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Kolaviron protects apoptotic cell death in PC12 cells exposed to Atrazine

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Abstract

Kolaviron (KV), a natural biflavonoid obtained from the seeds of Garcinia kola, has been documented for its wide pharmacological window, including anti-apoptotic activities. However, the underlying mechanisms are poorly understood at the cellular level. This study investigates the anti-apoptotic activity of KV in PC12 cells, a rat pheochromocytoma, exposed to endocrine disruptor-atrazine (ATZ). KV (60 μM) treatment for 24 h shows significant anti-apoptotic responses in PC12 cells exposed to ATZ (232 μM) for 24 h. KV treatment recovers the ATZ-induced levels of malondialdehyde, reactive oxygen species (ROS), caspase-3 activity and depleted levels of glutathione and catalase activity. However, KV was found to be ineffective to restore the ATZ-induced expression (mRNA) and activity of glutathione-peroxidase (GSH-Px) and glutathione reductase (GR). KV treatment also demonstrates significant restoration in ATZ-induced alterations in the expression of apoptosis markers viz., p53, Bax, Bcl2, caspase-3, caspase-9, cyclooxygenase-2 (COX-2), c-Jun and c-fos. Flow cytometric analysis confirms the involvement of ROS in the mediation of ATZ-induced apoptosis in PC12 cells. Together, these data suggest that KV has the therapeutic potential against chemical-induced apoptotic cell death in the neuronal system.

Keywords: *Kolaviron, atrazine, oxidative stress, apoptosis, PC12 cells.*

Introduction

The contribution of environmental contaminants to the aetiology of neurodegenerative diseases, such as Parkinson's disease (PD), is now being broadly recognized [1]. Of importance, a recent twin study for PD incidence indicated that genetic factors do not play a major role in causing adult onset PD [2]. While several environmental factors have been implicated in the aetiology of PD, the evidence for a positive relationship between PD incidence and pesticide exposure is increasing [3–5]. In animal studies, mice exposed to the fungicide maneb, the herbicide paraquat or their combination exhibit PD symptomatology [6]. Other pesticides, such as dieldrin [7] and heptachlor [8], also cause dopaminergic perturbations *in vivo* and *in vitro*.

The herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-*S*-triazine, Figure 1A) is one of the widely used agricultural pesticides all over the world and now recognized to have been disrupting effects on the reproductive systems of mammals [9–12]. The neurotoxic potential of ATZ has also been observed in both *in vivo* and *in vitro* models [13–16]. Studies with the Chinese Hamster Ovary (CHO-K1) cell lines, Caco-2 intestinal cells, HepG2 and the normal human fibroblasts, DET 551 showed decreased cell growth after exposure to ATZ [17–20]. Other studies found associations between ATZ exposure and an

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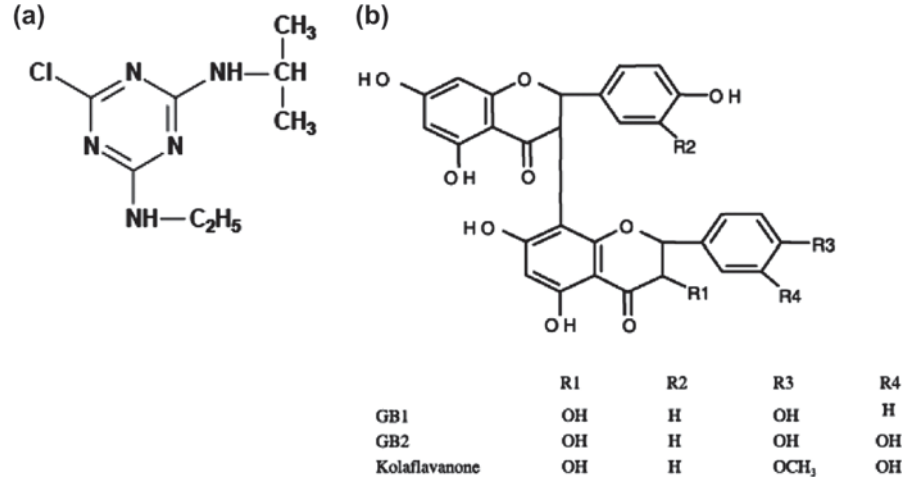

	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OCH_3	OH

Figure 1. Chemical structures of Kolaviron (A) and Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*S*-triazine) (B).

increased incidence of cancer in animal models and in humans [20,21]. Increased expressions of p53 have been suggested a biomarker for the occupational exposure to ATZ in both rodents and humans [22,23]. Furthermore, the role of oxidative stress and apoptosis in the toxicity of ATZ has been demonstrated in several model systems by us and others [16,24–27].

The protective potential of flavonoids in the neuronal system has been documented [28]. Flavonoids effectively scavenge free radicals and thereby protect neuronal cells from oxidative stress [29]. Consequently, there is growing interest in establishing therapeutic and dietary strategies to combat oxidative stress-induced damage to the central nervous system and attention is turning towards the potential neuroprotective effects of dietary antioxidants, especially flavonoids. Kolaviron (KV) is the most abundant Garcinia biflavanoids in *Garcinia kola* Heckel (Guttiferae). *Garcinia kola* Heckel is a valued edible nut in parts of Nigeria, West and Central Africa. Extractives of the plant have been employed in African traditional medicine for the treatment of bronchitis, throat infections, liver diseases and common cold and cough [30,31].

PC12 cells were used in the study to understand the protective mechanisms of KV against ATZ-induced apoptosis, since these cells have most of the functional expressions of neuronal cells and are extensively used in neurobiology studies [32,33].

Materials and methods

Reagents

Atrazine was purchased from Nantong Foreign Trade Meheco (China), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), neutral red and TMB/H_2O_2 substrate kit were purchased from Sigma Chemicals (St Louis, MO). Oligonucleotide Real Time primers were purchased from Integrated DNA technologies, Inc. (San Diego, CA). Rabbit polyclonal antibodies against Bax, c-Jun and c-Fos proteins were procured from Santa Cruz Biotechnology, Inc. (Santa cruz, CA, USA). Mouse monoclonal antibody gainst glyceraldehyde dehydrogenase (GAPDH) protein was procured from Millipore Corporation (Billerica, MA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Calbiochem (San Diego, CA). The culture wares were purchased from Nunc Inc. (Denmark). Nutrient mixture F-12 (Ham) culture medium, antibiotics/antimycotics, foetal bovine serum (FBS) and horse serum were purchased from Gibco BRL (Grand Island, New York, USA). Milli-Q and nuclease free water was used in all the experiments.

Extraction of kolaviron

Kolaviron was isolated according to the published procedure [30]. Briefly, the powdered seeds were extracted with light petroleum ether (bp 40–60°C) in a soxhlet for 24 h. The defatted dried marc was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethylacetate (6×300 ml). The concentrated ethylacetate yielded a golden yellow solid termed kolaviron (Figure 1B) which has been shown to consist of Garcinia biflavonoid GB-1 (3″,4″,4‴,5,5″,7,7″-heptahydroxy-3,8″ biflavanone), GB-2 (3″,4′,4‴,5,5″,5‴,7,7″-octahydroxy-3,8″-biflavanone) and kolaflavanone (3″,4′,4‴,5,5″,5′′′,7,7″octahydroxy-4′′′-methoxy-3,8″-biflavanone). Kolaviron was identified by direct comparison of the ^{1}H nuclear magnetic resonance

(NMR), ^{13}C NMR and electron ionization (EI)-mass spectral results with previously published data [30].

Cell culture and viability

PC12 cells (rat pheochromocytoma cell line) were originally procured from National Centre for Cell Sciences (Pune, India) and have been maintained at In Vitro Toxicology Laboratory, Indian Institute of Toxicology Research, Lucknow, India as per the standard protocol provided by the supplier. Briefly, cells were cultured in Nutrient Mixture F-12 (Ham), supplemented with 2.5% foetal bovine serum (FBS), 15% horse serum (HS), 0.2% sodium bicarbonate and antibiotic and antibiotic/antimycotic solution (100×, 1 ml/100 ml of medium, Invitrogen, Life Technologies, Grand Island, New York, USA). Cells were grown in 5% CO_2–95% atmosphere in high humidity at 37°C. Prior to experimental uses, cells were screened for integrity of established markers [34] and viability [35]. Batches showing more than 95% cell viability and passage number between 18–25 were used in the present studies. For the culture of cells, T-25 cm^2 flasks, 48 and 96 well culture plates were used depending on the type of assay/endpoint.

Cell treatment

Stock solutions of ATZ (100 mM) and KV (10 mM) were prepared in dimethyl sulphoxide (DMSO) and diluted to the experimental concentrations using culture medium with less than 0.01% DMSO in final volume. For cell viability assays, PC12 cells (1×10^4) were exposed to either ATZ (232 μM), KV (10, 30, 60 and 90 μM) or a combination of both ATZ and KV for 24 h. For enzyme assays, cells (1×10^6) received independent exposure and combination of ATZ (232 μM) and KV (60 μM) for 24 h. The exposure period for mRNA expression studies was 6 h. The parallel sets were also run under identical conditions and served as control. On the completion of respective incubation periods, medium was aspirated and cells were washed with phosphate-buffered saline, pelleted at 800 ×g and processed for further experimentation. For mRNA expression studies, a dose of KV (60 μM) for 6 h was selected as suggested safe to both human and rat cells [36,37].

Cytotoxicity studies

Cells (1×10^4 cells per well in 100 μl/well) were seeded in 96-well plates and treated with KV (10–90 μM) and ATZ (232 μM) alone or in combinations, as described previously for 24 h. After the treatment, the medium was removed and MTT (0.5 mg/ml, Sigma Chemical Company Pvt. Ltd., St. Louis, Missouri, USA) was added, followed by incubation at 37°C for 4 h in a CO_2 incubator. After a brief centrifugation, supernatants were carefully removed and dimethyl sulphoxide was added to the cells to solubilize the formazan crystals [38]. The colour developed was measured at 550 nm using Synergy HT multiplate reader (Bio-Tek, Highland Park, Winooski, VT, USA). The viability of cells was further followed by the NRU assay [39]. This assay was applied to determine the accumulation of the neutral red dye in the lysosomes of viable uninjured cells. Neutral red dye was added and the mixture was incubated at 37°C for 3 h. Neutral red solution was discarded and cells were washed with a solution containing 1% $CaCl_2$ and 0.5% formaldehyde. The cells were destained with 200 μL of destaining solution (1% glacial acetic acid; 50% ethanol; 49% distilled water) and the absorbance taken at 540 nm using a multiplate reader (Synergy HT, Bio-Tek). To determine the LDH activity released in the culture medium after treatment, 100 μl of conditioned media was added to 0.9 ml of a reaction mixture to yield a final concentration of 1 mmol/L pyruvate, 0.15 mmol/L NADH and 104 mmol/L phosphate buffer, pH 7.4. After being thoroughly mixed, the absorbance of the solution was measured at 340 nm at 60 s intervals with a microplate reader [40]. For trypan blue exclusion (TBE) assay, 60 μM KV was applied simultaneously with ATZ (232 μM) into the cell medium for 24 h to investigate the potential neuroprotective effect of the flavonoid against ATZ-mediated cell death. After treatment, the cell suspension was mixed with the same volume of 0.4% trypan blue solution (Invitrogen). Afterwards, the number of stained cells and the total number of cells were counted using a haemocytometer (Marienfeld, Germany). Untreated cultured cells (1×10^4 cells) were used as the 100% viability value. Experiments were repeated three times.

Estimation of biochemical parameters

The harvested cells (1×10^6 cells/ml) were suspended in 10 mM phosphate buffer (pH 7.4) and then lysed on ice by sonication twice for 30 s. The lysates were centrifuged at 5000×g for 15 min at 4°C to remove the cellular debris and the protein contents was determined by Bicinchoninic acid (BCA) protein assay (Lamda Biotech, St. Louis MO) using bovine serum albumin as standard. For detection of CAT activity, 50–100 μg of protein was added to 50 mM of phosphate buffer (pH 7.4) containing 100 mM(v/v) of H_2O_2 (Sigma, St Louis, MO). The reaction mixture was incubated for 2 min at 37°C and the rate of absorbance change (ΔA/min) at 240 nm was recorded, which indicated the decomposition of H_2O_2. Activities were calculated using the molar extinction coefficient of H_2O_2 at 240 nm, 43.59 L/molcm [41].

The activity of GSH-Px was assessed according to established methods of Rotruck et al. [42] which makes use of the reaction: $H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ (oxidized glutathione). The absorbance was determined at 412 nm. The enzymatic activity was expressed as μg GSH consumed/min/mg protein. GR activity was determined by the method of Staal et al. [43]. Briefly, 0.1 ml NADPH was added to the reaction mixture containing 0.5 ml cell extract, 0.7 ml sodium phosphate buffer, 0.5 ml of 25 mM EDTA and 0.2 ml of 12.5 mM oxidized glutathione. The absorbance was immediately read at 340 nm for 5 min at 60 s intervals in a microplate reader. Activity was expressed as μmol NADPH oxidized/min/mg protein. GSH is an important cellular non-enzymatic antioxidant. The reduced GSH was determined by measuring the reduction of 5, 5'-dithiobis-2- nitrobenzoate (DTNB) to the yellow coloured complex, 2-nitro-5-thiobenzoate according to the method of Sedlak and Lindsay [44]. The intensity of the yellow coloured complex formed is directly proportional to the amount of -SH groups and measured at 412 nm. The values were expressed as μg GSH/ml. Malondialdehyde (MDA) is a breakdown product of the oxidative degradation of cell membrane lipids and is generally considered an indicator of lipid peroxidation. In the present study, lipid peroxidation was thus evaluated by measuring MDA concentrations according to the method of Buege and Aust [45]. The method was based on the spectrophotometric measurement of the colour produced during the reaction to thiobarbituric acid (TBA) with MDA. MDA concentrations were calculated by the absorbance of thiobarbituric acid reactive substances (TBARS) at 532 nm and were expressed in pmol/ml. Analyses were performed in duplicates. Protein was measured by the Lowry reagent [46].

Measurement of intracellular oxidative stress

After treatment with ATZ (232 μM) and KV (60 μM) for 1, 6 and 24 h alone or in combination, the cells were incubated with 20 μM of the fluorescent dye 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Sigma) for 30 min. H_2DCF-DA is a non-polar compound which upon incorporation into cells is converted into a membrane-impermeable, non-fluorescent polar compound, H_2DCF, by the action of cellular esterases. The fluorescence intensity was measured with excitation and emission wavelengths of 485 nm and 528 nm, respectively, in a multiplate reader (Synergy HT, Bio-Tek). Furthermore, the fluorescence at 24 h culture period was readily detected by the fluorescence microscope (Nikon Eclipse 80i, Japan) and the fluorescence intensity analysed by Leica QWin Plus, version 2.7.1 (Leica Microsystems Imaging solution, Cambridge UK) The DCF fluorescence intensity was proportional to the amount of intracellularly formed ROS [47,48].

ATZ-induced ROS generation in PC12 cells was also assessed by using FACS. In brief, cells (5×10^4 per well) were seeded in a 6-well plate and exposed to ATZ (232 μM) with or without KV (60 μM) for 24 h. Following exposure, cells were washed twice with PBS and incubated for 30 min in the dark in incomplete culture medium containing DCFH-DA (20 μM). After twice washing with PBS, cells were read for fluorescence on a flow cytometer (BD-LSR II) and analysed by Cell Quest 3.3 software. Cells exposed to H_2O_2 (500 μM) for 1 h under identical conditions served as a positive control. The data are presented as the mean fluorescence obtained from a population of at least 10 000 cells.

Detection of apoptosis

Apoptosis was detected as earlier described by us [49]. Mitochondrial membrane potential, an early marker of apoptosis induction, was assessed using a flow-cytometer based MitolightTM Apoptosis Detection Kit (APT142, Chemicon, Billerica, MA, USA). Cells were exposed to ATZ (232 μM) alone or with KV (60 μM) for 24 h, then pelleted and re-suspended in 1 ml of pre-diluted Mitolight solution for 15 min at 37°C. The role of ROS in the induction of apoptosis was ascertained by pre-treating PC12 cells to diphenyleneiodonium chloride (DPI) (10 μM), 1 h prior to the exposure of ATZ (232 μM) for 24 h. Following incubation, uptake of Mitolight dye by living mitochondria was analysed by Flowcytometer (BD FACSCanto) equipped with the FACS Diva Version 6.0.0.software.

Isolation of total RNA and quantitative real time-polymerase chain reaction (QRT-PCR)

Total RNAs from PC12 cells exposed to ATZ and KV were isolated using Gene Elute mammalian total RNA Miniprep Kit (Catalogue No#RTN-70, Sigma) and following the protocol recommended by the supplier. Each total RNA sample was checked for integrity and DNA contamination by measurement of OD (Nanodrop ND-1000 Spectrophotometer, Wilmington, DE, USA) and size-fractionation of 18S and 28S rRNA on an agarose gel; 2μg of total RNA was reverse-transcribed into cDNA by Super Script III first strand cDNA synthesis Kit (Catalogue No#18080-051, Invitrogen Life Science). QRT-PCR was performed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The SYBR Green PCR Master Mix kits were used according to the supplier's instructions for quantification of gene expression. Rat specific primers for p53, caspase-3, caspase-9, Bax, Bcl-2 and the house keeping gene β-actin were designed using software Primer Express 3.0 (Applied Biosystems) and full genes sequences from National Center for Biotechnology Information

Entrez Nucleotide Database (www.ncbi.nlm.nih.gov/sites/entrez). COX-2, GSH-Px and GR primers were from previous publications [50,51]. Primer pairs used are as follows: β-actin F:ggaaatcgtgcgtgacattaaag and R:cggcagtggccatctctt; p53 F:ccccaccgcctgtaagatt and R:atgggtccggaggatacagat; p21 F:ttgtcgctgtcttgcactct and R:atctgcgcttggagtgatag; caspase-3 F: cagagctggactgcggtattga and R:agcatggcgcaaagtgactg; caspase-9 F:agccagatgctgtcccatac and R:caggagacaaaacctgggaa; Bax F:gggtggttgccctttctact and R:cccggaggaa gtccagtgtc; Bcl-2 F:ctgagtacctgaaccggcatc and R: gagcag cgtcttcagagacag; GSH-Px F: cggtttcccgtgcaatcagt and R: acaccggggaccaaatgatg; GR F: agcccacagcggaagtcaac and R: caatgtaaccggcacccaca; COX-2 F: aagggagtctggaacattgtgaac and R:caaatgt gatctggacgtcaaca. Thermal cycling conditions determined in ABI Prism 7900HT Sequence Detection System (SDS) were as follows: 95°C for 2 min; 1 cycle of 30 s denaturation at 94°C, 60 s annealing at 42°C and 90 s extension at 72°C; 40 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C and 90 s extension at 72°C; and 1 cycle of 10 min at 72°C. The calculation of the relative expression levels of each target was conducted based on the cycle threshold (Ct) method. The Ct for each sample was calculated using the SDS Relative Quantification Manager Software (Applied Biosystem) with an automatic fluorescence threshold (ΔR_n) setting. The relative expression ratio (R) of a target gene is expressed in a sample vs a control in comparison to β-actin gene and calculated based on the following equation: $\Delta Ct = (Ct, \text{target gene} - Ct, \beta\text{-actin})$; $\Delta\Delta Ct = \Delta Ct$ treatment $- \Delta Ct$ control; $R = 2^{-\Delta\Delta Ct}$.

Measurement of caspase-3 activity

To measure apoptosis enzymatically, caspase-3 activity in cell extracts was measured using N-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA). PC12 cells were plated on T75 cm^2 flask (Nunc, Germany) at a density of 1×10^6/flask for 24 h at 37°C and 5% CO_2. After 24 h, medium was removed and replaced with fresh medium with containing 232 μM ATZ and 60 μM KV alone or in combination. After the completion of the 24 h treatment period, Lysates was made using celLytic buffer (Sigma). Five microlitres of the lysate was used for protein concentration determination using the Bicinchoninic acid (BCA) protein assay (Lamda Biotech,

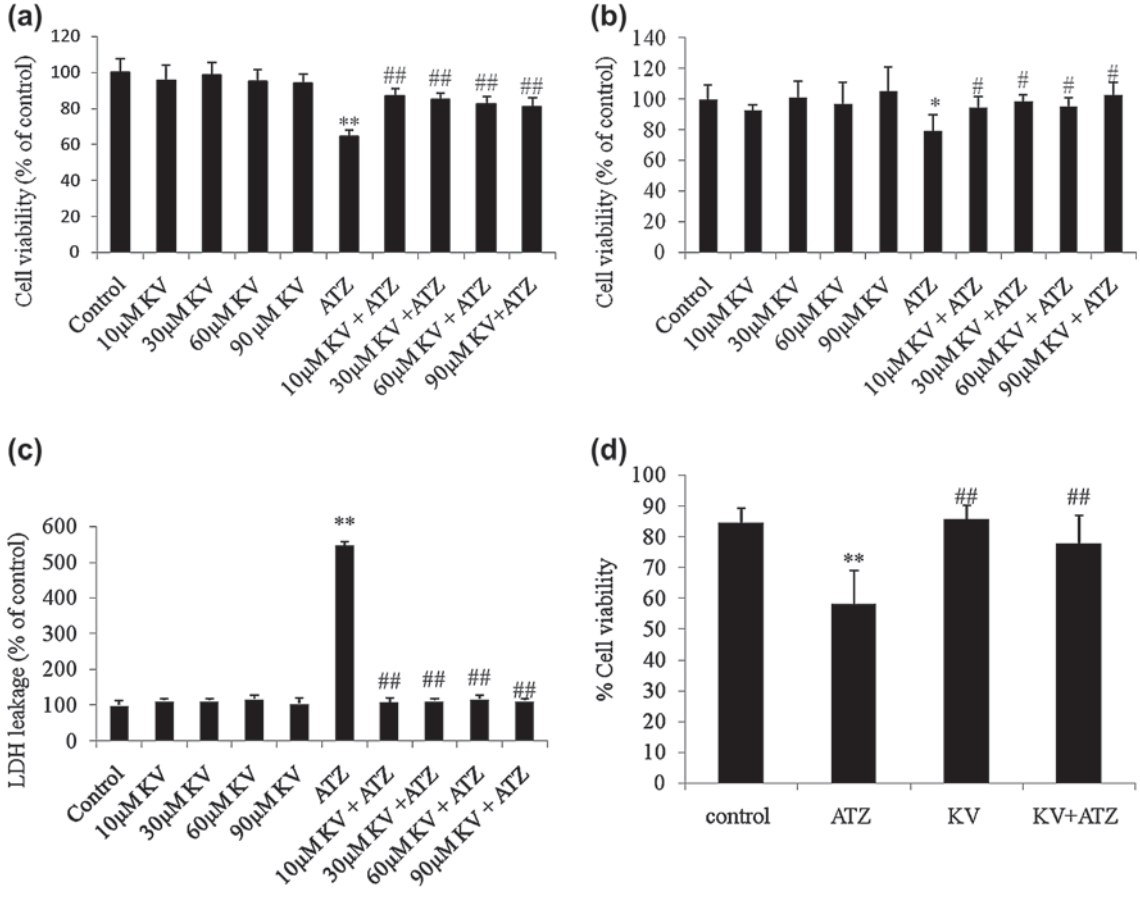

Figure 2. Protective effect of kolaviron (KV) in PC12 cells exposed to Atrazine (ATZ). (A) MTT reduction assay, (B) Neutral red uptake, (C) Lactate dehydrogenase leakage and (D) Trypan blue exclusion assay in PC12 cells exposed to 232 μM for 24 h in the presence and absence of KV treatment. Data are expressed as a percentage of values in untreated control sets and are mean ± SE of three independent experiments for six replicates per experiment. *Control vs ATZ-exposed, #ATZ-exposed vs KV-treated. */# $p < 0.05$, **Control vs ATZ-exposed, ##ATZ-exposed vs KV-treated. **/## $p < 0.001$.

St. Louis, MO). The cell extracts (50 µg protein/well) were added to a 96-well plate containing caspase assay buffer (50 mM HEPES, 20% glycerol and 100 mM dithiothreitol) and 200 µM DEVD-pNA colourimetric substrate and incubated at 37°C for 4 h. The OD was then read at 405 nm in a multiplate reader (Synergy HT, Bio-Tek). *p*-nitroanaline (pNA) is released from the substrate DEVD-pNA upon cleavage by DEVDase. Free pNA produces a yellow colour after cleavage that is proportional to the amount of DEVDase activity present in the sample. The increase in optical density positively correlates with the amount of caspase-3 activity. The experiment was done three times. The caspase assay had six replicates per sample.

Preparation of cell extract for western blotting analysis

After treatment of cells with ATZ (232 µM) in the presence or absence of KV (60 µM) for 24 h, the cells were lysed in 0.1 mL of CelLytic M Cell Lysis Reagent (Catalogue No# C2978, Sigma) supplemented with complete Protease Inhibitor Cocktail (Catalogue No# P8340, Sigma). Protein concentration of the cell lysates were determined by the method of Lowry et al. [46]. The same amount of cell lysate (100 µg of protein) was separated electrophoretically on a 12.5% SDS-polyacrylamide gel. The proteins on the gel were transferred to a polyvinylidene difluoride (PVDF) membrane at 250 mA for 3 h in transfer buffer (25 mM Tris buffer, 190 mM glycine, 20% methanol, 0.01% SDS). The membranes were blocked with 5% skim milk-TBST (20 mM Tris-base, 137 mM NaCl, 0.1% Tween-20, pH 7.5) overnight at 4°C, rinsed by 4–5 washes in TBST and subsequently incubated with primary antibodies (1:1000 or 500 dilutions) against Bax, c-*fos*, c-Jun and GAPDH at room temperature for 2 h. The membrane was washed four times in TBST and incubated with horseradish peroxidase-conjugated sheep anti-mouse antibody or donkey anti-rabbit antibody. After four washes with TBST, the immunoreactive bands were detected with TMB-H_2O_2 (Sigma) and visualized with a MultiImage Light cabinet (Alpha Innotech Corp., San Leandro, CA). Quantitation of expressed protein levels was done using AlphaEase^{TM} FC StandAlone V. 4.0.0 software (Alpha Innotech).

Statistical analysis

Results are expressed as Mean ± SE of six wells from each experiment from at least three independent experiments. One-way analysis of variance (ANOVA) and post-hoc Turkey's test was used to compare control and treated groups. p-values < 0.05 were considered statistically significant.

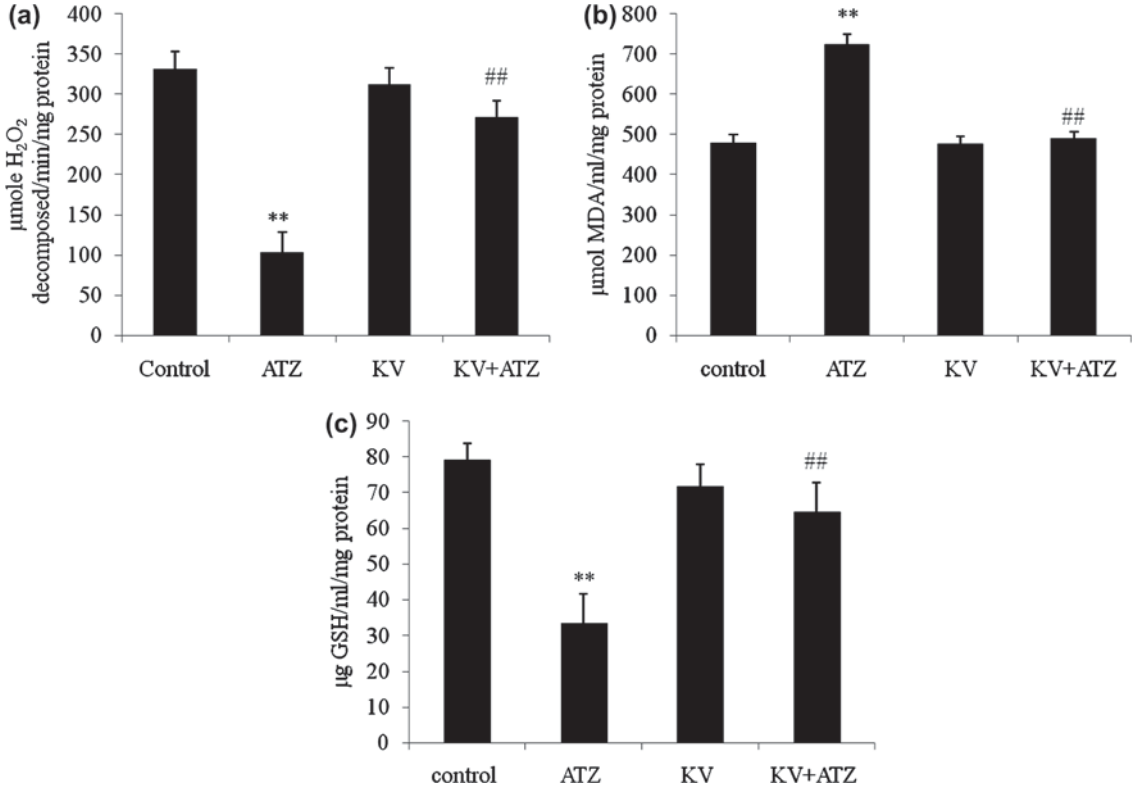

Figure 3. Ameliorative effect of kolaviron (KV) on (A) catalase activity, (B) malondialdehyde (MDA) levels and (C) reduced glutathione (GSH) levels in PC12 cells following the exposure to atrazine (ATZ) for 24 h. Data are mean ± SE of three independent experiments for six replicates per experiment. **Control vs ATZ-exposed, ##ATZ-exposed vs KV-treated. **/## $p < 0.001$.

Results

Protective effects of Kolaviron on the cytotoxicity of atrazine on PC12 cells

To evaluate whether KV protects neuronal PC12 cells from ATZ-induced cell death, we examined the direct cytotoxic effect of ATZ (232 μM) on PC12 cells in the presence and absence of various concentrations of KV (10–90 μM) for 24 h. The cell viability was measured by MTT and NRU assays. ATZ (232 μM) decreases the cell viability by 36% and 21% in MTT and NRU assays, respectively (Figures 2A and B). KV treatment significantly protects the loss of cell viability, which was dose-dependent. For NRU assay, cell viability restored up to 94%, 98%, 95% and 103% following the treatment of KV—10, 30, 60 and 90 μM for 24 h (Figure 2B). ATZ (232 μM) exposure for 24 h induces the LDH release more than 4-fold ($p < 0.05$) compared to unexposed cells. All the concentrations of KV used show significant recovery in ATZ-induced LDH release in the cells (Figure 2C). The data of TBE assay further confirms the cytoprotective potential of KV (60 μM) against ATZ (232 μM) exposure for 24 h in PC12 cells (Figure 2D). In totality, KV shows a dose-dependent protection in PC12 cells exposed to ATZ (232 μM) for 24 h.

Measurement of oxidative damage and mRNA expression of GSH-Px and GR

Cells exposed to ATZ (232 μM) for 24 h show a significant ($p < 0.05$) increase in the levels of MDA and decrease in the levels of GSH and catalase activity. KV (60 μM) treatment significantly recovers all these altered levels of MDA, GSH and catalase activity (Figures 3A–C). In general, KV (60 μM) treatment could not restore the ATZ-induced activity of GSH-Px and GR, factors known to be associated with the levels of GSH (Figures 4A and B). The finding was further confirmed by QRT-PCR analysis for mRNA expression of GSH-Px and GR genes. The results of mRNA show consistent findings observed at the activity level (Figures 4C and D).

Scavenging effects of kolaviron on intracellular ROS and apoptosis by atrazine

ATZ-induced generation of intracellular ROS and possible scavenging by KV were observed in using ROS-detecting fluorescence dye DCF-DA. Increased intensity of DCF fluorescence is used as an indicator of ROS generation. A time-dependent increase in the ROS generation was observed in PC12 cells exposed to ATZ with highest magnitude

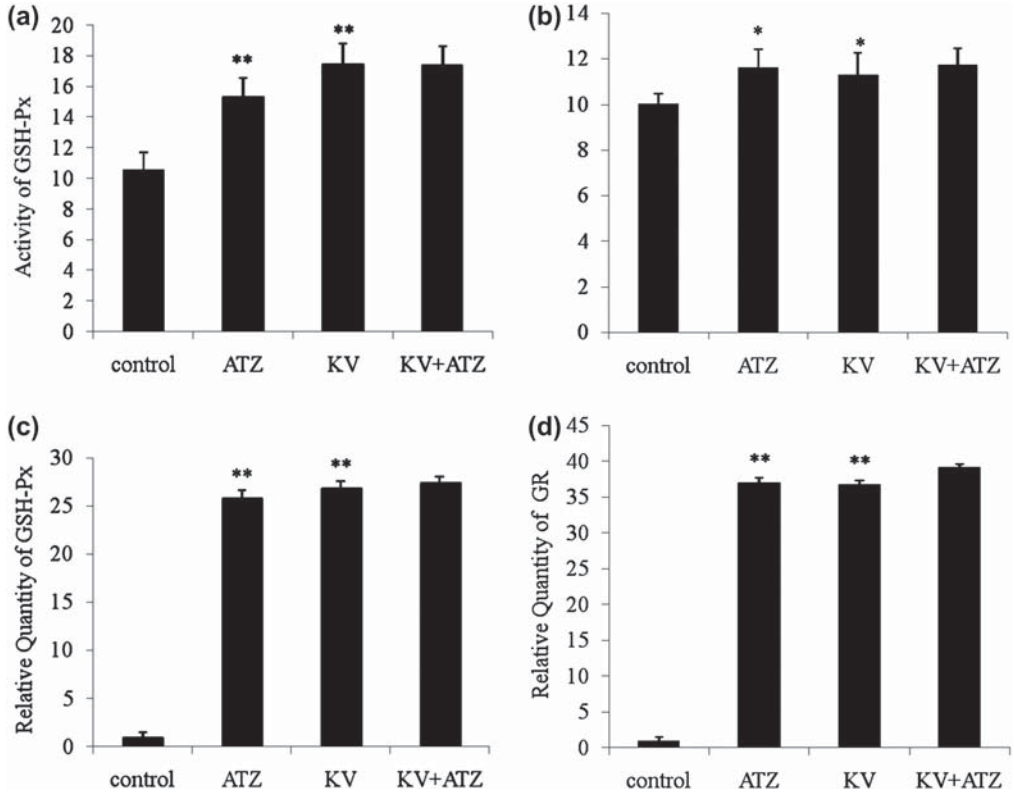

Figure 4. The effect of kolaviron (KV) on atrazine (ATZ)-induced changes of GSH-Px and GR activities (A, B) and mRNA expression levels (C, D) in PC12 cells after 24 h and 6 h culture period, respectively. Data are represented as mean ± SE of three independent experiments and six replicates per each experiment in the activity assay and three replicates for each sample in the gene expression study. **Control vs ATZ-exposed, ##ATZ-exposed vs KV-treated. **/## $p < 0.001$.

at 24 h. KV (60 μM) treatment for 24 h downs the ATZ-induced generation of ROS (146%) to near the control (108%) at 24 h (Figure 5A). The similar trend of fluorescence was observed in a quantitative assay of ROS generation carried out using fluorescence microscopy (Figure 5B). ATZ exposure of 24 h increases ROS-fluorescence signals by 6-fold, which came down to 2-fold in ATZ-exposed cells receiving KV treatment (Figure 5C). Our flowcytometric analysis for ATZ-induced ROS generation and KV mediated scavenging activity also confirms the results by showing a similar trend as observed in DCF-DA signalling studies (Figure 5D).

ATZ (232 μM) exposure for 24 h induces significant (~ 4-fold of control) apoptosis in PC12 cells. KV (60 μM) treatment for 24 h shows a significant effect on the lowering (~2-fold of control) of apoptosis. Apoptosis induction activities of ATZ were decreased significantly in the cells treated with ROS inhibitor, DPI (10 μM) for 1 h prior the exposure of ATZ for 24 h. System optimization was done by giving camptothecin (3 μg/ml) exposure for 6 h as a positive control, which induces 30% apoptosis in cells served as a positive control (Figure 6). The data suggest that ATZ treatment leads to an increase in ROS production, which may represent a critical step in ATZ-induced apoptosis and oxidative stress and that KV protected against ROS-mediated apoptosis and oxidative damage in PC12 cells.

The effects of kolaviron on the mRNA expression of COX-2 and apoptotic genes in atrazine exposed PC12 cells

We observe that ATZ exposure for 24 h up-regulates the levels of mRNA expression of Bax, p53, caspase-3, caspase-9 and COX-2 and down-regulates the mRNA expression of Bcl-2 genes in PC12 cells. KV

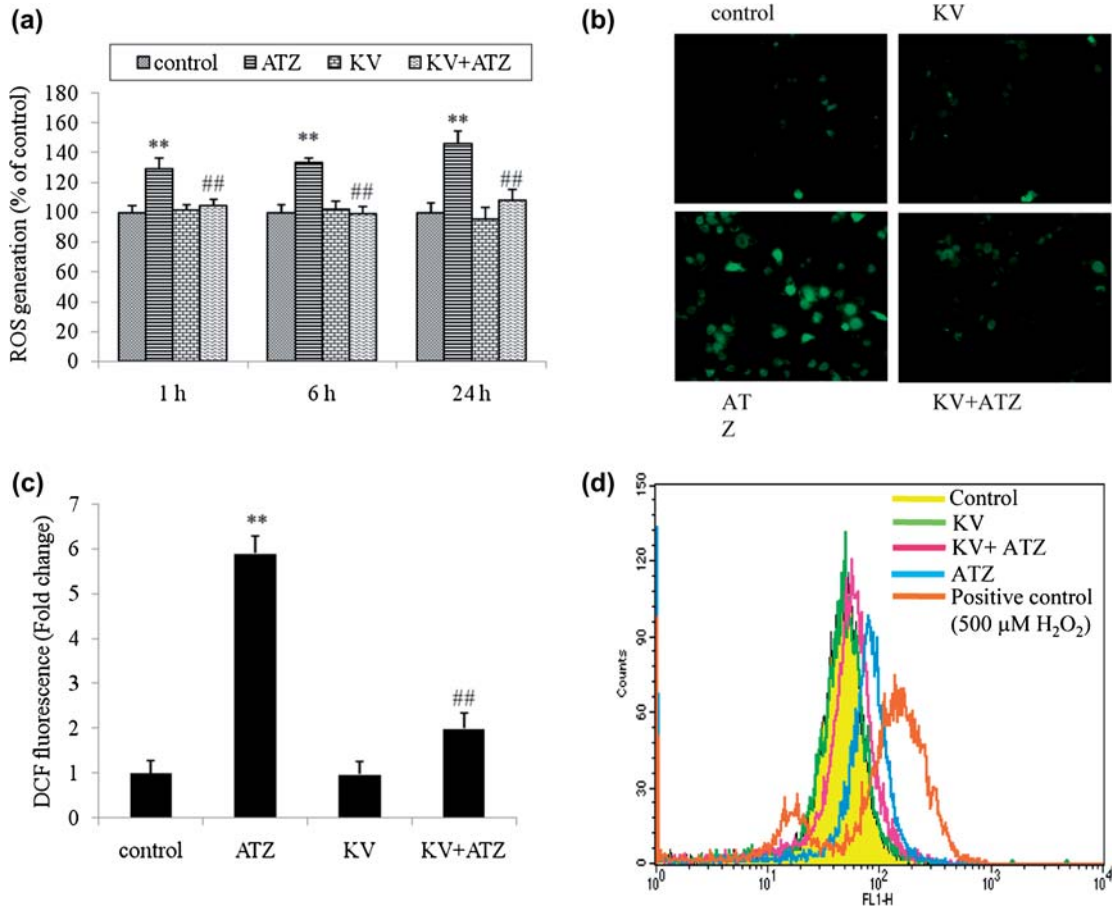

Figure 5. Atrazine-induced ROS generation and ameliorative effect of kolaviron biflavonoids (KV) treatment in PC12 cells for different time periods. Readings were taken on a multiwall plate reader (Synergy HT) at excitation wavelength 525 nm and emission wavelength 485 nm (A). Qualitative characterization of intracellular ROS generation by dichlorofluorescin diacetate (DCFH-DA) staining in KV- and ATZ-exposed cultured PC12 cells at 24 h (B). Images were snapped (×400) using a Nikon phase contrast cum fluorescence microscope (model 80i). Relative quantification of fold induction in ROS generation in PC12 cells following exposure to KV and ATZ alone or in combination for 24 h quantification of fluorescence images of for intracellular ROS generation was done by using Leica Q win500 image analysis software (C). KV mediated protective potential in the ROS generation in ATZ-treated PC12 cells were also estimated by FACS analysis (D). Values are represented as mean ± SE of three independent experiments. **Control vs ATZ-exposed, ##ATZ-exposed vs KV-treated. **/## $p < 0.001$.

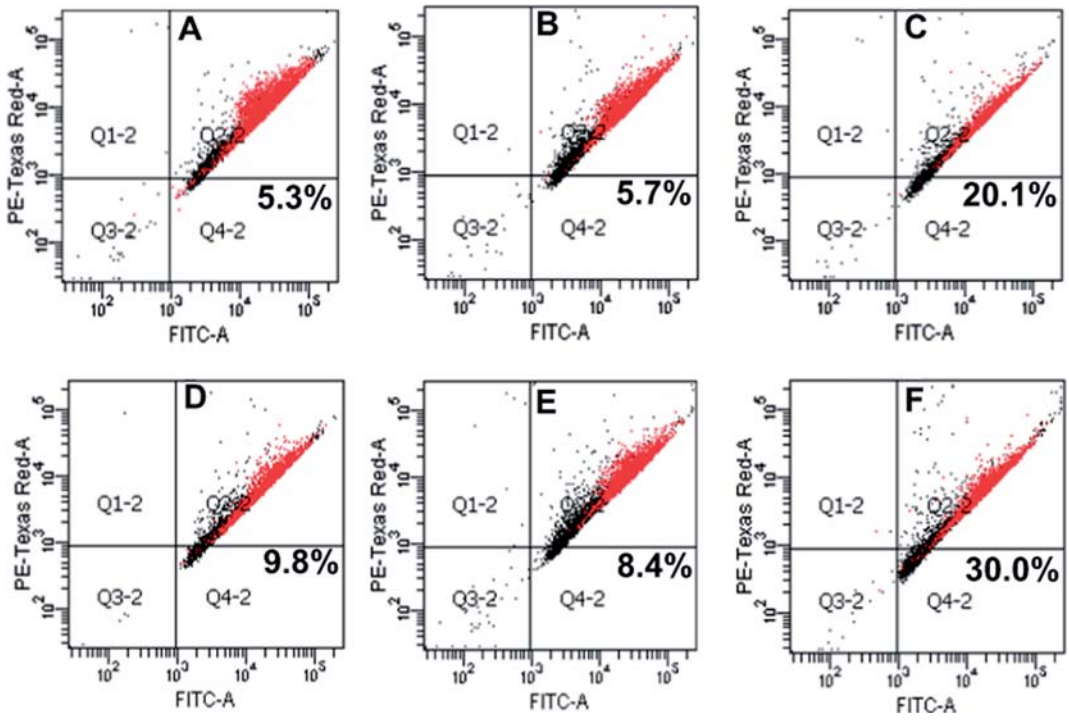

Figure 6. Apoptosis detection in PC12 cells exposed to atrazine (ATZ) and protective effect of kolaviron (KV) using MitolightTM apoptosis detection kit (Catalogue no. APT142, Chemicon). (A) Control cells; (B) PC12 cells exposed to KV (60 μM) for 24 h; (C) PC12 cells exposed to ATZ (232 μM) for 24 h; (D) PC12 cells exposed to ATZ (232 μM) + KV (60 μM) for 24 h; (E) Cells pre-treated with 10 μM DPI for 1 h and then exposed with ATZ (232 μM) for 24 h; (F) Experimental positive control-PC12 cells exposed to campothecin (3 μg/ml) for 6 h.

treatment for 24 h significantly shifts the altered levels of mRNA expression of Bax, p53, Bcl-2, caspase-3, caspase-9 and COX-2 toward the expression levels in unexposed control cells. Interestingly, the levels of mRNA expression for these genes were not affected significantly in ATZ unexposed cells treated to KV only for 24 h (Figure 7A).

The effects of kolaviron on caspase-3 activity, expression of apoptotic proteins in atrazine exposed PC12 cells

PC12 cells exposed to ATZ (232 μM) in the presence or absence of KV (60 μM) show that KV significantly reduces the ATZ-induced up-regulation of caspase-3 activity (Figure 7B). To investigate the possible role of the AP-1 transcription factors in ATZ-induced apoptosis, the expression of c-Fos and c-Jun were evaluated by Western blot analysis. ATZ shows significant down-regulation in the expression of c-Jun and c-Fos proteins. The treatment of KV was found effective in restoration in ATZ-induced down-regulation of c-Jun and c-Fos. As such KV treatment alone had no significant effect on the protein expression of these proteins when compared to unexposed control sets (Figures 8A and B). In a similar fashion, KV treatment was found to be effective in restoration of ATZ-induced elevated expression of Bax protein in PC12 cells (Figure 9).

Discussion

There are a number of studies showing the role of flavonoids in risk management in cancer, heart and liver diseases, however their role in brain functions is poorly understood [36,52]. In the present investigations, KV treatment for 24 h was found to be cytoprotective in a dose-dependent manner in PC12 cells exposed to ATZ (232 μM) for 24 h. It has also been reported the ATZ-induced cytotoxicity in variety of cell systems viz., in human neuroblastoma SH-SY5Y cells [16], CHO-K1 ovary cells [17], Caco-2 intestinal cells [18], normal human fibroblast (DET 551) and human peripheral blood mononuclear cells [19]. Similar to our finding observed with KV, other biflavonoids have also been reported to have protective potential against a variety of toxic insults in different cell types viz., in mouse P388 cells and human neuroblastoma SH-SY5Y cells [53,54], human glioma (A172) cells and in cardiomyocytes [55,56].

We observed that ATZ exposure decreases CAT activity and GSH levels while increasing MDA levels and increasing expression of mRNA levels and activities of GSH-Px and GR. The increased activities of glutathione-related enzymes (GSH-Px and GR) could be related to the increased formation of ROS as an adaptive defence mechanism to protect the critical redox functions [57]. Since ATZ reacts with sulphydryl

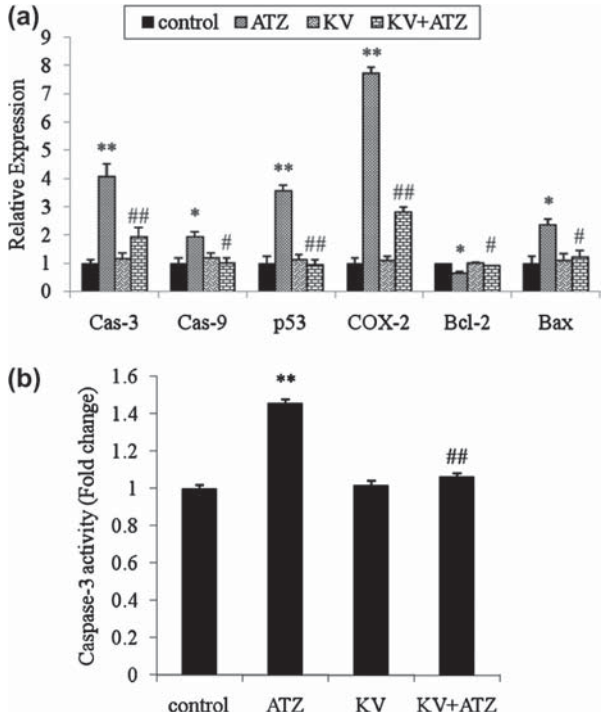

Figure 7. Real-time quantitative-PCR analyses of mRNA expression of caspase-3 (cas-3), caspase-9 (cas-9), p53, cyclooxygenase-2 (COX-2), Bcl-2 and Bax genes in PC12 cells exposed to kolaviron (KV) and atrazine (ATZ) alone or in combination for 6 h (A). Caspase-3 activity. Ameliorative effect of pre-treatment of KV on the activity of activated caspase-3 in PC12 cells exposed to ATZ (B). Each data point represents mean ± SE ($n = 6$) from three separate experiments. *Control vs ATZ-exposed, # ATZ-exposed vs KV-treated. */# $p < 0.05$; **Control vs ATZ-exposed, ##ATZ-exposed vs KV-treated. **/## $p < 0.001$.

groups of proteins including GSH [58] and ATZ phase-2 biotransformation is through conjugation with GSH mediated by glutathione-S-transferase (GST) [59], it seems likely that the depleted GSH levels may be related to the detoxification process. Toxic insult induced ROS generation and subsequent depletion in the levels of GSH and increased MDA levels and GST activity have already been reported in the case of neuronal cells [60,61]. Our results are in agreement with existing studies, suggesting that ATZ-mediated cytotoxicity might be due to the impaired antioxidant defences leading to peroxidative damage. Increased ROS generation is likely to be one among the confounding factors to induce the cytotoxicity [62].

KV treatment significantly recovers ATZ-induced alterations in the levels of ROS, MDA, GSH and catalase activity. It is suggested that KV may have antioxidant and free radical scavenging potential, which works against ATZ-induced stress in PC12 cells. Reports are there showing KV mediated restoration of GSH and antioxidant enzymes levels in gamma-radiation induced gastric ulcers [62,63]. Flavonoids possess *in vitro* antioxidant activity by directly scavenging intracellular ROS; however, more recently, it has been shown that they may also offer indirect protection by enhancing the activities of a number of protective enzymes [64–66]. The direct effect of flavonoids on both activity and DNA transcription of GSH-related enzymes have been shown [66]. The inability of KV to restore the ATZ-induced up-regulation in the mRNA expression and activity of GSH-Px and GR indicate that the antioxidant effects of KV are not regulated through these enzyme systems, rather routed through the scavenging activity of ROS. KV has been reported for its ROS scavenging activity in HepG2 cells and GST expression increasing potential in *in vivo* experiments [36,37,67,68]. Some antioxidant molecules are also reported to scavenge free radicals by raising the levels of GSH-Px and GR [69,70]. The structure of KV plays an important role in its antioxidant effect. Earlier studies on the structure–activity relationships of KV (Figure 1B) have shown that the presence of a hydroxyl group in position three (3-OH) of the C-ring can make it a potent inhibitor of lipid peroxidation [71]. Furthermore, the anti-peroxidative effect of KV may be linked to the presence of a C-4 carbonyl and C-5 and C-7 hydroxyl groups of the A-ring. However, it is suggested that the antioxidant activities of flavonoids are dictated both by their structural features and by their location in the membrane [72]. Flavonoids are known to anchor on the polar head of the main phospholipids. Hence, KV distributed on the surface of the lipid bilayers as well as in the aqueous phase could scavenge free radicals. Our FACS studies suggest that ROS generation in the present study may be a mediator for ATZ-induced apoptosis in PC12 cells. We confirmed the involvement of ROS in ATZ-mediated apoptosis by using an ROS inhibitor DPI, which reverses apoptosis (Figure 6). FACS data also indicates the ROS scavenging capacity of KV in ATZ-exposed PC12 cells (Figure 5D).

We have also observed that KV treatment significantly inhibits the transcriptional expression of caspase-3/9 and activity of caspase-3 cells exposed to ATZ. Apoptosis is a gene-regulated process that involves changes in the expression of anti- and/or pro-apoptotic genes. Bcl-2 acts as an anti-apoptotic gene in parts of the endoplasmic reticulum and mitochondrial membrane and, thus, has been shown to extend cell survival through blocking the apoptosis induced by cell death signals [73]. We also observe a KV mediated significant restoration in the ATZ-induced altered expression of Bcl-2, p53, Bax in PC12 cells. Such findings are indicative that ATZ-induced apoptosis may be mediated through mitochondria and regulated through caspase cascade [48,49,74].

Alteration in the expression of c-Fos and c-Jun are reported to be critical to the development of brain pathology and neurodegeneration associated with exposure to neurotoxins [75]. We demonstrate here that ATZ down-regulates the protein levels of c-Fos and c-Jun proto-oncogenes. This diminution of c-Fos

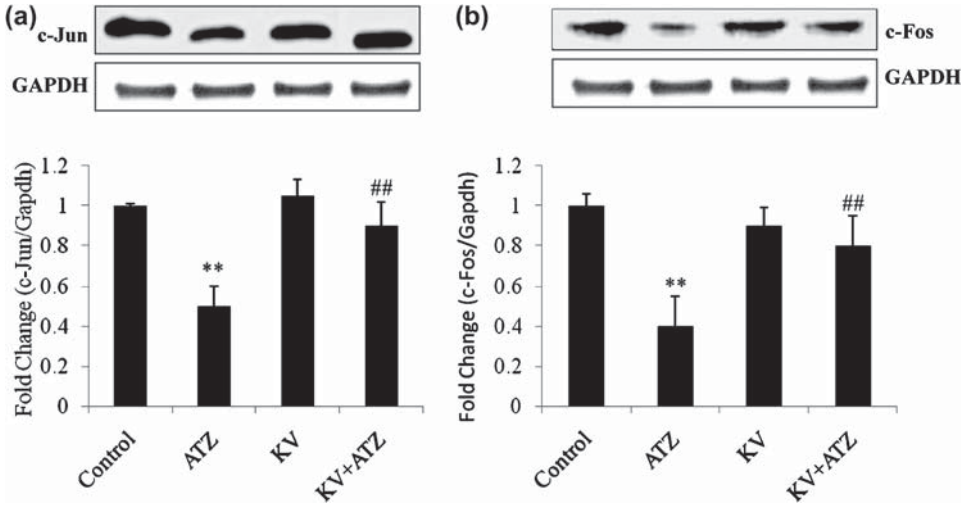

Figure 8. Protective effect of kolaviron (KV) on atrazine (ATZ)-induced alterations in the protein expression of c-Jun and c-Fos (A, B) in PC12 cells. Cells were exposed to KV and ATZ alone or in combination for 24 h. Cells were harvested and subjected to western blotting using respective antibodies. Relative optical density was determined by densitometric analysis. Quantification was done in Gel documentation System (Alpha Innotech) with the help of AlphaEase^{TM} FC StandAlone V. 4.0 software. Each data point represents mean ± SE from three separate experiments. *Relative to control. *Control vs ATZ-exposed, #ATZ-exposed vs KV-treated. */# $p < 0.05$.

and c-Jun expressions may be essentially due to the observed cell death. It is also reasonable to assume that ATZ might modify critical thiol groups in these proteins either by depleting GSH levels or by accumulating electrophillic metabolites such as ROS in PC12 cells leading to their down-regulated expression [76,77]. The recovery in the expression of the c-Fos and c-Jun in the KV + ATZ groups is probably due to the cytoprotective and antioxidant effects of KV. COX-2 like other early response gene products can be induced rapidly and transiently by pro-inflammatory mediators and mitogenic stimuli including cytokines and oncogenes [78,79]. ATZ up-regulated the mRNA expression of COX-2, which could be a result of inflammatory response due to oxidative stress via modifying gene transcription of numerous oxidative stress-sensitive transcription factors, such as NF-kB [80]. The ability of KV to suppress the increased mRNA expression levels of COX-2 could then be related to its anti-inflammatory effects as well as its antioxidant properties [52].

In summary, ATZ exposure for 24 h induces significant alterations associated with cytotoxicity and apoptosis in PC12 cells. KV exposure was found to be capable of significant restoration of most of the ATZ-induced alterations in these cells. This study suggests the therapeutic potential of KV to restore ATZ-induced neuronal damage and brain injury.

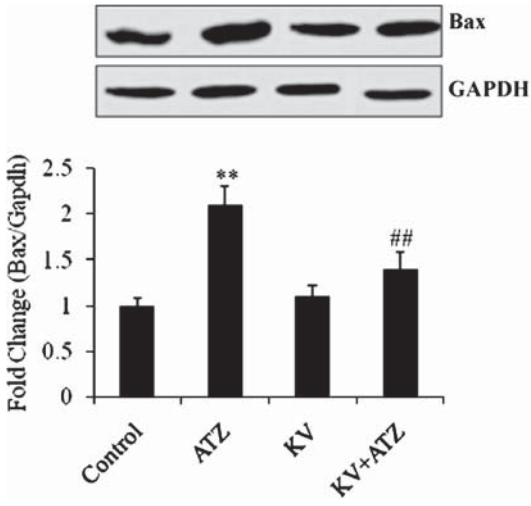

Figure 9. Protective effect of kolaviron (KV) on atrazine (ATZ)-induced alterations in the protein expression of apoptosis marker Bax in PC12 cells. Cells were exposed to KV and ATZ alone or in combination for 24 h. Cells were harvested and subjected to SDS-PAGE and western blotting. Relative optical density was determined by densitometric analysis. Quantification was done in a Gel documentation System (Alpha Innotech) with the help of AlphaEaseTM FC StandAlone V. 4.0 software. Each data point represents mean ± SE from three separate experiments. *Control vs ATZ-exposed, #ATZ-exposed vs KV-treated. */# $p < 0.05$.

Declaration of Interest

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