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# New insights on the neuroprotective potential of grape seed extract: Evidences-based on experimental animal study

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ABSTRACT

This study was constructed to explore the neuroprotective significance of grape seed extract (GSE) in management of neurodeterioration produced by Ethanol (EtOH) inhalation in rats. Fifty adult female rats were enrolled in this study and divided into 5 equal groups. The experimental period lasted 12 weeks, after which nitric oxide (NO), hydrogen peroxide  $(H_2O_2)$ , dopamine (DA), adrenaline (AD), noradrenaline (NA), brain derived neurotrofic factor (BDNF), B-cell lymphoma 2 (Bcl-2) levels were assayed in the brain. Additionally, brain Bcl-xl gene expression level was determined using RT-PCR. Immunohistochemical technique was used for assessment of survivinimmunopositive cells intensity in the brain tissue. Furthermore, histopathological investigation of brain tissue was carried out. The results indicated that inhalation of EtOH elicited significant elevation in brain NO,  $H_2O_2$ and Bcl-2 levels, accompanied with significant depletion in brain DA, AD, NA and BDNF levels. Significant upregulation in brain Bcl-xl gene expression level was also recorded in EtOH-challenged groups. In addition, significant elevation in the intensity of survivinimmunopositive cells in the brain has been detected due to EtOH inhalation. Histological investigation of brain tissue section of rats in EtOH- challenged groups showed pronounced pathological alterations. However, pre-treatment of EtOH challenged groups with grape seed extract resulted in significant improvement in the biochemical, molecular and immunohistochemical parameters. These findings were documented by histological investigation of brain tissue which revealed the ability of grape seed extract to restore the structural organization of the brain. In conclusion, the present study clearly indicated that grape seed extract represented a good neuroprotective candidate against EtOH- induced neuropathy due to its powerful antioxidant activity, anti- apoptotic potential and neurotrophic effect.

Keywords: Ethanol inhalation, neuropathy, grape seed extract, oxidative stress, apoptosis.

#### **INTRODUCTION**

Ethanol is a volatile, flammable, colorless liquid with a slight chemical odor. It is used as an antiseptic, a solvent and a fuel due to its low freezing point. Ethanol is also called Ethyl alcohol, drinking alcohol, or simply alcohol which is the principal type of alcohol found in alcoholic beverages, produced by the fermentation of sugars by yeast. It is a neurotoxic agent[1] with structural formula, CH3CH2OH and it is often abbreviated as C2H5OH, C2H6O or EtOH. Ethanol is miscible with water and it is a good general solvent. It is found in paints, tinctures, markers, and personal care products such as mouthwashes, perfumes and deodorants [2].

EtOH exposure has been shown to induce serious functional and structural abnormalities in the CNS and the most affected regions are corpus callosum, cerebellum, hippocampus and basal ganglia[3].*In vitro* studies, EtOH elicited mitochondrial dysfunction and oxidative stress in both astrocytes [4]and immature neurons[5]. Also, EtOH could damage the brain by inducing neuron apoptosis in the cortex, cerebellum, hippocampus, and dorsal as well as median raphe *in vitro* and *in vivo*[6].

Antioxidants have been found to significantly mitigate the neurotoxic effects of EtOH[7].Grape seeds (*Vitisvinifera*) are waste products of the grape juice industry. The seeds contain lipids, proteins, carbohydrates and flavonoids. Grape seed extract (GSE) has been reported to possess a broad spectrum of pharmacological effects such as antioxidative, anti-inflammatory, and antimicrobial activities, as well as cardioprotective and hepatoprotective potentials. GSE has ability to scavenger oxygen free radicals, inhibit lipid peroxidation, suppress the formation of the inflammatory cytokines, reduce the alterations in cell membranes receptors and modulate gene expression[8]. As it is rich in bioactive phytochemicals like flavonoids, polyphenols, proanthocyanidins, anthocyanins, procyanidines and the stilbene derivative, resveratrol which have the ability to cross the blood-brain barrier and diffuse through the central nervous system [9].

This study was planned to elucidate the neuroprotective significance of grape seed extract against neurodeterioration induced by EtOH inhalation in rats.

## MATERIALS AND METHODS

#### 1- Materials

1- Ethanol (EtOH) (CH<sub>3</sub>-CH<sub>2</sub>-OH): was purchased from Aldrich Chemica Co., GmbH (Germany).

**2- Grape Seed Extract (GSE)**: Form: powder, Part: seed extraction, Type: solvent extraction; grade 95%, Model Number: grape seed extract, Assay: proanthocyanidins by  $UV \ge 95\%$ . The used extract solvent was water and mEtOHyl alcohol 70%. This product was purchased from Sigma Company (St. Louis, Massouri, USA).

#### 3- Animals

Fifty adult female albino rats of Wistar strain weighing 150-170 g were obtained from a breeding stock maintained in the Animal House of the National Research Centre, Giza, Egypt. Animals were maintained in stainless steel wire meshed cages under environmentally controlled conditions with respect light, temperature or air humidity and fed with standard laboratory food and water *ad libitum*. All protocols and procedures were approved by Institutional EthicsCommittee of National Research Centre, Giza, Egypt and experiments were performed as per guideline of National Research Centre Ethical Committee for medical research.

#### 4- Experimental Set-up

After an acclimatization period of 10 days, rats were randomly allocated into 5 groups (10 rats /group).**Gp** (1): Normal healthy animals served as negative control group. **Gp** (2):EtOH-challenged group (300 ppm); the animals in this group were exposed to 300 ppm (low dose) of EtOH by inhalation for 6 hours daily, 5 days / week[10]. **Gp**(3): EtOH-challenged group (600 ppm); the animals in this group were exposed to 600 ppm (high dose) of EtOH by inhalation for 6 hours daily, 5 days / week. **Gp**(4) GSE+ EtOH 300 ppm; the animals in this group were orally administered with GSE (150mg/kg b.wt)[11]prior exposure to EtOH (300 ppm), **Gp**(5): GSE + EtOH 600 ppm; the animals in this group were orally administered with GSE (150mg/kg b.wt) prior exposure to EtOH (600 ppm) by inhalation. The experiment lasted for 12 weeks.

#### **Inhalation protocol**

1. Exposure Method: Whole body inhalation exposure method.

2. Inhalation period: Inhalation of EtOH was conducted daily for 6 hours/5 days/ week for 12 weeks to mimic the EtOH inhalation seen in human abusers.

3. Inhalation doses: The doses used in the present study were 300 ppm and 600 ppm of EtOH.

At the end of the experimental period, rats were fasted overnight and subjected to diethyl Ether anaesthesia. Then the rats were sacrificed by cervical dislocation and the brain was harvested, weighed, washed in saline solution and blotted dry. Then each brain was divided into two portions; the first portion was divided into two portions, one collected in liquid nitrogen and stored at  $-80^{\circ}$  C for molecular genetics analyses and the other portion was preserved in formalin saline (10%) for histological investigation and immunohistochemical examination. The second portion was homogenized immediately to give 10% (w/v) homogenate in ice-cold medium containing 50 mMTris-Hcl (pH 7.4) and 300 mM sucrose [12] for biochemical analyses.

## $\Pi$ -*Methods*

#### **1-Biochemical Analyses**

Brain NO and  $H_2O_2$  levels were determined by spectrophotometric method using kit purchased from Bio-diagnostic `Co., Egypt, according to the method described by Montgomery and Dymock[13] and Aebi, [14]respectively. Brain Bcl-2, was quantified by ELISA using Bcl-2 assay kit purchased from Glory Science Co., LTD, TX, USA following the method of Barbareschi *et al.* [15]. Brain BDNF level was assayed by ELISA using BDNF assay kit purchased from Ray Biotech, Inc. Co., according to the method described by Laske*et al.*[16]. Brain DA, AD and NA levels were assayed by ELISA using 3 CAT ELISA fast track assay kit purchased from Labor Diagnostika Nord GmbH & Co, KG according to the manufacturer's instructions.

## 2- Molecular Analysis for Bcl-xl Gene Expression Using Semi-Quantitative Real Time PCR

#### Isolation of total RNA

Total RNA was isolated from brain tissue of female rats by the standard TRIzol® reagent extraction method (Invitrogen, USA). Then, the complete  $Poly(A)^+$  RNA was reverse transcribed into cDNA in a total volume of 20 µL using RevertAid<sup>TM</sup> First Strand cDNA Synthesis Kit (MBI Fermentas, Germany).

#### Semi-Quantitative Real time-polymerase chain reaction (RT-PCR)

An iQ5-BIO-RAD Cycler (Cepheid, USA) was used to determine rat cDNA copy number. PCR reactions were set up in 25  $\mu$ l reaction mixtures containing 12.5  $\mu$ l 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5  $\mu$ l 0.2 M forward primers, 0.5  $\mu$ l 0.2 M reverse primer, 6.5  $\mu$ l distilled water, and 5  $\mu$ l of cDNA template.

#### Table (1): Primers sequences for RT-PCR

		-
Gene	Primer sequence $(5 - 3)$	Ref
Bcl-xl	F: CAGTGAAGCAAGCGCTGAGA	[17]
	R: ACTTGCAATCCGACTCACCAA	
GAPDI	F:TGGAGTCTACTGGCGTCTTCAC	C[17]
	R: GGCATGGACTGTGGTCATGA	
F: forward primer; R: reverse primer.		

The reaction program: denaturation at 95.0°C for 15 sec; annealing at 55.0°C for 30 sec and extension at 72.0°C for 30 sec. At the end of each RT-PCR, a melting curve was performed at 95.0 °C to check the quality of the used primers. The gene expression was calculated using the formulae of Bio-Rad laboratories Inc.[18].

#### 3- Immunohistochemical (IHC) Examination of the Intensity of survivin Immunopositive Cells

Brain samples of rats in the different studied groups were fixed in 10% formalin buffer for 24 hours. Washing was done in tap water then, ascending grade of Ethyl alcohol was used for dehydration. Specimens were cleared in xylene and embedded in paraffin for 24 hours. Sections were cut into 4  $\mu$  thick by slidge microtome then, fixed on positive slides in 65°C oven for 1 hr according to Bancroft *et al.*[19].Image analysis was performed using the image J, 1.4 1a NIH, (USA) analyzer for determination of optical density (O.D)that reflects the immunostaining intensity of survivin positive cells.

#### 4-Histological Investigation

The prepared paraffin blocks for IHC examination were sectioned on the microtome (4M thickness) and stained with haematoxylin and eosin stain [20].

#### **5-Morphometric Analysis**

Using an image analyzer system, five randomly chosen non overlapping high-power fields (original magnification ×400, light microscope) from the cortex and hippocampus were examined separately from each section. Dead neurons and purkinje cells were counted and calculated for the negative control animals, animals challenged with EtOH 300 ppm and 600 ppm as well as animals treated with grape seed extract (GSE) prior exposure to EtOH 300 ppm and 600 ppm.

#### Statistical analysis

In the present study, all results were expressed as Mean + S.E of the mean. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 21, followed by least significant difference(LSD) to compare significance between groups [21].

## RESULTS

#### 1- Biochemical Results

The data in Figs. (1 & 2) showed that there is significant increase (P< 0.05) in brain levels of NO and  $H_2O_2$  in EtOH challenged groups in comparison with the negative control group. While, pre-treatment with GSE in groups challenged with EtOH (300 ppm or 600 ppm) produces significant decrease (P< 0.05) in brain NO Fig. (1) and  $H_2O_2$ Fig. (2) levels versus the corresponding untreated EtOH challenged groups.







Fig. (2)

Figs. (1 and 2): Effect of pre-treatment with grape seed extract (GSE) on brain oxidative stress biomarkers in EtOH challenged rats at dose 300 ppm and 600 ppm

\* significant difference at P< 0.05</li>
 a: significant difference versus negative control group
 b:significant difference versus EtOH challenged group (300 ppm)
 c:significant difference versus EtOH challenged group (600 ppm)

The results in Fig. (3)Indicated that EtOH inhalation elicits significant increase (P< 0.05) in brain Bcl-2 level, in concomitant with significant decrease (P< 0.05) in brain BDNF level Fig. (4)relative to the negative control group. On the other hand, pre-treatment with GSE in EtOH challenged groups (300 ppm or 600 ppm) results in significant reduction (P< 0.05) in brain Bcl-2 level Fig. (3)accompanied with significant elevation (P< 0.05) in BDNF level Fig. (4)when compared with the corresponding EtOH challenged groups.









Figs. (3 & 4): Effect of pre-treatment with grape seed extract (GSE) on brain antiapoptotic marker (Bcl-2) and neurotrophic factor (BDNF) levels in EtOH challenged groups at doses 300 and 600 ppm.

\* significant difference at P< 0.05 a:significant difference versus negative control group b:significant difference versus EtOH challenged group (300 ppm) c:significant difference versus EtOH challenged group (600 ppm)

The data in Figs. (5,6 & 7) revealed that daily EtOH inhalation evoked significant reduction (P< 0.05) in brain DA(Fig. 5), AD(Fig. 6) and NA (Fig.7) levels in comparison with the negative control group. Meanwhile, pre-treatment with GSE in groups challenged with EtOH (300 ppm or 600 ppm) produces significant increase (P< 0.05) in brain, DA (Fig. 5)NA(Fig. 6) and AD(Fig. 7) levels when compared with the corresponding EtOH challenged groups.



Fig. (5): Effect of pre-treatment with grape seed extract (GSE) on brain dopamine (DA) level in EtOH challenged rats at dose 300and 600 \* significant difference at P < 0.05

a:significant difference versus negative control group b:significant difference versus EtOH challenged group (300 ppm) c:significant difference versus EtOH challenged group (600 ppm)



Fig. (6): Effect of pre-treatment with grape seed extract (GSE) on brain adrenaline (AD) level in EtOH challenged rats at dose 300and 600 ppm

\* significant difference at P< 0.05 a:significant difference versus negative control group b:significant difference versus EtOH challenged group (300 ppm)

*c:significant difference versus EtOH challenged group (500 ppm) c:significant difference versus EtOH challenged group (600 ppm)* 



Fig. (7): Effect of pre-treatment with grape seed extract (GSE) on brain noradrenaline (NA) level in EtOH challenged rats at dose 300and 600 ppm

\* significant difference at P< 0.05 a:significant difference versus negative control group b:significant difference versus EtOH challenged group (300 ppm) c:significant difference versus EtOH challenged group (600 ppm)

# 2. Molecular Genetic Results

#### 2.1. Expression of Bcl xl gene

The present findings in Fig. (8) showed that EtOH inhalation induces significant increase (P < 0.05) in the expression level of Bcl-xl gene in EtOHchallenged groups versus the negative control ones. In contrast, pre-treatment with GSE causes significant decrease (P < 0.05) in brain expression level of Bcl-xl gene in groups challenged with EtOH 300 ppm or 600 ppm relative to the corresponding EtOH challenged groups.



Fig. (8): Effect of pre-treatment with grape seed extract (GSE) on Bcl-xl gene expression level in brain tissue of EtOH challenged groups at doses 300 and 600 ppm measured by semi-quantitative real time -PCR.

\* significant difference at P< 0.05 a:significant difference versus negative control group b:significant difference versus EtOH challenged group (300 ppm) c:significant difference versus EtOH challenged group (600 ppm)

#### **3-Immunohistochemical Examination Results**

Fig. (9) represented the effect of pre-treatment with GSE on intensity of surviving immunopositive cells in the brain tissue of EtOH challenged groups. EtOH inhalation in rats (300 ppm or 600 ppm) elicits significant increase (P< 0.05) in the intensity of surviving immunopositive cells in brain tissue as compared to the negative control. On the other side, EtOH challenged groups pre-treated with GSE reveal significant (P< 0.05) decrease in the intensity of surviving immunopositive cells in brain tissue relative to the corresponding EtOH challenged groups.



Fig. (9): Effect of pre-treatment with grape seed extract(GSE) on intensity of survivinimmunopositive cells in the brain tissue of EtOH challenged groups at doses 300 and 600 ppm

\* significant difference at P< 0.05 a:significant difference versus negative control group b:significant difference versus EtOH challenged group (300 ppm) c:significant difference versus EtOH challenged group (600 ppm)

## 4- Histological and Morphometric Results

Microscopic examination of hematoxylin and eosin-stained braintissue section of rat in the negative control group shows the normal appearance of the cerebral cortex and cerebellum. The larger cells are neurons (red arrow). The pink substance between cells is the neuropil. A few capillaries are also seen (black arrow)(**Fig.10**).

Microscopic investigation of brain tissue section of rat in EtOH challenged group at dose 300ppm, shows extensive dark neuron; some appear apoptotic and others appeared with corkscrew dendrites. Hemorrhage in the meninges is observed and thrombotic vessels appear with membrane bound vacuoles (**Fig. 11**). Tangible degeneration of Purkinje cells in the cerebellum has been observed (**Fig. 12**). The morphometric analysis reveal an increase in the number of dead neuron in the cortex and hippocampus (**Table 1**), in association with the decrease in the number of Purkinje cells in the cerebellum as compared to the negative control (**Table 2**).

Microscopic examination of brain tissue section of rat in EtOH challenged group at dose 600 ppm shows signs of neurodegeneration (gliosis) in the cerebellum. The congestion of cerebral blood vessel is also noticed. Extensive dark picnoticneurons have been detected; some appear with corkscrew dendrites and others appear apoptotic. Hemorrhage in the meninges is seen (**Fig. 13**). Marked degeneration of Purkinje cells has been detected (**Fig. 14**). The morphometric analysis reveals an increase in number of dead neuron in the cortex and hippocampus (**Table 1**), in concomitant with the decrease in the number of Purkinje cells in the cerebellum as compared to negative control (**Table 2**).

Microscopic investigation of brain tissue section of rat in the group treated with GSE prior EtOH inhalation at dose 300ppm shows some improvement in the pathological changes as compared to those in the corresponding untreated ones. This improvement appears in the form of normal structure of granular cells as GSE can abolish the formation of dark neuron. The decreased size of pyramidal cells with darkening of their nuclei and mild thickening of the meninges are also seen (**Fig. 15**). Observable decrease in the number of dead neurons in the cortex and hippocampus has been recorded using morphometric analysis (**Table 1**). Also, tangible increase in the number of Purkinje cells has been detected as compared to that in the corresponding untreated ones (**Table 2**).

Microscopic examination of brain tissue section of rat in the group treated with GSE prior EtOH inhalation at dose 600 ppm shows some improvement in the pathological alterations as compared to those in the corresponding untreated ones. This improvement appears in the form of normal structure of granular cells in association with identifying the deeply easinophilic material of the plaque formation (**Fig. 16**). The morphometric measurement by image analysis of the cortex and hippocampus reveals a decrease in the number of dead neurons (**Table 1**). Also, an increase in the number of Purkinje cells has been demonstrated as compared to that in the corresponding untreated ones (**Table 2**).



(Fig.10): Photomicrograph of brain tissue section of rat in the negative control group.Showing the larger cells are neurons(red arrow). The pink substance between cells is the neuropil. A few capillaries are also seen (black arrow). (Hx&Ex400)



(Fig.12): Photomicrograph of brain tissue section of rat in EtOH challenged group at dose 300 ppmshowing degeneration of somepurkinje cells (arrow). (Hx&Ex400)



(Fig.11):Photomicrograph of brain tissue section of rat inEtOH challenged group at dose 300 ppm.showingextensive dark picnotic nuclei in neurons (blue curved arrow), some appeared apoptotic(yellow arrow).Hemorrhage in meninges (curved red arrow) and thrombotic vessels show a vessel with membrane bound vacuoles (star).(Hx&Ex200)



(Fig.13): Photomicrograph of brain tissue section of rat in EtOH challenged group at dose 600 ppmshowed signs of neurodegeneration the (gliosis) in cerebellum (double red arrow), congested of cerebral blood vessel(curved black arrow). Extensive dark neuron (red arrow), some appeared as corkscrew dendrites (black arrow) and apoptotic neuron (yellow arrow), hemorrhage in meninges were (black seen arrow).(Hx&Ex200)



(Fig.14): Photomicrograph of brain tissue section of rat in EtOH challenged group at dose 600 ppmshowing more degeneration of purkinje cells (arrow).(Hx&Ex400)

![](_page_9_Figure_4.jpeg)

(Fig.15): Photomicrograph of brain tissue section of rat treated with GSE prior EtOHinhalayionat dose 300 ppmshowing dilatation and congestion of many blood capillaries (blue arrow) with mild thickening of meninges above the surface (red arrow), decrease in size of pyramidal cells with darkening of their nuclei (black barrow) the granular cells appeared normal.(Hx&Ex200)

![](_page_9_Picture_6.jpeg)

(Fig.16): Photomicrograph of brain tissue section of rat treated with GSE prior EtOH inhalation at dose 600 ppmshowing normal appearance of granular layer cells (blue arrow), deeply eosinophilic material of the plaques formation (red arrow). (Hx&Ex400)

 Table (1):Effect of grape seed extract (GSE) on the number of dead neurons in the cortex and hippocampus of EtOH challenged group at doses 300 and 600 ppm

Parameters Groups	Number of dead neurons in cortex (µM) (n=5)	Number of dead neurons in hippocampus $(\mu M)$ (n=5)
Negative control	12	10
EtOH 300 ppm	51	58
EtOH 600 ppm	76	92
GSE + EtOH 300 ppm	32	25
GSE + EtOH 600 ppm	39	36

 Table (2):Effect of grape seed extract (GSE) on the number of Purkinje cells in the cerebellum of EtOH challenged group at doses 300 and 600 ppm.

Parameter	Number of Purkinje Cells in cerebellum
Groups	(µM) (n=5)
Negative control	30
EtOH 300 ppm	15
EtOH 600 ppm	10
GSE + EtOH 300 ppm	25
GSE + EtOH 600 ppm	21

#### DISCUSSION

The CNS is one of the major targets for alcohol and alcohol abuse may cause cognitive impairment with permanent structural brain damage [22].

The present data revealed that inhalation of EtOH significantly increased the levels of NO and  $H_2O_2$  in rat brain. The present findings are in harmony with the earlier study of Calabrese *et al.*[23]who postulated that brain exposure to EtOH is associated with oxidative perturbation of cellular oxidant/antioxidant balance of the brain towards the formation of ROS such as NO,  $H_2O_2$ , 'HO and superoxide anion radical ('O<sub>2</sub>) [24]. The proposed mechanism for the increased brain level of NO due to EtOH inhalation is the potentiation of iNOS as well as eNOS[25].

A conceivable importance of EtOH effect is the inhibition of mitochondrial respiratory chain components which stimulates the production of reactive species. This could be the origin of increased brain levels of  $H_2O_2$  and  $O_2$  due to EtOH exposure. Research studies have provided evidence that oxidative stress may be one mechanism by which EtOH produces these events [26]. Moreover, NADH is known to generate superoxide anions radical ( $O_2$ ), the immediate precursor of  $H_2O_2[27]$ , and EtOH has been found to induce alcohol dehydrogenase E (AdhE) which increases NADH level[28], with consequent generation of  $O_2$  and  $H_2O_2$ .

The data presented in the current work revealed the potent protective effect of GSE against EtOH inhalation as evidenced by the significant reduction in brain NO and  $H_2O_2$  levels of the treated rats. Several studies suggested that GSE has a critical role as neuroprotective delegate [29], through inhibition of calcium signals and nitric oxide radical formation. Terra *et al.* [30]demonstrated that GSE strongly down regulates iNOS mRNA expression, with consequent reduction in iNOS protein and in turn NO production. Moreover, compounds such as resveratrol, quercetin and catach in which are enriched in grape skin and seeds [31]have shown their ability to inhibit cytokine-induced NO production in astrocytes [32].

GSE is a potent inhibitor of  $H_2O_2$ -induced oxidative stress and activation of NF- $\kappa$ B and MAPK signaling in human lens epithelial (HLE) cells [33]. These investigators explained that the antioxidant property of GSE contributes to the reduction of  $H_2O_2$ -induced phosphorylation of MAPKs through modulation of ROS. Therefore, the reduction of MAPK and NF- $\kappa$ B signaling pathways could potentially be promoted by GSE to activate certain antioxidantresponsive genes to protect against  $H_2O_2$ -induced oxidative stress in HLE cells [33].

Although the mechanism(s) by which alcohol exposure could induce deleterious changes in the brain have not been fully elucidated, animal studies suggested that apoptosis is likely to be involved [34].In view of our data, EtOH exposure experienced significant upregulation in brain Bcl-2 protein as well as Bcl-xl gene expression level. Biochemical and molecular data indicated that EtOH, even in physiologic concentrations, can produce rapid lipid peroxidation followed by activation of NF- $\kappa$ B in the microvascular wall of the brain [35] and cerebral vascular muscle cells [36]. The activation of NF- $\kappa$ B is achieved *via* membrane oxidation and cellular entry of Ca21 oxygenfree radicals[35].In line with this evidence, Caroline *et al.* [37] as well as detected an increase in NF- $\kappa$ B in intact rat brain as soon as 15 min after acute EtOH administration. Thus, it could be suggested that EtOH-induced lipid peroxidation and activation of nuclear transcription factors particularly NF- $\kappa$ B probably play a key role in alcohol-induced significant amplification in brain Bcl-2 protein level as well as Bcl-xl gene expression level. As these molecules we well knew NF- $\kappa$ B- dependent genes [38].

In light of the current data, pre-treatment of EtOH challenged groups with GSE significantly down regulated brain Bcl-2 protein level and Bcl-xl gene expression level. GSE has been found to activate phosphatidylinositol 3-kinase (PI3K $\rightarrow$ pAkt), a pro-survival pathway, and reduce apoptotic death by modulating the activation of NF- $\kappa$ B pathways [33]. In this context, it could be suggested that the active phytochemicals of GSE (procyanidins and proanthocyanidins) are responsible for this effect as they have a well known scavenging activity for ROS and hence they can suppress the expression of NF- $\kappa$ B and its dependent genes including Bcl-2 and Bcl-xl.

Survivin is a novel multi-functional protein that inhibits apoptosis *via* inhibition of activated caspases [39]. In the present setup, EtOH inhalation in rats produced significant increase in surviving immunopositive cells in the brain. Upregulation of survivin may result from adaptation of the neurons to stress (mainly hypoxia) induced by EtOH inhalation [40, 41]. Furthermore, Zhu *et al.*[38] study clearly demonstrated the involvement of NF-κB and PI3-kinase pathways in the upregulation of brains urvivin expression.

In the present setting, pre-treatment with GSE in EtOH challenged rats exhibited significant decrease in surviving immunopositive cells in the brain. In line with these findings, Feng *et al.*[42] provided a clear document for the effect of GSE as a neuroprotective agent against hypoxia. GSE significantly reduces hypoxia-induced oxidative

stress and the increased brain ROS as well as thiobarbituric acid reactive substances (product of lipid peroxidation). Thus, the present data indicated that the suppression of free radicals after hypoxic ischemia due to EtOH inhalation by GSE is the potentunderlying mechanism for its neuroprotection.

Brain-derived neurotrophic factor (BDNF) is a member of a family of neurotrophic factors (NTFs) which includes nerve growth factor, neurotrophin-3, neurotrophin-4/5, neurotrophin-6 and neurotrophin-7 [43]. The present study showed that EtOH inhalation elicited significant depletion in brain BDNF level rats. *In vitro* study revealed that EtOH exposure inhibits the secretion of BDNF and neurotrophin-3 from cultured cerebellar granule cells (CGC)[44]. Further study, demonstrated that EtOH exposure blocks BDNF signaling in cultured CGC[45].

The results of this study showed that the pre- treatment with GSE prior EtOH inhalation reversed the drop in brain BDNF level of rats. This finding could be attributed to the active constituents in GSE mainly resveratrol as Rai *et al.*[46]confirmed the neuroprotective effect of resveratrol *via* its ability to enhance BDNF level in stressed rats. In another *invivo* study, resveratrol has been shown to enhance hippocampal BDNF mRNA, providing its neuroprotective effect [47].

In his study, prolonged EtOH inhalation evoked significant reduction in brain levels of DA, AD and NA of rats. These results come in line with the study of Vasconcelos *et al.*[48]which demonstrated that rats chronically exposed to EtOH reveal significant alterations in the levels of monoamines (DA, NA) and their metabolites in hippocampus and striatum. Also, Rothblat*et al.* [49]reported that DA and NA levels are significantly decreased in the striatum of rats chronically received alcohol. Furthermore, Budygin *et al.*[50] stated that EtOH exerts a profound effect on dopaminergic neurons, resulting in a suppression of DA neurotransmission in rat striatum. In addition, EtOH has been found to induce cellular and molecular maladaptive changes in dopaminergic reward pathways, including alterations in DA release [51].

Regarding the effect of EtOH inhalation on brain NA level in rats, the current result is in conformity with that of Vasconcelos *et al.*[48]who recorded significant changes in NA level in the striatum after repeated administration of EtOH. Rossetti *et al.*[52] found that EtOH at high dose (2 g/kg) inhibits NA outflow in the frontal cortex. Research investigators have suggested that the decreased cortical NA output may reflect the sedative-hypnotic properties of EtOH at high doses. In fact, EtOH has been found to affect the regional distributions of NA and DA in rodent brain. EtOH could change the activity of their related enzymes, tyrosin hydroxylase (TH), dopamine-Q-hydroxylase (DBH) and monoamine oxidase (MAO) in various regions of rat brain[53].

Oxidative stress is implicated in the neuronal cell death occurs in physiological settings and in neurodegenerative disorders[54]. Thus, it has been suggested that oxidative stress- induced degeneration in adrenergic neurons is the main cause of downregulation of AD level in the brain of EtOH- challenged rats as shown in the present study.

Pre-treatment with GSE prior EtOH- inhalation in rats produced significant increase in brain neurotransmitters (DA, NA and AD) levels. These findings are probably ascribed to the active components in GSE mainly resveratrol which possesses neuroprotective action against 1-methyl-4-phenyl pyridium ion (MPP + )-induced oxidative stress and cellular death [55]. Resveratrol has been shown to prevent the depletion of striatal DA as well as TH activity. Moreover, berry anthocyanin (polyphenols) has been found to inhibit monoamine oxidases (MOAs), providing neuroprotective effects as it is well known that the suppression of MOA activity leads to the increased neural levels of 5 HT, NA and DA [56].

The elevated brain NA level in rats treated with GSE prior exposure to EtOH inhalation in the current study is in agreement with other study which indicated that GSE can increase the biosynthesis of NA [57]. Measurement of NA after proanthocyanidin administration (50 mg/kg) showed an increase in both frontal cortex and hippocampus[58]. Regarding the enhancing effect of GSE on brain AD level in rats inhaled EtOH, this effect could be attributed to its resveratrol content which rescues adrenergic neurons from the damaging impact of EtOH due to its powerful antioxidant activity. This leads to increasing central AD level as shown in the present study.

In the current study, inhalation of EtOH at dose level 300 or 600 ppm induced extensively dark picnotic nuclei in the neurons and some neurons appeared with corkscrew dendrites. Morphometric measurement showed significant increase in number of dead neuron. Sings of neurodegeneration (gliosis) in the cerebrum, especially at 600 ppm have been observed. Therefore, histological alterations in the brain architecture observed in the present experimental setting are dose dependent. Histopathological findings of the present work are in agreement with Mitra*et al.*[59], Phachonpai *et al.*[60] and Asari *et al.* [61]who reported that chronic alcohol exposure is associated with neuronal damage in the hippocampus and the neurons appeared with corkscrew dendrites. These findings supported those of Mitra and Mukherjee [62]who stated that neuronal population, in particular, is vulnerable to alcohol which induces

neuronal damage and neuronal loss (death). A substantial increase in oxidative stress is considered to be the most important factor in the neurodegenerative action of EtOH. Shirpoor *et al.* [63]reported that oxidative stress plays a crucial role in alcohol-induced brain damage, mainly by induction of apoptosis. Ramachandran *et al.* [64]and Heaton *et al.* [65]proved that alcohol administration induces apoptotic death in primary cortical neuron cultures as measured by increased release of cytochrome c from mitochondria and increased caspase-3 activity.

In this work, the exposure of rats to EtOH by inhalation produced degeneration of Purkinje cell. Purkinje cells represent a unique cellular profile in the cerebellum and are the only output cells of the cerebral cortex. Interestingly, Purkinje cells are highly susceptible to a variety of abnormal conditions. In human, Purkinje cells are affected in a variety of diseases ranging from malnutrition to exposure to toxic agents such as alcohol and lithium [66]. Results of this study come in line with Heaton *et al.*[67]and pierce *et al.*[68] who reported that EtOH induces reduction in Purkinje cells. Also Kumar *et al.*[69]found that EtOH exposure leads to significant loss of cerebral Purkinje and granule cells. The loss in purkinje cells as a consequence of EtOH exposure has been explained by Pierce *et al.*[70] and Light *et al.*[71]who indicated that EtOH exposure involves cleavage of DNA and activation of caspase-3 enzyme in the Purkinje cells. Thus EtOH could induce Purkinje cells apoptosis. The main cellular damage caused by EtOH results from ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, proteins, and DNA. Lipid peroxidation plays a crucial role in alcohol-induced brain damage mainly by induction of apoptosis. Although many factors can induce apoptosis, it is generally believed that ROS serve as a common initiator of the apoptotic process [72].

In the current work, the pre-treatment with GSE prior EtOH inhalation restored the pathological changes of the brain of treated rats. According to Sato *et al.* [73],GSE could block cell death signaling mediated through the proapoptotic transcription factors. This means that GSE has antiapoptotic property in addition to its anti-inflammatory actions, oxygen free radical scavenging property, and anti-lipid peroxidation activity. Hwang *et al.*[74] stated that GSE provides powerful antioxidant efficacy that inhibits DNA oxidative damage in the gerbil forebrain ischemia model and hence it shows a potent neuroprotective effect[75]. Moreover, Haibo *et al.*[76] data showed that there is a significant decrease in the number of cells undergoing apoptosis along with the attenuation of oxidative stress in rat groups pre-treated with GSE prior exposure to methylmercury.

#### CONCLUSION

Based on the current results, it could be concluded that ethanol inhalation displayed negative impacts on the central nervous system. A protection afforded by grape seed extract was likely attributed to the powerful antioxidant activity, antiapoptotic potential and neurotrophic property of the active ingredients of this extract.

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