Antioxidant potential of different grape cultivars against Fenton-like reagent-induced liver damage \textit{ex-vivo}

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The phytochemicals present in the grapes are responsible for nutraceutical and health benefits due to their antioxidant properties. These phytochemicals, however, vary greatly among different cultivars. In this study, we evaluated the antioxidant potential and protective role of four different Indian grape (\textit{Vitis vinifera}) cultivars extracts, namely Flame seedless (Black grapes), Kishmish chorni (Black with reddish brown), Red globe (Red) and Thompson seedless mutant (Sonaka, Green) against the Fenton-like reagent (200 \(\mu\)mole H\(_2\)O\(_2\), 2 mmole ascorbate, 25 \(\mu\)mole FeSO\(_4\))-induced liver damage. Non-enzymatic antioxidants, such as glutathione (GSH) levels and activities of antioxidant enzymes, such as glutathione S-transferase (GST) and superoxide dismutase (SOD), as well as total antioxidant capacity (TAC) were highest in the grape seed, followed by skin and pulp. Among edible parts of different cultivars, skin of Flame seedless (Black) cultivar showed highest antioxidant potential, while the Thompson seedless the least potential. These antioxidants were found to be significantly \((P<0.01)\) correlated with the levels of total phenol, flavonoids and ascorbic acid. Fenton-like reagent treatment significantly \((P<0.001)\) decreased GSH content by 39.1\% and activities of catalase (CAT) by 43.2\% and glutathione reductase (GR) by 60\%, while increasing thiobarbituric acid reactive substances (TBARS) and nitric oxide levels by 2.13-fold and 0.64-fold, respectively and GST activity by 0.81-fold. Pre-treatment with grape seed extracts showed the best hepatoprotective action against Fenton-like reagent-induced damage, followed by the extracts of skin and pulp of any cultivar. Thus, our study showed the significant amounts of antioxidants were in grape seed, followed by its skin and pulp, which varied among the cultivars and was associated with the protective action of grape extracts against Fenton-like reagent-induced liver damage \textit{ex-vivo}.

**Keywords:** Antioxidant, Fenton’s reagent, Fenton-like reagent, Glutathione, Grapes, Liver, Total Antioxidant capacity

Reactive oxygen and nitrogen species (RONS) are constantly generated in cells during normal oxidative processes through a variety of pathways, including both enzyme-catalyzed reactions and non-enzymatic reactions\(^1\). The imbalance between reactive oxygen species (ROS) generation and natural antioxidant defense system leads to an overall increase of the intracellular ROS levels, resulting to oxidative stress\(^5\). The resulting oxidative stress is implicated in several human diseases\(^3,4\).

Epidemiological studies have revealed the association between the consumption of antioxidant-rich plant foods and prevention of oxidative-stress-related diseases\(^5,6\). Fruits and vegetables are sources of phytochemicals that constitute rich antioxidant molecules\(^7,8\). Grapes, one of the most popular, widely cultivated and consumed fruits in the world, are rich in phytochemicals\(^9\). It is well-known that the grape skins, seeds and stems are rich source of polyphenols, including flavonoids, phenolic acids and stilbenes\(^10\). The phytochemical composition of grapes, however, varies greatly among different cultivars\(^11\).

About twenty cultivars of grapes are grown under a variety of soil and climatic conditions in India\(^12\). Indian grape cultivars vary in their skin colour, seed and polyphenol content and antioxidant potency\(^9\). In our recent study, we have demonstrated the scavenging property of extracts from grape skin.

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**Abbreviations:** BSA, bovine serum albumin; CAT, catalase; CDNB, 1-chloro-2,4-dinitro benzene; DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5′-dithiobisnitrobenzoic acid; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione S-transferase; NBT, nitroblue tetrazolium; NEDD, naphthylethylene diamine dihydrochloride; NO, nitric oxide; PBS, phosphate buffered saline; PMS, phenazine methosulfate; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances.
and pulp against free radicals and attenuation of H$_2$O$_2$-induced liver tissue damage ex vivo$^{13}$. Though H$_2$O$_2$ is an oxidizing agent, but has limited reactivity, as it is unstable and decomposes to O$_2$ and H$_2$O. Hence, it is necessary to examine the efficacy of the antioxidants from different grape cultivars against stronger oxidizing agent-induced tissue damage ex vivo. However, H$_2$O$_2$ is particularly effective when it reacts with ferrous ion (Fe$^{2+}$) to produce Fenton’s reagent, a very powerful oxidizer. Therefore, in the present study, we have assessed the antioxidant potential of different grape (Vitis vinifera) cultivars in relation to their protective action against the highly reactive oxidizing agent, such as Fenton-like reagent-induced liver damage ex vivo.

Materials and Methods
Bovine serum albumin (BSA) and thiobarbituric acid reactive substance (TBARS) (Loba Chemie), Aluminum chloride hexa hydrate (from Alfa-Aesar), Folin-Ciocalteu’s reagent, sodium pyrophosphate and gallic acid (Merck India Ltd.), Tris buffer (Qualigens), yeast glutathione reductase (GR) (Sigma Chemical Co., St. Louis, USA) and 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiobisnitro benzoic acid (DTNB), naphthylethylene diamine dihydrochloride (NEDD), NADH, phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), reduced glutathione (GSH), 2, 4-dinitrophenyl-hydrazine (DNPH) and other chemicals (Sisco Research Laboratory, India) were used.

Grape analysis
Commonly available four grape (Vitis vinifera) cultivars, including ‘Flame seedless’ (black), ‘Kishmish chorni’ (black with reddish brown), ‘Red globe’ (Red) and ‘Thompson seedless’ (Sonaka, Green) were purchased from the local market and authenticated from the Department of Fruits and Orchard Management, Faculty of Horticulture, Bidhan Chandra Krishi Viswavidyalaya, Nadia.

The skin, pulp and seed of grapes were separated by squeezing the fruits. Total phenol$^{14}$, flavonoids$^{15}$, ascorbic acid$^{16}$ and GSH$^{17}$ content and glutathione S-transferase (GST, EC 2.5.1.18)$^{18}$ and superoxide dismutase (SOD, EC 1.15.1.1)$^{19}$ activities were determined.

Total antioxidant capacity (TAC) determination
Grape extracts (0.3 ml) were mixed with 3 ml of reagent solution (0.6 M H$_2$SO$_4$, 28 mM sodium phosphate, 4 mM ammonium molybdate mixture). The tubes were incubated for 90 min at 95°C. The mixture was cooled to room temperature and absorbance was recorded at 695 nm against blank. TAC was expressed as ascorbic acid equivalents using standard ascorbic acid solution$^{20}$, with the linear equation $Y = 0.044X + 0.033$, $r^2 = 0.998$, where $Y$ represents the absorbance at 695 nm and $X$ the concentration of ascorbic acid equivalent.

Ex vivo studies
Fresh samples of goat liver were collected from a local slaughter house, plunged into cold sterile phosphate buffered saline (PBS) and maintained at 4°C till its use within 2 h of collection. Goat liver slice (1 g) of 1 mm thickness was mixed with 4.0 ml of sterile PBS in each of twelve flasks. Fenton-like reagent (200 µM H$_2$O$_2$, 2 mM ascorbate, 25 µM FeSO$_4$)$^{21}$ and/or the grape extract (10 mg) were added and incubated at 37°C with mild shaking for 1 h. Appropriate controls were also established under identical experimental condition. After incubation, the liver slices were washed with PBS, homogenized and the homogenate was used for the chosen biochemical assay of protein$^{22}$, TBARS$^{23}$, nitrite$^{24}$, GSH$^{25}$, as well as activities of catalase (CAT; EC 1.11.1.6)$^{26}$, glutathione reductase (GR; EC 1.6.4.2)$^{27}$, GST$^{18}$ and glutathione peroxidase (GPx; EC 1.11.1.9)$^{28}$.

Statistical analysis
Analyses of polyphenols and antioxidant parameters were performed in triplicate, while those performed on the liver slices were replicated five times and the results were expressed as the mean ± standard error (SE). Significant differences were assessed through the one-way analysis of variance (ANOVA), followed by the Tukey test for individual differences using SPSS, version 13 (SPSS Inc., Chicago, IL). A $P$<0.05 was set to establish the statistical significance.

Results
Antioxidants, such as GSH content and the GST and SOD activities were found to be significantly higher in grape seed, followed by its skin and pulp (Table 1). Among edible parts of different cultivars, skin of Flame seedless (Black) cultivar showed the highest antioxidant property. Though substantial amount (10.3 to 24.3 mg in 100 mg fresh wt) of GSH content was present in seed, skin and pulp, it was significantly higher ($P< 0.05$) in skin than in pulp of all the cultivars, except red globe (Table 1). The
activities of GST and SOD significantly differed ($P<0.001$) in grape seed and skin as compared to its pulp among different cultivars (Table 1). The activities of antioxidant enzymes and levels of non-enzymatic antioxidant were found to be significantly ($P<0.01$) correlated with the levels of total phenols, flavonoids or ascorbic acid (Table 2).

Grape seed also showed the highest antioxidant capacity, followed by its skin (Fig. 1). Flame seedless (Black) cultivar skin showed the highest antioxidant capacity among the skin of selected cultivars in this study (Fig. 1).

Grape extracts protected against oxidative stress induced by the Fenton-like reagents in intact liver tissue $ex$ $vivo$ (Table 3). The Fenton-like reagent treatment significantly ($P<0.001$) decreased GSH content by 39.1%, activities of CAT by 43.2% and GR by 60%, and increased TBARS and nitric oxide levels 2.13-fold and 0.64-fold, respectively and GST activity 0.81-fold (Table 3). The treatment of liver tissue with skin of Flame seedless (Black) or Red globe cultivar increased GSH content by 24.6%, while treatment of the tissue with grape seed of Kishmish chorni or Red globe increased GSH content by 32.8%, as compared to the same in the Fenton-like reagent-treated liver slice.

### Table 1—GSH content and activities of GST and SOD in extracts of different grape cultivars

![Table 1](image1.png)

### Table 2—Correlation between phytochemicals with antioxidants

![Table 2](image2.png)

Fig. 1—Total antioxidant capacity of different grape cultivars

![Fig. 1](image3.png)
Table 3—Effect of grape extracts on reduced GSH, TBARS nitrite content and activities of CAT, GPx, GR and GST in Fenton-like reagent-treated intact liver tissue \textit{ex vivo} 
[Values are mean ± SE of 5 observations]

<table>
<thead>
<tr>
<th></th>
<th>GSH$^a$</th>
<th>TBARS$^b$</th>
<th>Nitrite$^c$</th>
<th>Catalase$^d$</th>
<th>GPx$^e$</th>
<th>GR$^f$</th>
<th>GST$^g$</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.4 ± 0.06</td>
<td>0.18 ± 0.006</td>
<td>0.45 ± 0.01</td>
<td>22.2 ± 0.66</td>
<td>0.39 ± 0.01</td>
<td>0.3 ± 0.01</td>
<td>0.32 ± 0.006</td>
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<td>Fenton-like reagent</td>
<td>1.46 ± 0.04$^a$</td>
<td>0.56 ± 0.02$^a$</td>
<td>0.74 ± 0.014$^a$</td>
<td>12.6 ± 0.68$^a$</td>
<td>0.21 ± 0.01$^a$</td>
<td>0.12 ± 0.01$^a$</td>
<td>0.59 ± 0.017$^a$</td>
</tr>
<tr>
<td>Grape cultivars</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Thompson seedless</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(Green)</td>
<td>Skin</td>
<td>1.84 ± 0.08$^{ad}$</td>
<td>0.31 ± 0.015$^{adg}$</td>
<td>0.62 ± 0.01$^{ad}$</td>
<td>14.8 ± 0.37$^a$</td>
<td>0.26 ± 0.004$^{adf}$</td>
<td>0.2 ± 0.006$^{adh}$</td>
</tr>
<tr>
<td></td>
<td>Pulp</td>
<td>1.56 ± 0.08$^d$</td>
<td>0.39 ± 0.006$^{ad}$</td>
<td>0.64 ± 0.02$^{ae}$</td>
<td>13.4 ± 0.51$^a$</td>
<td>0.22 ± 0.01$^a$</td>
<td>0.15 ± 0.004$^a$</td>
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<tr>
<td>Flame seedless</td>
<td></td>
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<td></td>
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<tr>
<td>(Black)</td>
<td>Skin</td>
<td>1.82 ± 0.05$^{ae}$</td>
<td>0.26 ± 0.006$^{adh}$</td>
<td>0.56 ± 0.02$^{bd}$</td>
<td>16.8 ± 0.37$^a$</td>
<td>0.3 ± 0.01$^{ad}$</td>
<td>0.22 ± 0.004$^{adh}$</td>
</tr>
<tr>
<td></td>
<td>Pulp</td>
<td>1.64 ± 0.04$^a$</td>
<td>0.33 ± 0.01$^{ad}$</td>
<td>0.57 ± 0.02$^{ad}$</td>
<td>14.4 ± 0.24$^a$</td>
<td>0.26 ± 0.004$^{ad}$</td>
<td>0.17 ± 0.004$^a$</td>
</tr>
<tr>
<td>Kishmish chorni</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Black with Reddish Brown)</td>
<td>Skin</td>
<td>1.8 ± 0.03$^{ae}$</td>
<td>0.29 ± 0.01$^{ad}$</td>
<td>0.59 ± 0.024$^{ad}$</td>
<td>15.8 ± 0.37$^{ae}$</td>
<td>0.28 ± 0.01$^{ad}$</td>
<td>0.18 ± 0.01$^a$</td>
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<tr>
<td></td>
<td>Pulp</td>
<td>1.68 ± 0.04$^a$</td>
<td>0.36 ± 0.01$^{ad}$</td>
<td>0.62 ± 0.01$^{ad}$</td>
<td>13.6 ± 0.4$^a$</td>
<td>0.24 ± 0.005$^a$</td>
<td>0.15 ± 0.005$^a$</td>
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<tr>
<td>Red globe</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Seed</td>
<td>1.94 ± 0.05$^{ae}$</td>
<td>0.26 ± 0.005$^{ad}$</td>
<td>0.56 ± 0.02$^{ad}$</td>
<td>18 ± 0.7$^{ad}$</td>
<td>0.23 ± 0.01$^a$</td>
<td>0.19 ± 0.012$^{adh}$</td>
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<tr>
<td></td>
<td>Skin</td>
<td>1.82 ± 0.04$^{ae}$</td>
<td>0.33 ± 0.006$^{ad}$</td>
<td>0.62 ± 0.01$^{ad}$</td>
<td>15 ± 0.55$^a$</td>
<td>0.28 ± 0.01$^{ad}$</td>
<td>0.21 ± 0.01$^{ad}$</td>
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<tr>
<td></td>
<td>Pulp</td>
<td>1.56 ± 0.05$^a$</td>
<td>0.38 ± 0.01$^{ad}$</td>
<td>0.65 ± 0.01$^{af}$</td>
<td>15.4 ± 0.51$^a$</td>
<td>0.25 ± 0.01$^a$</td>
<td>0.14 ± 0.006$^a$</td>
</tr>
<tr>
<td></td>
<td>Seed</td>
<td>1.96 ± 0.07$^{ae}$</td>
<td>0.26 ± 0.005$^{ad}$</td>
<td>0.554 ± 0.017$^{ad}$</td>
<td>18.8 ± 0.37$^{bd}$</td>
<td>0.22 ± 0.01$^a$</td>
<td>0.2 ± 0.01$^{ad}$</td>
</tr>
</tbody>
</table>

$P$ values: $^a<0.001$, $^b<0.01$, $^c<0.05$ compared to control group; $^d<0.001$, $^e<0.01$, $^f<0.05$ compared to Fenton-like reagent treated group; $^g<0.001$, $^h<0.01$, $^i<0.05$ compared to pulp of the same cultivar.

$\alpha$, µg/mg tissue; $\beta$, µmole MDA formed/min/100 mg tissue; $\gamma$, nM/100 mg tissue; $\delta$, µmole H$_2$O$_2$ decomposed/min/ mg protein; $\xi$, µmole NADPH breakdown/ min/100 mg protein; $\phi$, µmole CDNB conjugate formed/ min/mg protein

and GR activities of liver tissue by 22.2% and 16.6%, respectively. In contrast, the seeds of Red globe enhanced the activities of CAT and GR by 49% and 66%, respectively in liver tissue. Furthermore, with seeds of Kishmish chorni or Red globe cultivar showed better GST protective action with liver slices, as compared to the same with other extracts (Table 3).

**Discussion**

Plants have developed an antioxidant system for protection against potentially toxic effects of xenobiotics and ROS$^{39}$. GSH is also a plant-derived natural antioxidant that can reduce the ROS$^{40}$. Earlier study has reported GSH levels in 28 different grape (\textit{Vitis vinifera}) varieties ranging from 56 to 372 µmole/kg (17-114 mg/kg)$^{31}$, whereas in our study GSH levels ranged from 10.3 to 18.3 mg/100 g fresh weight (Table 1). In another study, the maximum level of GSH has been reported to be 628.57 nmole/g tissue in grapes$^{32}$.

Plant GSTs are a family of multi-functional enzymes involved in the intracellular detoxification of xenobiotics and toxic compounds produced endogenously$^{33}$. The higher level of GST activity in seeds and skin of grape extracts in this study (Table 1) suggested that they were able to detoxifying higher concentrations of toxins in the presence of sufficient GSH. Since SOD apparently protects the cell against oxygen toxicity$^{34}$ and limits hydroxyl radical formation$^{35}$, our study demonstrated that grape seed, followed by its skin possessed higher antioxidant property than its pulp in a cultivar (Table 1). However, determination of TAC of a sample is more useful than the knowledge of specific antioxidant species$^{20}$. Our study revealed that majority of TAC was found in the grape seeds, followed by skin and lesser content in pulp (Fig. 1). Moreover, antioxidant potential varied among cultivars.

In the presence of reduced transition metals, such as Fe and Cu, H$_2$O$_2$ can be converted into the highly reactive hydroxyl radical (HO\(^\cdot\)) through the Fenton reaction or Haber-Weiss reaction$^{36}$. Hydroxyl radical is believed to be the etiological agent for a large number of diseases$^{37,38}$. Owing to its reducing properties, ascorbic acid added in the Fenton system strongly increases the rate of OH\(^\cdot\) radical production
by regenerating Fe$^{2+}$ from Fe$^{3+}$ and might probably minimize or even suppress the interference of some scavengers$^{39,40}$. 

Ascorbic acid + Fe$^{3+}$ → Oxidized ascorbic acid + Fe$^{2+}$

Fe$^{2+}$ + H$_2$O$_2$ → Fe$^{3+}$ + OH$^-$ + *OH (Fenton reaction)

This extremely reactive species reacts at a high rate ($k \approx 10^{9} - 10^{10}$ M$^{-1}$s$^{-1}$) with all surrounding molecules, such as proteins, lipids, nucleic acids and sugars, causing cell injury or death$^{41}$. 

The precision-cut liver slices were used as an in vitro (ex vivo) model in the present study due to their simplicity, ease of preparation, retention to normal organ architecture and ability to obtain multiple slices from each organ$^{26}$. Free radical formation resulted in increased lipid peroxidation and increased nitrite level indicated increased oxidative and nitrosative stress$^{26}$, due to Fenton-like reagent in this study (Table 3). The depletion in GSH due to Fenton-like reagent in this study (Table 3) sensitized the tissue to oxidative injury. Increased level of GSH and decreased levels of TBARS and nitrite due to grape extract treatment elicited a protective response against the toxic manifestations of Fenton-like reagent.

Significant decrease in CAT and GPx activities in sliced liver by Fenton-like reagent might be possibly due to free radical-dependent inactivation of the enzyme or due to loss of NADPH$^{43}$. Increased GST and decreased GPx and GR activities, followed by GSH depletion are important factors sustaining a pathogenic role for oxidative stress$^{43}$. Reversal of GPx, GR and GST by the grape extracts of different cultivars at varying degree (Table 3) suggested that the hepatoprotective role of grape against Fenton-like reagent depends on its source of extracts and cultivars.

The antioxidant potential of grape extracts varied among cultivars and Flame seedless (black) cultivar showed the highest potential, while Thompson seedless (Sonaka, green) cultivar the least potential. We also found that grape seed was a better source of antioxidants and antioxidant capacity in grapes were found to be associated with hepatoprotective effect of grape extracts against Fenton-like reagent. Significant correlations between the antioxidants and total phenolics observed in grape extracts (Table 2) were in agreement with other study$^{45}$. 

In conclusion, our study demonstrated the presence of significant amounts of antioxidants in grape seed, followed by its skin and pulp, which also varied with cultivars and was associated with the protective effect against strong oxidant, such as Fenton-like reagent-induced tissue damage.

Acknowledgement

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