Figure 2C).

Cell necrosis did not correlate with apoptosis. $CoCl_2$ exposure generated most necrotic cells followed by $CdCl_2$ and $HgCl_2$ (Figure 2D).

ROS positive cells were quantified by using the fluorometric probe H_2DCFDA. Its fluorescent intensity is proportional to intracellular ROS production.[17,18] In this study, ROS production varied across the different metal groups (Figure 2B). It strongly increased to about 40% when $PbCl_2$ was added to PC12 cells (Figure 2E). Values for the $CdCl_2$ and $HgCl_2$ groups were significantly different from the control group but the percentage of ROS positive cells in $CoCl_2$ group was the lowest and there was no difference from controls (Figure 2E).

Natural compounds

Antioxidant and cell viability. The natural compounds selected (Figure 3A) showed potential antioxidant activity in a dose-dependent manner in the ABTS assay (Figure 3B). Compared with the other natural compounds, EGCG and L-Ascorbic acid showed the strongest effect on inhibiting ABTS+, which may be related to their high hydroxyl content. By contrast, caffeine and alpha tocopherol (Vitamin E), which lack hydroxyl in their structure, were found to be the weakest of all tested substances on ABTS+ scavenging.

The results of cell viability studies showed that PC12 cells were not affected by any of the natural compounds up to 80 µg/ml. However, with the exception of resveratrol, all natural compounds at 320 µg/ml significantly reduced cell viability compared with controls. Therefore 80 µg/ml

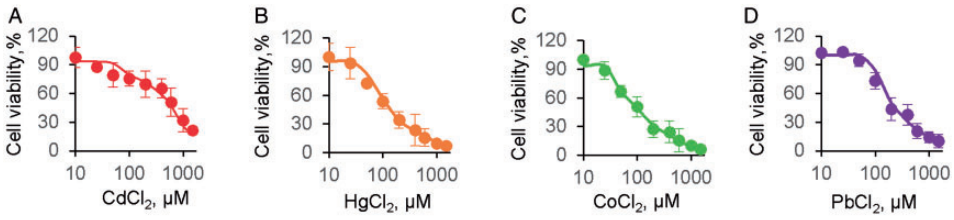

Figure 1. The viability of PC12 cells exposed to various metal salts using the AlamarBlue cell viability assay. (A) cadmium chloride [$CdCl_2$]; (B) mercuric chloride [$HgCl_2$]; (C) cobalt chloride [$CoCl_2$]; and (D) lead chloride [$PbCl_2$]; (E) Half-maximal inhibitory concentration (IC_{50}) for each metal salt was obtained by fitting the cell viability curves to the Hill equation.[13]

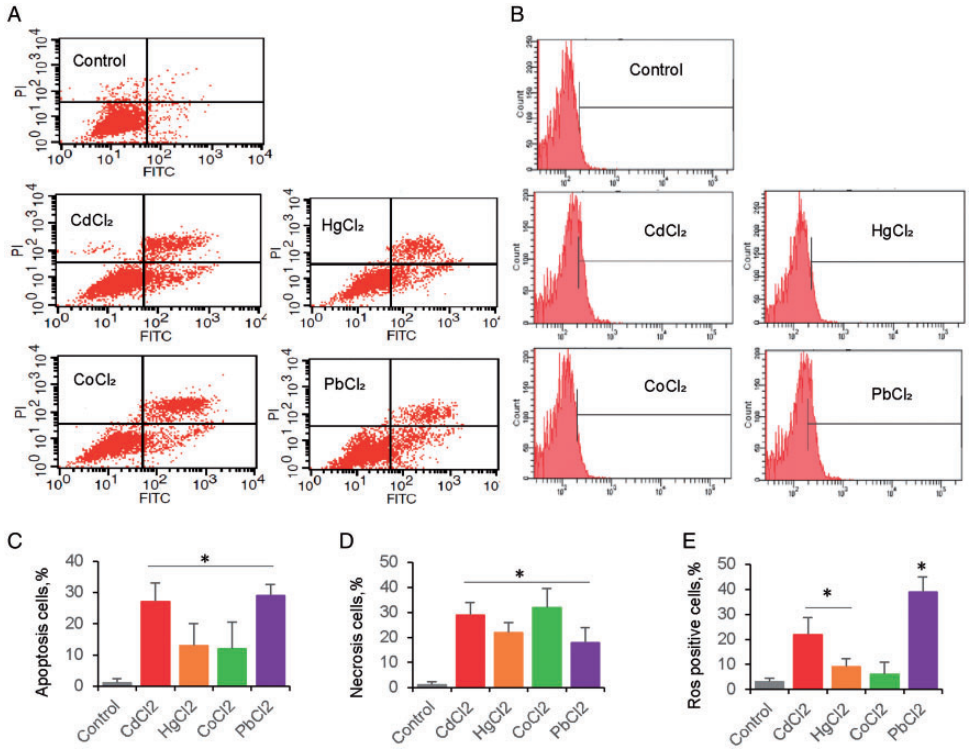

Figure 2. PC12 cell damage induced by metal salts.
(A) PC12 cell apoptosis and necrosis were measured using Annexin V and propidium iodide double staining technique after 24 h incubation. (B) ROS positive cells were quantified by using the fluorometric probe H_2DCFDA (C) Percentage of cell apoptosis, (D) Percentage of cell necrosis (E) Percentage of cell ROS positive cells.
*$P < 0.05$ compared with control group.

was selected as the dose for natural compounds when used for co-incubation with metal salts.

Metal salts combined with natural compounds

Cell apoptosis, necrosis and ROS production. PC12 cells were incubated with IC_{50} concentration of the four metal salts ($CdCl_2$, $HgCl_2$, $CoCl_2$ and $PbCl_2$) and 80 µg/ml of all six natural compounds for 24 hours to investigate any potential influence on cell damage (Figures 4A–D). While all six natural compounds tested attenuated cell damage to some extent, EGCG and L-ascorbic acid appeared to be the most effective substances in protecting PC12 cells.

Western blot analysis

The expression of mitochondrial apoptosis-related proteins was evaluated using Western blot analysis. EGCG protected against lead-induced cell damage (Figure 5A–D). The expression of Bax was upregulated in the lead group compared with the control group. By contrast, the level of

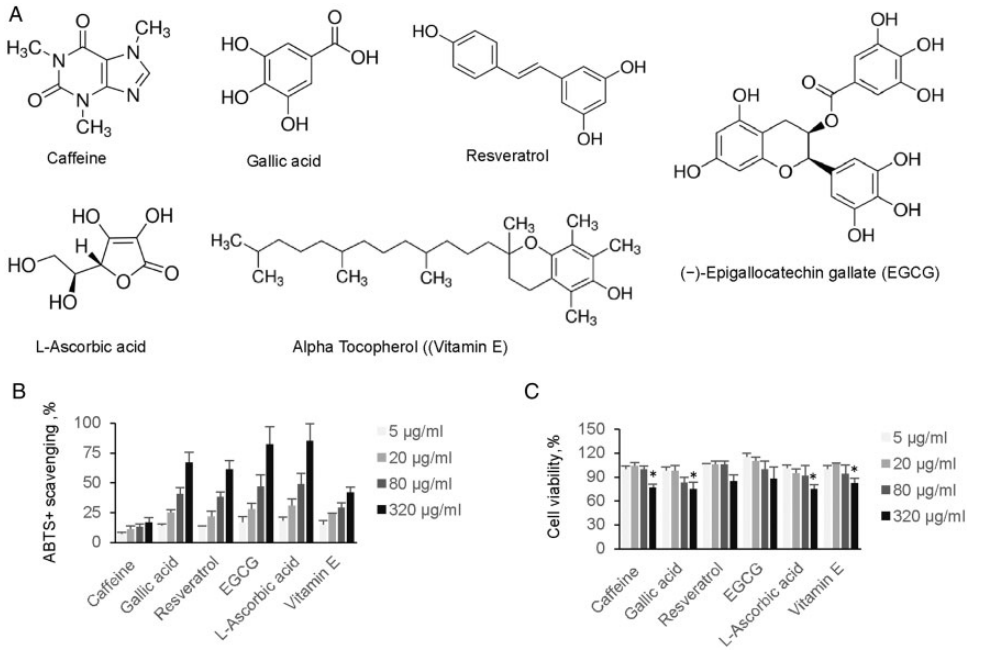

Figure 3. Natural compounds.
(A) Chemical structure of the six natural compounds. (B) Free radical scavenging activity of the natural compounds assessed by ABTS+ assay method. (C) PC12 cell viability exposed to various natural compounds using the AlamarBlue cell viability assay. $*P < 0.05$ compared with control group (control data are not shown in the figure but were 100%).

Bcl-2 was downregulated in the lead group compared with the control group, resulting in an increase in the ratio of Bax to Bcl-2 which was reduced by the addition of ECOG (Figure 5B). In addition, compared with the control group, there was an obvious activation of intracellular cytochrome C and activation of caspase-3 in the cells incubated with $PbCl_2$and again these were reduced by the addition of ECOG (Figure 5C and D).

Hierarchical clustering

PCA analysis was used to investigate the most influential factors in the data sets.[19,20] EGCG and L-ascorbic acid were located within the lower segment of the PCA analysis both in the cell apoptosis and necrosis indicating potential similar mechanisms for those two compounds against metal salts damage (Figure 6A and 6B). Caffeine, resveratrol and alpha tocopherol (Vitamin E) were closely distributed in the analysis of ROS production (Figure 6C).

Discussion

In this study we used PC12 cell lines as the neuronal model and investigated the protective effects of several natural compounds widely present in plants and fruit (i.e., caffeine, gallic acid, resveratrol, EGCG, L-ascorbic acid and vitamin E) on heavy metal-induced neuronal cell damage. We found that exposure of PC12 cells to the four metal salts (i.e., $CdCl_2$, $HgCl_2$, $CoCl_2$ and $PbCl_2$), led to several cell damage events, including apoptosis, necrosis and increased ROS production. Addition of

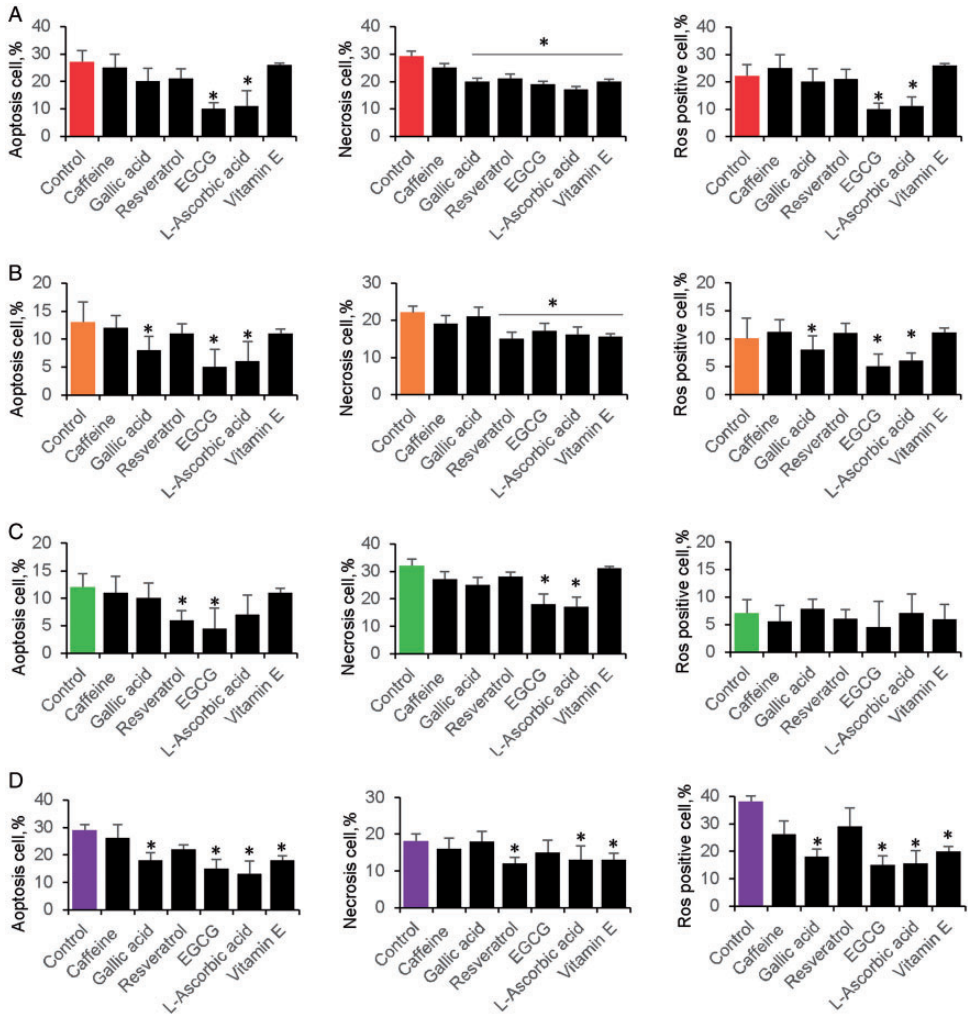

Figure 4. PC12 cell damage (apoptosis, necrosis and ROS positive cells) after natural compounds were combined with metal salts.
(A) cadmium chloride [$CdCl_2$]; (B) mercuric chloride [$HgCl_2$]; (C) cobalt chloride [$CoCl_2$]; and (D) lead chloride [$PbCl_2$];
*$P < 0.05$ compared with control group.

the natural compounds, particularly EGCG and L-ascorbic acid, to the PC12 cells attenuated the deleterious effects of the metal salts. Our findings confirm those from previous studies that have shown the protective effect of antioxidants from natural compounds in various cell lines exposed to pollutants, especially metals.[21–23]

Environmental pollutants, such as metals, can cause toxicological effects which may result in central nervous system pathology.[1–6] Studies have shown that heavy metals produce ROS which causes DNA damage, lipid peroxidation and depletion of protein sulfhydryls.[24] Natural compounds, such as plant

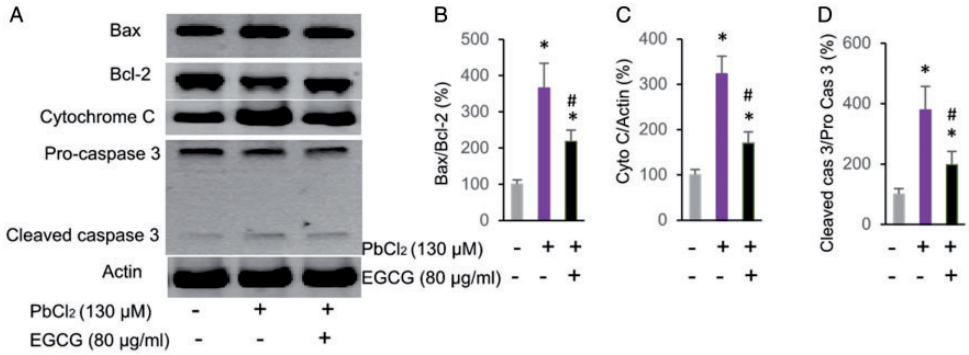

Figure 5. (A) Effects of lead chloride [$PbCl_2$] and epigallocatechin gallate (EGCG) on the expression levels of the Bax, Bcl-2, cytochrome C and caspase-3 determined via Western blot analysis.
(B) The relative expression of Bax/Bcl-2. (C) The relative expression of cytochrome C/Actin. (D) The relative expression of cleaved caspase 3/Pro caspase 3.
*$P < 0.05$ compared with control group.
#$P < 0.05$ compared with cells incubated with $PbCl_2$ alone.

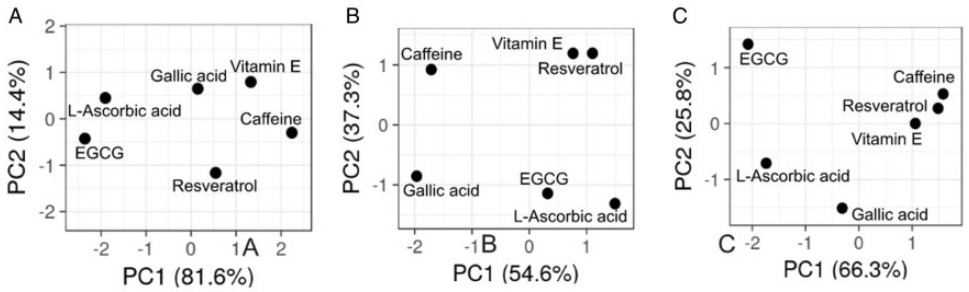

Figure 6. Principle component analysis. PC1, first principal component, PC2, second principal component. The percentages on the *x* and *y* axes denote the amount of variance in the dataset described.
(A) Cell apoptosis and (B) necrosis (C) ROS production.

polyphenols, have been shown to have potential protective effects against neuronal system diseases.[9–12] However, their specific bioactivities remain unknown. Although, multiple cell signal pathways may be involved in the aetiology of neurodegeneration, one hypothesis is that heavy metals activate the nuclear factor κB (NF-κB) pathway which in turn leads to stimulation of chronic inflammatory processes,[25,26] and natural compounds are able to protect cells by the suppression of NF-κB activation.[27] Another possibility is that natural compounds protect against the potential mitochondrial dysfunction caused by the heavy metals.[28] [Indeed, mitochondria are involved in the regulation of a number of apoptotic processes.[29] In this study we found that cells incubated with lead produced an imbalance in mitochondrial cell apoptotic proteins (i.e., Bax, Bcl-2, Cytochrome C, Caspase-3 and β-actin) and that the natural compound, EGCG attenuated this effect.

A novel aspect of this study was the use of hierarchical clustering analysis to indicate the relative bioactivity of the natural compounds against the various heavy

metals. Results from a PCA analysis showed the various effects of the natural compounds on metal toxicity. Overall, the natural compounds did provide protection against the metal-induced PC12 cell damage. These data suggest that natural compounds such as caffeine, gallic acid, resveratrol, EGCG, L-ascorbic acid and vitamin E may have therapeutic potential against metal-induced neurodegenerative disease. Although these data are interesting, they were obtained from one *in vitro* experiment and so further research is required both *in vitro* and *in vivo* to confirm these findings and explore more fully the potential neuroprotective mechanisms of natural compounds.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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