Protective effect of black tea extract during chemotherapeutic drug induced oxidative damage on normal lymphocytes in comparison with cancerous K562 cells

Debjani Ghosh, Subrata Kumar Dey, Chabita Saha

Abstract— Daunomycin and adriamycin have been widely used as chemotherapeutic drugs against varied number of cancers, however, non-targeted cytotoxicity has been limiting their therapeutic ratios. In the present study, protective effect of black tea, rich in natural antioxidants has been investigated against drug induced oxidative damage in normal lymphocytes and compared with erythroleukemic K562 cells. Pre-treatment with black tea extract (BTE) significantly reduced loss of cell viability, generation of ROS, mitochondrial dysfunction, DNA damage, activation of caspase-3 and apoptosis in normal lymphocytes compared to K562 cells. HPLC analysis confirms that extracellular BTE penetrate the cell membrane in both types of cells and also regulate the activity of endogenous antioxidant enzymes. The changes in the mRNA expression of bax, bcl2, p53 and Nrf2 were also followed to evaluate regulation of drug induced apoptosis by BTE. The findings demonstrate that black tea is a promising chemo-protective agent which can be supplemented along with chemotherapeutic drugs to reduce oxidative damage to non-targeted cells.

Index Terms— Antioxidants, Apoptosis, Black Tea, Chemotherapeutic drugs, Oxidative stress, Reactive oxygen species, Apoptosis.

1 INTRODUCTION

MYELOID leukemia is a heterogeneous group of diseases characterized by uncontrolled proliferation of neoplastic hematopoietic precursor cells and impaired production of normal hematopoiesis leading to neutropenia, anemia, and thrombocytopenia [1]. The anthracyclines are among the most effective anticancer treatments ever developed and are effective against more types of cancer than any other class of chemotherapeutic agents. Among them daunorubicin (DNM) and adriamycin (ADR) are the major antitumor agents widely used in the treatment of myeloid leukemia [2-5]. However, their mechanism of action is still not fully understood. It is generally postulated that most of these drug induced cytotoxicity is related to DNA intercalation, their interaction with nuclear topoisomerase II and generation of free radicals like reactive oxygen species (ROS) [6-8]. Therefore, it is believed that chemotherapeutic drugs are capable of inducing oxidative stress mediated apoptosis in malignant cells. The major limitation of these drugs is non-targeted cytotoxicity thus compromising therapeutic ratios. The use of antioxidants in the form of dietary supplement during conventional chemotherapy can be a potential measure to reduce non-targeted cytotoxicity generated by the drugs.

Black and green teas are rich in flavonoids like thearubigin, catechins, epicatechins etc which are known to have much higher antioxidant activity than vitamin C. Tea polyphenols are effective scavengers of reactive oxygen species (ROS) *in vitro* and may also function indirectly as antioxidants through their effects on transcription activities [13]. The anticarcinogenic effects of tea polyphenols have been amply demonstrated in a number of animal models involving tumors of the lung, digestive tract, prostate, bladder, mammary glands and skin [14]. Use of green tea as pro-oxidant is documented for prostate and breast cancer where epigallocatechin gallate (EGCG) had been identified to be the active component [15, 16]. Therefore, tea polyphenols can act like a double edged sword owing to their dual role as antioxidants and pro-oxidants but question remains regarding the relevance of their use as a supplement during cancer therapy. To this end we have demonstrated that black tea extract (BTE) (5 µg/ml) as an efficient radio-protector for V79 cells [17] and normal lymphocytes [18].

In extension to these findings, in the present study, the protective effect of BTE was tested during chemotherapeutic drug (DNM/ADR) induced oxidative damage in normal lymphocytes and compared with cancerous K562 cells. The effect of BTE against drug induced apoptosis and cell viability in both types of cells was evaluated. In the cells, BTE uptake was monitored by HPLC and intracellular ROS levels and restoration of mitochondrial membrane potential (MMP) were rec-

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orded flow cytometrically. The effect of BTE on the drug induced endogenous antioxidant enzyme activity of SOD, CAT and GST was monitored. The mRNA expression of transcription factor NF-E2-related factor 2 (Nrf-2) was also studied as it plays a critical role in trans-activating phase II enzyme expression and thus has become an important therapeutic target for antioxidants against oxidative stress [19-22].

2 MATERIALS AND METHODS

2.1 Ethical statement

The design of experiments with human blood samples were approved by Institutional Ethics Committee by West Bengal University of Technology and informed written consent had been obtained from each person.

2.2 Chemicals

Adriamycin (ADR), Daunomycin (DNM), dimethyl sulphoxide (DMSO), Histopaque HP 1077, 2′, 7′ Dichlorofluorescein diacetate (DCFDA), Rhoadamine 123 (Rh123), 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), Epicatechin (EC), Epicatechin gallate (ECG), Epigallocatechin (EGC), Epigallocatechin gallate (EGCG), Caffeine, Quercetin, Myricetin, Kaempferol, Pyrogallol, 1 chloro 2, 4 dinitrobenzene (CDNB) and 4′ 6 diamidino -2-phenylindole (DAPI) were obtained from Sigma Aldrich Company (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) medium, Fetal Bovine Serum (FBS) and Pen-Strep antibiotic were purchased from Gibco (Grand Island, NY). Annexin V-FITC apoptosis detection kit and caspase-3 detection kit were obtained from BD Biosciences (Franklin Lakes, NJ). All other reagents used were of analytical reagent grade. All the experimental solutions were prepared in MilliQ water.

2.3 Cell line and culture

K562 cells were obtained from National Centre for Cell Sciences (NCCS), Pune, India and were maintained in RPMI medium supplemented with 10% FBS and 1% Pen-Step antibiotic in 25 cm^2. Normal human lymphocytes were isolated from peripheral blood using HP 1077 according to the method of Boyum, [23]. All cells were maintained in 37°C in a humified atmosphere of 5 % CO_2 in air.

2.4 Preparation of Black Tea Extract (BTE)

Preparation of hot water black tea extract (BTE) was done as described previously by Ghosh et al. [18]. Briefly, Tea leaves (1 gm) were infused in 50 ml of boiling Milli-Q water for 15 minutes. Then the infusion was centrifuged at 12,000 rpm for 10 mins and the filtered supernatant (using 0.22 µm syringe filter) was lyophilized. 1 mg/ml lyophilized hot water BTE in Milli-Q water was used as stock solution from which experimental solutions were made by dilution.

2.5 Treatment procedure

K562 cells and normal lymphocytes were treated with or without BTE prior to the exposure of the drugs. The stock solutions of the drugs were prepared in phosphate buffer (pH-6.5) and 5 % DMSO was used as and when required. Stock solutions were further diluted by the same to the experimentally required concentrations and stored at 4°C. Clinically used concentration of these drugs (300 nM) was used in all studies.

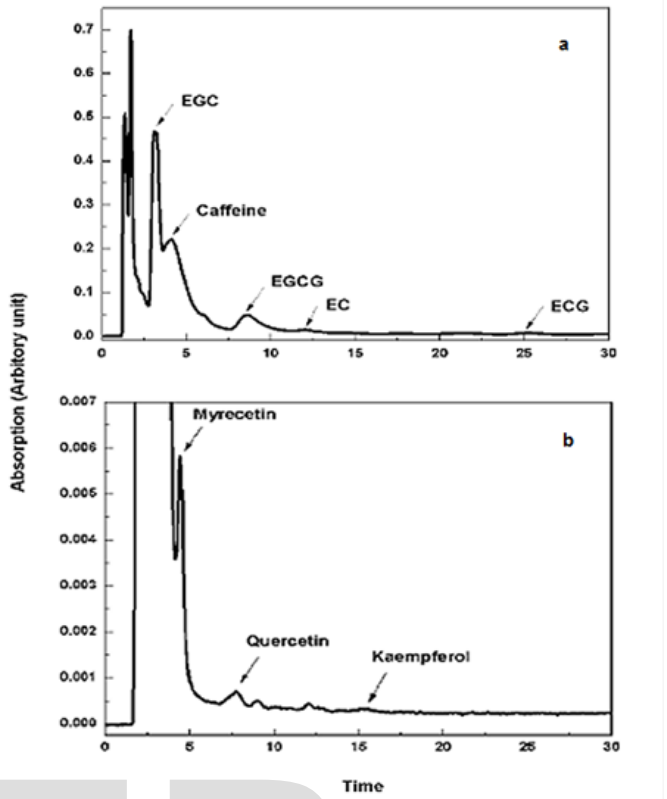

Fig 1. Chromatograms of BTE in a mobile phase consisting of (a) 50 % methanol w/ orthophosphoric acid at 210 nm for identification of catechins and caffeine and (b) 20 % methanol w/ trifluoacetic acid at 365 nm for identification of flavanols.

2.6 Analytical determination of tea polyphenols present in BTE using HPLC

Various polyphenols present in the BTE used were analysed by Waters HPLC system equipped with a photodiode array detector. A Waters NovaPak C18 (3.9 mm x 150 mm, 4 µm) reversed phase (RP) column with a guard column also packed with Waters NovaPak C18 was used in all the experiments. Tea catechins and caffeine were separated by isocratic elution system with a flow rate of 1.0 ml/min using a mobile phase of water/methanol/orthophosphoric acid (79.9/20/0.1 v/v) and UV detection (210 nm) whereas 50% (v/v) methanol (pH 2.5 with Trifluoroacetic acid) was used at 365 nm for detecting flavanols like quercetin, myricetin etc [24, 25]. Detection and tentative identification of major tea polyphenols was accomplished by comparing their retention time and UV spectrum with those of the references standards.

2.7 Cellular uptake of tea catechins by HPLC

Cellular uptake of tea catechins in normal lymphocytes and K562 cells was assessed by HPLC. Briefly, both types of cells

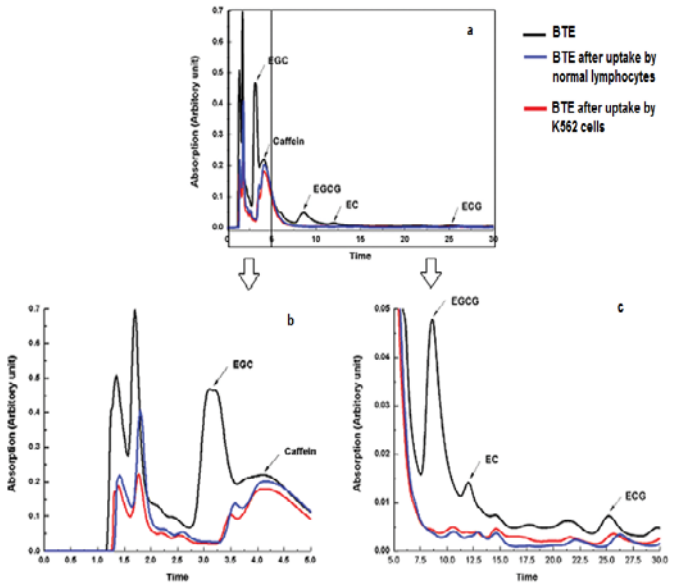

Fig 2. Overlaid chromatograms of 2.5 mg/ml BTE for 0-30 mins (a) in native state and after incubation of normal lymphocytes and K562 cells in it for 30 mins, separately. Zoomed chromatograms of the same from (b) 0 to 5 mins and (c) 5 to 30 mins.

($5x10^5$ cells/ml) were incubated in PBS (pH 7.4) with BTE (2.5 mg/ml) separately for 30 mins at 37°C. The experimental solutions were then centrifuged so that the cells settled at the bottom. The supernatant from both types of test solutions was filtered using 0.22 µm syringe filter and the filtrate was analyzed using the same HPLC settings, used for identifying the polyphenols of BTE. The chromatograms of both the samples were compared with the chromatogram of native samples of BTE (2.5mg/ml).

2.8 Assesment of cell cytotoxicity by MTT assay

Cytotoxicity was measured by viability and cell proliferation assay by measuring the ability of the cells to cleave the soluble compound 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into an insoluble salt. The ability of cells to cleave MTT is indicative of the degree of mitochondrial/cellular respiration within those cells. Following the cell treatment protocol, the level of MTT was quantified by the method described by Mosmann [26] with slight modification. Briefly, the cells were treated with various concentrations of BTE (0, 1, 5, 10 µg/ml) for 30 mins at 37°C. After washing twice with PBS (pH 7.4), cells were treated with ADR/DNM for 24 hrs. At the end of the stipulated time the cells were washed again and incubated in 1 ml PBS (pH 7.4) with MTT (50 µl of 5 mg/ml stock solution) into each sample at 37°C for 2 hrs. After incubation, the purple coloured precipitate of formazan salt was dissolved in 150 µl of DMSO. Absorbance of each sample was recorded at 540 nm with a Varian Spectrophotometer with a reference serving as blank.

2.9 Flow cytometric measurement of intracellular ROS and MMP

The intracellular ROS and MMP in K562 cells and normal lymphocytes were examined by flow cytometry, using DCFDA and Rh123 respectively [27, 28]. Briefly, the cells (5x105 cells/ml) were treated with various concentrations of BTE (0, 1, 5, 10 µg/ml) for 30 mins at 37°C. The cells were then washed twice in PBS (pH 7.4) and incubated in drugs (ADR/DNM) for 2 hrs. After incubation the cells were washed again and re-suspended in PBS (pH 7.4) and incubated with 10 µM DCFDA/Rh123 at 37°C for 30 mins. The detected changes of ROS and MMP were then analyzed by flow cytometry (Becton Dickinson FACSAria) using an excitation wavelength of 488 nm. At least 20,000 events were analyzed for each experiment. Gating was done from the FSC-SSC plot of normal lymphocytes and K562 cells respectively.

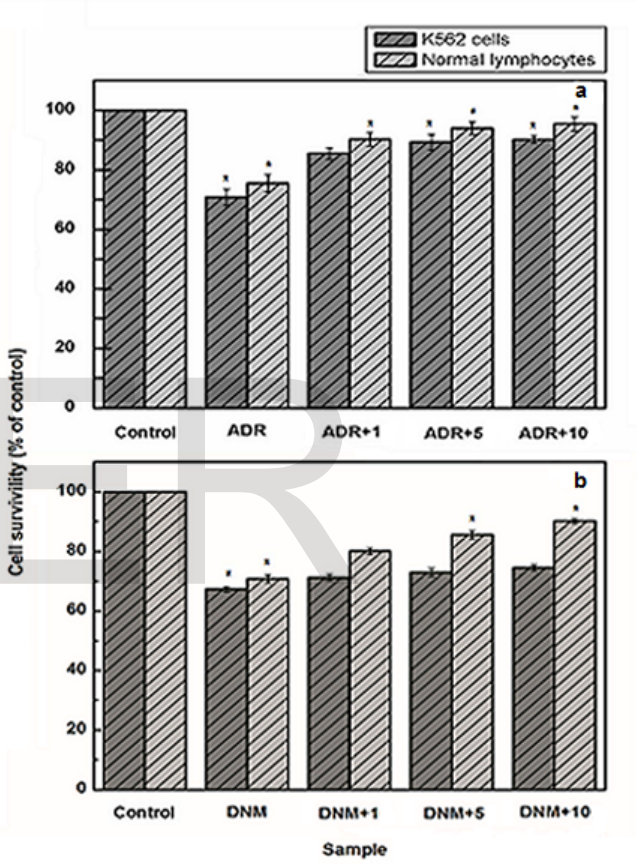

Fig 3. Histograms representing changes in cell survivability (% of control) on treatment of normal lymphocytes and K562 cells with BTE (0, 1, 5, 10 µg/ml) and exposed to (a) ADR (b) DNM. *Significantly different at p<0.05 compared to untreated control or treated without BTE treatment.

2.10 Single cell gel electrophoresis for DNA damage

Comet assay was performed under alkaline conditions according to the procedure described by Singh et al. [29]. Cell suspensions of 5×10^5 cells in 1 ml RPMI 1640 media were treated with 5 µg/ml of BTE separately for 30 mins at 37°C in a CO2 incubator. Cells were washed twice with PBS (pH 7.4) and re-suspended in 1 ml media and treated with ADR or DNM separately for 2 hrs. After treatment, the cells were washed and again re-suspended in 0.5 % LMP (low melting point) agarose. Then the cells were spreaded on the slides coated with NM (normal melting) agarose and further analysed according to the procedure used in our previous studies [30].

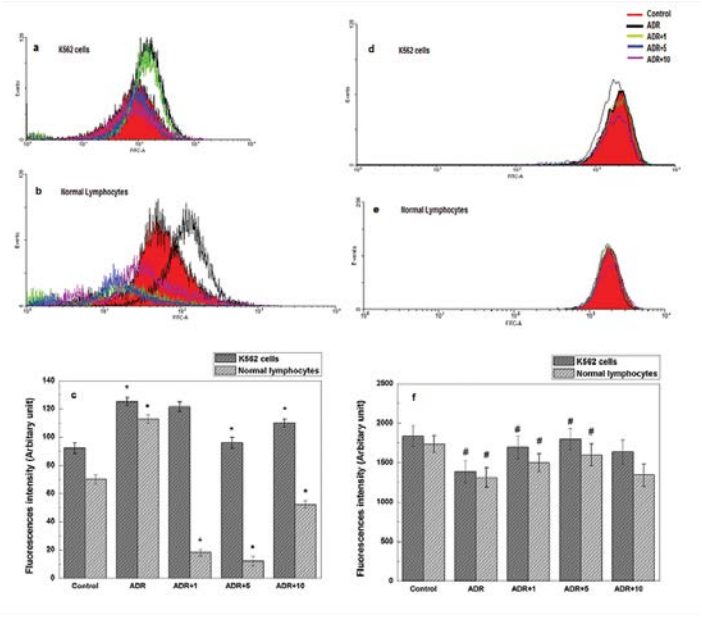

Fig 4. Illustration of flow cytometric traces for intracellular ROS and MMP measurements: (a) & (d) in K562 cells and (b) & (e) in normal lymphocytes using DCFDA and Rh123 respectively; induced by ADR treatment after pre-treatment with BTE (0, 1, 5, 10 µg/ml). The same results are represented as histograms (c) ROS population and (f) MMP changes in normal lymphocytes and K562 cells. *Significantly different at $p<0.001$ and # at $p<0.05$ compared to untreated control or treated without BTE treatment.

2.11 Measurement of apoptosis by Annexin V FITC-PI staining

Apoptosis was measured using Annexin V-PI Apoptosis detection kit. After drug exposure with or without prior BTE treatment, K562 cells and normal lymphocytes were washed twice with PBS (pH 7.4). Then cells were stained with PI and FITC conjugated-Annexin V fluos in the binding buffer for 30 mins at room temperature. Percentage of apoptosis was assessed using a BD FACS Calibur (San Jose, CA, USA) equipped with 488 nm argon laser light source, 515 nm band-pass filter for FITC fluorescence and 623 nm band-pass filter for PI fluorescence and data were analysed using CellQuest software on flow cytometry. A total of 20,000 events were acquired and the cells were properly gated for analysis.

2.12 Fluorescence microscopic analysis of apoptotic cells

Changes in cellular chromatin of treated and untreated cells of both types were visualized after the cells were fixed in 3 % paraformaldehyde, washed in PBS (pH 7.4) and stained by the DNA-intercalating fluorescent probe DAPI (0.1µg/ml in PBS, pH 7.4) [31]. In each experiment, the presence or absence of apoptotic nuclei in samples of 300 cells was scored by two independent observer using fluorescence microscopy.

2.13 Measurement of caspase-3 levels

Caspase-3 assay was determined by using the caspase-3 activity assay kit. Briefly, after exposure of the drugs with or without BTE treatment, cells (1 x 10^5 cells/ml) were washed twice in PBS (pH 7.4). Each cell sample was resuspended in cell lysis buffer comprising 10 mM Tris-HCL, 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.5), 130 mM NaCl, 1% Triton X. In 1ml protease assay buffer (1X HEPES buffer) 50 µl cell lysate was added along with caspase-3 fluorogenic substrate Ac-DEVD-AMC and incubated at 37°C for 1 hr in the dark. The fluorescent intensity of AMC liberated from Ac-DEVD-AMC by caspase-3 was measured in a Perkin Elmer Spectrofluorimeter (USA) using excitation at 380 nm and an emission wavelength range of 420-460 nm.

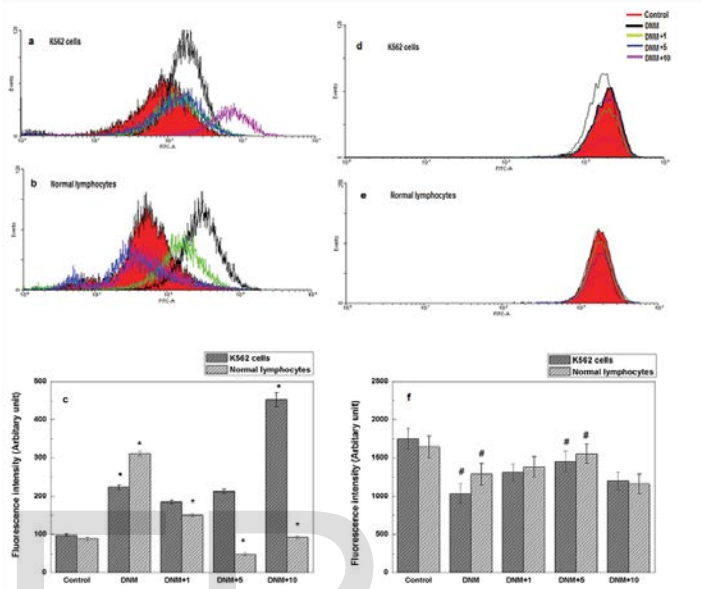

Fig 5. Illustration of flow cytometric traces for intracellular ROS and MMP measurements: (a) & (d) in K562 cells and (b) & (e) in normal lymphocytes using DCFDA and Rh123 respectively; induced by DNM treatment after pre treatment with BTE (0, 1, 5, 10 µg/ml). The same results are represented as histograms (c) ROS population and (f) MMP changes in normal lymphocytes and K562 cells. *Significantly different at $p<0.001$ and # at $p<0.05$ compared to untreated control or treated without BTE treatment.

2.14 mRNA expression studies of apoptotic genes and Nrf2 by Reverse Transcription Polymerase Chain Reaction (RTPCR)

Total RNA was extracted from drug treated samples with or without BTE treatment as well as control of both types of cells using RNA extraction kit, Genei, according to manufacturer's instructions and RNA was quantified using Varian Spectrophotometer. Oligo (dT) 18 primed reverse transcription (RT) was performed with First Strand cDNA Synthesis Kit (Fermentas) in 20 µl reaction volume (37°C, 1h) by taking equal amount of RNA (1 µg) from each sample [32]. After reverse transcription, Taq polymerase and each sample along with dNTPs (Total volume 30 µl) were placed in a thermal cycler. The forward and reverse primer sets used for each gene of interest (bax, bcl2, p53, Nrf2) were provided in Table 1 taking GAPDH as control. PCR amplification was conducted using pre-calibrated denaturation, annealing and polymerization thermal protocols. Following the PCR analysis, 10 µl aliquots of the reaction mixtures were dissolved on 1.2 % agarose gel containing ethidium bromide to identify the DNA amplicons generated.

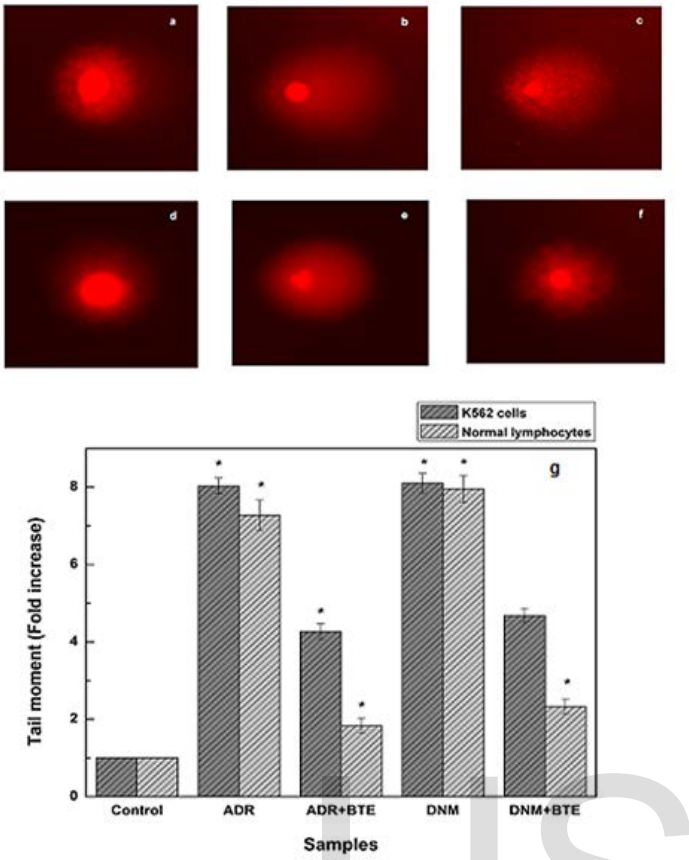

Fig 6. Images of comets comparing (a) untreated control with (b) DNM treated samples alone and with (c) BTE pre-treatment in normal lymphocytes and same in (d-f) K562 cells; (g) histograms illustrating the effect of BTE (5 µg/ml) on genotoxicity of normal lymphocytes and K562 cells when treated with ADR and DNM. *Significantly different at $p<0.05$ compared to untreated control or treated without BTE treatment.

2.15 Biochemical analysis of anti-oxidant enzymes

CAT activity. CAT activity was assessed by the method of Luck [33], wherein the breakdown of H_2O_2 is measured. Briefly, assay mixture consists of 1 mL of H_2O_2, phosphate buffer and 100 µl of the cell lysate. The change in absorbance of the reaction mixture was recorded for 5 min at 30 sec interval at 240 nm using Varian spectrophotometer.

SOD activity. SOD activity was assayed by the method of Marklund and Marklund [34] based on pyrogallol auto-oxidation inhibition. Auto-oxidation of pyrogallol in Tris–HCL buffer (50 mM, pH 7.5) was measured by increase in absorbance at 420 nm using spectrophotometer and expressed in percentage of control.

GST activity. GST activities were measured in cell lysate by determining the increase in absorbance at 340 nm with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate and the specific activity of the enzyme was expressed as mU/ml of lysate [35].

2.16 Statistical analysis

Data were expressed as the means ± standard error. Statistical evaluation of the data was performed by one way ANOVA using Bonferroni test. Error bars indicate the standard error for N = 4 independent experiments.

3 RESULTS

3.1 Permeability of BTE in normal lymphocytes and K562 cells

Acidic mobile phase [36] was used for the complete separation of catechins present in BTE. The chromatogram of catechins and caffeine was recorded at 210 nm using 20 % methanol with 0.1% orthophosphoric acid as mobile phase [24] for better separation and is shown in Fig 1a. All major tea polyphenols and caffeine were identified using the standards. The tea catechins namely epigallocatechin (EGC), caffeine, epicatechin (EC), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) were identified by their retention times of 3.1, 4.1, 8.6, 12.0 and 25.0 mins, respectively. Tea flavanols in the extract were also detected and identified in the extract using acidic mobile phase at 365 nm as shown in Fig 1b. The three major flavanols namely myricetin, quercetin and kaempferol were identified by their retention times of 4.6, 8.1 and 15.0 mins, respectively. The chromatogram of native sample of BTE was compared with the chromatograms obtained after both types of cells were incubated in the extract separately for 30 mins and are shown in Fig 2a. On comparison of the absorbance units of the chromatograms it is observed that BTE was permeable to both normal lymphocytes and K562 cells *in vitro* (Fig 2b-c).

3.2 Effect of BTE on drug induced cytotoxicity of K562 cells and normal lymphocytes

Effects of BTE on drug induced cytotoxicity in normal lymphocytes and K562 cells were evaluated by MTT assay. Normal lymphocytes and K562 cells when exposed to ADR/DNM, cell survivability decreased to 75.4 ± 2.8 % / 70.7 ± 1.3 % and 70.7 ± 2.7 % / 67.2 ± 0.8 % respectively whereas drug induced cytotoxic effects were attenuated in the presence of BTE (1 µg, 5 µg or 10 µg/ml). Survivability of normal lymphocytes when treated with 5 µg/ml of BTE increased to 95.3 ± 2.2 % / 90.1 ± 0.8 % for ADR and DNM respectively (Fig 3a). These findings strongly establish the chemo-protective effects of BTE and corroborate with our earlier findings [17]. Protection was also observed for K562 cells after BTE treatment on exposure to drugs, which is less compared to normal cells (Fig 3b).

3.3 Effect of BTE on drug induced alteration of redox status and MMP levels

Chemotherapeutic drugs like ADR and DNM exposure generate ROS that can react with nucleic acids to incapacitate the function of DNA and RNA through oxidative damage [37-39]. Moreover, mitochondrial membrane permeabilization consti tutes an early event in the apoptotic process, which leads to the disruption of the inner transmembrane potential and release of soluble intermembrane proteins [40]. Although the connection between ROS and MMP is not always straight forward, some sporadic results suggests that ROS is an important contributor to the decrease of MMP [41]. From the results it is

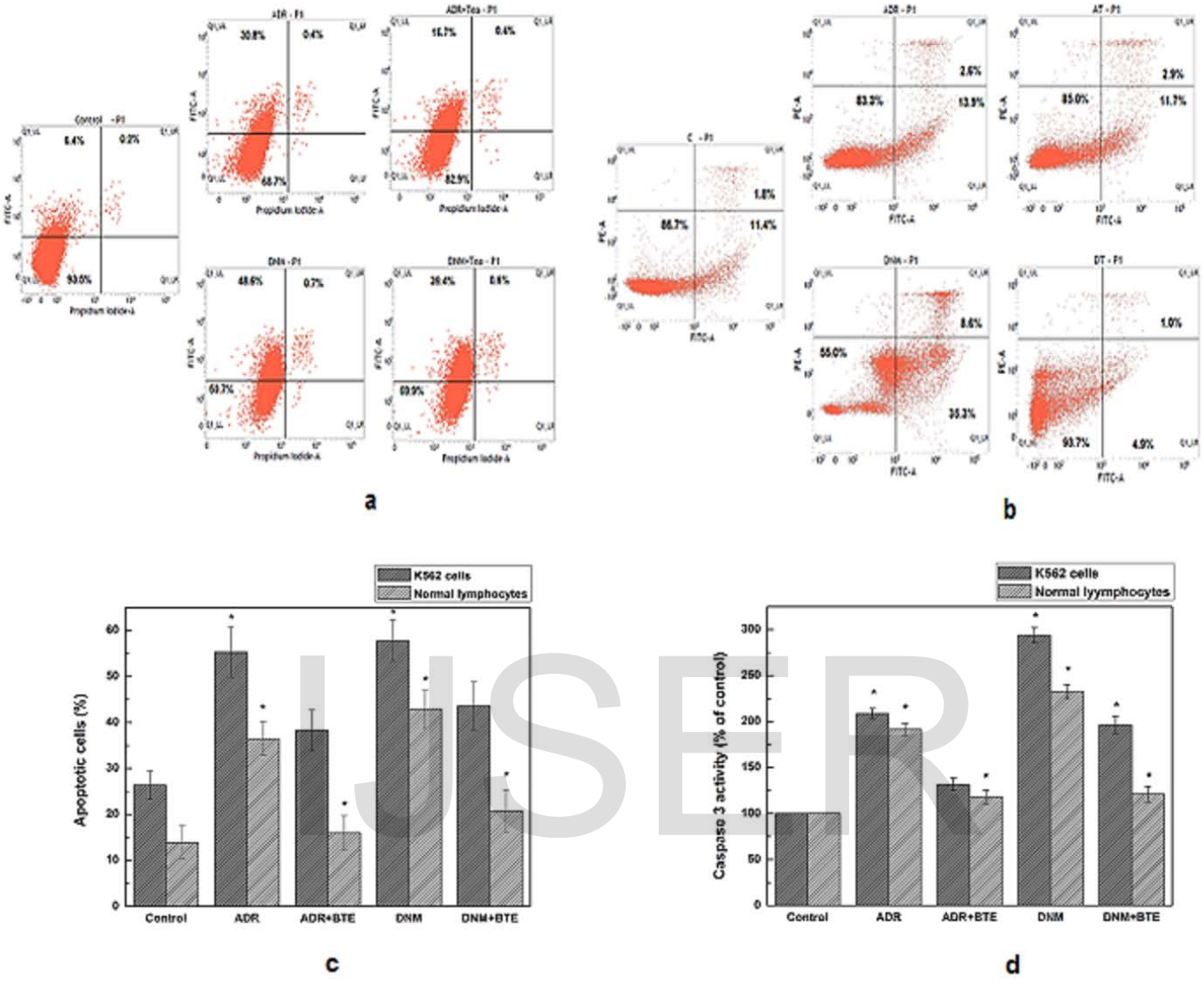

Fig 7. Effect of BTE on ADR/DNM induced apoptotic cell population on (a) K562 cells and (b) normal lymphocytes observed using flow cytometry; (c) percentage of apoptotic cells examined by DAPI staining and (d) change in caspase-3 activity after BTE treatment on both K562 cells and normal lymphocytes. *Significantly different at $p<0.05$ compared to untreated control or treated without BTE treatment.

evident that, the basal level of ROS is significantly different in K562 cells compared to normal lymphocytes [42, 43] and exposure to ADR/DNM increases intracellular ROS population markedly in both noncancerous and cancerous cells whereas DNM is more efficient in generating ROS (Fig 4a-c and 5a-c). In normal lymphocytes pre-treated with various concentrations (1-10 µg/ml) of BTE [18], a significant dose-dependent decrease in ROS yield was observed compared to K562 cells. Exposure of normal lymphocytes and K562 cells to drugs also led to marked loss in the MMP as inferred from the flow cytometry which was restored efficiently in normal lymphocytes on BTE (1 and 5 µg/ml) treatment compared to K562 cells as shown in Figures 4d-f and 5d-f . High dose of BTE (10 µg/ml) showed toxic effect in both type of cells, which may act synergistically with drugs due to pro-oxidant activity of catechins.

3.4 Effect of BTE on drug induced apoptosis

Single cell gel electrophoresis (Comet assay). The alkaline comet assay was used to estimate the oxidative DNA lesions in normal lymphocytes and K562 cells. Hundred images of the comets were randomly selected for each test sample as shown in Fig 6. The comet tail moment is positively correlated with the level of DNA breakage and/or alkali labile sites in the cell.

On treatment with ADR/DNM, the TM values increased by 7.3 ± 0.3 / 7.9 ± 0.3 fold in normal lymphocytes and 8.0 ± 0.2 / 8.1 ± 0.2 fold in K562 cells, respectively (Fig 6b). It was observed that at a dose of 5 µg/ml of BTE, protection was conferred to normal lymphocytes, with TM as low as 1.8 ± 0.2 / 2.3 ± 0.1 fold against ADR and DNM respectively. K562 cells

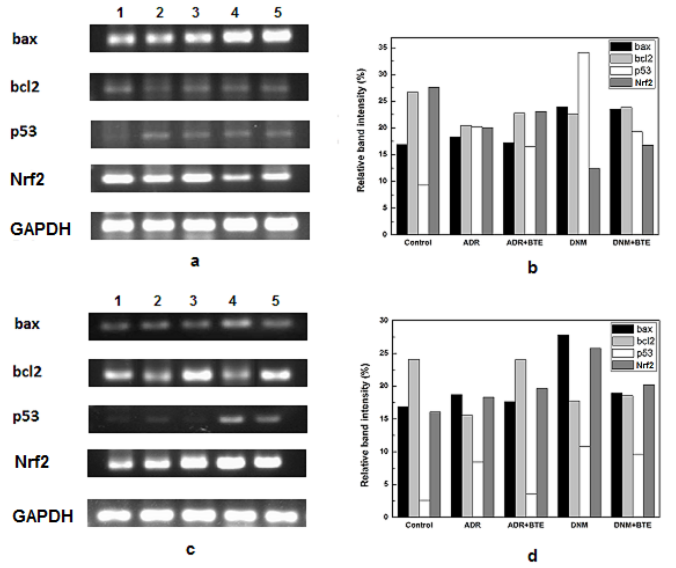

Fig 8. Effect of BTE on ADR/DNM induced change in mRNA expression of bax, bcl2, p53 and Nrf2 and relative band intensity in (a, b) K562 cells and (c, d) normal lymphocytes where lane 1 control, lane 2 ADR treated, lane 3 ADR+BTE treated, lane 4 DNM treated and lane 5 DNM+BTE treated.

when subjected to similar treatment did not result in such significant decrease in TM.

Annexin V FITC-PI staining. It is known that phosphatidylserine (PS) is flipped from the intra to extra plasma membrane leaflet during the early stage of apoptosis. Annexin V, with a high affinity for PS, can therefore be employed as a sensitive marker for early apoptosis [44]. By contrast, propidium iodide (PI) can conjugate to necrotic cells. Double staining of cells with annexin V-FITC and PI was assayed with flow cytometry to further determine the apoptosis of each sample. Fig 7a-b shows a typical quadrant analysis of both type of cells treated with or without BTE before drug exposure. Compared with the control, the apoptotic cell population increased from 11.4 % to 13.9 % / 35.3 % following ADR/DNM treatment alone in normal lymphocytes. However, a dramatic decrease to 11.7 % for ADR and 4.9 % for DNM was observed after 5 µg/ml BTE pretreatment in normal lymphocytes compared to K562 cells.

Microscopic analysis by DAPI staining. Effect of BTE on drug induced apoptosis was also verified by microscopic analysis by DAPI staining. Both K562 cells and normal lymphocytes had typical morphology of apoptosis such as nuclear chromatin condensation and nuclear fragmentation after exposure of drugs and the number of condensed nuclei increased (Fig 7c). However, when the cells were pre-treated with BTE, the number of condensed nuclei decreased in normal lymphocytes compared to K562 cells.

Caspase-3 activity. Caspases are the molecular machinery that drives apoptosis. They are responsible for the morphologic and biochemical characteristics of apoptotic cells. After 24 hr treatment of ADR/DNM, 191.6 % / 232.8 % increase in caspase-3 like activity was detected in normal lymphocytes (Fig 7d). In contrast, normal lymphocytes that were treated with BTE exhibited a significant decrease in caspase-3 activity (118 % and 121 % for ADR and DNM, respectively) compared to K562 cells (131.4 % and 196.4 % for ADR and DNM, respectively). Here it is evidenced that treatment with BTE result in the inhibition of drug induced apoptosis in normal lymphocytes when compared to K562 cells.

3.5 Effect of BTE on mRNA expression of apoptotic genes and Nrf2

The bcl2 family consists of both apoptotic and anti-apoptotic proteins. The balance between these proteins is critical in turn ing the cellular apoptotic machinery on and off [45]; any shift in the balance of pro- and anti-apoptotic factors will affect cell death. Members of bcl2 family are intimately involved in cell death processes that are caused by the anticancer drugs [46]. bcl2 is an anti-apoptotic protein while bax is pro-apoptotic [45]. Bcl2 and bax are anti and pro apoptotic genes respectively and their balance attributescell death or apoptosis.

In this study, we investigated the effect of BTE on the mRNA expression levels of bax, bcl2, p53 and Nrf2 in ADR/DNM treated K562 cells and normal lymphocytes using semi-quantitative RTPCR analysis. As shown in Fig 8, bax mRNA expression significantly increased in the ADR/DNM treated samples, however BTE treatment may reduce the bax mRNA expression level in normal lymphocytes compared to K562 cells. In contrast to bax, the level of bcl2 in the ADR/DNM treated samples decreased compared to untreated controls. However, bcl2 mRNA expression recovered after BTE treatment in normal lymphocytes compared to K562 cells. Similar to bax, p53 mRNA expression also increased in drug treated samples and reduced in BTE pre-treated samples more significantly in normal lymphocytes than in K562 cells. The expression of Nrf2 mRNA decreased in drug treated samples and recovered after BTE treatment in K562 cells but showed opposite results in normal lymphocytes as shown in Fig 8b and 8d.

Table 1: The synthetic primers used for RTPCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)	Amplicon size (bp)
bax	TTTCATCCAGGATCGAGCA	ATCCTCTGCAGCTCCATGTT	52.0	150
bcl2	GTCCAAGAATGCAAAGCACA	CCGGTTATCGTACCCTGTTC	53.0	163
p53	GAAGACCCAGGTCCAGATGA	CTGCCCTGGTAGGTTTTCTG	54.5	152
Nrf2	GCGACGGAAAGAGTATGAGC	ACCTGGGAGTAGTTGGCAGA	54.0	192

3.6 Effect of BTE on enzymatic antioxidant activity (biochemical estimations)

In the present study, the activity of major antioxidant enzymes like SOD, CAT, GST was measured to observe the effect of BTE on their antioxidant activity on drug treatment in K562 cells and normal lymphocytes. A significant increase in SOD, CAT and GST activity in the ADR/DNM treated cells was observed. On BTE treatment a decrease in enzymatic activity was observed in both types of cells but more efficiently in normal lymphocytes compared to K562 cells (Fig 9a-c).

4 DISSCUSION

In the present study, the detailed mechanism of action of BTE exerting chemo-protective effect was investigated and it is revealed that antioxidant efficacy is the possible pathway. The present findings are supported by our previous observations where antioxidant efficacy of BTE in cell free as well as in cellular system was documented [17, 18]. Results suggest that BTE is an efficient ROS scavenger and inhibit DNM/ADR induced intracellular ROS generation in normal lymphocytes compared to K562 cells (Fig 4a-c, 5a-c). Overproduction of ROS by these drugs can lead to severe impairment of cellular functions like DNA damage, up-regulation of endogenous antioxidant enzymes, mitochondrial dysfunction and eventually oxidative stress mediated apoptosis. Existence of such process is established by significant decrease in MMP after treatment with DNM/ADR. Pre-treatment with BTE was found to restore MMP more efficiently in normal lymphocytes compared to K562 cells due to the antioxidant activity of BTE (Fig 4d-f, 5d-f). DNA damage as a result of genotoxic effect of anticancer drugs comprising of single or double strand breaks and damage to bases and sugar and ultimately leads to chromosomal aberrations [47]. All the above mentioned lesions significantly contribute to the increased levels of primary DNA damage detected by single cell gel electrophoresis on exposure to DNM/ADR in both type of cells. Interestingly, BTE pre-treatment efficiently lowers such damage in normal lymphocytes compared to K562 cells (Fig 6). In addition, treatment with BTE prevented normal lymphocytes from undergoing apoptosis compared to K562 cells, as characterised by decrease in the number of apoptotic cells, condensation in the nuclei and activation of caspase-3 (Fig 7). All these healing effects are attributed to the antioxidant properties of the polyphenols present in BTE.

In order to further elucidate the molecular mechanism responsible for the protective effect of BTE against drug induced apoptosis. The mRNA expression levels of some critical genes associated with apoptosis such as bax, bcl-2 and p53 were investigated using RTPCR. The bcl-2 family of proteins regulates the release of cytochrome c and other intermembrane space proteins. While bcl-2 is one of the most important anti-apoptotic members in this family, it interacts with bax, a pro-apoptotic member, thereby preventing the release of cytochrome c and subsequent apoptosis. Earlier studies have suggested that ROS might be associated with bax activation in apoptosis induced by some stimuli [48, 49]. Increased expression of bax can induce apoptosis, while bcl-2 protects cells from apoptosis [45]. In the present study, BTE effectively

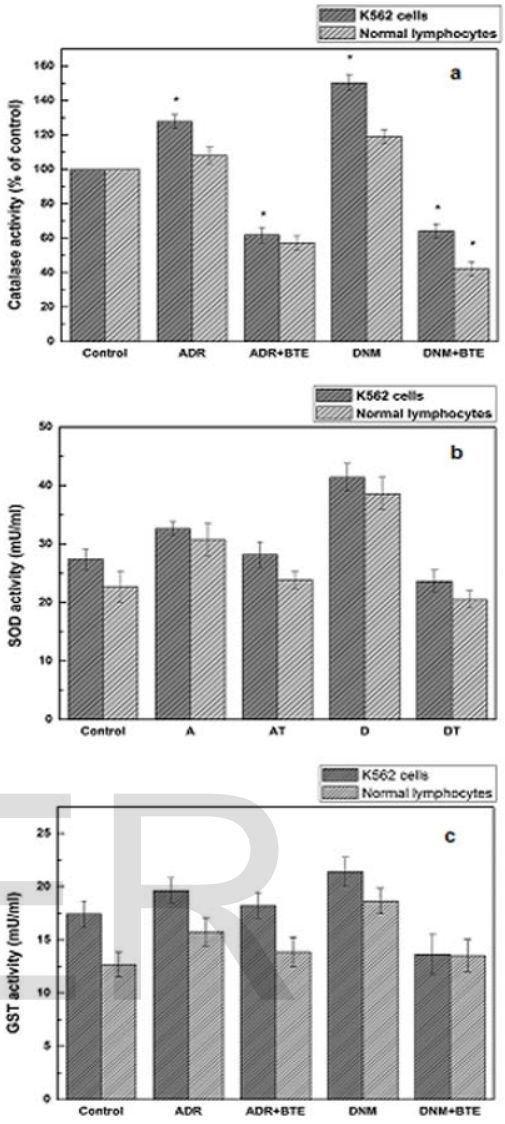

Fig 9. Effects of BTE on (a) SOD activity, (b) CAT activity and (c) GST activity on K562 cells and normal lymphocytes after 24hrs ADR and DNM treatment. *Significantly different at p<0.05 compared to untreated control or treated without BTE treatment.

suppressed programmed cell death by decreasing apoptotic features including caspase activation, increasing anti-apoptotic molecules (bcl-2), decreasing pro-apoptotic molecules (bax) and p53 when compared with drug alone (Fig 8). Here it is revealed that tea polyphenols can prevent the first injury step of apoptosis which subsequently decreases the overall effect of the drugs.

Activity of antioxidant enzymes (SOD, CAT and GST) may change due to increase in ROS population on drug exposure was examined along with the expression of Nrf2. From the observed activity assays it is established that SOD, CAT and GST activity increase on exposure to DNM/ADR to maintain a balance between excess ROS generated by the drugs and the basal level of endogenous antioxidant enzymes in both type of cells. Pre-treatment with BTE indirectly lowers the activity of these enzymes by scavenging ROS and limiting the

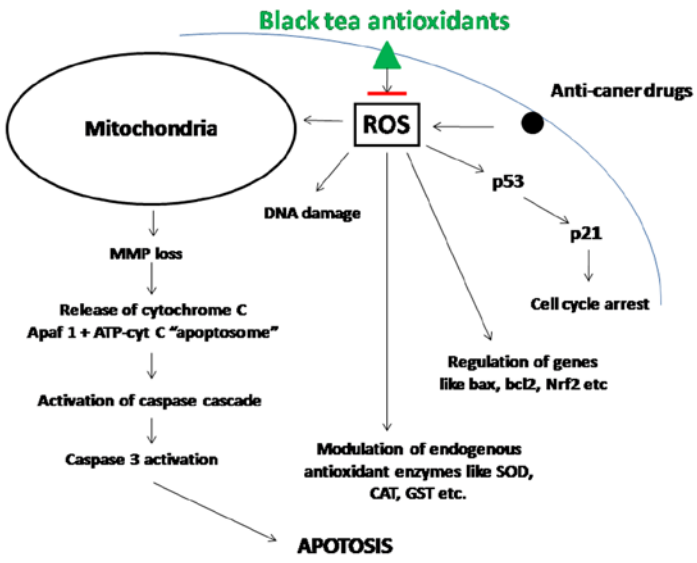

Fig 10. Proposed mechanism of action of chemo-preventive effect of black tea against anticancer drug induced oxidative damage in non-targeted cells.

overproduction (Fig 9). But the results are not significant enough indicating more complex relation between ROS generation, endogenous antioxidant enzyme activity and antioxidant supplements. Further study showed, mRNA expression of Nrf2 increased after exposure to the drugs and decreased when pre-treated with BTE in normal lymphocytes evidencing that cellular defense mechanism becomes active by producing excess endogenous antioxidant enzymes to combat excess ROS production by the drugs and less active when BTE already scavenge excess ROS. In K562 cells, mRNA expression of Nrf2 decreased after drug treatment and recovered after BTE pre-treatment highlighting complex mechanism in cancerous cells.

The present findings illustrate that the development of a beneficial or a detrimental cellular response by an antioxidant supplement will depend on the supplement's antioxidant or pro-oxidant characteristics, which in turn are a product of the cellular oxygen environment. Antioxidant or pro-oxidant activities depend on the redox potential of the individual molecule and the redox status of the cell. The present knowledge of use of antioxidants or any supplement, calls for more in vitro as well as in vivo studies on cancerous as well as noncancerous cells which will capture the various pathways of mechanisms of action (as represented in Fig 10) so that they can safely used as therapeutic supplements.

5 Conclusion

The current study demonstrated for the first time that the hot water black tea extract, exerts significant protective effects against chemotherapeutic drug (DNM/ADR) induced cytotoxicity by inhibiting ROS generation and mitochondrial dysfunction in normal lymphocytes compared to erythroleukemic K562 cells. Results suggest that BTE has the efficacy to inhibits drug induced ROS generation, restoration of MMP, increase in the number of viable cells, regulation of endogenous antioxidant enzymes, inhibition of mRNA expression of apoptotic genes, prevention in caspase-3 activation, reduction of DNA fragmentation and the total number of apoptotic cells in normal lymphocytes. This is attributed due to BTE uptake through cell membranes of both normal lymphocytes and K562 cells. The antioxidant activity of BTE at certain dose in K562 cells is lower than that observed in normal lymphocytes. The study highlight the chemo-protective properties of BTE against DNM and ADR induced oxidative stress to normal lymphocytes at a dose of 5 µg/ml at which marginal interfere with the cytotoxic effect of the drugs in K562 cells.

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