Scavenging and antioxidant properties of different grape cultivars against ionizing radiation-induced liver damage \textit{ex vivo}

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Ionizing radiation (IR) has become an integral part of the modern medicine—both for diagnosis as well as therapy. However, normal tissues or even distant cells also suffer IR-induced free radical insult. It may be more damaging in longer term than direct radiation exposure. Antioxidants provide protection against IR-induced damage. Grapes are the richest source of antioxidants. Here, we assessed the scavenging properties of four grape (\textit{Vitis vinifera}) cultivars, namely Flame seedless (Black), Kishmish chorni (Black with reddish brown), Red globe (Red) and Thompson seedless mutant (Green), and also evaluated their protective action against γ-radiation-induced oxidative stress in liver tissue \textit{ex vivo}. The scavenging abilities of grape seeds [2,2-diphenyl-1-picrylhydrazyl (DPPH) (IC$_{50}$=0.008±0.001 mg/mL), hydrogen peroxide (IC$_{50}$=0.49 to 0.8 mg/mL), hydroxyl radicals (IC$_{50}$=0.08±0.008 mg/mL), and nitric oxide (IC$_{50}$=0.8±0.08 mg/mL)] were higher than that of skin or pulp. Gamma (γ) radiation exposure to sliced liver tissues \textit{ex vivo} from goat, @ 6 Gy significantly (P <0.001) decreased reduced glutathione (GSH) content by 21.2% and also activities of catalase, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione s-transferase (GST) by 49.5, 66.0, 70.3, 73.6%, respectively. However, it increased thiobarbituric acid reactive substances (TBARS) by 2.04-fold and nitric oxide level by 48.6% compared to untreated group. Further increase in doses (10 or 16 Gy) of γ-radiation correspondingly decreased GSH content and enzyme activities, and increased TBARS and nitric oxide levels. Grape extract treatment prior to ionizing radiation exposure ameliorated these effects at varying extent. The seed extracts exhibited strong antioxidant potential compared to skin or pulp extracts of different grape cultivars against oxidative damage by ionizing radiation (6 Gy, 10 Gy and 16 Gy) in sliced liver tissues \textit{ex vivo}. Grape extracts at higher concentration (10 mg extract/g liver tissue) showed stronger antioxidant potential against lower dose (6 Gy) of ionizing radiation. Our results suggest that grape extracts could serve as a potential source of natural antioxidant against lower doses of IR-induced oxidative stress in liver tissues \textit{ex vivo}.

\textbf{Keywords:} Gamma (γ) radiation, ROS, \textit{Vitis vinifera}

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\textit{Abbreviations:} DPPH, 2,2-diphenyl-1-picrylhydrazyl; NADH, 1,4-dihydro-nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate buffered saline; PMS, phenazine methosulfate.

\textbf{Note}

In medicine, ionizing radiation (IR) can be beneficial in diagnosis and therapy, particularly cancer. However, it is a known human mutagen and carcinogen. The associated risks due to stochastic (low dose related to chronic effects) and deterministic (high dose causing acute effects) effects make it necessary to protect patients from potential harm. The use of IR is compromised by the radio-sensitivity of normal tissues. Exposure of cells to IR results in significant biological effects, and are not restricted only to the irradiated cells, but also to non-irradiated bystander or even distant cells. “Bystander effects” (BE) are now recognized as an indispensable component of tissue response related to deleterious effects of IR. The BE may be more damaging in the longer term than direct radiation exposure.

Radiation-induced liver injury following conventionally fractionated radiotherapy is a well known phenomenon. Depending on the variables mentioned, IR up to 35 gray (Gy) is given to the human liver in a limited volume, without major liver function disturbances. A number of studies suggest that biological effects of IR including bystander effects appear to be associated with upregulation of oxidative metabolism induced by reactive oxygen species (ROS).

In this context, agents that could prevent damage to normal cells and tissues caused by the direct and bystander effects of radiation gain importance. Phytochemicals from various plants possess a variety of biological activities including antioxidant potential, and are considered to be promising and safe source. Studies suggest that naturally occurring non-toxic phytochemicals, including curcumin, parthenolide, genistein, gossypol, ellagic acid, withaferin, plumbagin and resveratrol, have shown considerable activity. Grapes are rich in flavonoids and phenolics, anthocyanins and resveratrol predominantly and have nutraceutical and health benefits, essentially due to their antioxidant properties. Moreover, combinations of antioxidants have been shown to be more effective than single antioxidant, because of their synergistic effects. We have also demonstrated the protective effect of grapevine fruit extract against radiation-induced oxidative stress and apoptosis in human lymphocytes.
In this study, we explored extracts of different grape (Vitis vinifera) cultivars as natural antioxidants for its capacity to scavenge reactive oxygen and nitrogen species and can effectively protect cells and organisms from oxidative damage against various doses of γ-radiation-induced oxidative stress in liver tissue ex vivo.

Materials and Methods

Chemicals

Naphthylethylene diamine dihydrochloride (NEDD) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) from SIGMA Chemical Co; bovine serum albumin (BSA) (fraction V) from Loba Chemie; 2-deoxy-D-ribose, from Alpha-Aesar; 5,5′-dithiobisnitrobenzoic acid (DTNB), glutathione reductase (GR), 2,4-dinitrophenylhydrazine (DNPH), from SRL Chemical Co. were used. All other chemicals were purchased from SRL India.

Preparation and analysis of extracts

Commonly available four grape (Vitis vinifera L.) cultivars, namely ‘Thompson seedless’ (Green), ‘Red globe’ (Red), ‘Flame seedless’ (Black), and ‘Kishmish chorni’ (Black with reddish brown) were purchased from local market, and were authenticated by the Department of Fruits and Orchard Management, Faculty of Horticulture, Bidhan Chandra Krishi Viswavidyalaya, Nadia. Immediately after collection, grapes were washed thoroughly and the skin, pulp and seeds were separated and were individually crushed and lyophilized (concentrated). The concentrated extracts were stored at −20°C for further study.

Determination of DPPH•/OH•/H2O2/NO• scavenging activity

DPPH/hydroxyl radical and hydrogen peroxide scavenging activity of aqueous solution of different concentration of grape extracts were determined as described earlier. To determine nitric oxide scavenging activity, the reaction was initiated by adding 2 mL sodium nitroprusside (100 mM), 0.5 mL phosphate buffered saline (PBS, pH 7.4), 0.5 mL of aqueous grape extracts of different concentration at 25°C for 30 min. Later, 0.5 mL Griess reagent (1% sulphanilamide, 2% H3PO4, and 0.1% NEDD) was added and incubated for another 30 min. The absorbance was read at 546 nm against the reagent blank (without extract) in spectrophotometer.

The scavenging activities were expressed as a percentage of DPPH•/OH•/H2O2/NO• scavenged from the following equation: \( \frac{A_0 - A_s}{A_0} \times 100 \), where \( A_0 \): Absorbance of control and As: Absorbance in presence of grape extract (sample). IC50 value was calculated from the graph plotted between percentage of inhibition and concentration of the sample. Ascorbic acid and rutin were used as reference compounds.

Ex vivo studies

The goat liver was collected fresh from a local slaughter house, plunged into cold sterile PBS and maintained at 4°C till its use within 2 h of collection. Goat liver slice of 1 mm thickness (1 g) was mixed with 4 mL of sterile PBS in each of ten flasks. The grape extract (2.5, 5 and 10 mg) from seed, skin or pulp of each cultivar were added and incubated at 37°C with mild shaking for 1 h. Appropriate controls were also set up. The samples were irradiated at UGC-DAE Consortium for Scientific Research, Kolkata Centre, Salt Lake City, Kolkata at a rate 3.05 kGy/h. The study was approved by the Animal Ethics Committee of the Dept of Zoology, Kalyani University as per CPCSEA guidelines. After irradiation, samples were incubated for an hour at room temperature (25°C), and the goat liver slices were homogenized in the incubation medium and each homogenate was used for assaying thiobarbituric acid reactive substances (TBARS), nitrite, GSH, as well as activities of CAT (EC 1.11.1.6), GST (EC 2.5.1.18), glutathione peroxidase (GPx; EC 1.11.1.9), glutathione reductase (GR; EC 1.6.4.2) and glutathione S-transferase (GST; EC 2.5.1.18).

Statistical analysis

Scavenging activity of grape extracts were analyzed in triplicate, while those performed on the liver slices were replicated 6 times and the results are expressed as the mean ± standard error (SE). Statistical significance was established by one-way analysis of variance (ANOVA), followed by Tukey test for individual differences using SPSS, version 13.0 (SPSS Inc., Chicago, Ill.). Value of \( P < 0.05 \) was set to establish the statistical significance.

Results and Discussion

The extracts of various grape cultivars exhibited concentration-dependent scavenging abilities of DPPH radical, hydroxyl radical (OH•), hydrogen
peroxide (H$_2$O$_2$) and nitric oxide (NO). Effectiveness of antioxidant properties was determined using the half inhibition concentration [IC$_{50}$] of grape extracts, and was found inversely correlated. The activities as observed in this study were in the order of seeds, followed by skin and then pulp (Table 1). The grape seed extracts exhibited the highest OH$^\bullet$ scavenging ability with IC$_{50}$ value of 0.008 ± 0.001 mg/mL. However, the IC$_{50}$ value of scavenging H$_2$O$_2$ for Red globe grape seed extract was 0.5 mg/mL, while the pulp of Thompson seedless exhibited 17.7 mg/mL (Table 1). On the contrary, the NO scavenging of Red globe grape seed extract (0.8 mg/mL) was more than that of skin of Flame seedless (1.81 mg/mL). However, the scavenging activities of all factions were significantly less (P < 0.05) than those of standard compounds such as ascorbic acid and rutin (Table 1).

The scavenging properties in grape extracts were evaluated against γ-irradiation-induced oxidative stress in intact liver tissues ex vivo (Fig. 1 a-g). Different doses (6, 10 or 16 Gy) of γ-irradiation showed varying degrees of oxidative stress. Gamma radiation treatment at a dose of 6 Gy significantly (P < 0.001) decreased GSH content by 21.2% (Fig. 1a) and activities of catalase by 49.5% (Fig. 1d), GPx by 66% (Fig. 1e), GR by 70.3% (Fig. 1f), and GST by 73.6% (Fig. 1g); whereas TBARS increased by 204% (Fig. 1b) and NO level by 48.6% (Fig. 1c), respectively compared to the untreated group. Further increase in doses (10 or 16 Gy) of γ-irradiation correspondingly decreased GSH content and enzyme activities, while increasing TBARS and NO level (Data not shown).

Table 1—In vitro effects of grape extracts from different cultivars in terms of inhibitory concentration (IC$_{50}$= mg/ mL) on various parameters

<table>
<thead>
<tr>
<th>Grape cultivars</th>
<th>Scavenging</th>
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<tbody>
<tr>
<td></td>
<td>DPPH*</td>
</tr>
<tr>
<td>Thompson seedless (Green)</td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>1.195±0.017</td>
</tr>
<tr>
<td>Pulp</td>
<td>11.6±0.46</td>
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<tr>
<td>Flame seedless (Black)</td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>0.736±0.032</td>
</tr>
<tr>
<td>Pulp</td>
<td>2.55±0.232</td>
</tr>
<tr>
<td>Kishmish chorni (Black with Reddish Brown)</td>
<td></td>
</tr>
<tr>
<td>Pulp</td>
<td>1±0.115</td>
</tr>
<tr>
<td>Seed</td>
<td>0.008±0.001</td>
</tr>
<tr>
<td>Skin</td>
<td>1.34±0.038</td>
</tr>
<tr>
<td>Red globe</td>
<td></td>
</tr>
<tr>
<td>Pulp</td>
<td>4.963±0.145</td>
</tr>
<tr>
<td>Seed</td>
<td>0.008±0.001</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.003±0.006</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.004±0.0003</td>
</tr>
</tbody>
</table>

Values are mean±standard errors (SE) of means of 3 experiments; ‘-‘, not determined
increased activities of catalase by 17.2% (Fig. 1d), GPx by 16.6% (Fig. 1e), GR by 50% (P<0.05) (Fig. 1f) and GST by 40% (Fig. 1g) compared to 6 Gy \( \gamma \)-irradiated group. Interestingly, higher concentration (10 mg/ g liver tissue) of same grape extracts (skin of Flame seedless cultivar) increased activities of catalase by 46.5% (Fig. 1d), GPx by 41.6% (Fig. 1e), GR by 87.5% (Fig. 1f) and GST by 100% (Fig. 1g) compared to 6 Gy \( \gamma \)-irradiated group (P<0.05). Though grape seed extracts at a higher dose (10 mg/ g liver tissue) effectively ameliorated lower dose (6 Gy) of radiation, failed to protect at higher radiation doses (10 or 16 Gy).

IR is responsible for oxidative stress by generating ROS\(^{31}\), including superoxide anions, hydroxyl radicals, singlet oxygen and hydrogen peroxide\(^{32}\). These free radicals react with critical cellular components, such as DNA, RNA, proteins and membranes, leading to cell dysfunction and death\(^ {33,34}\). Hydroxyl radical, an extremely reactive free radical formed in biological systems, has the potential of reacting with all cellular macromolecules and induce tissue damage\(^{35}\). Hydrogen peroxide (H\( _2\)O\( _2\)) is a non-radical ROS in living organisms and has ability to penetrate cell membranes, inactivate enzymes by oxidation of thiol groups, and initiate lipid peroxidation\(^{36}\). Nitrogen monoxide (NO\(^{3+}\)) is a diffusible free radical that acts as an effector molecule in diverse biological systems including vasodilation, inhibition of platelet aggregation and regulation of cell mediated toxicity\(^ {37}\). The NO\(^{3+}\) radical production at a sustained level results in direct tissue toxicity and contributes to the vascular collapse, whereas chronic nitric oxide radical expression is associated with various inflammatory conditions\(^{38}\). The reaction between NO\(^{3+}\) with superoxide radical generates highly reactive peroxynitrite anion (ONOO\(^{\cdot}\)), which is highly toxic for living cells\(^ {39}\). Hence, more than one type of antioxidant measurement was performed in this study to take into account the various mode of scavenging action of grape extracts.

Antioxidant capacity of each extract measurements can be related to the capacity of extracts to either directly transfer hydrogen to a radical (DPPH) or to act as competitor to the peroxy radicals\(^ {39}\). Our study
showed that extracts from grape seeds, followed by skin of Flame seedless cultivars were more potent as scavengers than other extracts. These scavenging activities of grape extracts were found to be significantly correlated with the phenolic concentration. Studies suggested that grapes are rich in polyphenols, of which seeds contained higher amounts of total polyphenols than skin extracts, followed by pulp. The DPPH results showing the highest antioxidant activity of V. vinifera seeds in this study is in agreement with other studies. The potential OH· scavenging abilities of grape extracts in our study could be due to either the active hydrogen donor ability of hydroxy substitution from phenolic substances or the phenolic compounds of grape extracts might have reacted with NO· to generate phenoxy radicals to scavenge this radical.

The precision cut liver slices were used in this study as an ex vivo model due to its simplicity, ease of preparation, retention to normal organ architecture, and the ability to obtain multiple slices from each organ. The decreased activities of antioxidant enzymes (CAT, GR, GPx and GST), GSH level, and the increased levels of TBARS and NO in the liver tissues ex vivo (Figs. 1 a-g) could be attributed to enhanced production of ROS. Other studies also observed that exposure to γ-radiation resulted in decreased activity of antioxidant enzymes and lipid peroxidation. In addition, the present study has shown that the antioxidant potency of the grape extracts was highest in seeds, followed by skin and pulp at a dose of 10 mg extract/g liver tissue. Polyphenols present in the grape seed extract may support the better antioxidant activity and scavenging of both free radicals and reactive oxygen species, both in vivo and in vitro by the extract. Another study has also demonstrated that grape seed pro-anthocyanidins (GSPs) protect against radiation-induced lung injury (RILI) through scavenging free radicals and modulating RILI-associated cytokines. Further, quality of grape berries greatly depends on skin colour, which is influenced by the anthocyanin profile. Among the edible parts, skin of Flame seedless cultivar showed more antioxidant potential than other cultivars, indicating that cultivars also determine the quality.

In conclusion, our study showed that while the antioxidant potential of grape extract is dependent on the part of the berry, as well as cultivars, its efficacy is directly related to the concentration of the extract, but inversely related to dose of radiation exposure.

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