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Mitigating effects of apigenin on edifenphos-induced oxidative stress, DNA damage and apoptotic cell death in human peripheral blood lymphocytes

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ABSTRACT

Edifenphos (EDF) is an Organophosphorus pesticide and used in agriculture for pest control. However, EDF has been shown to accumulate in agricultural products and causes hazards to human health. Although reports are available regarding environmental impact of EDF, toxic effects of EDF on human cellular system especially immune cells have not been elucidated. In this study, genotoxicity and cytotoxicity of EDF on human peripheral blood lymphocytes and its amelioration by apigenin (dietary flavonoid) was investigated. We demonstrated that EDF inhibited cell viability, and induced oxidative stress and DNA damage in lymphocytes. In addition, results indicate that EDF induced apoptosis in lymphocytes concurrent with ROS generation, loss of mitochondrial membrane potential, up-regulation of Bax and caspase-9/-3 activation. Mechanistically, incubation of lymphocytes with N-acetylcysteine (ROS scavenger) abrogated the ROS generation and apoptosis caused by EDF. These findings suggest that ROS generation by EDF acts as an upstream signal leading to DNA damage and apoptosis in lymphocytes. This study also showed that apigenin could potentially attenuate EDF-induced oxidative stress, DNA damage and apoptosis in lymphocytes. Collectively, these results suggest that EDF exerts cytotoxicity and DNA damage in lymphocytes, and apigenin could be a potent dietary anti-oxidant regimen against EDF-induced toxicity on human health.

1. Introduction

Edifenphos (EDF) (O-ethyl-S, S-diphenyldithiophosphate) (Fig. 1A) is an organophosphate pesticide (OPs) and used as a fungicide in rice agricultural fields (Ahmad and Ahmad, 2018a,b; Kodama et al., 1980). OPs have been categorised as toxicological class I (extremely toxic) by the U.S. Environmental Protection Agency (Tiwari et al., 2013). EDF exposure is toxic to living organisms and such toxicity is attributed to decreased blood cholinesterase activity, inhibition of phosphatidylcholine biosynthesis and suppression of immune response (El-Gendy et al., 1998; Binks et al., 1993; Poul, 1983). Recent study from our lab has also demonstrated the toxic effects of EDF towards human serum albumin (most abundant protein in humans) at the molecular level (Ahmad and Ahmad, 2018a,b).

It has been found that EDF accumulates in different agricultural products such as vegetables and rice (Salamzadeh et al., 2018; Shakouri et al., 2014). The comprehensive evaluation of the EDF profile provides a basis for risk management by controlling pesticide use in different regions around the world and among susceptible populations. Many different techniques have been used to determine EDF profile (data usage, retention, percent recovery) in rice, vegetables, cattle, fish and water (Fujikawa et al., 2009; Hashemi et al., 2017; Ko et al., 2014; Pareja et al., 2011; Shakouri et al., 2014; Yadolahi et al., 2012). The data of EDF profile obtained via techniques include as follows: (1) rice – 20.5 min retention time, % recovery = 85 (spiked at 0.25 μg/g) (Shakouri et al., 2014); paddy rice - concentration (2.1–6.1 mg/kg) (Pareja et al., 2011); tomatoes – 31.3 min retention time, % recovery time = 75.4 (at concentration level 2.5 mg/kg) (Yadolahi et al., 2012); fish – logBCF (BCF stands for bioconcentration factor) = 1.52 (Fujikawa et al., 2009); cattle - % recovery = 96.3 (spiked at 6 mg/kg) (Ko et al., 2014); water = 0.1506 ppb (Hashemi et al., 2017). This information provides us an inference that food safety remains a public issue all over the world, and thus efforts on the large scale should be taken to fill the gaps. In addition, GC-MS analysis has revealed the EDF profile in serum and blood of human population in different countries (Chang et al., 2017; Musshoff et al., 2002).

Although substantial information is available regarding ecological and environmental impact of EDF, knowledge of the toxicity of EDF on human cellular system is insufficient. Further, it has been found that EDF causes chromosomal aberrations in in vivo mouse model (Jayashree et al., 1994). In general, the potential harmful effects of pesticides takes place on entering into systemic blood circulation.

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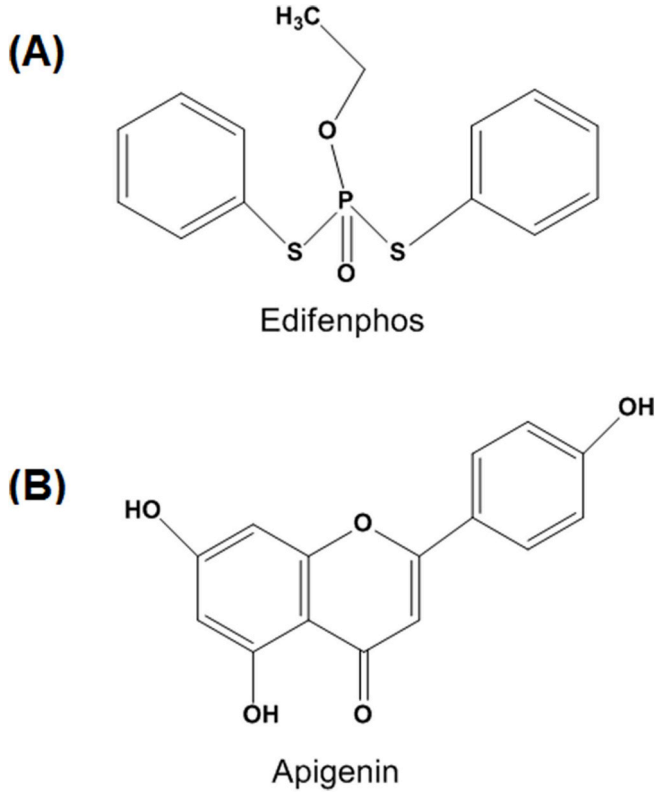

Fig. 1. Chemical structures of edifenphos (A) and apigenin (B).

Hence, it was pertinent to identify the genotoxic and cytotoxic effects of EDF on human peripheral blood lymphocytes.

Apigenin (APG) (4′,5,7-trihydroxyflavone) (Fig. 1B) is a dietary flavonoid belonging to the flavones structural class, present in abundance in fruits and vegetables (Cao et al., 2010). APG has long been considered to have various biological activities such as anti-oxidant, anti-inflammatory and anti-mutagenic (Birt et al., 1986; Cos et al., 1998; Gerritsen et al., 1995). Recently, investigators have found that APG protected rat cortical neuron against neurotoxicity by the inhibition of the caspase pathway (Wang et al., 2001). These findings suggest that APG possesses the excellent scavenging reactive oxygen species (ROS) and inhibiting inflammatory effects, indicating its potential ability as a food supplement to protect against varied toxicants in the foods. In addition, APG is also reported having the protective effect on ischemia/reperfusion-induced rat hepatic necrosis through the regulation of Fas/FasL pathway (Tsalkidou et al., 2014).

Keeping in view the above facts, the present study was aimed to investigate whether EDF induces genotoxic and cytotoxic effects in human peripheral blood lymphocytes in vitro, and later to explore the molecular mechanism of cytotoxicity of EDF. This study would provide an appropriate cytotoxicity model of EDF to study the mitigatory potential of any antioxidant under such stresses. Specifically, our objective was to elucidate the ameliorative and mitigatory functions of apigenin under EDF-induced toxicities in human lymphocytes.

2. Materials and methods

Edifenphos, apigenin, agarose (low melting and normal melting), trypan blue, N-acetylcysteine (NAC), DAPI, methylthiazolydiphenyl tetrazolium bromide (MTT), annexin V-FITC apoptosis detection kit, RPMI 1640, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), dihydroethidium (DHE), Rhodamine 123 and histopaque-1077 were procured from Sigma Chemical Co. (St. Louis, MO, USA). Rest of the chemicals purchased were of highest grade available. Antibodies to Bcl-2, Bax and β-actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). A 3 mM stock solution of EDF was prepared in dimethyl sulfoxide (DMSO). DMSO solution was added to the reaction media at the final concentration of 1% (v/v), which was the highest concentration of DMSO used in the treated samples.

2.1. Isolation and culture of lymphocytes

Experiments on human blood lymphocytes were approved by the Institutional Ethical Committee of the Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, India (714/02/a/CPCSEA). Fresh blood was taken from venipuncture from healthy non-smoking donor with written consent, and the blood so obtained was added in heparinized tubes. Then, blood was diluted in Ca^{2+} - and Mg^{2+} - free PBS. Peripheral blood lymphocytes were isolated from diluted blood using histopaque-1077 method. Isolated lymphocytes were suspended in RPMI 1640 medium supplemented with L-glutamine, 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin. The viability of isolated lymphocytes was checked using trypan blue exclusion test (Pool-Zobel et al., 1993).

2.2. MTT assay for cell viability in lymphocytes

Cell viability was determined using MTT assay (Mosmann, 1983). Briefly, isolated lymphocytes were seeded in a 96-well culture plate at a concentration of 1×10^4 cells per well. Lymphocytes were treated with increasing concentration of edifenphos (EDF) for 4 h at 37 °C in 5% CO_2. After treatment, 10 µl of MTT solution (5 mg/ml) was added to each well and reincubated for 3 h at 37 °C to develop color. Later, 100 µl of DMSO was added to each well to dissolve formazan crystals and absorbance was read at 570 nm using ELISA plate reader (Bio-Rad, USA). Viability of EDF-treated cells was expressed as a percentage of untreated cells (100%). IC_{50} value of EDF for lymphocytes was calculated as the concentration at which 50% of cell viability of cells is inhibited. Subsequently, lymphocytes were pre-treated with doses of 5 µM apigenin or 10 µM apigenin or 5 mM N-acetylcysteine (NAC) (ROS scavenger) for 4 h at 37 °C in 5% CO_2. After this pretreatment, lymphocytes were exposed to IC_{50} concentration of EDF for 4 h at 37 °C in 5% CO_2 and MTT assay was performed again to determine the viability of treated lymphocytes.

2.3. Estimation of intracellular ROS generation

ROS generation, namely hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$), was detected in lymphocytes using molecular probes DCFH-DA and DHE, respectively (Dikalov et al., 2002; Wang; Joseph, 1999). Briefly, lymphocytes were treated in the following groups: (1) Control cells; (2) 5 µM apigenin or 10 µM apigenin alone for 4 h at 37 °C in 5% CO_2; (3) IC_{50} concentration of EDF for 4 h at 37 °C in 5% CO_2; (4) Pretreatment of lymphocytes with 5 µM apigenin or 10 µM of apigenin or 5 mM N-acetylcysteine (NAC) (ROS scavenger) for 4 h, and then treatment with IC_{50} concentration of EDF for 4 h at 37 °C in 5% CO_2. After treatment, cells were stained with 20 µM DCFH-DA for 30 min and 10 µM DHE for 20 min at 37 °C. ROS production in lymphocytes was determined using microplate reader (Bio-Rad, USA) at 485 nm (excitation wavelength) and 528 nm (emission wavelength) for DCFH-DA, and 596 nm (excitation wavelength) and 620 nm (emission wavelength) for DHE. Intracellular ROS generation in lymphocytes was also analyzed using fluorescence microscope (BX43, Olympus, Japan).

Hydroxyl radical generation on treatment with EDF was detected by the method of Quinlan and Gutteridge (1987). In this method, calf thymus DNA (ctDNA) (300 µg) was used as a substrate and generation of malondiaaldehyde from deoxyribose radicals was measured by recording absorbance at 532 nm.

2.4. Lipid peroxidation assay

TBARS generation in treated lymphocytes is an indicator of lipid peroxidation. Lipid peroxidation in lymphocytes was estimated using the method of Ramanathan et al. (1994). Cell pellet of control and treated lymphocytes was obtained, washed in PBS and then dissolved in 0.1 N NaOH. Then, 0.5 ml of 10% TCA and 0.5 ml of 0.6 M TBA was added to the reaction mixture and finally incubated in boiling water for 10 min. Absorbance was read at 532 nm and the OD was converted to nanomoles of TBA-reactive substances using molar extinction coefficient.

2.5. Protein carbonylation assay

Protein carbonyl content in lymphocytes was estimated using 2,4-dinitrophenylhydrazine (2,4-DNPH) method (Nakagawa et al., 2007). In this method, cells were lysed and blended with 10 mM 2,4-DNPH in 2 M HCl. Tubes were incubated at room temperature for 1 h and then 0.5 ml of 20% TCA was added to each tube. After this, tubes were incubated on ice for 10 min and then centrifuged at 12,000g for 10 min. Protein pellets so obtained was washed three times with 1 ml ethanol-ethyl acetate mixture (1:1 v/v). After washing, protein pellets were dissolved in 6 M guanidine HCl (pH 2.3) and then centrifuged at 12,000g for 10 min at 4 °C. Absorbance of supernatant was recorded at 370 nm and carbonyl content was calculated using molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

2.6. Comet assay

DNA breakage in treated lymphocytes was determined using comet assay. Comet assay was performed according to the protocol of Singh et al. (1988). Comet assay protocol consists of following steps: (1) layering of cells on agarose coated slides; (2) cell lysis; (3) un-winding of DNA in alkaline electrophoretic solution; and (4) neutralization step. After complete protocol, slides were stained with EtBr and covered with cover slips. Cells were visualised in fluorescence microscope (CX41, Olympus, Japan) and scored with image analysis system (KOMET 7.1; Kinetic Imaging, UK). Tail length was measured in micrometers to determine extent of DNA damage.

2.7. DAPI staining

DAPI staining was performed to detect nuclear morphology in control and treated lymphocytes. Briefly, cells were incubated with DAPI dye (10 μg/ml) for 20 min at 37 °C in the dark. Images were then captured using fluorescence microscope (BX43, Olympus, Japan).

2.8. Scanning electron microscopy (SEM)

Surface morphology of lymphocytes was determined using scanning electron microscopy as described by Polliack et al. (1973). Briefly, control and treated cells were collected and centrifuged at 10,000 rpm for 10 min. The pellet obtained was washed twice with PBS and then fixed in 1.5% glutaraldehyde solution (pH 7.5) for 30 min, and then postfixed with 1% osmium tetraoxide for 1 h. After fixing, samples were centrifuged again at 10,000 rpm and cell pellet was washed with PBS. Samples were evenly spread on a glass slide, dehydrated in graded alcohol for 5 min and finally critically dried with CO_2. Slides were gold-palladium coated and examined using JEOL JSM-5310 scanning electron microscope (Tokyo, Japan).

2.9. Mitochondrial membrane potential (MMP) analysis

Rhodamine 123 (Rh123), a cationic lipophilic dye was used to detect mitochondrial membrane potential in lymphocytes. The MMP of a cell is proportional to the amount of Rh123 uptake by the mitochondria (Baracca et al., 2003). Briefly, cells were treated in the different reaction groups as mentioned above. After treatment, cells were harvested and washed with twice with PBS. Then, cells were incubated in 20 μM Rh123 for 30 min at room temperature in the dark. Rh123 fluorescence intensity in lymphocytes was evaluated using microplate reader (Bio-Rad, USA) at 505 nm excitation wavelength and 525 nm emission wavelength.

2.10. Determination of caspase-3 and caspase-9 activities

Caspase-3 and caspase-9 activities in untreated and treated lymphocytes were determined using caspase-3 and caspase-9 colorimetric kit, respectively. Briefly, untreated and treated lymphocytes were lysed in 50 μl cold lysis buffer for 10 min. The tubes were centrifuged at 10,000g for 5 min and then supernatant was transferred to a new tube. After this, the protein concentration was quantified in samples. Two hundred microgram of protein was added to 50 μl of 2X reaction buffer (containing 10 mM DTT) to each sample. Then, 5 μl of 4 mM Ac-LEHD-pNA (susbtrate for caspase-9) and DEVD-pNA (substrate for caspase-3) was added and incubated at 37 °C for 2 h. Finally, the absorbance was recorded at 405 nm in a microplate reader (Bio-Rad, USA). Caspase activity in cells was calculated as OD (inducer)/OD (control) and data was expressed as % of control values.

2.11. Western blotting analysis

Lymphocytes were harvested and lysed with ice-cold lysis buffer (150 mM NaCl, 50 mM of pH 7.4 Tris, 1 mM EDTA, 1% Triton X-100, 0.5% SDS, 0.01% PMSF) The cell lysate was centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to new tubes and the concentration of the protein was quantified. Equal amount of lysates (~40 μg) were resolved on an SDS-PAGE and separated proteins were transferred to PVDF membrane (Milipore). Membranes were blocked with 5% non-fat milk in PBS for 1 h at room temperature. Thereafter, the membranes were washed with PBS and incubated with primary antibody for Bcl-2, Bax and β-actin at 4 °C overnight. The target proteins were finally detected using HRP-conjugated secondary antibody. Protein expression was visualised by ECL detection reagent and developed by exposure to X-ray films.

2.12. Statistical analysis

Results are expressed as mean ± SEM for three independent experiments for each assay. Statistical analysis was performed via one-way analysis of variance (ANOVA) using GraphPad Prism software (v 5.01). The significance was set at $p < 0.05$.

3. Results

3.1. Cell viability detection in lymphocytes

MTT method was used to detect the cytotoxic effect of edifenphos on lymphocytes. As shown in Fig. 2A, edifenphos inhibited the cell viability of lymphocytes in a dose-dependent manner. The IC_{50} value for edifenphos on lymphocytes was found to be 17.2 ± 2.1 μM after 4 h treatment. To ascertain the role of ROS in edifenphos cytotoxic action, MTT assay was performed again with IC_{50} concentration of EDF in the presence of N-acetylcysteine (NAC). NAC is a well known ROS-scavenger (Aruoma et al., 1989). Results showed that pretreatment of NAC significantly inhibited the cytotoxic action of edifenphos (Fig. 2B). This result confirmed that ROS plays a significant role in the cytotoxic action of edifenphos. Further, we also checked the inhibitory effect of edifenphos on cell viability in the presence of apigenin (a plant-derived compound). Firstly, treatment with apigenin alone at both

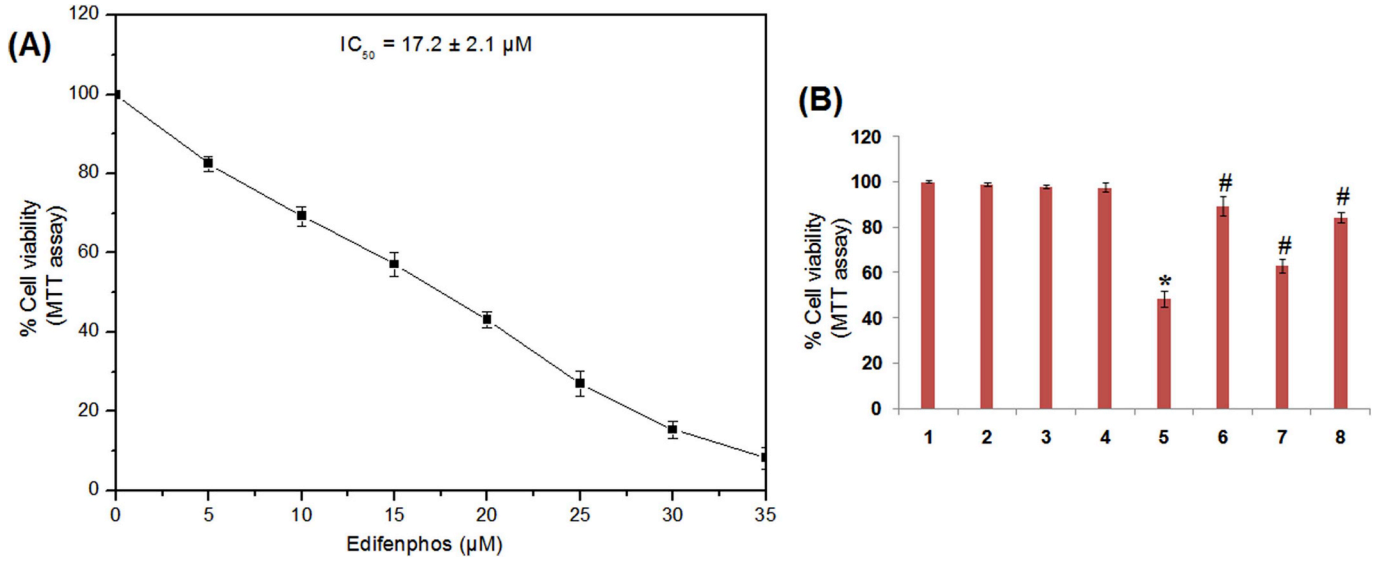

Fig. 2. MTT assay to measure cell viability of lymphocytes. (A) Effect of increasing concentrations of edifenphos (EDF) on the cell viability of lymphocytes and determination of IC_{50} concentration of EDF. Values expressed as mean ± SEM of three independent experiments. (B) Effect of N-acetylcysteine (ROS scavenger) and apigenin treatments on EDF induced cell viability inhibition in lymphocytes. (1) Control, (2) Control Vehicle (DMSO), (3) Apigenin (5 μM), (4) Apigenin (10 μM), (5) EDF (IC_{50}), (6) EDF (IC_{50}) + NAC (5 mM), (7) EDF (IC_{50}) + Apigenin (5 μM), (8) EDF (IC_{50}) + Apigenin (10 μM). Values expressed as mean ± SEM of three independent experiments. *P < 0.05 with respect to control group and #P < 0.05 with respect to EDF (IC_{50}) treated cells.

concentrations i.e. 5 μM and 10 μM, did not affect the cell viability of lymphocytes (Fig. 2B). Further, pretreatment of apigenin at concentrations 5 μM and 10 μM markedly increased the cell viability and reduced the cytotoxic effect of edifenphos (Fig. 2B). Overall, we infer that edifenphos cytotoxic effects involve ROS generation, and apigenin plays an important role in decreasing the cytotoxic action induced by edifenphos.

3.2. Effect of edifenphos on ROS generation

Intracellular ROS generation, namely H_2O_2 and ($O_2^{\cdot-}$), was monitored in treated lymphocytes using DCFH-DA and DHE dyes, respectively. In DCFH-DA assay, fluorescent micrograph showed an increased level of hydrogen peroxide generation in edifenphos-treated lymphocytes (appearance of green fluorescence) (Fig. 3A, Panel 2). On the other hand, pretreatment with NAC abolished hydrogen peroxide generation in edifenphos-treated cells (no green fluorescence) (Fig. 3A, Panel 3). We also found that pretreatment with apigenin at concentrations 5 μM and 10 μM markedly abrogated the hydrogen peroxide generation induced by edifenphos (no green fluorescence) (Fig. 3A, Panels 4 and 5). DHE assay revealed a significant level of edifenphos induced superoxide anion generation in lymphocytes (appearance of red fluorescence) (Fig. 3B, Panel 2). Pretreatment with NAC and apigenin largely quenched the generation of intracellular superoxide (no red fluorescence) in edifenphos-treated cells (Fig. 3B, Panels 3–5). We also found that apigenin treatment alone at 5 μM and 10 μM concentrations did neither generate hydrogen peroxide (no green fluorescence) nor superoxide anions (no red fluorescence) in lymphocytes (Supplementary Figs. S1 and S2). Quantitative ROS (hydrogen peroxide and superoxide anion) generation results obtained via multi-plate reader were in agreement with the above mentioned fluorescence microscopy results (Fig. 3C and D).

Fig. 4 demonstrates the results of hydroxyl radical assay. We found that edifenphos treatment also induced significant levels of hydroxyl radical production in lymphocytes. However, hydroxyl radical production by edifenphos treatment was significantly suppressed in the presence of NAC and apigenin (Fig. 4). In addition, apigenin treatment alone did not induce the generation of hydroxyl radical in lymphocytes (Fig. 4).

3.3. Estimation of lipid peroxidation in lymphocytes

Lipid peroxidation as a biomarker of oxidative stress was estimated in lymphocytes. As shown in Fig. 5A, exposing lymphocytes to IC_{50} concentration of edifenphos leads to increase in the formation of TBARS (lipid peroxidation). On the other hand, a considerable decrease in TBARS generation induced by edifenphos treatment was observed in the presence of NAC and apigenin. In addition, apigenin alone at concentrations 5 μM and 10 μM did not induce TBARS formation in lymphocytes. These results suggest that edifenphos triggered off lipid peroxidation most probably as a result of ROS generation, and apigenin essentially served as an antioxidant to quench edifenphos-induced lipid peroxidation in lymphocytes.

3.4. Estimation of protein carbonylation in lymphocytes

Protein carbonyl content as a result of oxidative damage of proteins was measured using 2,4-DNPH method. Results clearly demonstrate that edifenphos treatment significantly increased the total carbonyl content in the cells (Fig. 5B). Incubation of cells with NAC resulted in diminishing the edifenphos induced protein carbonyl content. Likewise, protein carbonyl content was also inhibited by apigenin pretreatment at 5 μM and 10 μM concentrations in edifenphos-treated cells (Fig. 5B). Interestingly, apigenin treatment alone did not affect the carbonyl content in lymphocytes (Fig. 5B).

3.5. Analysis of DNA damage in lymphocytes

DNA damage in lymphocytes was evaluated using comet assay and the results are shown in Fig. 6. As shown in this figure, the extent of DNA breakage in lymphocytes was significantly enhanced on treatment with edifenphos. On the other hand, pretreatment with NAC resulted in significant inhibition of DNA damage induced by edifenphos in lymphocytes. We also found that apigenin prevented edifenphos induced DNA damage in lymphocytes. Apigenin alone treatment did not lead to any DNA damage in lymphocytes. Taken together, we can infer that edifenphos induces ROS generation in lymphocytes that causes oxidative DNA damage, and apigenin acts as an antioxidant to protect against the oxidative DNA damage in these cells.

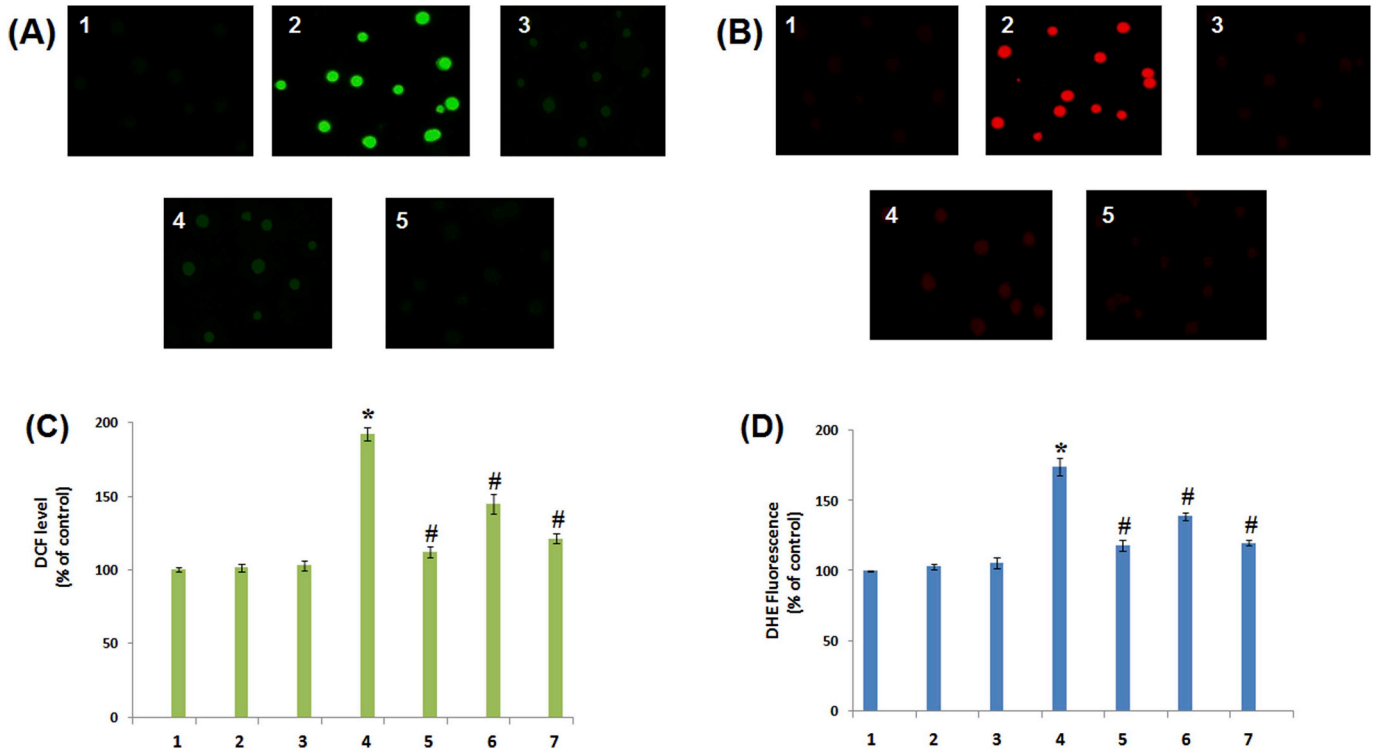

Fig. 3. (A) Photomicrographs showing the intracellular ROS generation (H_2O_2) via DCFH-DA assay in lymphocytes. Panel 1: Control cells, (2) Panel 2: EDF (IC_{50}), Panel 3: EDF (IC_{50}) + NAC (5 mM), Panel 4: EDF (IC_{50}) + Apigenin (5 μM), Panel 5: EDF (IC_{50}) + Apigenin (10 μM). (B) Photomicrographs showing the intracellular ROS generation (O_2^-) via DHE assay in lymphocytes. Panel 1: Control cells, (2) Panel 2: EDF (IC_{50}), Panel 3: EDF (IC_{50}) + NAC (5 mM), Panel 4: EDF (IC_{50}) + Apigenin (5 μM), Panel 5: EDF (IC_{50}) + Apigenin (10 μM). (C) Bar graph representation of data from DCFH-DA assay in lymphocytes. (1) Control, (2) Apigenin (5 μM), (3) Apigenin (10 μM), (4) EDF (IC_{50}), (5) EDF (IC_{50}) + NAC (5 mM), (6) EDF (IC_{50}) + Apigenin (5 μM), (7) EDF (IC_{50}) + Apigenin (10 μM). *P < 0.05 with respect to control group and #P < 0.05 with respect to EDF (IC_{50}) treated cells. (D) Bar graph representation of data from DHE assay in lymphocytes. (1) Control, (2) Apigenin (5 μM), (3) Apigenin (10 μM), (4) EDF (IC_{50}), (5) EDF (IC_{50}) + NAC (5 mM), (6) EDF (IC_{50}) + Apigenin (5 μM), (7) EDF (IC_{50}) + Apigenin (10 μM). *P < 0.05 with respect to control group and #P < 0.05 with respect to EDF (IC_{50}) treated cells.

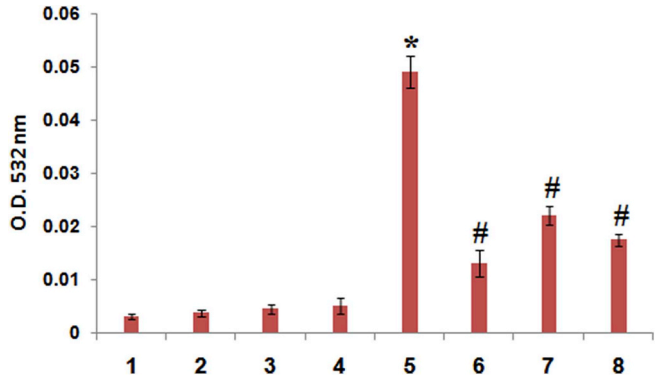

Fig. 4. Detection of hydroxyl radical generation in treated lymphocytes. (1) Control, (2) Control vehicle (DMSO), (3) Apigenin (5 μM), (4) Apigenin (10 μM), (5) EDF (IC_{50}), (6) EDF (IC_{50}) + NAC (5 mM), (7) EDF (IC_{50}) + Apigenin (5 μM), (8) EDF (IC_{50}) + Apigenin (10 μM). Values expressed as mean ± SEM of three independent experiments. *P < 0.05 with respect to control group and #P < 0.05 with respect to EDF (IC_{50}) treated cells.

3.6. DAPI staining in lymphocytes

DAPI staining was performed to detect apoptotic nuclei in treated lymphocytes. Apoptotic cells are characterized by chromatin condensation and nuclear fragmentation (Saraste and Pulkki, 2000). Results of DAPI staining clearly shows that untreated lymphocytes have round nuclei and there was no fragmentation and condensation (Fig. 7). On treatment with edifenphos, DNA fragmentation was observed in lymphocytes indicating the induction of apoptosis. Pretreatment with NAC abrogated DNA fragmentation induced by edifenphos in lymphocytes. Further, we also found that apigenin pretreatment was able to suppress DNA damage in edifenphos treated lymphocytes.

3.7. Morphological assessment by scanning electron microscopy (SEM)

SEM was employed to detect morphological changes in treated lymphocytes (Fig. 8). Results clearly show that the untreated cells are round and the surface morphology is homogenously smooth (Fig. 8, Panel A). Lymphocytes treated with edifenphos led to the development of blebs on membrane surface (Fig. 8, Panel B) along with a clear-cut plasma membrane disruption (Fig. 8, Panel C). Addition of NAC and apigenin inhibited the cellular damage induced by edifenphos and largely reversed the cell morphology to spherical shape (no blebs and membrane damage) (Fig. 8, Panels D–F). In addition, we also affirmed that apigenin treatment alone did not affect the surface morphology in lymphocytes, and the cells retained the spherical shape with no blebs and membrane damage (Supplementary Fig. S3).

3.8. Analysis of mitochondrial membrane potential (MMP) in lymphocytes

Loss of mitochondrial membrane potential is a characteristic feature of apoptosis induction in cellular system (Ly et al., 2003). To explore the effect of edifenphos on MMP in lymphocytes, fluorescent probe Rhodamine 123 (Rh123) was used. As shown in Fig. 9, Rh123 fluorescence intensity was significantly decreased in lymphocytes on treatment with IC_{50} concentration of edifenphos. Further, we found that pretreatment of NAC and apigenin ameliorated the loss of MMP (decrease in Rh123 fluorescence intensity) induced by edifenphos in

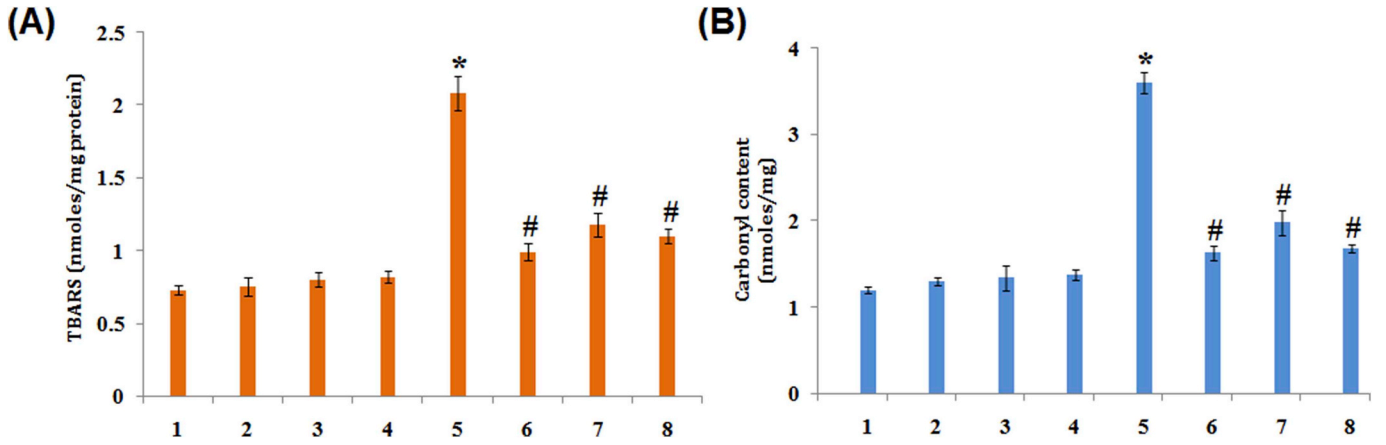

Fig. 5. Estimation of lipid peroxidation (A) and protein carbonylation (B) in control and treated lymphocytes. (1) Control, (2) Control Vehicle (DMSO), (3) Apigenin (5 μM), (4) Apigenin (10 μM), (5) EDF (IC_{50}), (6) EDF (IC_{50}) + NAC (5 mM), (7) EDF (IC_{50}) + Apigenin (5 μM), (8) EDF (IC_{50}) + Apigenin (10 μM). Values expressed as mean ± SEM of three independent experiments. *P < 0.05 with respect to control group and #P < 0.05 with respect to EDF (IC_{50}) treated cells.

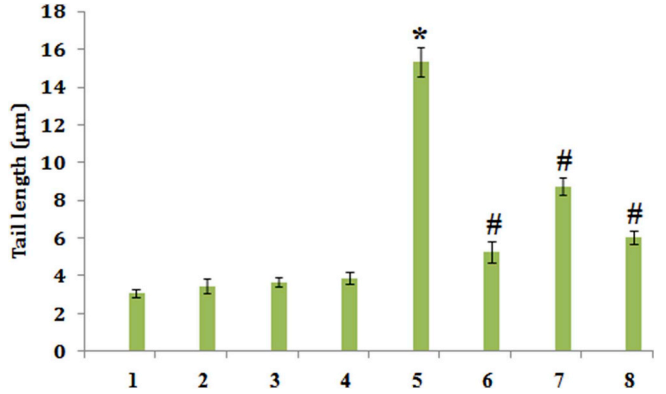

Fig. 6. Analysis of DNA damage by comet assay. Data is expressed in terms of tail length (μm). (1) Control, (2) Control vehicle (DMSO), (3) Apigenin (5 μM), (4) Apigenin (10 μM), (5) EDF (IC_{50}), (6) EDF (IC_{50}) + NAC (5 mM), (7) EDF (IC_{50}) + Apigenin (5 μM), (8) EDF (IC_{50}) + Apigenin (10 μM). Values expressed as mean ± SEM of three independent experiments. *P < 0.05 with respect to control group and #P < 0.05 with respect to EDF (IC_{50}) treated cells.

lymphocytes. These results confirm that edifenphos induces ROS generation which causes loss of MMP in lymphocytes, and apigenin has a potential to attenuate edifenphos-induced effect on MMP.

3.9. Caspase-3 and caspase-9 activities in lymphocytes

Caspase-3 and caspase-9 activities in lymphocytes were evaluated using caspase colorimetric detection kits. As shown in Fig. 10, caspase-3 and caspase-9 activities were significantly increased on treatment with IC_{50} concentration of edifenphos. However, pretreatment of lymphocytes with NAC and apigenin significantly inhibited the caspase-3/-9 activation via edifenphos treatment (Fig. 10). These results indicate that edifenphos treatment induces ROS generation which leads to caspase-3/-9 activation in lymphocytes. Our results also suggest that apigenin attenuates edifenphos-induced caspase activation in lymphocytes.

3.10. Analysis of Bcl-2 and Bax expression in lymphocytes

Bcl-2 and Bax expressions were evaluated in lymphocytes using western blotting (Fig. 11). Blotting results clearly show that expression of Bax (pro-apoptotic protein) and Bcl-2 (anti-apoptotic protein) was significantly up-regulated and down-regulated, respectively on treatment with IC_{50} concentration of edifenphos. On the other hand, addition of NAC and apigenin under our experimental conditions decreased Bax activation and increased Bcl-2 expression in edifenphos-treated lymphocytes. Based on our results of MMP, caspase-3/-9 activities and Bcl-2/Bax expression results, we suggest that edifenphos induces mitochondrial-mediated apoptotic pathway in lymphocytes, and apigenin plays a significant mitigatory role in this system.

4. Discussion

Edifenphos (EDF) belongs to the group of organophosphate pesticide and has been used for pest control in rice fields (Ahmad and Ahmad, 2018a,b; Kodama et al., 1980). EDF has been reported to accumulate in different agricultural products (vegetables and rice) (Salamzadeh et al., 2018; Shakouri et al., 2014). In general, increasing application of pesticides has raised a concern of harmful effects to humans and other living species. Further, large scale usage of pesticides increases the prospect of interaction and damage to genetic material (Kaur et al., 2011). As a result, it is prudent to perform an elaborate genotoxic study of pesticides. Till date, there is no study revealing the genotoxic and cytotoxic effects of EDF on human cellular system especially human lymphocytes. Hence, this study investigated the genotoxic and cytotoxic behaviour of EDF on human peripheral blood lymphocytes under in vitro conditions.

Our study showed that EDF decreases the cell viability of lymphocytes in a dose-dependent manner. We also demonstrated that the underlying mechanism of cytotoxic effects of EDF relies largely on the mitochondrial apoptotic pathway. Earlier literature reveals that enhanced ROS production is responsible for damaging cellular biomolecules (protein, DNA) (Abdollahi et al., 2004; Lukaszewicz-Hussain, 2010). In this context, we evaluated the intracellular ROS generation (superoxide anion and hydrogen peroxide) in lymphocytes on treatment with EDF. Our results showed that EDF was potent enough to induce superoxide anion and hydrogen peroxide in lymphocytes, and N-acetylcysteine was effective in abrogating the ROS generation. Malondialdehyde (MDA), a product of lipid peroxidation, has been extensively used as marker of oxidative stress (Ahmad and Ahmad, 2017; Khan et al., 2018; Yoshida et al., 2013; Zafar et al., 2016). Thus, EDF treated lymphocytes were also checked for MDA levels and our findings clearly showed that ROS mediated MDA formation was significantly increased in lymphocytes by EDF treatment. This effect of increased lipid peroxidation was inhibited in the presence of NAC, a ROS scavenger. Hence, we can suggest that EDF induces oxidative stress in lymphocytes. We further provide evidence that EDF exposure causes DNA damage in lymphocytes. Interestingly, DNA damage induced by

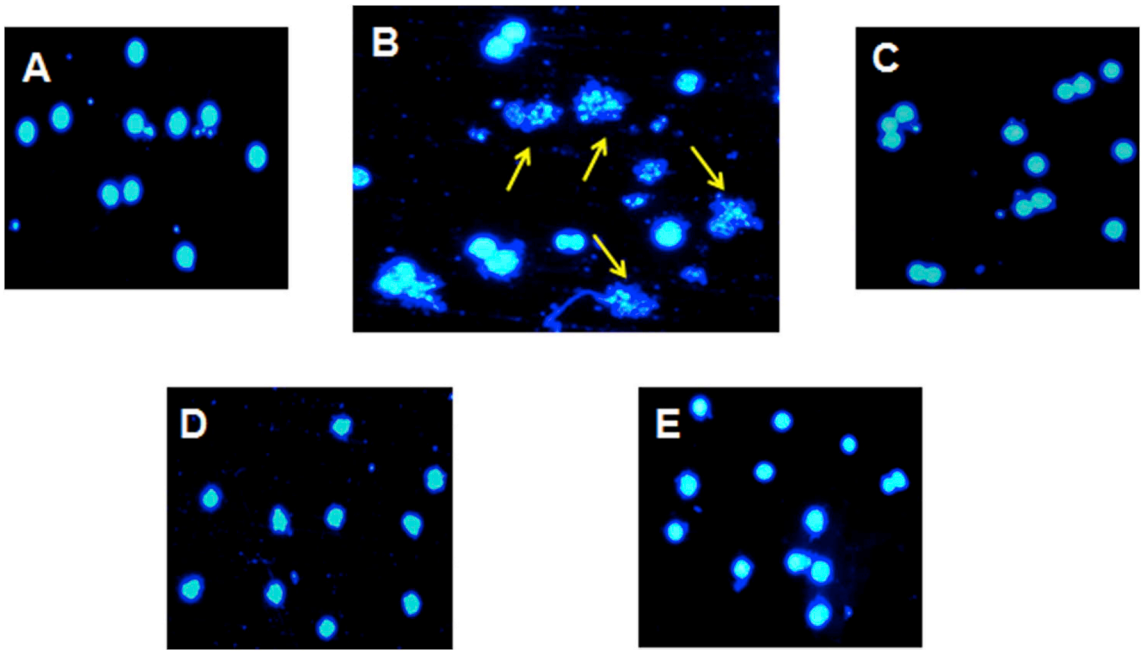

Fig. 7. DAPI staining for apoptosis analysis in lymphocytes. Panel A: Control, Panel B: EDF (IC_{50}), Panel C: EDF (IC_{50}) + NAC (5 mM), Panel D: EDF (IC_{50}) + Apigenin (5 μM), Panel E: EDF (IC_{50}) + Apigenin (10 μM). Arrows in panel B indicate DNA fragmentation.

EDF was significantly inhibited by N-acetylcysteine. This outcome provides an inference that ROS generation induced by EDF is responsible for its genotoxic action.

Apoptosis induction in cellular system is characterized by DNA fragmentation (Elmore, 2007). DAPI staining and comet assay results confirmed the onset of apoptosis as evidenced by DNA fragmentation in EDF-treated lymphocytes, and NAC treatment was effective in inhibiting the DNA damage. Overall, it can be inferred that ROS generation by EDF plays a key role in DNA damage and apoptosis induction in lymphocytes.

Later, we also deciphered the molecular mechanism underlying apoptosis induction by EDF in lymphocytes. Apoptosis in cells can be initiated through two pathways; the intrinsic pathway which is mediated by mitochondria and extrinsic pathway which requires the cell surface receptor (Fulda and Debatin, 2006; Fulda, 2014). Mitochondrial-dependent intrinsic apoptotic pathway is regulated by the members of the Bcl-2 family i.e. pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins, and the ratio of Bax/Bcl-2 determines the cell survival or cell death (Lopez and Tait, 2015). It is widely characterized that Bax protein targets mitochondria membrane permeability, and the major consequence of the change of permeability is the loss of mitochondrial membrane potential (ΔΨm) (Er et al., 2006). This disruption of mitochondrial membrane potential is the key point in apoptosis and further facilitates the activation of caspases 9/3 cascade and apoptosis induction (Anuradha et al., 2001; Chen et al., 2007). In view of this, Rh123 staining results clearly showed that EDF treatment causes loss of

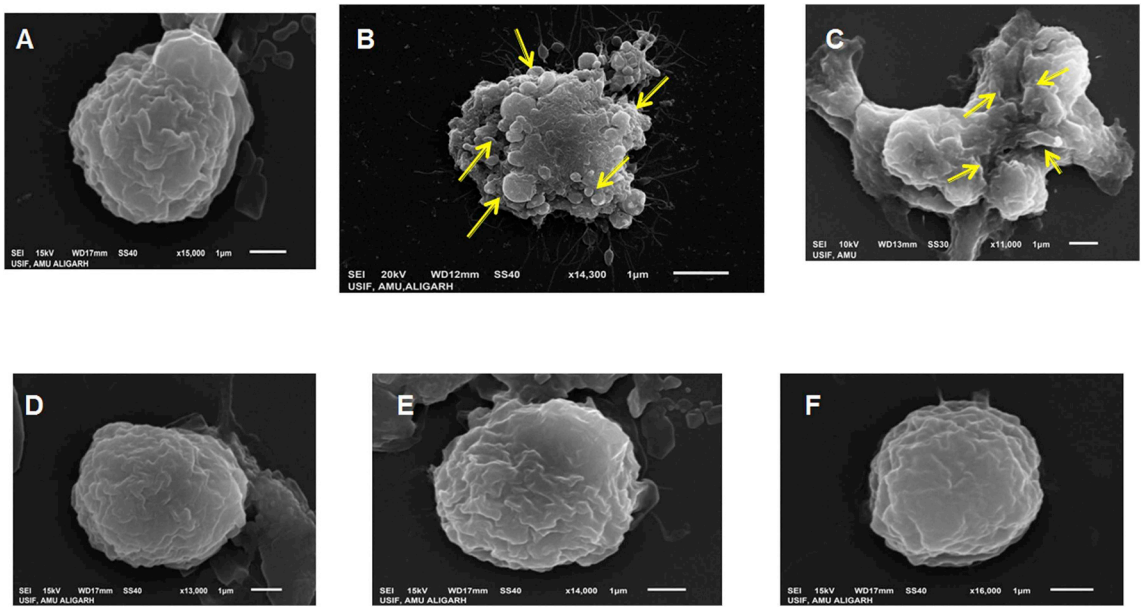

Fig. 8. Scanning electron microscopy of control and treated lymphocytes. Panel A: Control, Panels B and C: EDF (IC_{50}), Panel D: EDF (IC_{50}) + NAC (5 mM), Panel E: EDF (IC_{50}) + Apigenin (5 μM), Panel F: EDF (IC_{50}) + Apigenin (10 μM). Arrows in panels B and C indicate blebbing and membrane damage, respectively.

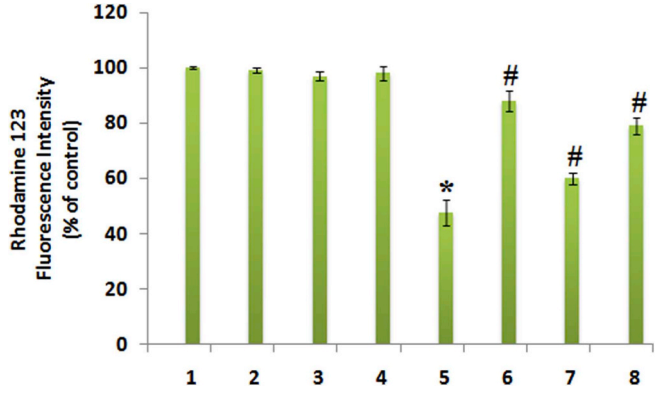

Fig. 9. Estimation of mitochondrial membrane potential (MMP) via Rhodamine 123 staining in lymphocytes. (1) Control, (2) Control vehicle (DMSO), (3) Apigenin (5 μM), (4) Apigenin (10 μM), (5) EDF (IC_{50}), (6) EDF (IC_{50}) + NAC (5 mM), (7) EDF (IC_{50}) + Apigenin (5 μM), (8) EDF (IC_{50}) + Apigenin (10 μM). Values expressed as mean ± SEM of three independent experiments. *P < 0.05 with respect to control group and #P < 0.05 with respect to EDF (IC_{50}) treated cells.

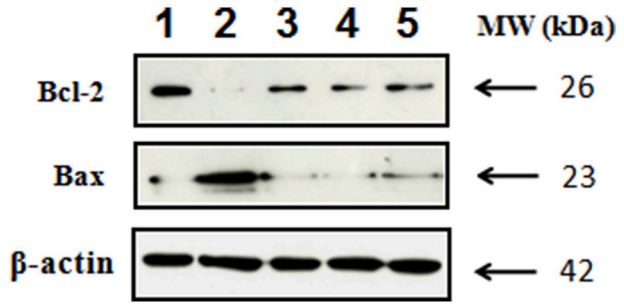

Fig. 11. Western blot showing the expression levels of Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) in control and treated lymphocytes. (1) Control, (2) EDF (IC_{50}), (3) EDF (IC_{50}) + NAC (5 mM), (4) EDF (IC_{50}) + Apigenin (5 μM), (5) EDF (IC_{50}) + Apigenin (10 μM). β-Actin is used as a loading control.

mitochondrial membrane potential (ΔΨm) in lymphocytes. Further, we also found that loss of mitochondrial membrane potential by EDF in lymphocytes was abrogated in the presence of NAC. Our results also showed that EDF down-regulates Bcl-2 protein expression and up-regulates Bax protein expression in treated lymphocytes. This effect was reversed to up-regulation of Bcl-2 and down-regulation of Bax in EDF-treated lymphocytes by NAC. Also, increased caspase 9/3 activity was observed in EDF-treated lymphocytes, and NAC was able to inhibit the activities of both the caspases. Overall, we can infer that EDF induces ROS generation which increases Bax/Bcl-2 ratio concomitant with the loss of ΔΨm and consequently the activation of caspases 9/3 to trigger apoptotic cascade in lymphocytes.

Flavones are plant-derived polyphenolic compounds that are commonly used in the diet. Apigenin is a flavone which is ubiquitously distributed in leaves, vegetables, stems and fruits of various vascular plants (Yan et al., 2017). It is cell permeable and exhibits a variety of pharmacological properties including antioxidant effects (Choi et al., 2004; Patel et al., 2007). Earlier studies have shown that apigenin exhibits antigenotoxic effects against anticancer drugs in cultured human lymphocytes (Siddique et al., 2008), and also in mouse-bone marrow cells (Siddique and Afzal, 2009a,b). Apigenin has also shown to exhibit protective effects against genotoxicity of hydrogen peroxide in cultured human lymphocytes (Siddique and Afzal, 2009a,b).

In accordance with the notion pertaining to protection of apigenin against oxidative stress as mentioned above, the present study in fact was also aimed to investigate the effect of apigenin on ROS-dependent EDF-induced cytotoxic and genotoxic effects in human lymphocytes. Pretreatment of apigenin results in dose-dependent increase in cell viability of EDF-treated lymphocytes concomitant with a proportional decline in their ROS generation. In addition, we also found that apigenin pretreatment was able to inhibit EDF-induced lipid peroxidation and protein carbonylation as well as DNA damage in lymphocytes. Pretreatment of apigenin was also effective in inhibiting the mitochondrial-mediated apoptosis induced by EDF in lymphocytes. All these results lead us to suggest that underlying mechanism of the protective action of apigenin against EDF-induced toxicity probably operates through free-radical scavenging activity of apigenin. The free radical scavenging property of apigenin may be attributed to the hydroxyl groups at 4th, 5th and 7th position of the molecule (Noroozi et al., 1998). The antioxidant property of apigenin may also be attributed to the double bonds between carbon atoms 2 and 3 of the carbon ring (Ratty and Das, 1988). Thus, apigenin may be used as a potent antioxidant regimen against organophosphate pesticide induced cytotoxic and genotoxic effects in human cellular systems.

In conclusion, our data provides evidence that EDF decreases cell viability, induces DNA damage and cell death via activation of mitochondrial mediated intrinsic apoptotic pathway in lymphocytes. The EDF-induced DNA damage and apoptosis is mediated by elevated levels of ROS production. Overall, we propose that EDF-induced ROS generation is an upstream event which then leads to oxidative stress, DNA damage and apoptotic cell death. Further, the same study also demonstrated that apigenin potentially attenuates EDF-induced oxidative

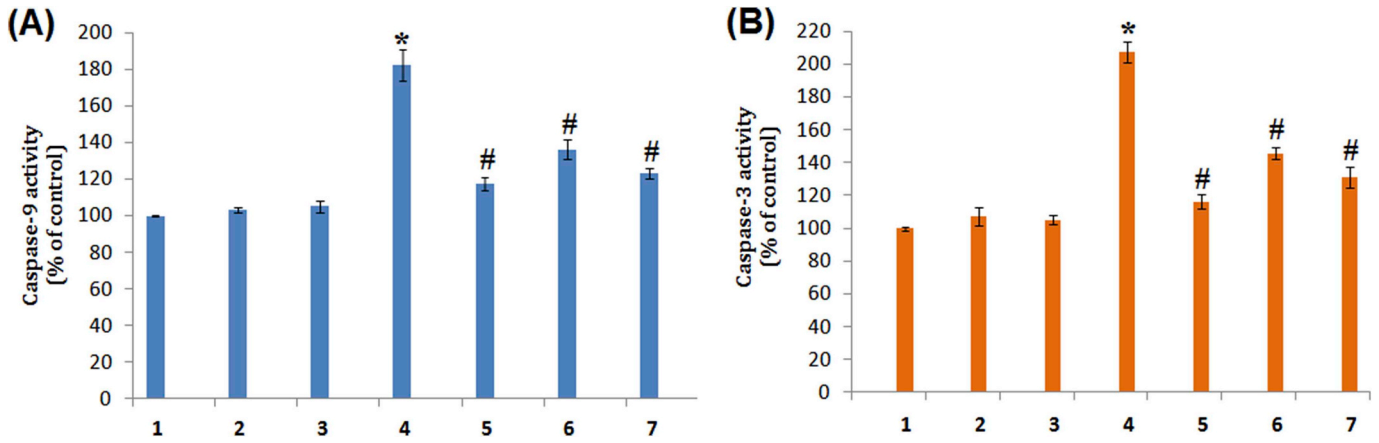

Fig. 10. Estimation of apoptosis via caspase-3 activity (A) and caspase-9 activity (B) in lymphocytes. (1) Control, (2) Apigenin (5 μM), (3) Apigenin (10 μM), (4) EDF (IC_{50}), (5) EDF (IC_{50}) + NAC (5 mM), (6) EDF (IC_{50}) + Apigenin (5 μM), (7) EDF (IC_{50}) + Apigenin (10 μM). Values expressed as mean ± SEM of three independent experiments. *P < 0.05 with respect to control group and #P < 0.05 with respect to EDF (IC_{50}) treated cells.

stress and apoptosis in human lymphocytes. This study supports the view of using apigenin as a dietary supplement against EDF-induced cytotoxic and genotoxic effects. Further, investigations are in progress in our laboratory to test the in vivo toxicity of EDF and examine the protective effect of apigenin in rodent model.

Conflicts of interest

The authors confirm that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.03.034.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.03.034

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