

## Dietary grapes (*Vitis vinifera*) feeding attenuates ethanol-induced oxidative stress in blood and modulates immune functions in mice

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Ethanol metabolism is known to induce overwhelming production of reactive oxygen species (ROS) and also to cause associated immune dysfunction. Several interventional agents of plant origin, in particular fruits and vegetables have been used to counteract these alterations induced by ethanol. In this study, we investigated the efficacy of dietary feeding of skin and flesh of grapes (*Vitis vinifera* L.) on the alterations in immune and vascular functions in mice with liver abnormalities induced by chronic ethanol consumption. Results revealed that feeding of both grape skin and flesh (2.5 g/kg body wt/day) effectively attenuated the oxidative stress and alterations in immune function and angiogenesis induced by chronic ethanol consumption (1.6 g/kg body wt/day for 12 weeks) in mice. The antioxidant actions of the grape skin and flesh as observed in this study might be attributed to the polyphenols present in the grapes.

**Keywords:** Cytokines, Ethanol, Grape, Oxidative Stress, Polyphenols, *Vitis vinifera* L.

Ethanol metabolism has been established to cause the formation of overwhelming levels of reactive oxygen species (ROS) and to alter the cellular antioxidant defense system<sup>1</sup>. Chronic ethanol feeding is also associated with immunomodulatory activities, such as the alterations in cytokine levels in tissues including plasma, lung, liver and brain. These cytokines play critical roles in cellular communication, activation, inflammation, cell death, cell proliferation, migration and healing mechanisms<sup>2</sup>. These alterations lead to the structural modifications and consequently result in severe dysfunctions in cells and tissues<sup>1</sup>. Tissue repair is a dynamic compensatory cell proliferation and tissue regeneration response stimulated in order to overcome acute toxicity and recover organ/tissue structure and function. It is a complex process governed by intricate cellular signaling involving a

number of chemokines, cytokines and growth factors leading to pro-mitogenic gene expression and cell division<sup>3</sup>.

Several interventions have been put forward to counteract the vulnerability of the oxidative challenges during alcohol consumption. Fruits and vegetables in diet are known to have an important role in maintaining physiological redox equilibrium. These foods are the sources of several antioxidants<sup>4</sup>. Resveratrol (3, 4', 5-trihydroxystilbene), a naturally occurring polyphenol phytoalexin compound can effectively prevent ethanol-induced oxidative challenges, immunomodulatory activity and angiogenesis process<sup>5</sup>. It has been reported to protect the kidney, heart and brain from ischemic-reperfusion injury<sup>6</sup>. Resveratrol and few other polyphenols are present only in grapes and are virtually absent from commonly consumed fruits and vegetables<sup>7</sup>. Piceatannol (3,5,3',4'-tetrahydroxy-trans-stilbene), another polyphenol found in grapes is known as a protein kinase inhibitor that modifies multiple cellular targets, exerting immunosuppressive and antitumorigenic activities in several cell lines<sup>8</sup>.

Studies have revealed that active polyphenolic compounds present in the freeze-dried grape powder (FDGP) including epicatechin, cyanidin and quercetin dramatically decrease the taurodeoxycholic acid-induced production of ROS. Combination of four

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**Abbreviations:** ALP, alkaline phosphatase; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; DPPH<sup>•</sup>, 1,1-Diphenyl-2-picrylhydrazyl; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione S-transferase; NEDD, N-(1-naphthyl)ethylenediamine dihydrochloride; ROS, reactive oxygen species; SOD, superoxide dismutase; TAS, total antioxidant status; TBA, 2-thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

polyphenolic compounds (epicatechin, cyanidin, quercetin and resveratrol) of FDGP has been shown to exhibit anti-apoptotic effects<sup>9</sup>. Grape juice has also been demonstrated to prevent platelet aggregation, LDL oxidation, oxidative damage to DNA, coronary diseases, and atherosclerosis<sup>4</sup>. Therefore, in the present study, we have investigated the efficacy of the alcoholic extracts of grape (*Vitis vinifera* L.) skin and flesh on oxidative stress and alterations in immune function and angiogenesis in mice exposed to chronic ethanol consumption with liver damage.

### Materials and Methods

Ethanol was purchased from Bengal Chemicals Limited, Kolkata, India. The cytokine kits (Becton, Dickinson Biosciences and Co., Franklin Lake, USA), transforming growth factor (TGF)-1 and vascular endothelial growth factor (VEGF)-A ELISA kits (Bender Med Systems, Austria) were used. The other chemicals used were: trichloroacetic acid (TCA) (Thomas Baker, Mumbai, India), 2-thiobarbituric acid (TBA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (HiMedia Lab. Pvt. Ltd., Mumbai, India), N-(1-naphthyl)ethylenediamine dihydrochloride (NEDD) (SRL, Mumbai, India), 1-chloro-2,4-dinitrobenzene (CDNB) (Aldrich, Milwaukee, WI). All other chemicals were purchased from Sisco Research Laboratory, India, Sigma Chemical Co., St. Louis, USA, and E. Merck, India.

### Preparation and analysis of grapes

Grapes (*Vitis vinifera* L.) were purchased from local market. The skin and flesh of grapes were separated by squeezing the fruits. The skin was soaked in absolute ethanol for 24 h, the supernatant decanted and the process repeated twice. Water was removed from the flesh *in vacuo* and the residue was extracted as done for the skin. The individual extracts were concentrated in vacuum.

Ascorbic acid<sup>10</sup>, polyphenols<sup>11</sup>, total flavonoids<sup>12</sup> and sugar<sup>13</sup> contents were determined. ROS scavenging activity of grape extracts was measured from their ability to quench 1,1-diphenyl-2-picrylhydrazyl (DPPH<sup>•</sup>)<sup>7</sup>.

### Animals and treatment

The male BALB/c mice (8–10 weeks old, 20–30 g) were housed in plastic cages inside a pathogen-free well-ventilated room, maintained under standard husbandry condition. The mice had free access to standard diet<sup>14</sup> and water *ad libitum*. The animals

were weighed daily and their general conditions including liquid intake were recorded. The experiments were approved by the Animal Ethics Committee of the institution in accordance with the CPCSEA guideline.

The mice were divided into the following four groups of 6 each: Group I: control mice were fed isocaloric glucose solution instead of ethanol (1.6g/kg body wt/day); Group II: mice fed with ethanol (1.6/kg body wt/day); Group III: mice fed with ethanol (1.6/kg body wt) and grape skin (2.5 g/ kg body wt) per day; Group IV: mice fed with ethanol (1.6/kg body wt) and grape flesh (2.5 g/kg body wt) per day.

Ethanol was diluted with distilled water to get desired concentration. Grape skin and flesh were separately dissolved in ethanol and fed orally by intragastric infusion technique. After 12 weeks, blood samples were collected from retroorbital plexus of mice. Blood ethanol concentration was determined with an ethanol assay kit (Sigma). Serum was separated and used for estimation of protein<sup>15</sup>, albumin<sup>16</sup>, aminotransferases<sup>17</sup>, alkaline phosphatase (ALP) and total antioxidant status (TAS). The cytokines (such as TNF- $\alpha$ , IL-2, IL-4, IL-10, IFN- $\gamma$ , VEGF-A and TGF-1) levels in serum were estimated using representative Sandwich ELISA kits according to manufacturer's instruction.

The whole blood samples collected into vacutainers containing EDTA were centrifuged at  $1000 \times g$  for 10 min at 2°C. After removing the plasma and buffy coats, the packed erythrocyte was washed with 4.0 ml cold normal saline, centrifuged at  $3000 \times g$  for 15 min at 2°C and hemolysed by adding 30 ml chilled double-distilled water<sup>18</sup>. The hemolysate was used for assaying nitrite<sup>19</sup>, TBARS<sup>20</sup>, reduced glutathione (GSH) content<sup>21</sup>, as well as activities of catalase (CAT, EC 1.11.1.6)<sup>14</sup>, superoxide dismutase (SOD; EC 1.15.1.1)<sup>22</sup>, glutathione reductase (GR; EC 1.6.4.2)<sup>23</sup>, glutathione S-transferase (GST; EC 2.5.1.18)<sup>24</sup> and glutathione peroxidase (GPx; EC 1.11.1.9)<sup>25</sup>.

### Statistical analysis

All data were analyzed using the statistical package SPSS (version 11.0, SPSS Inc., Chicago, IL). Results were expressed as mean  $\pm$  SD (standard deviation). The sources of variation for multiple comparisons were assessed by the analysis of variance (ANOVA), followed by Post-hoc test.

## Results

The total phenolics, flavonoids and ascorbic acid contents were much higher in the grape skin compared to those present in the grape flesh (Table 1). Free radical scavenging activity was found to be higher in grape skin than flesh (Table 1). On the other hand, no observable change was noted in physical appearance in the animals which had

normal food and water. No significant change was noted on body weight gain or blood ethanol level among ethanol-treated and ethanol with grape-derived supplementation-fed mice were not significantly different (Table 2).

Chronic ethanol treatment significantly decreased serum protein, albumin and total antioxidant status by 6.39%, 14% and 55%, respectively, whereas significantly increased globulin level (14.65%) and activities of AST (8.82-fold), ALT (7.37-fold) and ALP (2.04-fold) (Table 3). Ethanol with grape skin supplementation significantly increased albumin level by 8.7%, compared to ethanol-treated group (Table 3). Grape skin or grape flesh supplementation in ethanol significantly increased TAS level by 100.6% and 55.5% respectively and decreased activities of AST by 43% and 31.9% respectively, ALT by 39.4% and 25.6% respectively and ALP by 39.3% and 24.9%, respectively in comparison to ethanol-treated group (Table 3).

Table 1—Total polyphenols, flavonoids, ascorbic acid content and scavenging activities of skin and flesh from grapes

[For each estimation, n = 4]

	Skin	Flesh
Total polyphenols (mg/100 g fresh wt)	219.3	42.8
Total flavonoids (mg/100 g fresh wt)	53.9	14.7
Ascorbic acid (mg/100 g fresh wt)	48.4	18.2
Sugar content (g/100 g fresh wt)	16.8	22.3
DPPH* (free radical scavenging, %)	46.3	32.4
DPPH*, 1,1-Diphenyl-2-picrylhydrazyl		

Table 2—Body weight gain and plasma alcohol and protein level of normal, ethanol-fed, and ethanol with of grape skin or flesh  
[Values represent mean  $\pm$  SD of three independent experiments with 6 mice in each group]

Parameters	Normal	Ethanol-treated	Ethanol + Grape skin-treated	Ethanol + Grape flesh-treated	F-variance	Significance
Weight gain (%) after 12 weeks	13.98 $\pm$ 0.93	14.1 $\pm$ 0.87	13.98 $\pm$ 0.67	13.88 $\pm$ 0.58	0.077	0.971
Ethanol consumption/day (g/kg)	Nil	1.6	1.6	1.6	-	-
Plasma alcohol level (mM)	-	53.33 $\pm$ 3.56	54.5 $\pm$ 2.74	54.5 $\pm$ 3.2	576.871	<0.001

No significant change in any parameter was observed among different groups of animals

Table 3—Effects of grape skin and flesh on protein fractions, and liver specific enzyme activities and total antioxidant status in serum of chronic ethanol-fed mice

[Values represent mean  $\pm$  SD of 6 rats in each group]

Parameters	Normal	Ethanol-treated	Ethanol + Grape skin-treated	Ethanol + Grape flesh-treated	F-variance	Significance
Protein (g%)	4.38 $\pm$ 0.11	4.1 $\pm$ 0.14 <sup>b</sup>	4.26 $\pm$ 0.16 (+3.9)	4.23 $\pm$ 0.1 (+3.17)	4.593	0.013
Albumin (g%)	3.21 $\pm$ 0.11	2.76 $\pm$ 0.15 <sup>a</sup>	3 $\pm$ 0.14 <sup>c</sup> (+8.69)	2.96 $\pm$ 0.13 <sup>c</sup> (+7.24)	10.867	<0.001
Globulin (g%)	1.16 $\pm$ 0.08	1.33 $\pm$ 0.05 <sup>b</sup>	1.25 $\pm$ 0.05 (-6.01)	1.25 $\pm$ 0.05 (-6.01)	7.246	0.002
AST (IU/L)	19 $\pm$ 2	186.6 $\pm$ 14.12 <sup>a</sup>	106.3 $\pm$ 7.63 <sup>ad</sup> (-43.03)	127 $\pm$ 8.74 <sup>adg</sup> (-31.93)	301.365	<0.001
ALT (IU/L)	19.17 $\pm$ 2.04	160.5 $\pm$ 7.86 <sup>a</sup>	97.17 $\pm$ 3.71 <sup>ad</sup> (-39.45)	119.3 $\pm$ 7.63 <sup>adf</sup> (-25.67)	324.137	<0.001
ALP (IU/L)	41.8 $\pm$ 2.64	127 $\pm$ 4.86 <sup>a</sup>	77 $\pm$ 4.33 (-39.37)	95.3 $\pm$ 7.86 <sup>adf</sup> (-24.96)	612.45	<0.001
TAS (mmol/L)	3.45 $\pm$ 0.1	1.55 $\pm$ 0.08 <sup>a</sup>	3.11 $\pm$ 0.2 <sup>cd</sup> (+100.64)	2.41 $\pm$ 0.24 <sup>adf</sup> (+ 55.48)	144.621	<0.001

Values in the parentheses are % increase (+) or decrease (-), compared to the experimental control (ethanol-treated) group.

P values: <sup>a</sup><0.001, <sup>b</sup><0.01, <sup>c</sup><0.05 compared to control; <sup>d</sup><0.001, <sup>e</sup><0.05 compared to ethanol-fed, <sup>f</sup><0.001, <sup>g</sup><0.05 compared to ethanol with grape skin-fed group.

Table 4—Effects of grape skin and flesh on non-enzymatic and enzymic activities in hemolysate and total antioxidant status (TAS) in serum of chronic ethanol-fed mice

[Values represent mean $\pm$ SD of 6 rats in each group]						
Parameters	Normal	Ethanol-treated	Ethanol + Grape skin-treated	Ethanol + Grape flesh-treated	F-variance	Significance
GSH ( g/mg protein)	3.23 $\pm$ 0.12	1.78 $\pm$ 0.06 <sup>a</sup>	2.58 $\pm$ 0.07 <sup>ad</sup> (+44.94)	2.3 $\pm$ 0.08 <sup>adg</sup> (+29.21)	275.343	<0.001
TBARS (nmol/ml)	3.21 $\pm$ 0.07	5.98 $\pm$ 0.14 <sup>a</sup>	4.75 $\pm$ 0.13 <sup>ad</sup> (-20.56)	5.11 $\pm$ 0.17 <sup>adh</sup> (-14.56)	421.637	<0.001
Nitrite (nM)	0.18 $\pm$ 0.02	0.26 $\pm$ 0.02 <sup>a</sup>	0.21 $\pm$ 0.01 <sup>e</sup> (-19.23)	0.23 $\pm$ 0.02 <sup>b</sup> (-11.53)	18.963	<0.001
Superoxide dismutase (U/mg hemoglobin)	3.46 $\pm$ 0.16	2.93 $\pm$ 0.12 <sup>a</sup>	3.25 $\pm$ 0.15 <sup>e</sup> (+10.92)	3.13 $\pm$ 0.08 <sup>b</sup> (+6.82)	16.8	<0.001
Catalase (mmol H <sub>2</sub> O <sub>2</sub> decomposed/mg protein /min)	0.3 $\pm$ 0.01	0.25 $\pm$ 0.01 <sup>a</sup>	0.28 $\pm$ 0.01 <sup>cf</sup> (+12)	0.28 $\pm$ 0.01 <sup>cf</sup> (+12)	13.238	<0.001
Glutathione reductase (nmol NADPH oxidized/min/mg protein)	0.28 $\pm$ 0.02	0.17 $\pm$ 0.01 <sup>a</sup>	0.25 $\pm$ 0.01 <sup>cd</sup> (+47.05)	0.22 $\pm$ 0.01 <sup>ad</sup> (+29.41)	48.986	<0.001
Glutathione peroxidase (U/g hemoglobin)	2.95 $\pm$ 0.1	2.11 $\pm$ 0.07 <sup>a</sup>	2.51 $\pm$ 0.09 <sup>ad</sup> (+18.95)	2.53 $\pm$ 0.1 <sup>ad</sup> (+19.9)	75.12	<0.001
Glutathione s- transferase (nmol CDNB conjugate formed/mg protein/min)	0.16 $\pm$ 0.01	0.28 $\pm$ 0.01 <sup>a</sup>	0.23 $\pm$ 0.01 <sup>ad</sup> (-17.85)	0.24 $\pm$ 0.01 <sup>ad</sup> (-14.28)	238.378	<0.001
TAS	3.45 $\pm$ 0.1	1.55 $\pm$ 0.08 <sup>a</sup>	3.11 $\pm$ 0.2 <sup>cd</sup> (+100.64)	2.41 $\pm$ 0.24 <sup>adf</sup> (+55.48)	144.621	<0.001

Values in the parentheses are % increase (+) or decrease (-), compared to the experimental control group.

P values: <sup>a</sup><0.001, <sup>b</sup><0.01, <sup>c</sup><0.05 compared to control; <sup>d</sup><0.001, <sup>e</sup><0.01, <sup>f</sup><0.05 compared to ethanol-fed, <sup>g</sup><0.001, <sup>h</sup><0.01 compared to ethanol with grape skin-fed group.

Chronic ethanol administration significantly ( $P<0.001$ ) increased TBARS (86.3%) and nitrite (44.4%) levels and GST activity (75%), but significantly ( $P<0.001$ ) decreased GSH content (44.9%) and activities of SOD (15.3%), CAT (16.6%), GR (39.3%) and GPx (28.5%) compared to normal group (Table 4). Supplementation with grape skin or flesh to ethanol-fed mice significantly ( $P<0.001$ ) lowered TBARS level by 20.56% and 14.56% respectively, GST activity by 17.85% and 14.28% respectively, and nitrite levels by 19.23% and 11.53%, respectively, while significantly ( $P<0.05$ ) elevated GSH content by 44.9% and 29.2% respectively, GR activity by 47% and 29.41% respectively and GPx activity by 18.95% and 19.9% respectively, compared to the experimental group. While both treatment

significantly ( $P<0.05$ ) reduced CAT activity by 12%, grape flesh did not show any significant effect on nitrite level or SOD activity (Table 4).

Ethanol administration significantly ( $P<0.001$ ) increased the levels of IL-10 (4.32 fold), TNF- (3.3 fold), IFN- (2.16 fold), TGF- 1 (0.75 fold) and VEGF-A (1.8 fold), but reduced IL-4 (47.6%), compared to the normal mice (Fig. 1). Treatment with grape skin or flesh significantly ( $P<0.001$ ) increased IL-4 (41.75% and 50.25%, respectively), but reduced TNF- (46.32% and 35.04%, respectively), IFN- (38.18% and 31.24%, respectively), TGF- 1 (19.64% and 14.28%, respectively) and VEGF-A (39.83% and 30.78%, respectively), compared to the ethanol-treated group (Fig. 1).

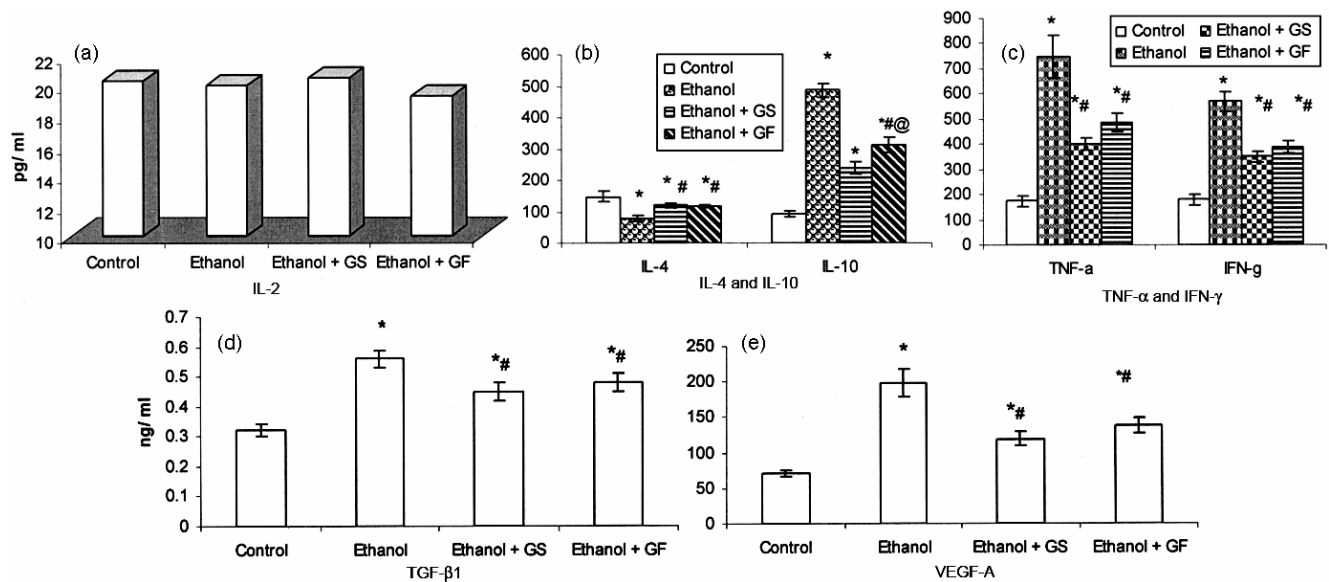


Fig. 1—Effects of grape skin and flesh on immunologic parameters in serum of chronic ethanol-fed mice [Values represent mean  $\pm$  SD of 6 rats in each group. *P* values: \* $<0.05$  compared to control; # $<0.05$  compared to ethanol-fed, @ $<0.05$  compared to ethanol with grape skin-fed group]

## Discussion

Higher content of the total phenolics, flavonoids and ascorbic acid in the grape skin compared to those present in the grape flesh in this study (Table 1) was in agreement with other reports<sup>7</sup>. Although limited literature makes comparison of doses difficult, the dose of grape skin or flesh used in this study was based on previous study<sup>7</sup>. Moreover, since the control mice were fed with isocaloric glucose solution, their nutritional status remained the same, as evident from the weight gain of the respective groups (Table 2).

Chronic ethanol consumption causes hepatic injury which can be diagnosed from the serum globulin level and transaminase activities (AST and ALT)<sup>26</sup>. Hypoalbuminemia is a common feature of chronic alcohol-induced liver damage<sup>27</sup>. Significantly decreased albumin level and increased liver marker enzymes activities in response to chronic ethanol exposure (Table 3) suggested that these animals suffered from liver damage. Significantly decreased serum TAS level (Table 3) indicated that ethanol-induced damage was associated with oxidative stress. Although administration of grape skin and flesh to the ethanol-fed mice decreased the levels of serum AST and ALT, as well as globulin (Table 3), these parameters were not restored to normal levels.

Glutathione protects the cells by altering cellular redox status and acting as a cofactor for

antioxidant enzymes<sup>28</sup>. Depletion of GSH due to ethanol exposure (Table 4) renders the cell more susceptible to oxidative stress<sup>29</sup>. Elevated Lipid peroxidation is considered as the primary mechanism of cell membrane damage<sup>30</sup>. Though nitric oxide (NO<sup>\*</sup>) provides beneficial role, such as blood vessel relaxation, proliferation and migration of endothelial cells as well as angiogenesis, it is also cytotoxic at a high concentration, due to the genesis of peroxynitrite<sup>31,32</sup>. Significantly elevated TBARS and nitrite levels (Table 4) in this study further indicated that chronic ethanol administration caused oxidative stress in the mice. SOD catalytically dismutates superoxide radical anion to H<sub>2</sub>O<sub>2</sub>, while CAT and GPx convert H<sub>2</sub>O<sub>2</sub> to water and oxygen within cells<sup>33</sup>. The increased GST activity and decreased activities of GPx, GR, SOD and CAT (Table 4) are important factors sustaining a pathogenic role for oxidative stress. Supplementation with grape skin or flesh to ethanol-fed mice reduced TBARS and nitrite levels, increased SOD and CAT activities, partially reversed the activities of GR, GPx and GST and significantly restored the GSH level (Table 4).

Alcohol intake stimulates the production of Th1 type proinflammatory cytokines (e.g. IFN- $\gamma$  and IL-2) that enter into the circulation<sup>34</sup>. The low levels of GSH and increased level of serum Th1 type IFN- $\gamma$  (Fig. 1c) might augment the proinflammatory

cytokine TNF- $\alpha$  level<sup>33</sup> as observed in this study (Fig. 1c). TNF- $\alpha$  triggers the production of other cytokines that recruit inflammatory cells and initiate a healing response that includes fibrogenesis<sup>35,36</sup>. Elevated TGF- $\beta$  1 level due to ethanol exposure (Fig. 1d) is implicated as a trigger for collagen deposition and fibrosis<sup>37</sup>. Th1 immunity is attenuated by the Th2 immune reaction<sup>38</sup>. The markedly increased IL-10 level (Fig. 1b) due to ethanol exposure in this study might inhibit the production of nitric oxide and proinflammatory cytokines<sup>39</sup>. However, chronic ethanol exposure reduced the serum IL-4 level (Fig. 1b).

Liver damage can develop as a consequence of any imbalance between Th1 and Th2 immune reactions in the liver<sup>40</sup>. The chronic ethanol consumption leads to hypoxia<sup>41,42</sup>, which might induce production of VEGF-A (Fig. 1e) to stimulate the growth of new blood vessels to meet the increased oxygen demand<sup>43</sup>. Interestingly, both extracts from grape could apparently produce a protective and ameliorating effect against liver tissue damage by regulating cytokines and restoring Th1/Th2 balance. Other study has also shown that grape powder extract (GPE) attenuates TNF- $\alpha$ -induced expression of inflammatory genes<sup>44</sup> and LPS-mediated inflammation<sup>45</sup>.

Grape contains large amounts of phytochemicals, including resveratrol, quercetin, proanthocyanidins, anthocyanidin, procyanin, myricetin, kaempferol, catechin, and epigallocatechin gallate<sup>46</sup>. These phytochemicals have antioxidative and anti-inflammatory activities, as well as hepatoprotective effects<sup>47</sup>. Combined phytochemicals are found to be more effective than individual in grape<sup>48</sup>. Though grape skin and flesh significantly reversed ethanol-induced alterations, grape skin showed higher protective effects compared to grape flesh in this study. Our study also showed that grape skin contains higher amount of polyphenolics, flavonoids, antioxidants and free radical scavenging properties than grape flesh. It is generally assumed that the beneficial effects of the grape products might be due to the presence of the polyphenols. In fact some polyphenols are able to decompose H<sub>2</sub>O<sub>2</sub>, and thereby reducing the damage induced by oxidative agents<sup>4</sup>. Therefore, further study is needed to identify the role of other ingredients in grapes.

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