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Radioprotective properties of apple polyphenols: An *in vitro* study

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Abstract

Present study was undertaken to evaluate the radioprotective ability of total polyphenols extracted from edible portion (epicarp and mesocarp) of apple. Prior administration of apple polyphenols to murine thymocytes significantly countered radiation induced DNA damage (evaluated by alkaline halo assay) and cell death (trypan blue exclusion method) in a dose dependent manner maximally at a concentration of 2 and 0.2 mg/ml respectively. Apple polyphenols in a dose dependent fashion inhibited both radiation or Fenton reaction mediated 2-deoxyribose (2-DR) degradation indicating its ability to scavenge hydroxyl radicals and this activity was found to be unaltered in presence of simulated gastric juice. Similarly apple polyphenols in a dose dependent fashion scavenged DPPH radicals (maximum 69% at 1 mg/ml), superoxide anions (maximum 88% at 2 mg/ml), reduced Fe^{3+} to Fe^{2+} (maximum at 1 mg/ml) and inhibited Fenton reaction mediated lipid peroxidation (maximum 66% at 1.5 mg/ml) further establishing its antioxidative properties. Studies carried out with plasmid DNA revealed the ability of apple polyphenols to inhibit radiation induced single as well as double strand breaks. The results clearly indicate that apple polyphenols have significant potential to protect cellular system from radiation induced damage and ability to scavenge free radicals might be playing an important role in its radioprotective manifestation. (Mol Cell Biochem **288:** 37–46, 2006)

Key words: alkaline halo, apple polyphenols, radioprotection, reactive oxygen species

Introduction

Exposure of cells to low linear energy transfer (LET) radiation leads to increased generation of reactive oxygen species. As results of which the balance between pro- and antioxidants shifts towards former resulting in severe oxidative stress and ultimately cell death [1–3]. An agent which can modulate the shift towards antioxidant can be of immense help to cellular system in dealing with the oxidative stress and in augmenting recovery [4]. Several molecular drugs of synthetic and natural origin are being tried in several experimental models to mitigate the radiation injury [5, 6]. Among different molecular radioprotectors WR-2721 and related compounds have been found to be most promising [7]. However, severe side effects such as nausea, vomiting, hypotension and neurotoxicity associated with most of the radioprotective agents at therapeutic levels have restrained their use [5, 8]. In view of this search for newer and more effective agents is inevitable.

In the last decade natural products have received maximum attention owing to their wide utility in traditional medicine systems like Ayurveda. A number of herbal preparations both in wholesome form or their components have been shown to

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render radioprotection both *in vivo* and *in vitro* systems [9–12]. The possible beneficial health effects of diet containing fruits, vegetable and certain beverages such as wine and tea have lead to renewed interest in flavonoids and other plant phenolics to be used for radioprotection [13, 14].

Apple *(Pyrus malus*; family Rosaceae) is one of the main sources of flavonoids because of various factors including availability, diversity of cultivars and variety of conditionings (fresh fruit, juice, cider, mashed apples etc). Most of the investigations on the health effects of apple have been focused on their lipid-lowering effects [15], chemopreventive action and more recently, on their anti-oxidative properties [16]. The main classes of polyphenols found in apple are flavonoids, such as flavonols (quercetin, as glycosides), flavanols ([+]-epicatechin, [+]-catechin, and their oligomers, procyanidins), anthocyanins, dihydrochalcones (phloridzin and phloretin) and phenolic acids (chlorogenic acid) etc. [17, 18].

Keeping in view of its beneficial antioxidative properties and worldwide consumption present study was undertaken to evaluate the radioprotective properties of apple polyphenols under *in vitro* conditions.

Material and methods

Chemicals

pUC18, sodium carbonate were obtained from Genetix, India. Agarose, 2,2′ bipiridyl, 2-deoxyribose, DPPH, ethidium bromide, ferric chloride, ferrous ammonium sulfate, Na_2-EDTA, nitroblue tetrazolium, NADH, phenazine methosulfate, potassium chloride, sodium chloride, sodium lauryl sulfate, sodium lauryl sarcosine, thiobarbituric acid, tricholoroacetic acid, triton-x-100, were obtained from Sigma Chemical Co, MO, USA. All other chemicals of standard make and purity were used.

Animals

Swiss albino strain 'A' male mice (6–8 weeks) weighing 25 ± 2 g were maintained under standard laboratory conditions (25 ± 2 °C; photoperiod 12 h light/dark cycle) and fed standard animal food pellets (Amrut Laboratory feed, India) and water *ad libitum*. Four to five animals were kept in polypropylene cages. All experiments involving animals were done following Animal Ethics Committee Rules and Regulations of the institute.

Irradiation

For experiments requiring exposure to varied doses of γ-radiation, chemical solutions, different cell suspension and tissue homogenates were kept on ice and irradiated with γ-source (Co^{60}) procured from Board of Radiation and Isotope Technology (BRIT), India. Dose rate was determined following Fricke's method and was found to be 3.56 kGy/h [19].

Plant material and extraction of polyphenols

Fresh red delicious apples were obtained from local market and used immediately for extraction of polyphenols. Apple polyphenols were extracted according to the method of Silvina and Frei [20]. Briefly, edible portion of six apples (skin and flesh) was chopped into small pieces and grounded to a fine powder in liquid nitrogen in a blender. The resulting powder (about 10 g) was extracted with 30 ml 100% acetone, sonicated for 5 minutes and centrifuged for 10 minutes at 1000 × g. The residual powder was reextracted twice with 30 ml of 70% acetone following the above procedure. The aqueous acetone fractions were pooled and subsequently extracted with 140 ml of chloroform. After centrifugation (1000 × g 20 minutes) the mixture was partitioned in separator funnel. The chloroform fraction was discarded and the aqueous acetone fraction was centrifuged and evaporated under reduced pressure.

HPLC profile of apple polyphenols

HPLC of apple polyphenol was performed with Simadzu HPLC system using reverse phase C-18 column and UV detector. Methanol: water 50:50; v/v was used as mobile phase and flow rate was maintained at 1 ml/min.

Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteau technique [21]. Briefly, 100 μl aliquot of apple polyphenols was mixed with 500 μl Folin-Ciocalteau reagent. To this 400 μl sodium carbonate (7.5%) was added and incubated at 20 °C for 30 minutes. Thereafter absorbance was read at 765 nm. Total phenolic content (mg/g) was expressed as gallic acid equivalents (GAE), using a standard curve of freshly prepared gallic acid solution.

Preparation of liver homogenate and thymocytes cell suspension

Six to eight weeks old strain 'A' male mice were sacrificed by cervical dislocation, dissected and abdominal cavity was perfused with 0.9% saline. Whole liver was taken out, visible clots were carefully and maximally removed and weighed. A 10% homogenate was prepared in phosphate buffer saline

(PBS) pH-7.4 using potter Elvejham homogenizer and filtered to get a clear homogenate. Similarly thymic lobes were excised and processed on frosted slides to get single cell suspensions and thereafter resuspended in PBS.

Cell survival assay

Cell survival after different treatments was evaluated by trypan blue dye exclusion method. Briefly, thymocytes were washed (2–3 times) with ice-cold phosphate buffered saline and centrifuged (1000 rpm, 5 min, 4 °C). The cell pellet was resuspended in incomplete RPMI and 10 μl of the cell suspension was mixed with 10 μl trypan blue solution (0.04% w/v), loaded on to a hemocytometer (Precicolor, HBG, Germany). Cells were counted under light microscope (Olympus CK 30, Japan), dead cells retained the dye while the viable cells excluded trypan blue and appeared bright. Survival in different treatment groups was calculated as percentage viability = no. of viable cells/ no. of viable cells + no. of non-viable cells × 100.

Estimation of hydroxyl radicals

Scavenging of radiation (100 Gy) or Fenton reaction (100 μM $FeSO_4$) induced hydroxyl radicals by apple polyphenols was quantified using 2-deoxyribose as marker substrate [22]. Briefly, 1 ml of reaction volume containing, 5 mM 2-deoxyribose and varied concentrations of apple polyphenols, were mixed either with 100 μM $FeSO_4$ or exposed to 100 Gy followed by incubation for 1 h at 37 °C. Thereafter 2 volumes of solution containing 25% TCA and 1% TBA in 0.1 N NaOH was added, incubated in boiling water bath for 20 minutes, cooled and the absorbance of the resulting pink colored chromogen was measured at 532 nm.

Effect of gastric juice on OH radical scavenging activity of apple polyphenols

Effect of gastric juice on OH radical scavenging potential of apple polyphenols was evaluated by incubating apple polyphenols with simulated gastric juice for 30 min at 37 °C. Thereafter, the extract was used for evaluating hydroxyl radical scavenging ability using 2-deoxyribose degradation assay. The simulated gastric fluid consisted of 2 g of NaCl, 3.3 g of pepsin, dissolved in 7 ml of HCl and water was added to make 1000 ml. The pH of gastric fluid was 1.2.

Estimation of chemically generated superoxide anions

Superoxide anion scavenging ability of apple polyphenols was carried out by employing NBT reduction assay [23]. Briefly, reaction mixture containing of 0.52 M sodium pyrophosphate, pH 8.3,186 μM phenazine methosulphate, 300 μM nitroblue tetrazolium and 780 μM NADH was mixed with varied concentrations of apple polyphenols and incubated for 90 seconds at 30 °C. The purple colored chromogen formed was measured spectrophotometrically at 560 nm.

DPPH radical scavenging activity

DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging ability of apple polyphenols was measured according to the method of Shimada et al, 1992 [24]. Methanolic solutions of DPPH and apple polyphenols were mixed so that the final mass ratio of extract to DPPH was 2:1. Thereafter the samples were incubated for 15 minutes in dark at 37 °C and the decrease in the absorbance at 517 nm was measured against methanol.

Reducing potential of apple polyphenols

100 μl of phosphate buffered saline (pH 7.4) containing 20 μg of ferric chloride, varied concentrations of apple polyphenol were mixed and incubated at room temperature for 5 min. The amount of Fe^{2+} was quantified by adding 100 μl of 2,2′-bipiridyl (100 mM) and the absorbance of resulting chromogen was measured at 532 nm (Fe^{2+}-bipiridyl complex) [25].

Inhibition of lipid peroxidation

2 ml of liver homogenate (10%) was taken in a series of 35 mm petridishes to which desired amount of apple polyphenols were added and mixed gently to form homogeneous solution. Lipid peroxidation was initiated by adding 100 μl of 15 mM ferrous ammonium sulphate and thereafter petridishes were incubated at 37 °C. After 30 min, 100 μl of this reaction mixture was taken in a tube containing 1.5 ml of 10% TCA. After 10 min tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated in a hot water bath at 85 °C for 30 min to complete the reaction. The intensity of pink colored complex formed was measured at 535 nm [26]. The percentage inhibition of lipid peroxidation was calculated by comparing control ($FeSO_4$ control) with treated ($FeSO_4$ + polyphenols) groups.

Apple polyphenols and plasmid DNA damage

Effect of apple polyphenols on radiation induced DNA strand breaks was studied using plasmid DNA [27]. Briefly 1 μg of pUC 18 DNA was incubated with varied concentration of

apple polyphenols (in a total volume of 20 μl) for 10 minutes at 37 °C. Thereafter the reaction mixture was exposed to 20 Gy on ice. After irradiation DNA was stabilized by adding 1/10th volume of 10 × TE (100 mM tris, 10 mM EDTA) followed by addition of 5 μl of 5 × loading buffer. DNA samples were electrophoresed on a 1.2% agarose gel in 0.5 × TBE, stained with ethidium bromide (0.5 μg/ml), visualized and photographed.

Alkaline halo assay

DNA strand breaks in individual cells were detected using alkaline halo assay [28]. After different treatments thymocytes were suspended (2×10^4/ml) in 0.5% low melting agarose solution in PBS (pH 7.4) and immediately pipetted on to slides precoated with 0.1% normal agarose and spread uniformly. The slides were put on steel plates and cooled from below by ice cubes to accelerate gelling. After 5 minutes the slides were immersed in icecold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM tris, 1% sodium lauryl sarcosine, 5% DMSO and 1% triton-x-100, pH 10) and left in refrigerator for 30 minutes. Thereafter the slides were incubated in alkali buffer (0.3 M NaOH and 1mM EDTA; pH 13) for 20 min at 4 °C followed by washing with neutralization buffer (0.4 M tris, pH 7.4). Measurements were done from centre of the core to any point on the perimeter of the halo and at least 100 cells were counted from each treatment.

Statistical analysis

The data presented as mean ± s.d. of three separate experiments and each experiment comprised of three parallel measurements. Significance was determined by Student's t-test and probability level of <5% was considered significant.

Results

Total polyphenol content

Total polyphenol content in the apple extract was found to be 28 mg/g in terms of gallic acid equivalent.

HPLC profile

The HPLC profile of apple extract using a mobile phase (50% methanol: 50% water) revealed 10 peaks (Fig. 1).

Cell survival

5 Gy gamma radiation induced significant ($p < 0.05$) cell killing and surviving fraction was reduced to 53% (Fig. 2) in comparison to untreated control (93%). Pre-irradiation administration of increasing concentrations of apple polyphenols, significantly countered radiation induced cell death and maximum cell survival (87.6%) was observed at 0.2 mg/ml. However apple polyphenols alone, at all concentration tried (0.02–0.20 mg/ml) did not show any toxic effects (data not shown in figure).

Hydroxyl radical scavenging activity

Apple polyphenols significantly ($p < 0.05$) inhibited Fenton or 100 Gy induced 2-deoxyribose degradation in a dose dependent manner (Fig. 3). Maximum inhibition was observed

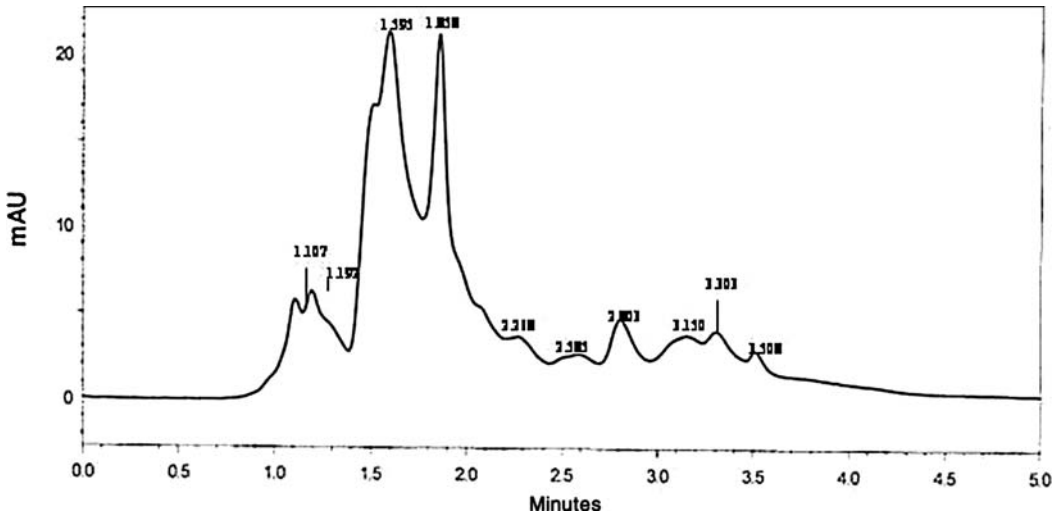

Fig. 1. Chromatogram of apple polyphenols resolved using mobile phase methanol:water: (50:50) v/v at a flow rate of 1 ml/min.

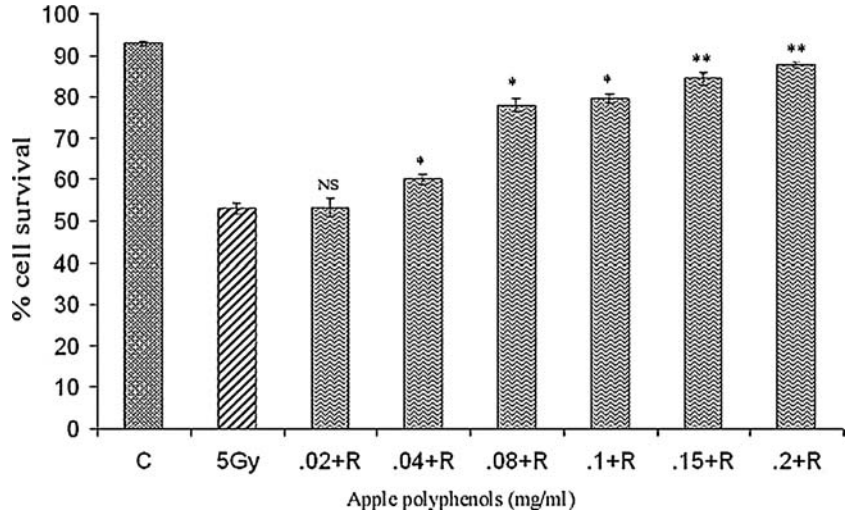

Fig. 2. Mouse thymocytes exposed to 5 Gy gamma radiation in absence or presence of varied concentrations of apple polyphenols. Thereafter cell viability was estimated using trypan blue exclusion method and expressed as % trypan blue negative cells. All values are mean ± s.d. of three parallel observations and comparison was made between radiation alone and radiation + apple polyphenols groups. (NS not significant, $^{*}p < 0.05$, $^{**}p < 0.01$).

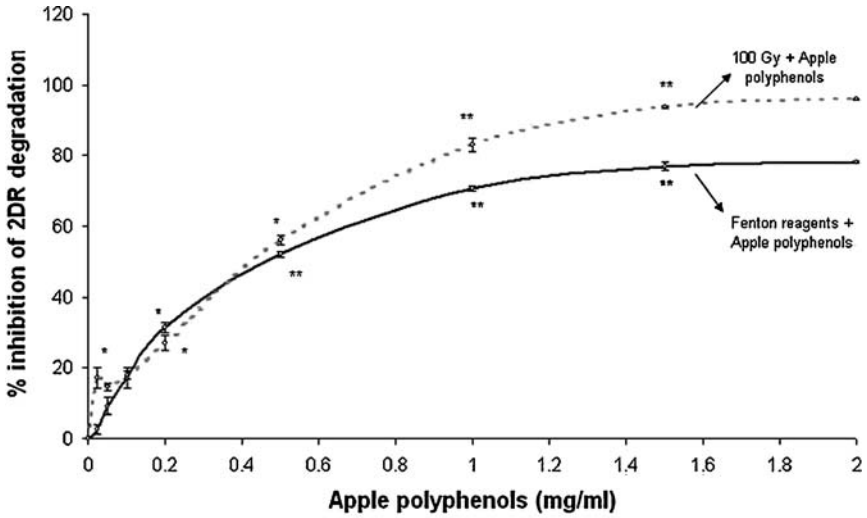

Fig. 3. Effect of varied concentration of apple polyphenols on 100 Gy induced or Fenton reaction mediated hydroxyl radical generation and subsequent degradation of 2-deoxy ribose as measured by % inhibition in the formation of TBARS. Statistical significance was checked between Fenton reaction or radiation alone group with Fenton reagents + apple polyphenols or radiation + apple polyphenols respectively. ($^{*}p < 0.05$, $^{**}p < 0.01$).

at a concentration of 1.5 mg/ml (90%) and 1 mg/ml (72%) for radiation and Fenton reaction mediated 2-deoxyribose degradation respectively. Scavenging of radiation induced hydroxyl radicals by apple polyphenols was unaltered in presence (93.5%) or absence (90%) of gastric juice (Fig. 4).

Superoxide anion scavenging activity

Figure 5 shows that increasing dose of apple polyphenols inhibited superoxide anions in a proportionate manner and maximal inhibition was observed at a concentration of 2 mg/ml (88%; $p < 0.05$).

DPPH radical scavenging activity

Increasing concentrations of apple polyphenols significantly scavenged DPPH radicals in a dose dependent fashion and maximally (69%) at a concentration of 1 mg/ml (Fig. 6).

Reducing potential

The reducing potential of apple polyphenols, evaluated as their ability to reduce ferric ions to ferrous forms and formation of a colored complex with 2,2′-bipiridyl, revealed a dose dependent increase in the reduction of ferric ions. Maximum

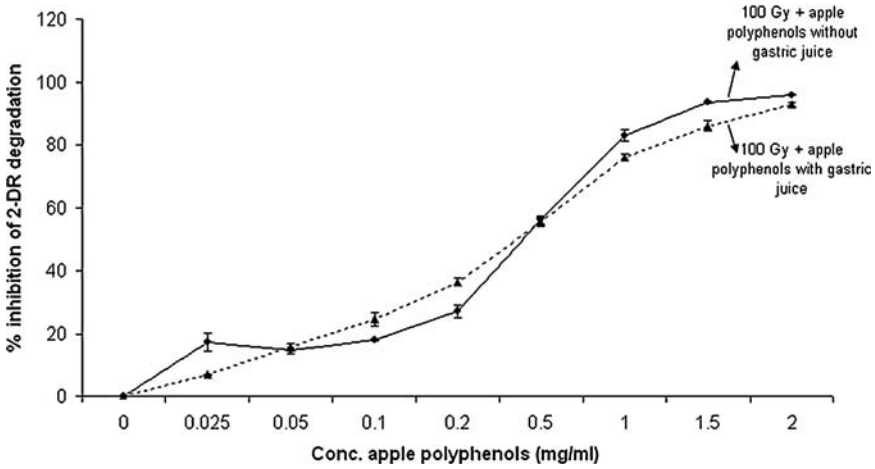

Fig. 4. Effect of simulated gastric juice on hydroxyl radical scavenging ability of apple polyphenols. 2-doexyribose in presence or absence of gastric juice treated apple polyphenols were exposed to 100 Gy and inhibition of 2-deoxyribose degradation was measured as % inhibition of formation of TBARS.

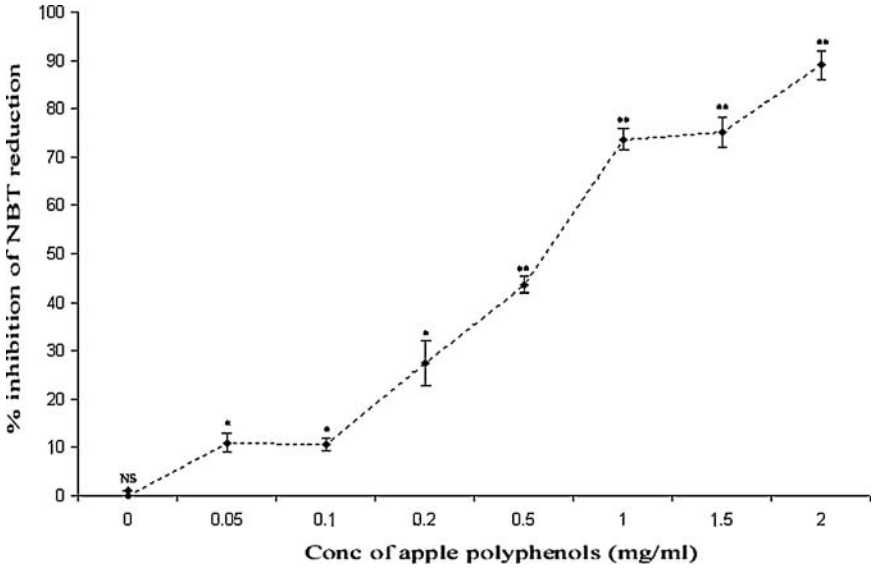

Fig. 5. Effect of apple polyphenols on chemically induced superoxide anions. The inhibitory effect upon superoxide anions is measured as % inhibition in NBT reduction. The absorbance at 560 nm was recorded in triplicate and experiments were repeated thrice. Values are expressed as mean ± s.d. (NS not significant, $^{*}p < 0.05$, $^{**}p < 0.01$).

reduction (absorbance 1.15) was observed at a concentration of 1 mg/ml (Fig. 7).

Inhibition of lipid peroxidation

Apple polyphenols rendered dose dependent inhibition of Fenton reaction mediated lipid peroxidation in liver homogenate and maximum inhibition (66%) was observed at a concentration of 1.5 mg/ml (Fig. 8). At higher concentrations (>1.5 mg/ml) saturation was observed and did not exhibited any increase in inhibition (data not shown in Figure).

Plasmid DNA damage study

Exposure of pUC 18 DNA to 20 Gy under the buffer conditions used in the present study (10 mM phosphate buffer, pH 7.4) resulted in a significant amount of single as well as double strand breaks as can been seen from the formation of relaxed and linear topoisomers (lane-2; Fig. 9). However,

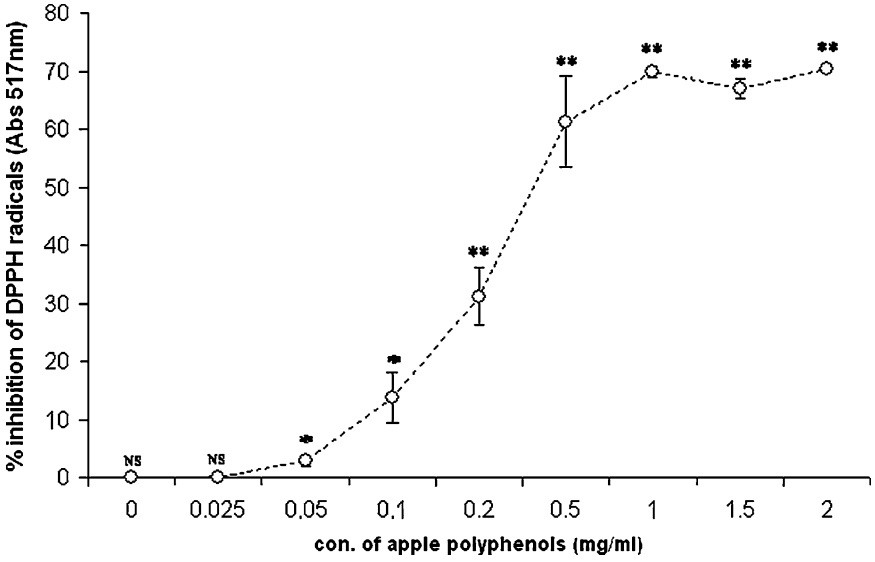

Fig. 6. Scavenging activity of apple polyphenols against DPPH. radical. The absorbance at 517 nm was recorded in triplicate and each experiment was repeated thrice. The values are expressed as mean ± s.d. (NS not significant, $^{*}p < 0.05$, $^{**}p < 0.01$).

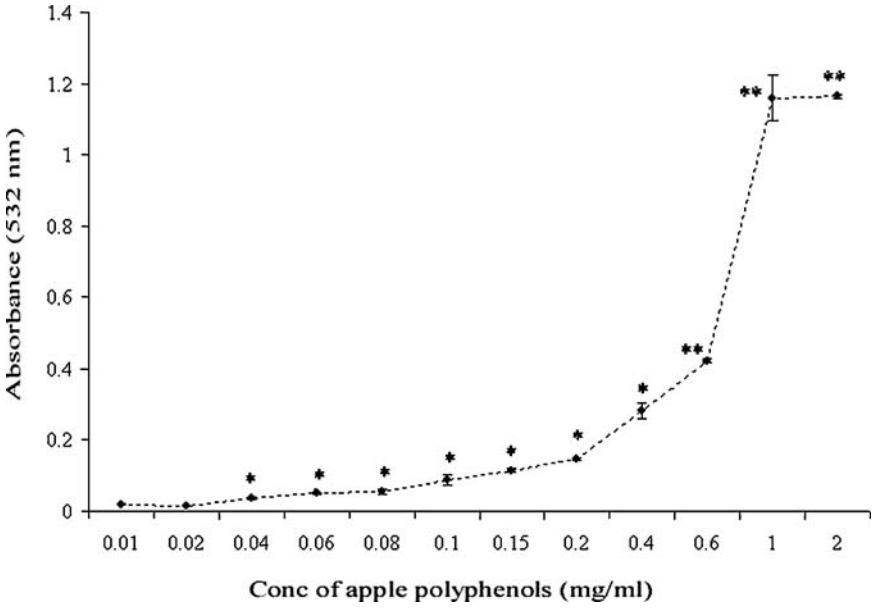

Fig. 7. Reducing power of apple polyphenols. The absorbance at 532 nm was recorded in triplicate and each experiment was repeated thrice. The values are expressed as mean ± s.d. ($^{*}p < 0.05$, $^{**}p < 0.01$).

apple polyphenols inhibited radiation induced strand breaks in a concentration dependent manner (0.02 to 1 mg/ml) and retained fast migrating supercoiled form (lanes 4–13).

Alkaline halo assay

Normal thymocytes without any treatment exhibited a halo of diameter 9 ± 2 microns (Fig. 10A). Radiation (20 Gy) alone significantly induced strand breakage resulting in a large halo of diameter 40 ± 3 microns (Fig. 10B). Apple polyphenols in a concentration dependent fashion inhibited radiation induced DNA strand breaks and at a concentration of 2 mg/ml, it almost completely blocked the strand breakage and the halo diameter found to be almost equal to that of untreated control (l2 ± 2 micron) (Fig. 10 C, D, E)

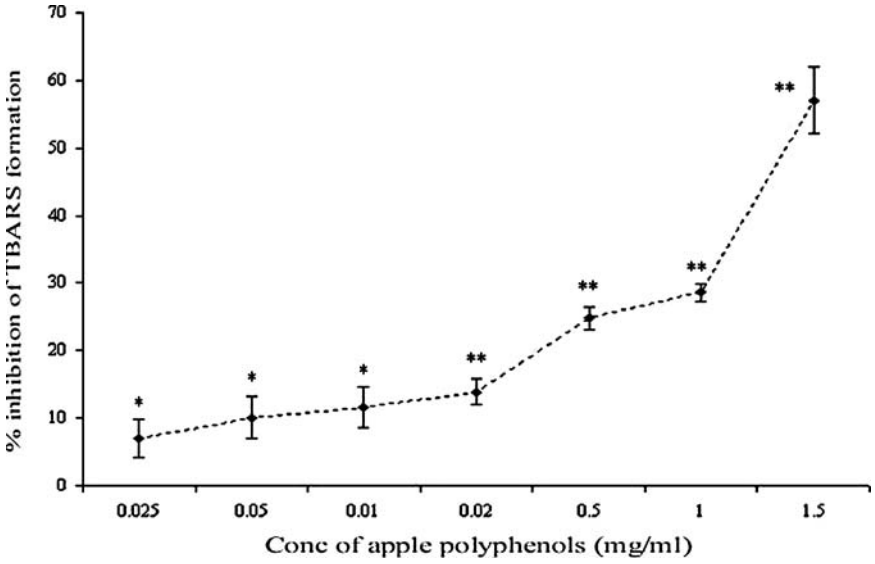

Fig. 8. Effect of varied concentrations of apple polyphenols on Fenton reaction mediated lipid peroxidation in mice liver homogenate. Each experiment was performed in triplicate and was repeated three times. ($^{*}p < 0.05$, $^{**}p < 0.01$).

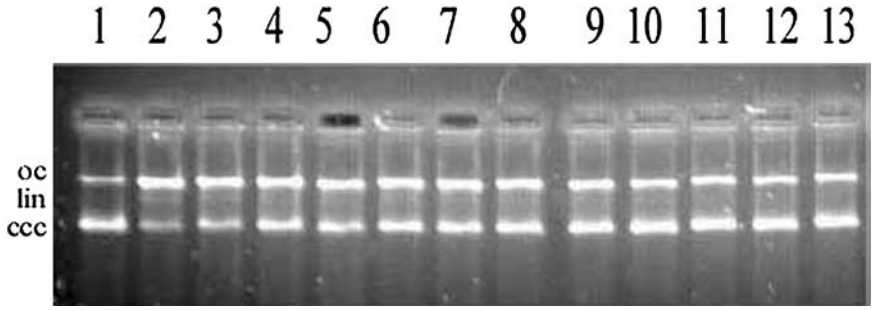

Fig. 9. Effect of varied concentrations of apple polyphenols on radiation induced DNA strand breaks evaluated as conversion of fast migrating supercoiled (ccc) plasmid DNA to slow migrating linear (lin) and relaxed form (oc). Lane 1- Control, Lane 2–20 Gy, Lane 3–13 increasing concentrations (0.02–1 mg/ml) of apple polyphenols + 20 Gy.

Discussion

Present study was aimed at understanding the role of apple polyphenols in ameliorating radiation induced oxidative damage under *in vitro* condition. Exposure of thymocytes to 5 Gy gamma radiation resulted in a significant reduction in cell survival as measured by employing trypan blue exclusion method (Fig. 2). This could be attributed to generation of reactive oxygen species and oxidation of critical bio macromolecules [29]. However, pre-irradiation administration of apple polyphenols resulted in significant reduction in radiation induced cell killing (Fig. 2) clearly establishing its protective efficacy. In order to obtain insight into its mode of action, antioxidant and free radical scavenging ability of apple polyphenols were probed. The inhibition of 2-deoxy ribose degradation, a simple and reliable technique to assess the scavenging ability of an agent [30, 31], clearly demonstrated its OH radical scavenging potential (Fig. 3).

Oral intake of drugs or agents results in inevitable exposure to varied pH and macromolecule breaking enzymes in gastrointestinal tract which are known to have profound effect on their bioactivity. In order to examine the effect of gastrointestinal milieu on apple polyphenols, the effect of simulated gastric juice on its hydroxyl radical scavenging activity was studied. Results have shown that the activity decreased merely by 3% (Fig. 4) indicating gastric juice has no significant effect upon free radical scavenging activity of apple polyphenols and can be administered orally. Superoxide anions constitute an important fraction of radiation induced ROS and known to disrupt normal cellular functions. Apple

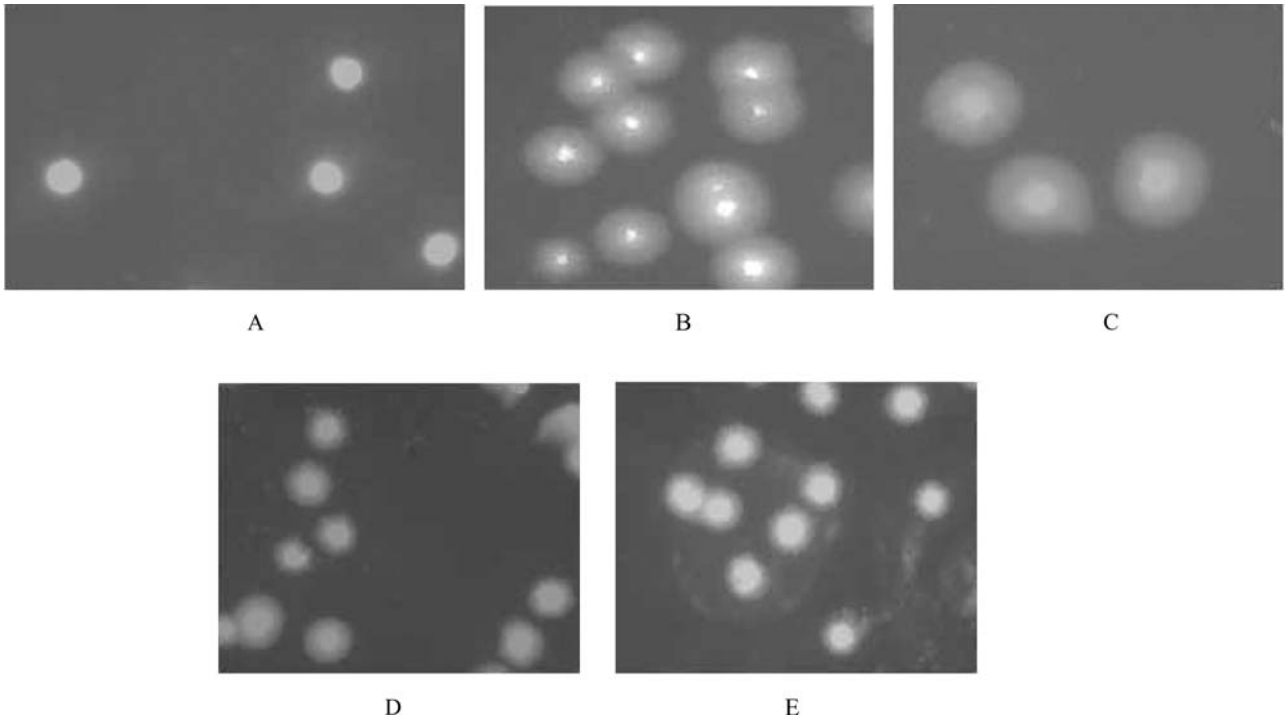

Fig. 10. Effect of varied concentration of apple polyphenols on 20 Gy induced DNA strand breakage as measured by alkaline halo assay in mouse thymocytes (A) Control (B) 20 Gy (C) 0.5 mg/ml +20 Gy (D) 1.5 mg/ml +20 Gy (E) 2.0 mg/ml +20 Gy.

polyphenols significantly ($p < 0.05$) scavenged superoxide anions in dose dependent manner and maximally (88%) at 2 mg/ml (Fig. 5). To further strengthen the radical scavenging potential of polyphenols, DPPH radical scavenging ability was studied. At a dose of 1 mg/ml apple polyphenols significantly scavenged (69%) DPPH radicals (Fig. 6) which can be attributed to phenolic hydroxyl groups of apple polyphenols [17, 18].

The antioxidant activity of natural compounds is known to have a direct correlation with their power to act as reducing agent. Apple polyphenols were found to reduce Fe^{+3} to Fe^{+2} and maximum reduction was observed at 1mg/ml (Fig. 7). The reducing ability of apple polyphenols can be attributed to the presence of phenolic compounds like quercetin, phloridzin, epicatechins, rutin, chlorogenic acid, caffeic acid etc in apple extract [32]. In post irradiation scenario cellular free metal ion content is known to increase and catalyses Fenton reaction mediated generation of hydroxyl radicals there by exaggerating radiation induced oxidative stress [33]. Apple polyphenols have moderately (66%) inhibited Fenton reagent mediated lipid peroxidation in a dose dependent manner (Fig. 8) indicating its ability to prevent metal ion mediated oxidative stress.

DNA being the major site of radiation-induced damage, the capacity of polyphenols to prevent DNA damage was investigated using plasmid DNA (pUC 18) and mice thymocytes. Induction of single or double strand breaks in plasmid DNA resulted in conversion of supercoiled form into slow migrating relaxed form or linear form with intermediate migration respectively. This simple assay has been widely used to study effects of various pro- and anti-oxidant properties of a number of compounds [27]. In corroboration with cell survival and free radical generation, apple polyphenols inhibited radiation induced strand breaks (ssb and dsb) in plasmid DNA (Fig. 9). Similarly apple polyphenols protected cellular DNA from radiation induced damage as revealed by alkaline halo assay of murine thymocytes (Fig. 10). Scavenging of radiation induced hydroxyl radicals, superoxide anions, ability to act as a reducing agent seems to be responsible for mitigating radiation induced DNA damage and cell death.

This study reports for the first time radioprotective potential of apple polyphenols *in vitro* and scavenging of radiation-induced free radicals appears to be contributing towards its over all radioprotective ability. This study warrants further investigation *in vivo*.

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