Grapevine fruit extract protects against radiation-induced oxidative stress and apoptosis in human lymphocyte

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Ionizing radiation (IR) causes oxidative stress through overwhelming generation of reactive oxygen species (ROS) in the living cells leading the oxidative damage further to biomolecules. Grapevine (*Vitis vinifera* L.) possess several bioactive phytochemicals and is the richest source of antioxidants. In this study, we investigated *V. vinifera* for its phytochemical content, enzymes profile and, ROS- and oxidant-scavenging activities. We have also studied the fruit extract of four different grapevine viz., Thompson seedless, Flame seedless, Kishmish chorni and Red globe for their radioprotective actions in human lymphocytes. The activities of ascorbic acid oxidase and catalase significantly (*P* <0.01) differed among extracts within the same cultivar, while that of peroxidase and polyphenol oxidase did not differ significantly. The superoxide radical-scavenging activity was higher in the seed as compared to the skin or pulp of the same cultivar. Pretreatment with grape extracts attenuated the oxidative stress induced by 4 Gy γ-radiation in human lymphocytes *in vitro*. Further, γ-radiation-induced increase in caspase 3/7 activity was significantly attenuated by grape extracts. These results suggest that grape extract serve as a potential source of natural antioxidants against the IR-induced oxidative stress and also inhibit apoptosis. Furthermore, the protective action of grape depends on the source of extract (seed, skin or pulp) and type of the cultivars.

**Keywords:** Antioxidants, Caspase 3/7, Ionizing radiation, Polyphenols, Radioprotective agents, ROS, *Vitis vinifera*.

Ionizing radiation (IR) is widely used in modern medicine for diagnostic and therapeutic purposes, including cancer treatment. Increased utilization of γ-radiation for medical and industrial purposes has lead to increased radiation hazards in humans. In radiotherapy of cancer, normal cells are also damaged during the exposure γ-radiation.

Radiation hazards are mainly manifested by radiolysis of body water. Low Linear Energy Transfer (LET) radiations act by generating the free radicals. These free radicals interact with different components of the cells including cell membrane and different intracellular molecules like DNA, RNA and proteins, resulting in cellular dysfunction and mortality. Subcellular membranes, in view of their high content of polyunsaturated fatty acids, are major targets of oxidative damage and their adverse alterations can lead to undesirable consequences. IR induces the production of reactive oxygen species (ROS) and promotes changes in the expression of several apoptotic proteins, leading to elevated apoptosis.

Therefore, it is crucial to identify an effective radioprotective agent capable of protecting against the radiation-induced cell death by scavenging the free radicals. Although several synthetic compounds have been found to be good radioprotectors, their practical application remains limited due to the high systemic toxicity at their optimum protective dose, which accentuated the search for either less or non-toxic radioprotective compounds of biological origin. Plants rich in antioxidants and minimally toxic in nature are known for use in amelioration of the damage caused by oxidative stress. Flavonoids, a family of natural products present in many fruits, vegetables, and beverages, exhibit pharmacological properties including the anti-inflammatory, hepatoprotective, and antioxidant actions. These natural products of plant origin also react with and scavenge free radicals and reactive molecules produced by oxidative stress. These interactions are facilitated by their polyphenol ring structure with hydroxyl and methoxyl groups, or both that can specifically bind to or react with a base or other groups in the DNA backbone, as well as trap or scavenge the ROS. These polyphenolic compounds considerably mitigate the damaging effects of ionizing radiation.
irradiation at the molecular, cellular, and tissue levels\textsuperscript{15-17}. Radioprotective actions of antioxidative phytochemicals such as sesamol from sesame seed, apple, and curcumin from turmeric and apigenin have been reported\textsuperscript{7,18-21}. Currently, health benefit of grape polyphenols is gaining a great deal of attention\textsuperscript{17,22-25}. Grapes contain a variety of phytochemicals and antioxidants including resveratrol, anthocyanins, catechin, quercetin, flavans, and several phenolics\textsuperscript{17,22-27}. Grape polyphenols have been demonstrated to maintain endothelial function, prevent platelet aggregation, suppress LDL oxidation, and to protect against the oxidative DNA damage, coronary diseases and atherosclerosis\textsuperscript{17,24-26}. In vitro and in vivo studies have shown that grapes have strong antioxidant activity, inhibit cancer cell proliferation, suppress platelet aggregation, and lowering cholesterol\textsuperscript{17,22-25}. Grape phytochemicals in combination have been shown to be more effective than individual molecules in isolation in exerting their beneficial actions\textsuperscript{28}. The immune system provides the first line of defense against exposure to environmental hazards. Blood and other components receive a significant dose of radiation during radiation exposure\textsuperscript{29}. The immune system cells are the most radiosensitive cells in the body\textsuperscript{30}. Most of the existing reports on the radioprotective role of polyphenols are based on the animal studies, and so far no report has been made on the protective actions of grape polyphenols against the radiation-induced cellular damage, in particular as a radioprotector, in the human lymphocytes.

Hence, in the present study we evaluated the radioprotective effect of grape extract against γ-radiation (IR)-induced oxidative stress and apoptosis in the human lymphocytes. Yu and Little\textsuperscript{31} have already reported the role of caspase-3 in mediation of apoptosis induced by different stimuli in human lymphoblast cell lines. Here, radioprotection of the human lymphocytes by the extracts of seed, pulp and skin of different cultivars of grape was established by determining the activities of the oxidative stress-related enzymes and levels of non-enzymatic antioxidants, and caspase 3/7 activity to assess the apoptosis.

**Materials and Methods**

**Chemicals**— DTNB (5,5′-Dithiobisnitro benzoic acid), NADH, NADPH, 5-methylenazinium methosulfate (PMS), reduced glutathione (GSH), oxidized glutathione (GSGG), glutathione reductase (GR), nitroblue tetrazolium (NBT) were obtained from the SRL Chemicals. Trypan blue was purchase from the Sigma Chemical Co. (St. Louis, MO). HiSep LSM 001 was procured from the Hi Media Laboratories (Mumbai, India). The Caspase-Glo®3/7 assay kit was supplied by the Promega, Madison, WI, USA. Other reagents used were of analytical grade. All other chemicals were purchased from the Merck India Ltd or SRL India.

**Sources of grape cultivars**— Commonly available four grape (V. vinifera L.) cultivars, namely the ‘Thompson seedless’ (green), ‘Red globe’ (red), ‘Flame seedless’ (black), and ‘Kishmish chorni’ (black with reddish brown) were purchased from the local market, and were authenticated by the Department of Fruits and Orchard Management, Faculty of Horticulture, Bidhan Chandra Krishi Viswavidyalaya, Nadia, India. The seed, skin and pulp of grapes were carefully manually separated from undamaged and disease-free berries.

**Ascorbic acid oxidase (AAO; EC 1.10.3.3) activity assay**— Grape (5 g) was crushed and the extract was made up to 100 ml with distilled water. The substrate (2 ml of 50 mg ascorbic acid in 100 ml distilled water) was mixed with 2 ml of the enzyme extract and 3 ml of distilled water, and incubated at 28°C for 20 min. The enzyme reaction was stopped with 5 ml of 10% TCA solution. Control contained 2 ml of the substrate, 5 ml of distilled water, and 5 ml of 10% TCA solution. The enzyme mixture was titrated against the dye solution (15 mg of 2,6-dichlorophenol indophenol in 50 ml of distilled water and 20 mg NaHCO3). Final volume of the solution was 100 ml, filtered and kept in a coloured glass bottle) till a faint blue color persisted for at least 15 s and the AAO enzyme activity was expressed as mg ascorbic acid decomposed/g tissue/min\textsuperscript{32}.

**Polyphenol oxidase (PPO; EC 1.14.18.1 or EC 1.10.3.2) activity assay**— The grape extract (500 mg) homogenized in 2 ml of the extraction medium containing Tris-HCl (50 mM, pH 7.2), sorbitol (0.4 M) and NaCl (10 mM) was centrifuged at 3000×g for 10 min and the supernatant was used for the PPO activity assay. The enzyme extract (0.2 ml) was added to 2.5 ml of phosphate buffer (0.1 M, pH 6.5) containing 0.3 ml of catechol solution (0.01 M), and the change in absorbance was recorded at 495 nm for every 30 s up to 5 min\textsuperscript{33}. One unit of PPO activity was expressed as the change in absorbance of 0.1/ min/ ml of the enzyme extract\textsuperscript{32}.

**Catalase (CAT; EC 1.11.1.6) activity assay**— An extract of ~50 ml was made from 5 g of grapes with distilled water, stained through a linen cloth, and the
filtrate was used for the CAT activity assay. Distilled water (5 ml), 1 ml of H$_2$O$_2$ substrate, and 2 ml of the enzyme extract were mixed and incubated for 15 min at 28±1°C. A blank without the enzyme was prepared with 7 ml of water and 1 ml of H$_2$O$_2$. The enzyme activity was stopped by adding 5 ml of 10% H$_2$SO$_4$ to the reaction mixture. Residual H$_2$O$_2$ content of the mixture was determined by titration against 0.05 N KMnO$_4$ solution till a faint pink colour persisted for at least 10 s and the CAT activity was expressed as mg H$_2$O$_2$ decomposed/g fresh weight/min determined.$^{35}$

**Peroxidase (POD; EC 1.11.1.7) activity assay**—A 20% homogenate was prepared in 0.1 M phosphate buffer (pH 6.5) centrifuged, and the supernatant was used for the assay of POD activity. To 2.5 ml of pyrogallol solution (0.05 M in 0.1 M phosphate buffer, pH 6.5), 0.02 ml of the enzyme extract was added, and the reaction was initiated by mixing 0.5 ml of H$_2$O$_2$ (1% in 0.1 M phosphate buffer, pH 6.5). The change in absorbance was recorded every 30 s up to 3 min in a spectrophotometer (Systronics). One unit of peroxidase is defined as the change in absorbance/min at 430 nm.$^{36}$

**Grape extract preparation**—Grape extracts were lyophilized (concentrated) and stored at 4°C in light-tight containers until used. Lyophilized extract was used for the assay of ROS-scavenging action. For experiments with γ-irradiation, 50 mg of each lyophilized grape extract was dissolved in 500 µl of 1X sterile PBS.

**Superoxide anion radical scavenging activity (SRSA) assay**—Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by reduction of nitroblue tetrazolium (NBT) into a purple colored formazan. In this experiment, the superoxide anion was generated in 3 ml of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 µM) solution and 0.3 ml of different concentration the chosen grape extracts. The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at ambient temperature, absorbance was recorded at 560 nm.$^{37}$ The percent inhibition was calculated from the following formula:

% inhibition = 
\[
\frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100
\]

**Blood sample preparation**—Fresh blood samples were aseptically collected in heparinized tubes from healthy male donors (22-30 years) who had no history of disease or recent administration of any drug. One hour prior to irradiation, each blood sample (4 ml) was treated with the individual chosen grape extract (60 µl supernatant) from seed, skin or pulp of each cultivar. One set of blood sample without any extract was irradiated and was considered as experimental control, while another set without any treatment was considered as normal control. Preliminary studies were carried out to ensure whether this concentration did not have any toxic effect using the trypan blue exclusion technique.$^{29,38}$

**In vitro γ-irradiation**—The samples were irradiated at @ 4 Gy in a $^{60}$Co γ-radiation unit at the UGC-DAE Consortium for Scientific Research, Kolkata Centre, Salt Lake City, Kolkata at a dose rate of 3.05 kGy/h. The study was approved by the Institutional Ethics Committee [No. F-24/Pr/CMJNMH/IEC/14/93(4)] as per the ICMR guideline (ECR/674/Inst/WB/2014, dated 31/10/2014). After irradiation, samples were incubated for 1 h at 25°C and later transported to the laboratory on ice. Lymphocytes were then isolated using the HiSepLSM 001 gradient.

**Biochemical assays**—Lymphocytes were suspended in 50 mM PBS containing containing 130 mM KCl and 10 mM Dithiothreitol and centrifuged at 20000 ×g for 15 min at 4°C. The supernatant was taken for protein estimation$^{39}$, thiobarbituric acid reactive substances (TBARS)$^{40}$, GSH$^{41}$, and activities of superoxide dismutase (SOD)$^{42}$, CAT$^{43}$ and glutathione peroxidase (GPx)$^{44}$.

**Caspase Glo® 3/7 assay**—Lymphocytes were lysed with NT2 buffer (500 µl 1 M Tris-cl pH 7.4; 300 µl 5M NaCl; 10 µl 1 M MgCl$_2$; 5 µl 100% NP 40) and centrifuged at 10000 RCF at 4°C for 10 min. The protein content in the supernatant was determined. Each sample containing 10 µg protein was mixed with the substrate, Caspase activity was determined according to the manufacturer’s instructions. Briefly, samples containing 10 µg protein were seeded onto 96-well plates (10 µg protein/ well). Volume was made up to 10 µl using NT2 buffer (500 µl 1 M Tris-cl pH 7.4; 300 µl 5M NaCl; 10 µl 1 M MgCl$_2$; 5 µl 100% NP 40). The caspase3/7 reagent (10 µl) was added to each well and incubated for 30 min at 25°C. Luminescence was then measured by Chameleon multimode plate reader (Hidex, Finland).
Statistical analysis— All the analyses related to grape polyphenols extracts were performed in triplicate, while lymphocyte related studies were performed five times, and the results are expressed as the mean ± standard error (SE). Statistical significance between groups was established by the one-way analysis of variance (ANOVA), followed by the Tukey test for individual differences. Values P < 0.05 were considered statistically significant.

Results

From the antioxidant enzyme profile established in different grape cultivars, AAO and CAT activities differed significantly (P < 0.01) among grape extracts within the same cultivar (Table 1). The seed extract showed the highest activity, followed by the extracts of skin and pulp. These activities also differed among different grape cultivars. Though significant difference in the POD activity was observed between the extracts of skin and pulp of the Thompson seedless (green) cultivar (Table 1); the PPO activity significantly differed in the Flame seedless (Black) cultivar (Table 1). However, the activity of these two enzymes did not show marked difference in the seed extract (Table 1). The grape extracts showed a scavenging activity of the superoxide radicals in a dose-dependent manner (Fig. 1). The IC$_{50}$ values for the superoxide-scavenging activities were in the order of seed, followed by skin and pulp (Table 2). The grape extract exhibited the highest superoxide-scavenging ability with the IC$_{50}$ value of 66.6 µg/ml in the Kishmish chorni seed, followed by the Red globe seed extract (70.8 µg/ml).

Table 1—Enzyme profiles of different grape cultivars

<table>
<thead>
<tr>
<th>Grape cultivars</th>
<th>Part</th>
<th>Ascorbic acid oxidase (mg ascorbic acid decomposed/g tissue/min)</th>
<th>Catalase (mg H2O2 decomposed/g fresh weight/ min)</th>
<th>Peroxidase (U/ min)</th>
<th>Polyphenol oxidase (U/ min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thompson seedless (Green)</td>
<td>Skin</td>
<td>6.07±0.12*</td>
<td>245 ±6.51*</td>
<td>0.29±0.02*</td>
<td>2±0.06</td>
</tr>
<tr>
<td></td>
<td>Pulp</td>
<td>2.31±0.02</td>
<td>83.33±2.73</td>
<td>0.18±0.01</td>
<td>2.03±0.09</td>
</tr>
<tr>
<td>Flame seedless (Black)</td>
<td>Skin</td>
<td>10.08±0.1*</td>
<td>309±8.62*</td>
<td>0.26±0.006</td>
<td>2.87±0.12**</td>
</tr>
<tr>
<td></td>
<td>Pulp</td>
<td>7.17±0.15</td>
<td>128±7.64</td>
<td>0.27±0.01</td>
<td>2.03±0.09</td>
</tr>
<tr>
<td>Kishmish chorni (Reddish Brown)</td>
<td>Skin</td>
<td>8.56±0.64*</td>
<td>279.33±29.06*</td>
<td>0.24±0.006</td>
<td>2.63±0.09</td>
</tr>
<tr>
<td></td>
<td>Pulp</td>
<td>6.03±0.18</td>
<td>108.67±5.46</td>
<td>0.23±0.003</td>
<td>2.4±0.06</td>
</tr>
<tr>
<td>Red globe</td>
<td>Seed</td>
<td>98±4.24</td>
<td>443±23.6</td>
<td>0.14±0.008</td>
<td>1.4±0.03</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>8.07±0.12**</td>
<td>204.67±6.9*</td>
<td>0.19±0.007</td>
<td>2.77±0.04</td>
</tr>
<tr>
<td></td>
<td>Pulp</td>
<td>2.49±0.03</td>
<td>107.33±5.81</td>
<td>0.17±0.003</td>
<td>2.5±0.06</td>
</tr>
<tr>
<td></td>
<td>Seed</td>
<td>82±3.39</td>
<td>378±16.47</td>
<td>0.12±0.01</td>
<td>1.3±0.01</td>
</tr>
</tbody>
</table>

Values are mean ± SE (standard error). P values: *<0.001, **<0.01 compared between flesh and pulp within the same grape cultivar.

*One unit of peroxidase is defined as the change in absorbance/ min at 430 nm

**One unit of PPO activity was expressed as the change in absorbance of 0.1/ min/ ml of the enzyme extract at 495 nm

Table 2—in vitro effects of grape extracts from different cultivars on Superoxide radical scavenging activity

<table>
<thead>
<tr>
<th>Grape cultivars</th>
<th>Part</th>
<th>SOD radical scavenging activity [inhibitory concentration (IC50=µg/ml)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thompson seedless (Green)</td>
<td>Skin</td>
<td>472.4±17</td>
</tr>
<tr>
<td></td>
<td>Pulp</td>
<td>2491.2±46</td>
</tr>
<tr>
<td>Flame seedless (Black)</td>
<td>Skin</td>
<td>159.9±12</td>
</tr>
<tr>
<td></td>
<td>Pulp</td>
<td>2242±43</td>
</tr>
<tr>
<td>Kishmish chorni (Reddish Brown)</td>
<td>Skin</td>
<td>183.7±11.5</td>
</tr>
<tr>
<td></td>
<td>Pulp</td>
<td>1931±74</td>
</tr>
<tr>
<td>Red globe</td>
<td>Pulp</td>
<td>2775±45</td>
</tr>
<tr>
<td></td>
<td>Seed</td>
<td>70.8±3</td>
</tr>
</tbody>
</table>

Values are mean ± standard errors (SE) of means of three experiments.
The grape extracts did not alter the viability of human lymphocytes. Exposure of lymphocytes to the $\gamma$-irradiation at a dose of 4 Gy significantly increased the extent of formation of TBARS, while decreased the level of GSH and activities of CAT, GPx and SOD in the lymphocytes as compared to the non-irradiated control counterparts (Fig. 2). However, these changes were significantly attenuated by the pretreatment with the grape seed ($P < 0.001$) and the skin extracts ($P < 0.05$) compared to the ionizing radiation exposed group. Moreover, protection offered by the seed extracts was found significantly better than that was offered by the pulp extract of the same cultivar.

Luminescence measurement revealed that caspase 3/7 activity in the human lymphocytes to assess the apoptosis increased upon $\gamma$-irradiation at 4 Gy compared to the control group (Fig. 3). Pretreatment of the lymphocytes with the grape extracts significantly attenuated radiation-induced increase in caspase 3/7 activity and the seed extracts showed higher inhibitory action.

**Discussion**

Grapes, one of the most widely cultivated and consumed fruits in the world, are rich in phytochemicals beneficial to human health. However, the biological importance and health benefit of grape extracts are mainly attributed to its antioxidant properties of grapes. The antioxidant enzymes and free radical scavengers may provide a defensive mechanism against the deleterious actions of ROS. Some of the antioxidant enzymes that are found to...
provide a protection against the ROS are CAT, POD and AAO. AAO catalyzes the four-electron reduction of molecular oxygen to water with concomitant one-electron oxidation of the substrate. Polyphenol oxidase (PPO) and peroxidase (POD) are two well known enzymes involved in the browning process, through the generation of hydrogen peroxide during the oxidation of phenolic compounds. PPO has also been implicated in the generation of ROS, oxygen-scavenging and pseudocyclic phosphorylation in the chloroplast and defense mechanism against the insects and plant pathogens. Although in the current study the PPO activity in the grape skin was found higher as compared to the pulp, its activity in the seed was found lower in the grape extracts, however seed extracts showed more scavenging properties than skin or pulp.

POD is one of the most thermostable enzymes responsible for performing the single electron oxidation on a wide variety of compounds, in the presence of hydrogen peroxide. POD reduces H₂O₂ to water while oxidizing a variety of substrates in a multistep reaction. Our current study showed lower activity of these enzymes in the grape extracts. However, the function of POD has been traditionally questioned mainly because of the low H₂O₂ content in the fruit and vegetable tissues and the relatively high catalytic power of PPO for the phenolics.

Hydrogen peroxide (H₂O₂) is an important signal molecule involved in the plant development and environmental responses. While plants contain several types of H₂O₂-metabolizing proteins, Catalase (CAT) is a highly active enzyme that does not require cellular reductants as it primarily catalyze a dismutase reaction. Higher catalase activity in seeds, followed by the skin and pulp in our study, may be associated with the scavenging (Table 2) and antioxidant property of the extracts.

Significantly higher AAO and CAT activities were observed in the seed, followed by the skin and pulp of the same grape cultivar in this study (Table 1), which could be associated with the scavenging (Table 2) and antioxidant property of the extracts. Radiation exposure alters the balance of endogenous defense systems, and the living cells have evolved the endogenous antioxidant defense mechanisms which include the non-enzymatic antioxidants like GSH, ascorbic acid, and the antioxidant enzymes such as the CAT, SOD and GPx. The decreased GSH levels in the γ-irradiated lymphocytes could be due to their utilization by the enhanced production of ROS, while the observed increased levels of TBARS in the γ-irradiated lymphocytes expose the attack of free radicals on the polyunsaturated fatty acid components of membrane lipids. The observed decrease in the activities of SOD, CAT and GPx in the γ-irradiated lymphocytes as observed in this study could be due to radiation-induced production of free radicals that impaired the antioxidant defense mechanisms. Pretreatment with the grape extracts of the γ-irradiated lymphocytes resulted in decreased lipid peroxidation, improved antioxidant status, and thereby protecting against damage to the lymphocytes in this study. The grape seed and skin contain several biologically active components, and the grape seed extract in particular possess a broad spectrum of pharmacological and therapeutic principles, including snake envenomation. The phytochemical composition of the grapes, however, varies greatly among different varieties. Our results also revealed that the grape seed extracts were more protective followed by the extracts of skin and pulp. Among varieties, the Flame seedless and the Kishmish chorni cultivars showed better free radical scavenging and antioxidant actions than that of the Thompson seedless or the Red globe cultivars.

Apoptosis is a key process in the development and maintenance of tissues of mammals, providing a mechanism to eliminate the damaged or unnecessary cells. Cellular response to the IR involves formation of free radicals that alter the cellular biochemistry, and subsequent induction of apoptotic death. It is
known that the IR induces a complex signaling apoptotic cascade post-exposure to low doses ultimately to eliminate the damaged cells from a population, specifically via the intrinsic pathway. The caspase-3 has been shown to be involved in mediating apoptosis induced by different stimuli. The increase in ROS and induction of caspase 3/7 activity (Fig. 3) in the IR-exposed lymphocytes vs. respective control as observed in the current study was in agreement with this hypothesis. From our observation, we had chosen the Thomson seedless and Kishmish chorni cultivars to study their effects on the IR-induced apoptotic events. A central effector of apoptosis, caspase-3, facilitates rather than suppresses the chemical- and radiation-induced genetic instability and carcinogenesis. These results are in agreement with our observation in the current study.

In conclusion, our results suggested that the grape extracts could serve as a potential source of natural antioxidants and significantly attenuate IR-induced oxidative stress and apoptosis in human lymphocytes. Furthermore, the protective activity of grape depends on the source of extract i.e. seed, skin or pulp, and the cultivars.

Acknowledgements

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