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Black tea extract protects against γ-radiation-induced membrane damage of human erythrocytes

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The membrane integrity of circulating red blood cells (RBCs) is compromised by the deleterious actions of γ-radiation in humans. Tea is the most widely consumed popular, inexpensive and non-toxic beverage rich in antioxidants. Here, we explored the radioprotective actions of black tea against the γ-radiation-induced membrane permeability of human erythrocytes. The phytochemical analysis of tea revealed the total polyphenol content to be 114.89±6.03 mg gallic acid equivalent/g dry wt. and flavonoid content, 34±0.11 mg catechin equivalent/g dry wt. of the extractable solid in the commercially available tea bags. Tea extracts showed potential scavenging of H_2O_2 and NO, appreciable extent of total antioxidant capacity and effective anti-hemolytic action. Tea extracts (15 μg/mL) significantly ameliorated the γ-radiation-induced increase of the levels of thiobarbituric acid-reactive substances (TBARS, an index of lipid peroxidation) in the RBC membrane ghosts. Stored blood showed higher levels of K^+ ion as compared to the normal blood which was elevated by γ-radiation. Membrane ATPase was inhibited by the exposure to γ-radiation. Treatment of RBCs with the tea extracts (15 μg/ml) prior to the exposure of γ-radiation significantly mitigated these changes in the erythrocyte membranes caused by the lower dose of radiation (4 Gy) as compared to that induced by the higher dose of γ-radiation.

Keywords: Antihemolytic activity, Antioxidants, ATPase, Gamma radiation, Membrane integrity, RBCs, ROS

The increasing utilization of γ-radiation in industry and modern medicine including diagnostic and therapeutic purposes causes radiation injury in humans[1]. Moreover, during radiotherapy for cancer, patients are exposed to different fractionated doses of γ- radiation. In the course of treatment, normal cells are also vulnerable to γ-radiation[2]. The effects of γ-irradiation are largely caused by water radiolysis, a process that produces reactive oxygen species (ROS). ROS can interact with critical macromolecules, such as DNA, proteins, carbohydrates, and lipids inducing cell damage[3]. It is also known that blood and other components also receive a significant dose of radiation during its exposure[4,5]. Circulating throughout the body, RBCs are representative reporter cells for the whole body exposure to γ-radiation. It has been generally accepted that peroxidation of RBC membrane lipids and proteins by radiation-induced ROS can damage membrane integrity resulting in severe cellular dysfunction[6].

Experimental studies on the membrane radiosensitivity focusing on the effect of energy deposition on cell death have been reported[7]. Though, the DNA molecule has been considered as the critical target of ionizing radiation in the cell[8], so far, not much attention has been paid to biological membranes. However, the cell membrane is a significant biological target of radiation[9]. Radiation-induced oxidation of membrane lipid and protein appear to be responsible for damaging the red cell membranes, leading to an increase in the permeability to monovalent and divalent ions[10,11]. Thus, the role of biological membrane in the manifestation and sequel of cell damage following radiation exposure must be considered[9].

Radioprotectors have an indispensable role in the clinical radiation therapy[2]. Due to their high systemic toxicity, application of several synthetic compounds as potent radioprotectors remains limited at their optimum protective dose[1]. These consequences emphasize the search for less toxic or non-toxic compounds from natural source as effective radioprotectors[12] like the natural products from the plant sources. Several phytochemicals from fruits and vegetables, rich in

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Abbreviations: HEPES, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid; H_2O_2, Hydrogen peroxide; NO, nitric oxide; PMSF, phenyl methyl sulfonyl fluoride, ROS, reactive oxygen species; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TAC, total antioxidant capacity

antioxidants and minimally toxic in nature have been investigated to counteract the damage caused by free radicals[13]. Grapevine extract has been reported to be a potential source of natural antioxidants not only against the IR-induced oxidative stress but also inhibit apoptosis[14]. Flavonoids, a family of natural products presents in the fruits, vegetables, and beverages, have shown pharmacological properties including the anti-inflammatory, hepatoprotective, and antioxidant actions[2,15]. Flavonoids extracted from *Rosa roxburghii* Tratt[16] and *Ocimum sanctum* Linn. (Holy Basil)[17] act as a radioprotector.

One such an excellent source of natural antioxidants, tea, is the most widely consumed, inexpensive, non-toxic, and a popular beverage[18]. Epidemiological studies suggest that consumption of tea may be associated with reduced risk of coronary heart disease, stroke and cancer-related deaths[19]. Depending upon the level of fermentation, tea can be categorized into three types: green (unfermented), oolong (partially fermented), and black (highly to fully fermented). In general, green tea has been found to be superior to other types of tea with respect to its antioxidant and health promoting benefits[20]. Green tea extract and its polyphenolic constituents have been shown to possess anticarcinogenic, antimutagenic, antihypertensive and antihepatotoxic actions[21]. Polyphenols from green tea reduce radiation-induced oxidative damage and apoptosis by restoring the redox status[22]. Remarkably, black tea is one of the most widely consumed beverages, particularly in India.

Therefore, in the current study, the radioprotective actions of black tea against the γ- radiation-induced alterations of the RBC membrane permeability were investigated.

Materials and Methods

Chemicals

Naphthylethelene diamine dihydrochloride and sodium heparin from Sigma (St Louis, MO, USA), Aluminum chloride hexahydrate from Alfa Aesar (UK), 2-thiobarbituric acid (TBA) from Loba Chemie, ammonium molybdate, HEPES, PMSF and Tris-HCl from the SRL Chemical (India), and Folin-ciocalteau's reagent and gallic acid from Merck India Ltd. were used. All other chemicals used were of analytical grade and procured from the Merck India Ltd. or SRL India.

Tea extract preparation

Two grams of tea leaves (2 g) procured from local market was added in 50 mL of boiling distilled water for 15 min and then it was centrifuged at 8500 ×g for 5 min and the supernatant was lyophilized. Lyophilized tea extract (1 mg/mL) dissolved in PBS (140 m*M* NaCl in 10 mM sodium phosphate, *p*H 7.4) was used as the stock solution, from which the experimental solutions were prepared by dilution.

The tea samples were analyzed to determine its polyphenol content[23], flavonoid content[24], scavenging activities of hydrogen peroxide[25], nitric oxide[1], total antioxidant capacity (TAC)[26,27] and antihemolytic activity[28].

Blood sample preparation

Isolation and γ-irradiation of cells and RBC membranes

Unutilized stored human whole blood units in acid-citrate-dextrose (ACD-A) solution from the Blood Bank of College of Medicine & JNM Hospital, WBUHS, Kalyani, Nadia was collected.

Preparation for γ-irradiation

Two groups of blood samples, one group for electrolyte analysis and the other group for membrane ghost preparation, were used. Each group consists of three sets. One hour prior to irradiation, each blood sample (4 mL) was treated with the tea extract (60 μL supernatant). One set of blood sample without any extract was prepared for irradiation as experimental control, while another set without any treatment or radiation exposure was considered as normal control. One group of each set was used for the electrolyte analysis, and another group for membrane ghost preparation.

In vitro γ-irradiation

The samples were irradiated at a dose of 4**,** 10, 20 and 50 Gy in a ^{60}Co γ-radiation unit at the UGC-DAE Consortium for Scientific Research, Kolkata Centre, Salt Lake City, Kolkata, with a dose rate of 2.334 kGy/h, incubated for an hour at 25°C and then transported to the laboratory on ice.

This study was approved by the Institutional Ethics Committee (No. F-24/ Pr/ CMJNMH/ IEC/ 16/ 1206) as per ICMR guideline (ECR/674/Inst/WB/2014, dated 31/10/2014).

Determination of Sodium and Potassium

Blood samples collected in 8 sets of test tubes of three each were irradiated for 6, 15, 30 and 77 s. After irradiation samples were centrifuged at 1500 × g for 5 min, plasma were separated, and Na^+ and K^+

level were measured using the electrolyte analyzer (Model: Roche Diagnostics 9180).

Preparation of erythrocyte membranes (Ghosts)

Cytoplasm-free membrane ghosts were prepared as described elsewhere with some modifications[29]. The blood concentrates were spun at 6500×g for 10 min at 4°C. The precipitates were re-suspended in a (final volume of 40 mL for 20 mL blood) solution of 20 m*M* Tris–Cl (*p*H 7.4), 130 m*M* KCl, and 0.6 mg/mL phenylmethylsulfonyl fluoride (PMSF). This suspension was centrifuged for 6500×g for 10 min at 4°C. The cell pellets were lysed by freezing in liquid nitrogen followed by thawing at room temperature. The lysed cells were then re-suspended with 5 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES; *p*H 7.4), 1 mM ethylenediamine tetraacetic acid (EDTA) and 0.6 mg/mL PMSF and centrifuged at 9000×g for 10 min at 4°C. This washing step was repeated four times, and the resulting pellets were suspended in (40 mL) 10 m*M* HEPES (*p*H 7.4), 130 m*M* KCl, 0.5 m*M* $MgCl_2$, and 0.05 m*M* $CaCl_2$. The solutions were spun at 9000×g for 10 min, re-suspended in a small volume of this last buffer, and stored under liquid nitrogen until use.

The total protein concentration[30] and lipid peroxidation[31] were assayed in the free ghost membranes.

ATPase Assays

Assay of total ATPase

The total ATPase activity in 0.1 mL aliquot of the homogenates were measured in a final volume of 2 mL containing 0.1 mL each of 0.1 *M* Tris–HCl (*p*H 7.4), 0.1 *M* NaCl, 0.1 *M* $MgCl_2$, 1 m*M* EDTA 0.01 *M* ATP and 1.5 mL of 0.1 *M* KCl. The reaction was stopped at 20 min by adding 1 mL of 10% TCA, centrifuged at 1500×g for 10 min[32], and the inorganic phosphorus (Pi) liberated was estimated in the protein-free supernatant.

Na^+K^+ATPase assay

Tissue homogenate (0.2 mL) was added to the mixture containing 1 mL of 184 m*M* Tris–HCl buffer (*p*H 7.5), 0.2 mL each of 50 m*M* $MgSO_4$, 50 m*M* KCl, 600 m*M* NaCl, 1 m*M* EDTA and 10 m*M* ATP and incubated for 15 min at 37°C. The reaction was then stopped by adding 1 mL of ice cold TCA (10%)[33]. The amount of Pi liberated was estimated in the protein free supernatant.

Ca^{2+}ATPase assay

Tissue homogenate (0.1 mL) was added to a mixture containing 0.1 mL each of 125 m*M* Tris–HCl buffer (*p*H 8), 50 m*M* $CaCl_2$ and 10 m*M* ATP. The contents were incubated at 37°C for 15 min. The reaction was terminated by the addition of 0.5 mL of ice cold TCA (10%) and centrifuged[34]. The amount of Pi liberated was determined in the protein free supernatant.

Mg^{2+}ATPases assay

Tissue homogenate (0.1 mL) was added to a mixture containing 0.1 mL each of 375 m*M* Tris–HCl buffer (pH 7.6), 25 m*M* $MgCl_2$, 10 m*M* ATP and 0.1 mL water. The contents were incubated at 37°C for 15 min and the reaction was stopped by adding 0.5 mL of TCA (10%)[35]. The Pi liberated was then determined in protein free supernatant

Determination of inorganic phosphate

Inorganic phosphorus present in the protein free supernatant was determined from the formation of phoshomolybdic acid by the reaction between phosphate and molybdic acid as the intensity of which is proportional to the phosphate ion concentration[36]. To suitable aliquots of the supernatant, 1 mL of ammonium molybdate reagent (2.5%; 2.5 g of ammonium molybdate dissolved in 100 mL 3 *N* sulphuric acid) was added. 0.4 mL of ANSA [0.5 g of ANSA was dissolved in 195 mL of 15% sodium metabisulphite and 5 mL of 20% sodium sulphate was added to attain complete solubility. The solution was filtered and stored in a brown bottle] was added after 10 min of incubation at room temperature. Standards (35.1 mg of potassium dihydrogen phosphate in 100 mL double distilled water) and blank were also treated in the above manner. The blue colour developed was measured spectrophotometrically after 20 min at 640 nm.

Statistical analysis

All the analyses of the tea extracts were performed in triplicate, while studies on erythrocyte were performed five times and the results were expressed as the mean ± SE using statistical software SPSS. Statistical significance between treatments was established through the one-way analysis of variance (ANOVA), followed by the Tukey's test for individual differences. A value of $P < 0.05$ was used to establish statistical significance.

Results

The total polyphenol content was 114.89±6.03 mg gallic acid equivalent/g dry weight and flavonoid content was 34±0.11 mg catechin equivalent/g dry wt. in the extractable solid. It was observed that the H_2O_2- and NO- scavenging actions increased with the increase in concentration of tea extract (Fig. 1). However, these changes were significant in case of H_2O_2- scavenging activity. However, in the NO-scavenging activity this change was significant at lower concentration, while it did not change significantly at higher concentration. Total antioxidant capacity was estimated 32 µg ascorbic acid equivalent/ mg dry wt., which was higher than the standard ascorbic acid. The antihemolytic activity was determined by comparing the hemolysis of the treated blood with tea extracts of different concentration with respect to the positive (water) and negative (PBS) control (Fig. 2). Tea extracts showed effective antihemolytic activity and its activity increased with the increase in concentration of the tea extract.

Our results showed that γ-irradiation increased the TBARS level in the membrane ghosts in comparison to the corresponding non-irradiated controls; the increase was irradiation dose- dependent (Fig. 3). Tea extracts (15 µg/mL) significantly ameliorated these changes at a lower radiation dose (4 Gy), but not at higher dose of radiation (Fig. 3).

We observed elevated level of K^+ in stored blood as opposed to the Na^+ level in the same (Table 1). Exposure to γ-radiation induced an increase in the K^+ values of the plasma (Table 1). Conversely, Na^+ levels did not change in the irradiated samples as compared to the controls (Table 1). Tea extracts (15 µg/mL) treatment prior to the γ-radiation significantly lowered the K^+ level as compared to the untreated irradiated group at a radiation dose of 4 and 50 Gy (Table 1).

Cytosolic ion concentrations are dependent on the correct functioning of membrane ATPases, namely, the Na^+,K^+–ATPase, Ca^{2+}–ATPase and Mg^{2+}–ATPase. γ-Radiation inhibited the ATPase activities in membrane ghosts. Tea extracts (15 µg/mL) pretreatment significantly protected total ATPase activity against lower γ-radiation dose (Table 1).

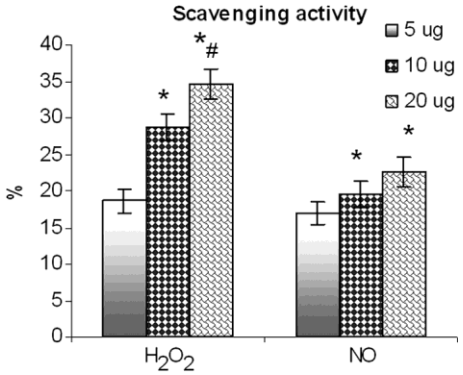

Fig. 1—Tea extracts scavenges on hydrogen peroxide and nitric oxide. [Values are mean ± SEM of 3 observations. P values: *<0.05 compared to control group; #<0.05 compared to 5 µg extract treated group]

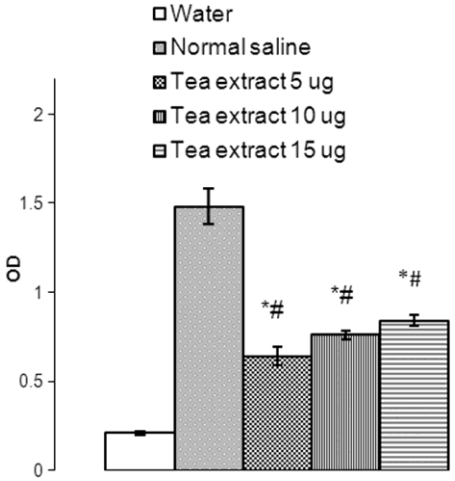

Fig. 2—Antihemolytic actions of tea extract. [Each value is represented as mean ± SE (n = 3). *P* values: *<0.05 compared to positive control group; #<0.05 compared to negative group. Notes: Water replaced extract/fractions to serve as a positive control since this treatment results in 100% hemolysis; -Phosphate buffered saline replaced extract/fraction to serve as a negative control since this treatment results in 0% hemolysis; Lower values are associated with increased cell lysis]

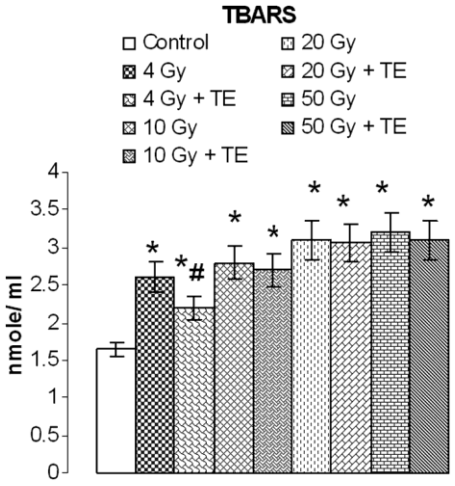

Fig. 3—Tea extracts modulates lipid peroxidation in γ-radiation-exposed RBC ghosts. [Values are mean ± SEM of 3 observations. P values: *<0.05 compared to non-irradiated group; #<0.05 compared to tea extract treated group of same radiation dose]

Table 1—γ-radiation Modulates Membrane activity in RBCs

	Na^+*	K^+*	Total ATPase#	Na^+, K^+ ATPase#	Ca^{2+} ATPase#	Mg^{2+}ATPase#
Control	135.4 ± 0.75	6.5 ± 0.10	222.4 ± 3.5	181.6 ± 3.17	193.2 ± 4.49	171.8 ± 3.02
4 Gy	132.6 ± 1.1	6.98 ± 0.07[b]	183.6 ± 2.48[a]	165.2 ± 3.32[b]	168.8 ± 2.72[a]	152.2 ± 2.65[a]
4 Gy + T.E	132.4 ± 0.81	6.56 ± 0.10[e]	208.4 ± 2.22[c,d]	174 ± 3.2	169.8 ± 2.51[a]	160 ± 3.03
10 Gy	131.4 ± 1.36	6.88 ± 0.05[c]	171.0 ± 1.18[a]	152.0 ± 2.28[a]	145.2 ± 3.0[a]	142.4 ± 2.56[a]
10 Gy + T.E	132.2 ± 0.86	6.52 ± 0.08	183.8 ± 2.33[a,e]	164.6 ± 2.96[b]	153.0 ± 3.3[a]	150.8 ± 2.41[a]
20 Gy	131.6 ± 1.43	6.88 ± 0.08[c]	165.4 ± 2.56[a]	149.6 ± 2.31[a]	138.8 ± 1.35[a]	126.8 ± 2.43[a]
20 Gy + T.E	132.4 ± 0.92	6.66 ± 0.74	179.4 ± 1.96[a,e]	155.6 ± 3.14[a]	144.8 ± 1.85a	127.2 ± 3.48[a]
50 Gy	132 ± 0.83	7.0 ± 0.07[b]	153.6 ± 3.01[a]	135.0 ± 1.84[a]	128.0 ± 2.23[a]	121.6 ± 1.63[a]
50 Gy + T.E	132.8 ± 0.86	6.62 ± 0.066[e]	150.0 ± 2.98[a]	138.0 ± 1.85[a]	135.2 ± 1.39[a]	122.6 ± 2.20[a]
F value	1.31	6.142	86.38	31.90	58.04	46.78
Significance	0.27	<0.001	<0.001	<0.001	<0.001	<0.001

*milli equivalent/L; #nmole inorganic phosphate liberated/mg protein/hour

[T.E., tea extract (15 µg/mL). Values are mean ± SEM of 5 observations. *P* values: [a]<0.001, [b]<0.01, [c]<0.05 compared to control group; [d]<0.001, [e]<0.05 compared to corresponding gamma radiation group]

Discussions

Among the dietary antioxidants, the phenolic compounds, secondary metabolites occurring in fruits and vegetables are the most abundant natural antioxidants[37]. Though the content of extractable polyphenol from tea bags in our study was less than ethanol extractable solid yield in tea leaf in another study[38], they are one of the main nutritional sources of bioactive compounds.

Most tea polyphenols exert their biological actions by scavenging the reactive oxygen species (ROS)[20]. In the present study, the H_2O_2-scavenging action was found to increase with the increasing concentration of the extract, but the nitrite-scavenging action did not change with the increasing concentration in our study. However, it has been reported that the scavenging activity is the highest among different scavenging actions of the black tea extract[39]. Therefore, determination of the TAC of a sample is more useful than the knowledge of specific species[26], and the antioxidant capacity showed by tea extract in our study is comparable to the other reported study[40]. The infusion/brewing time and the form of tea (loosely packed or bagged) have been shown to be important determinants of the antioxidant action and polyphenol content of the black tea infusions in addition to the variety, growing environment and manufacturing conditions[41]. The presence of primary antioxidants in the tea extracts also exhibited the antihemolytic action(s). It could be possible that the total phenols and flavonoids present in the extract might have caused its potent anti-haemolytic action[42].

The antioxidant potential and scavenging property of tea extracts seen *in vitro* were investigated on the radioprotective efficacy of tea on human RBCs and their membranes *in vitro* in this study. RBCs are easy to isolate and suitable for monitoring both the short- and long-term adverse effects of radiation.

Transfusion-associated graft-versus-host disease (T_A-G_VHD) is a rare, fatal disease that can be prevented by irradiating RBC with 25 Gy of gamma rays[29]. It is a common practice in blood banks to stock irradiated RBC units to utilize in several situations, including those involving immunocompromized patients, and direct and intrauterine transfusions[29]. In this study, we used stored blood and found lower levels of Na^+ and high levels of K^+ in the non-irradiated control set. It was reported that the storage of RBCs for 3 wk at 4°C under blood bank conditions resulted in a rise in intracellular Na^+ and a fall in intracellular K^+ with concomitant opposite changes in Na^+ and K^+ levels in the suspending plasma[43]. Biochemical analyses revealed a progressive decrease from day seven in the stored blood sodium, with a concomitant increase in potassium. These changes are attributable to the ongoing metabolism and deterioration of the RBCs while in storage[44]. ROS can also be produced in the non-irradiated RBCs during the low-temperature storage in the citrate-phosphate-dextrose-adenine-1 (CPDA-1) solution because of high glucose concentration, the exposure to light and agitation, the leakage of free radicals from leukocyte contamination, etc.[45]. These chemical activities lead to a complex RBC storage lesion that includes haemolysis and membrane loss[46].

Further, irradiation of blood induces an increase in the plasma K^+ in agreement with other studies[47,48]. Moreover, irradiation of RBCs resulted in the

enhancement of TBARS production as reported elsewhere[49]. However, tea extract treatment prior to irradiation protected erythrocytes from the irradiation-associated TBARS formation and cation leakage as observed in the current study.

Shifts in the cell membrane lipid composition[48] and cation exchange[43] might also alter the activity of the membrane pumps. Earlier reports show that γ-radiation can induce damage in the plasma membrane or associated proteins[48,50]. Two plasma membrane enzymes that are performing keyroles in erythrocyte ionic balance: Na^+,K^+–ATPase and Ca^{2+}–ATPase. The Na^+,K^+–ATPase is a heterodimer ($\alpha_2\beta_2$), where α (100 kDa) is the catalytic subunit and β (50 kDa) is a glycoprotein with regulatory and chaperone-like relevance. This enzyme maintains an asymetrical gradient of Na^+ and K^+ through plasma membrane, which is important to maintain the transport of glucose and amino acids; and thereby establish the resting membrane potential and regulate cell volume[48]. Ca^{2+}–ATPase, a monomer (140 kDa) is important to extrude Ca^{2+} ions from cytoplasm to the outside of cells, and controlling intracellular Ca^{2+} concentrations in homeostatic levels[48]. Another ATPase found in erythrocytes requires magnesium for its activity as a cofactor is Mg^{2+}-ATPase[51]. The effects of irradiation worsen the activities of these enzymes. The addition of tea extracts to blood prior to γ-irradiation could effectively protect total ATPase activity but not individual ATPase activity in stored blood as observed in our present study.

The development of effective radioprotectors is important for safe application of IR in medical practices, e.g. radiotherapy, nuclear medicine, and transfusion medicine. Inclusion of antioxidants to blood prior to the γ-irradiation treatment can be an effective alternative since this strategy can decrease lipid peroxidation[3]. In the present study, the radioprotective efficacy of tea in the human RBCs and their membranes *in vitro* were investigated. The preventative effects were observed *in vitro* at 15 µg tea extract/mL test solution in this study. In one study[52], black tea extract exerted maximum protection against radiation-induced damage in V79 cells at a maximum dose of 5 µg/ml. Different doses (0.005-500 µg/mL) of tea extract have prevented oxidative DNA damage due to γ-radiation (2 Gy) in human lymphocytes[53]. Tea contains 70% flavonoids such as catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate[2,54]. Since the polyphenolics function as good electron and hydrogen atom donors, and therefore, can terminate the radical chain reaction by converting the free radicals and ROS to more stable products, thereby considerably mitigating the IR effects at the molecular, cellular, and tissue levels[2,55,56]. Moreover, tea flavonoids are known to be rapidly absorbed into the circulation following oral ingestion[19]. Therefore, the tea extracts thus mitigated γ-radiation associated blood electrolyte disturbances and altered erythrocyte membrane total ATPase activity in stored blood at a lower dose as revealed by our present study.

One could hypothesize that the capacity of flavonoids to induce detoxifying enzymes is a major mechanism by which these compounds exert their preventive/protective action. However, under certain reaction conditions they can also display the pro-oxidant activity[57]. Antioxidative properties of flavonoids *in vitro* depend up on various experimental conditions, i.e. flavonoid concentration, biological system, incubation time, pH. Therefore, antioxidant dose and reaction state/condition are extremely crucial to formulate and use as the radioprotective agent.

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