



# **AUSTRALIAN FUNCTIONAL NUTRACEUTICAL FLAVOURS, FRAGRANCES & INGREDIENTS**

**Plant Extracts-Naturally Fermented Fruits and Vinegars  
Cold Pressed Oils-Essential Oils-Phenolic Rich Powders**

## **LITERATURE REVIEW HEALTH BENEFITS GRAPE SEED & SKIN EXTRACTS**



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## LITERATURE SUPPORTS THEORY THAT GRAPE EXTRACTS IMPROVE HEALTH AND WELLBEING

Botanical Innovations Grape seed and skin extracts are a natural plant products derived from grapes (*Vitis vinifera*) grown in Australia.

Botanical Innovations Grape seed and skin extracts contain concentrated phenolic rich phytonutrients including powerful antioxidants.

The phenols in grape seed and skin extracts are water soluble bioflavonoids that benefits the body by fighting free radicals. Grape seed extracts also contains vitamin E, flavonoids, anthocyanins and resveratrol.

**Grape seed and skin extracts are widely used for antioxidant properties and associated health benefits relating to:**

- **Age-related macular degeneration (AMD).**
- **Attention deficit-hyperactivity disorder (ADHD).**
- **Blood circulation problems in the legs**
- **Canker sores.**
- **Chronic fatigue syndrome (CFS).**
- **Constipation**
- **Coughs**
- **Diarrhea.**
- **Heart disease**
- **Heavy menstrual periods.**
- **Hemorrhoids**
- **High blood pressure**
- **High cholesterol levels.**
- **Liver damage.**
- **Nasal allergies**
- **Poor night vision**
- **Skin and breast cancer**
- **Varicose veins**

The following report is a review of current literature about the potential health benefits of Grape Seed and Grape Skin Extracts.

January 2017. The information contained in this datasheet has been gathered from publicly available source material and is intended to provide general information to readers. No therapeutic claim in relation to the product is intended. Whilst reasonable care has been taken in the preparation of this report, General Industry Pty Ltd trading as Botanical Innovations is not responsible for any reliance readers place on the information in the datasheet and does not represent or warrant that the information in the datasheet is complete or accurate. Readers rely on the information in the report at their own risk. Disclaimer: Reasonable care has been taken in preparing this document and the information provided herein is believed to be accurate.



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# BOTANICAL INNOVATIONS

*The Fusion of Botany and Technology to Create Natural Solutions*

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## **Grape Seed Extract Health Benefits – Published Research Papers**

Grape Seed Extract published research papers have found a links between taking grape seed and management of a number of health conditions including:

- Type 2 Diabetes
- Breast Cancer
- Liver Disease
- Neurodegenerative Diseases
- Cardiovascular conditions
- Osteoporosis
- Oxidative Stress



## **Health Benefits Of Grape Seed Extract To Type 2 Diabetes Conditions.**

In a published report in Diabetes Medical Journal of 2009 May entitled "Effects of grape seed extract in Type 2 diabetic subjects at high cardiovascular risk: a double blind randomized placebo controlled trial examining metabolic markers, vascular tone, inflammation, oxidative stress and insulin sensitivity." The researchers from Queen Alexandria Hospital in United Kingdom reported that grape seed extracts significantly improved markers of inflammation and glycaemia and a sole marker of oxidative stress in obese Type 2 diabetic subjects at high risk of cardiovascular events over a 4-week period, which suggests that grape seed extracts may have therapeutic benefits in decreasing cardiovascular risk.

## **Grape Seed Extract Benefits For Breast Cancer**

Researchers from The Department of Surgical Research, Beckman Research Institute of the City of Hope, Duarte California, USA, as published in the Journal of Cancer Research, June 2006 has reported that grape seed extract is an aromatase inhibitor and a suppressor of aromatase expression. Aromatase is the enzyme that converts androgen to estrogen and it is expressed at higher levels in breast cancer tissues than normal breast tissues. Grape seed extract contains high levels of procyanidin dimers that have been shown in laboratory to be potent inhibitors of aromatase. This report suggested that grape seed extract has potential benefits in the prevention/treatment of hormone-dependent breast cancer through the inhibition of aromatase activity as well as its expression.

## **Grape Seed Extract Benefits Nonalcoholic Liver Disease.**

In a report from Saudi Journal of Gastroenterology, grape seed extract has been found to improve liver function in patients with nonalcoholic fatty liver change. In a three month clinical studies, 15 patients with nonalcoholic liver disease were administered with grape seed extracts. The study suggested that grape seed extract significantly improved the grade of fatty liver change; and resulted in significant decrease in alanine aminotransferase in patients receiving the concentrate compared to those receiving vitamin C independently. The study describes the benefits of using grape seed extract for three months in patients with nonalcoholic fatty liver disease.





## **Grape Seed Extract Has Benefits For Neurodegenerative Diseases**

In a NCCAM funded research done at Mount Sinai School of Medicine, it was found that grape seed extract containing polyphenols may inhibit the misfolding of proteins in the brains that is linked to various neurodegenerative conditions including Alzheimer's disease. The results of their in vitro study showed that grape seed extract is capable of interfering with the generation of tau protein aggregates and also disassociating preformed aggregates, suggesting that grape seed extract may have beneficial effects to processes critical to the progression and onset of cognitive dysfunctions and neurodegeneration. The research concluded that grape seed extract is likely to be safe and well-tolerated in people, and may offer benefits as alternative therapy for Alzheimer's disease.

## **Grape Seed Extract Has Benefits For Cardiovascular System.**

In a review study done in Yale School of Medicine, New Haven CT, USA and published in the Journal of American Dietetic Association 2011 August, entitled "The effect of grape seed extract on cardiovascular risk markers: a meta-analysis of randomized controlled trials." It was reported that grape seed extract has beneficial effects on the cardiovascular system. Based on the currently available and reviewed literature, grape seed extract appears to significantly lower systolic blood pressure and heart rate, with no effect on lipid or C-reactive protein levels. Although it is suggested that larger randomized, double-blinded trials evaluating different dosages of grape seed extract and for longer follow-up durations are needed.

## **Grape Seed Extract Has Benefits For Platelet-Dependent Antithrombotic And Anti-Inflammatory Properties**

In a research study done in Whitaker Cardiovascular Institute and Evans Department of Medicine, Boston University School of Medicine, Boston, Massachusetts, USA. and was published in the Journal of Cardiovascular Pharmacology 2005 Oct, reported that grape seed and skin extracts inhibit platelet function and release of reactive oxygen intermediates. Grape seed extract contain polymeric flavonoids with antioxidant properties believed to be protective against cardiovascular events. Acute cardiac events are also associated with enhanced inflammation and thrombosis. In this study, the extracts from grape skins or seeds were examined for their anti-inflammatory properties and effect on platelet release of reactive oxygen intermediates. The results suggests that the extracts from purple grape skins and seeds inhibit platelet function and platelet-dependent inflammatory responses at pharmacologically relevant concentrations. These findings suggest potential benefits for platelet-dependent antithrombotic and anti-inflammatory properties.



## **Grape Seed Extract Benefits Dermal Wound Healing Of Redox-Active Grape Seed Proanthocyanidins**

In a laboratory research done in the Department of Surgery, Heart and Lung Research Institute, The Ohio State University Medical Center, Columbus, OH, USA found that topical application of grape seed extract accelerated wound contraction and closure. Grape seed extract treatment was associated with a more well-defined hyperproliferative epithelial region, higher cell density, enhanced deposition of connective tissue, and improved histological architecture. The study suggests that there are firm evidence to support that topical application of grape seed extract represents a feasible and productive approach to support dermal wound healing.

## **Grape Seed Extract Benefits Osteoporosis By Enhancing Bone Density And Strength In Experimental Animals**

In the Journal of Musculoskeletal Neuronal Interactions 2005, a research was published studying the mechanical assessment of effects of grape seed proanthocyanidins extract on tibial bone diaphysis in rats. The research was done in the Department of Paediatric Dentistry, Kyushu Dental College, Manazuru, Kokurakita-ku, Kitakyushu, Japan. The report presented data from a 6-week experimental period that showed the tibial cortical parameters were increased by grape seed extract treatment, suggesting a potential herapeutic application of this compound for treatment of bone debility.

## **Grape Seed Proanthocyanidines And Skin Cancer Prevention: Inhibition Of Oxidative Stress And Protection Of Immune System**

In a study reported in Molecular Nutrition and Food Research June 2008, suggested that the in vitro and in vivo studies of the possible protective effect of grape seed proanthocyanidins and the molecular mechanism for these effects in SKH-1 hairless mice produced a decreased UVB-induced skin tumor development in terms of tumor incidence, tumor multiplicity, and a decrease in the malignant transformation of papillomas to carcinomas. This study suggests that dietary grape seed extract supplementation could be useful in the attenuation of the adverse UV-induced health effects in human skin.





## **Hepatoprotection Benefits Of Grape Seed Extract**

In Toxicology and Applied Pharmacology. 2011 Aug, a report has been made that grape seed extract inhibited arsenic-induced rat liver injury through suppression of NADPH oxidase and TGF- $\beta$ /Smad activation. The study was made in Public Health College, Zhengzhou University, 450001, China. The report discussed that chronic arsenic exposure induces oxidative damage to liver leading to liver fibrosis. From the in vitro study, grape seed extract dose-dependently reduced arsenic-stimulated ROS production and NADPH oxidase activities.

## **Antiviral Activity Of Grape Seed Extract**

In Biology Research. 2002, it was reported that grape seed extract proanthocyanidins down regulate HIV-1 entry coreceptors, CCR2b, CCR3 and CCR5 gene expression by normal peripheral blood mononuclear cells. The study was made in the Departments of Medicine and Microbiology, Kaleida Health System, Buffalo General Hospital, Buffalo, NY, USA. It was suggested that the flavonoids and related polyphenols found in grape seed extract, in addition to their cardioprotective, anti-tumor, anti-inflammatory, anti-carcinogenic and anti-allergic activities, also possess promising anti-HIV effects.



## Published Research Abstracts

### Induction of cell cycle arrest and apoptosis by grape seed procyanidin extract in human bladder cancer BIU87 cells.

**Publication:** European Review of Medical Pharmacological Science. 2016 Jul;20(15):3282-91.

**Authors:** Liu J, Zhang WY, Kong ZH, Ding DG.

#### Objective

The aim of this study was to evaluate the effects of grape seed procyanidin extract (GSPE) on cell proliferation and apoptosis in human bladder cancer BIU87 cells and to investigate its molecular mechanism in vitro.

#### Materials and Methods

BIU87 cells were treated with different concentrations of GSPE for 24h in vitro while an untreated group was taken as control. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, Hoechst 33258 staining, flow cytometry, RT-PCR and Western blot were used to detect the anti-proliferation and apoptotic induction effects of GSPE on BIU87 cells.

#### Results

It was found that GSPE inhibited the cell growth through cell cycle arrest at G1 phase and induced cell apoptosis in BIU87 cells in a dose-dependent manner. Semi-quantitated RT-PCR and Western blot analyses indicated that GSPE increased caspase-3 ( $p<0.01$ ), but decreased the expression of cyclinD1, CDK4 and survivin ( $p<0.01$ ).

#### Conclusions

GSPE inhibits cell proliferation by inducing cell cycle arrest and apoptosis in BIU87 cells, and the effect may be related with its down-regulation of cyclinD1, CDK4.



## Protective effect of grape seed and skin extract against diabetes-induced oxidative stress and renal dysfunction in virgin and pregnant rat.

**Publication:** Biomed Pharmacother. 2016 Jul 23;83:584-592.

**Authors:** Oueslati N, Charradi K, Bedhiafi T, Limam F, Aouani E.

### Abstract

The present work deal with the effect of alloxan-induced diabetes on kidney oxidative stress and dysfunction of virgin and pregnant rat as well as the protection that may be afforded by high dosage GSSE (4g/kg) treatment.

Diabetes affected negatively several kidney function parameters as creatinemia, uremia, uricemia and proteinuria without affecting kidney index. Diabetes also induced an oxidative stress characterized by increased lipid and protein oxidation, a drop in antioxidant enzyme defenses as catalase, superoxide-dismutase, glutathione-peroxidase, an alteration in transition metals as free iron, copper, selenium and associated enzymes and an increase in calpain and acetyl-cholinesterase activities. Tremendously, GSSE treatment protected efficiently against all the deleterious effects of diabetes-induced kidney dysfunction in both virgin and pregnant animals.

High dosage GSSE is a safe and potent anti-oxidant that may be further tested in clinical trials for the long-term preservation of kidney function especially in multiple pregnancies.



## Scavenging and antioxidant properties of different grape cultivars against ionizing radiation-induced liver damage ex vivo.

**Publication:** Indian J Exp Biol. 2016 Apr;54(4):280-5.

**Authors:** Singha I, Das SK.

### Abstract

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 (*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  $\gamma$ -radiation-induced oxidative stress in liver tissue ex vivo.

The scavenging abilities of grape seeds [2,2-diphenyl-1-picrylhydrazyl (DPPH) ( $IC_{50} = 0.008 \pm 0.001$  mg/mL), hydrogen peroxide ( $IC_{50} = 0.49$  to  $0.8$  mg/mL), hydroxyl radicals ( $IC_{50} = 0.08 \pm 0.008$  mg/mL), and nitric oxide ( $IC_{50} = 0.8 \pm 0.08$  mg/mL)] were higher than that of skin or pulp. Gamma ( $\gamma$ ) radiation exposure to sliced liver tissues 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  $\gamma$ -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 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 extracts ex vivo.



## The effect of supplementation of grape seed proanthocyanidin extract on vascular dysfunction in experimental diabetes.

**Publication:** Journal of Medicinal Food 2011, Nov 14 (11) 1298-302

**Authors:** Okudan N1, Barışkaner H, Gökbel H, Sahin AS, Belviranlı M, Baysal H.

### Abstract

Increased oxidative stress and impaired endothelium-dependent relaxation could underlie many of the vascular complications associated with diabetes.

We aimed to investigate the effect of supplementation with grape seed proanthocyanidin extract (GSPE), a natural antioxidant, on vascular responses and oxidative stress in streptozotocin-induced diabetic rats.

Male Sprague-Dawley rats were divided into three groups: control rats, untreated diabetic rats, and GSPE (100 mg/kg, for 6 weeks)-supplemented diabetic rats. Thoracic aorta rings of the rats were mounted in organ baths, and relaxant responses to acetylcholine (ACh), A23187, and sodium nitroprusside (SNP) were assayed in tissues precontracted with 60 mM KCl. Plasma samples used for the measurement of malondialdehyde (MDA) level and superoxide dismutase (SOD) activity. The endothelium-dependent relaxations in response to ACh and A23187 were impaired, but endothelium-independent relaxation in response to SNP did not change in diabetic rats.

Supplementation with GSPE significantly improved the relaxant responses to ACh and A23187. The MDA level was significantly elevated and the plasma SOD activity was decreased in diabetic rats, but supplementation with GSPE attenuated the elevated MDA levels and increased plasma SOD activity.

Thus supplementation of GSPE may attenuate oxidative stress through the inhibition of lipid peroxidation and may restore endothelial function and reduce the risk of vascular disease in diabetes.



## Grape seed and skin extract mitigates heart and liver oxidative damage induced by a high-fat diet in the rat: gender dependency.

**Publication:** Canadian Journal of Physiology and Pharmacology 2013 Dec 91 (12) pp1076-85

**Authors:** Charradi K1, Mahmoudi M, Elkahoui S, Limam F, Aouani E.

### Abstract

Obesity is a public health problem contributing to morbidity and mortality from metabolic syndrome. It has long been recognized that there is a gender dependency in several obesity-related health risks.

Using a high fat diet (HFD) to induce obesity in Wistar rats, we studied the gender dependency of fat-induced oxidative stress in the heart and liver, with a special emphasis on the distribution of transition metals, as well as the protective effects of grape seed and skin extract (GSSE). HFD induced obesity in both male and female rats, characterized by increased body weight as well as relative liver mass in both genders, and increased relative heart mass in the males only.

HFD also provoked the accumulation of triglycerides and total cholesterol into the male hearts, and into the livers of both genders. HFD induced oxidative stress in the male hearts and also in the livers of both genders. Furthermore, HFD affected cardiac levels of copper in the males, and hepatic levels of copper and zinc in both genders, whereas HFD affected free iron in the male hearts and female livers, specifically.

In conclusion, HFD treatment altered transition metal homeostasis more drastically in the male heart than in the female liver, and GSSE efficiently protected these organs against fat-induced disturbances, regardless of gender.





## Grape seed extract alleviates high-fat diet-induced obesity and heart dysfunction by preventing cardiac siderosis.

**Publication:** Cardiovascular Toxicology 2011 Mar 11(1): 28-37

**Authors:** Charradi K1, Sebai H, Elkahoui S, Ben Hassine F, Limam F, Aouani E.

### Abstract

Obesity is a tremendous public health problem, characterized by ectopic accumulation of fat into non-adipose tissues, leading to oxidative stress and chronic inflammation, in which the heart is the most severely affected organ.

We used an experimental model of high-fat-diet (HFD)-induced obesity to analyze the link between oxidative stress and heart dysfunction. We also studied the cardioprotective effect of a grape seed and skin extract (GSE).

Exposure of rats to HFD during 45 days induced heart hypertrophy, inflammation as assessed by plasma CRP elevation and contractile dysfunction as revealed after ischemia/reperfusion of Langendorff-perfused hearts. HFD also induced cardiac steatosis and lipotoxicity, which are linked to an oxidative stress status, worsened by increased siderosis and resulting in Ca(2+) overload. Importantly, GSE alleviated all the deleterious effects of HFD treatment.

These studies suggest that GSE is a safe anti-obesity and cardioprotective agent that should also find potential applications in other inflammatory damaging conditions as stroke.



## Grape seed and skin extract prevents high-fat diet-induced brain lipotoxicity in rat.

**Publication:** Neurochemical Research Journal 2012 Sep 37(9) 2004-13

**Authors:** Charradi K, Elkahoui S, Karkouch I, Limam F, Hassine FB, Aouani E.

### Abstract

Obesity is related to an elevated risk of dementia and the physiologic mechanisms whereby fat adversely affects the brain are poorly understood.

The present investigation analyzed the effect of a high fat diet (HFD) on brain steatosis and oxidative stress and the intracellular mediators involved in signal transduction, as well as the protection offered by grape seed and skin extract (GSSE). HFD induced ectopic deposition of cholesterol and phospholipid but not triglyceride. Moreover brain lipotoxicity is linked to an oxidative stress characterized by increased lipoperoxidation and carbonylation, inhibition of glutathione peroxidase and superoxide dismutase activities, depletion of manganese and a concomitant increase in ionizable calcium and acetylcholinesterase activity. Importantly GSSE alleviated all the deleterious effects of HFD treatment.

Altogether our data indicated that HFD could find some potential application in the treatment of manganism and that GSSE should be used as a safe anti-lipotoxic agent in the prevention and treatment of fat-induced brain injury.



## **Resveratrol improves cardiovascular function and reduces oxidative organ damage in the renal, cardiovascular and cerebral tissues of two-kidney, one-clip hypertensive rats.**

**Publication:** Journal of Pharmacy and Pharmacology 2010 Dec62(12) 1784-93

**Authors:** Toklu HZ, Sehirli O, Erşahin M, Süleymanoğlu S, Yiğiner O, Emekli-Alturfan E, Yarat A, Yeğen BÇ, Sener G.

### **Objectives:**

The putative protective effects of resveratrol against oxidative injury in the heart, kidney and brain tissues of rats induced with the two-kidney, one-clip (2K1C) hypertension model were investigated.

### **Methods:**

Wistar albino rats were divided into sham-operated (n = 8) or 2K1C groups, in which rats received either resveratrol (10 mg/kg per day, i.p., n = 8), or saline (n = 8) starting at Week 3 after the surgery and continuing for the following 6 weeks. Indirect blood pressure recordings and echocardiographic images were made to evaluate cardiac function. At the end of Week 9 the animals were decapitated and plasma, heart, kidney and brain were taken for biochemical assays, while aortic rings were prepared for vascular reactivity studies.

### **Key Findings:**

2K1C hypertension resulted in increased blood pressure, aortic hypercontractility and reduced left ventricular function, leading to increased lipid peroxidation and myeloperoxidase activity, concomitant with significant reductions in tissue glutathione, superoxide dismutase, Na<sup>+</sup>/K<sup>+</sup>-ATPase and catalase activities in the cardiac, renal and brain tissues, indicating the presence of oxidative tissue damage in peripheral target organs. Elevated plasma levels of lactate dehydrogenase, creatine kinase, as well as reduced plasma levels of antioxidant capacity and nitric oxide further verified the severity of oxidative injury. A 6-week treatment with resveratrol reversed all the measured parameters, ameliorated hypertension-induced oxidative injury in the target organs and improved cardiovascular function.

### **Conclusions:**

Resveratrol improved cardiovascular function through the augmentation of endogenous antioxidants and the inhibition of lipid peroxidation by maintaining a balance in oxidant/antioxidant status, which also ameliorated hypertension-induced oxidative injury in the cardiac, renal and cerebral tissues.



## Polyphenolics in grape seeds-biochemistry and functionality.

**Publication:** Journal of Medicinal Food 2003 Winter 6(4) 291-9

**Authors:** Shi J1, Yu J, Pohorly JE, Kakuda Y.

### Abstract

Grape seeds are waste products of the winery and grape juice industry. These seeds contain lipid, protein, carbohydrates, and 5-8% polyphenols depending on the variety.

Polyphenols in grape seeds are mainly flavonoids, including gallic acid, the monomeric flavan-3-ols catechin, epicatechin, gallic acid, epigallocatechin, and epicatechin 3-O-gallate, and procyanidin dimers, trimers, and more highly polymerized procyanidins. Grape seed extract is known as a powerful antioxidant that protects the body from premature aging, disease, and decay. Grape seeds contain mainly phenols such as proanthocyanidins (oligomeric proanthocyanidins).

Scientific studies have shown that the antioxidant power of proanthocyanidins is 20 times greater than vitamin E and 50 times greater than vitamin C. Extensive research suggests that grape seed extract is beneficial in many areas of health because of its antioxidant effect to bond with collagen, promoting youthful skin, cell health, elasticity, and flexibility. Other studies have shown that proanthocyanidins help to protect the body from sun damage, to improve vision, to improve flexibility in joints, arteries, and body tissues such as the heart, and to improve blood circulation by strengthening capillaries, arteries, and veins.

The most abundant phenolic compounds isolated from grape seed are catechins, epicatechin, procyanidin, and some dimers and trimers.



## The effect of grape seed extract on cardiovascular risk markers: a meta-analysis of randomized controlled trials.

**Publication:** Journal of the American Dietetic Association 2011 Aug 111(8) 1173-81

**Authors:** Feringa HH, Laskey DA, Dickson JE, Coleman CI.

### Abstract

Recent animal studies have suggested that grape seed extract has beneficial effects on the cardiovascular system. Randomized trials in human beings have yielded conflicting results.

The objective of this systematic review was to assess the effect of grape seed extract on changes in blood pressure, heart rate, lipid levels, and C-reactive protein (CRP) levels. We searched MEDLINE (January 1, 1950, through October 31, 2010), Agricola (January 1, 1970, through October 31, 2010), Scopus (January 1, 1996, through October 31, 2010), and the Cochrane Central Register of Controlled Trials (through October 31, 2010) for randomized controlled trials in human beings of grape seed extract reporting efficacy data on at least one of the following end points: systolic or diastolic blood pressure, heart rate, total cholesterol, low-density or high-density lipoprotein cholesterol, triglycerides, or CRP.

A manual search of references from primary and review articles was performed to identify additional relevant trials. For all endpoints except CRP, the mean change in each parameter from baseline was treated as a continuous variable and the effect size was calculated as the weighted mean difference between the means in the grape seed extract and control groups. Data on CRP were pooled as a standardized mean difference. Nine randomized, controlled trials (N=390) met the inclusion criteria, and a meta-analysis was conducted. Upon meta-analysis, grape seed extract significantly lowered systolic blood pressure (weighted mean difference -1.54 mm Hg (95% confidence interval -2.85 to -0.22,  $P=0.02$ )), and heart rate (weighted mean difference -1.42 bpm (95% confidence interval -2.50 to -0.34,  $P=0.01$ )). No significant effect on diastolic blood pressure, lipid levels, or CRP was found. No statistical heterogeneity was observed for any analysis ( $I^2<39\%$  for all). Egger's weighted regression statistic suggested low likelihood of publication bias in all analysis ( $P>0.05$  for all), except for the effect on diastolic blood pressure ( $P=0.046$ ).

Based on the currently available literature, grape seed extract appears to significantly lower systolic blood pressure and heart rate, with no effect on lipid or CRP levels. Larger randomized, double-blinded trials evaluating different dosages of grape seed extract and for longer follow-up durations are needed.



## Grape Seed Extract – Published Research Papers

### Grape pomace extract exerts antioxidant effects through an increase in GCS levels and GST activity in muscle and endothelial cells

**Publication:** International Journal of Molecular Medicine 36: 433-441, 2015

**Authors:** Goutzourelas N, Stagos D, Housmekeridou A, Karapoulou C, Kerasioti E, Aliogiannis N, Skaltsounis A, Spandido D, Tsatsakis A, Kouretas D.

### The Protective Effect of Grape-Seed Proanthocyanidin Extract on Oxidative Damage Induced by Zearalenone in Kunming Mice Liver

**Publication:** International Journal of Molecular Sciences 2016, 17, 808;

**Authors:** Miao Long, Shu-Hua Yang, Jian-Xin Han, Peng Li, Yi Zhang, Shuang Dong, Xinliang Chen, Jiayi Guo, Jun Wang, and Jian-Bin He.

### Grape seed proanthocyanidin extract protects the retina against early diabetic injury by activating the Nrf2 pathway

**Publication:** Experimental and Therapeutic Medicine 11: 1253-1258, 2016

**Authors:** Yan Sun, Caimei Xiu, Wei Liu, Yuan Tao, Jianrong Wang, Yi Qu.

### Co-administration of Grape Seed Extract and Exercise Training Improves Endothelial Dysfunction of Coronary Vascular Bed of STZ-Induced Diabetic Rats

**Publication:** Iranian Red Crescent Medical Journal. 2013 October; 15(10): e7624.

**Authors:** Mohammad Badavi, Hassan Ali Abedi, Ali Reza Sarkaki, Mahin Dianat

### Neuroprotection of Grape Seed Extract and Pyridoxine against Triton-Induced Neurotoxicity

**Publication:** Oxidative Medicine and Cellular Longevity Volume 2016, Article ID 8679506, 8 pages

**Authors:** Heba M. Abdou and Mayssaa M. Wahby

### Grape Seed Proanthocyanidin Extract Ameliorates Diabetic Bladder Dysfunction via the Activation of the Nrf2 Pathway

**Publication:** PLOS ONE | DOI:10.1371/journal.pone.0126457 May 14, 2015

**Authors:** Shouzhen Chen, Yaofeng Zhu, Zhifeng Liu, Zhaoyun Gao, Baoying Li, Dongqing Zhang, Zhaocun Zhang, Xuwen Jiang, Zhengfang Liu, Lingquan Meng, Yue Yang, Benkang Shi.





Article

# The Protective Effect of Grape-Seed Proanthocyanidin Extract on Oxidative Damage Induced by Zearalenone in Kunming Mice Liver

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**Abstract:** Although grape-seed proanthocyanidin extract (GSPE) demonstrates strong anti-oxidant activity, little research has been done to clearly reveal the protective effects on the hepatotoxicity caused by zearalenone (ZEN). This study is to explore the protective effect of GSPE on ZEN-induced oxidative damage of liver in Kunming mice and the possible protective molecular mechanism of GSPE. The results indicated that GSPE could greatly reduce the ZEN-induced increase of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities. GSPE also significantly decreased the content of MDA but enhanced the activities of antioxidant enzymes SOD and GSH-Px. The analysis indicated that ZEN decreased both mRNA expression levels and protein expression levels of nuclear erythroid2-related factor2 (Nrf2). Nrf2 is considered to be an essential antioxidative transcription factor, as downstream GSH-Px,  $\gamma$ -glutamyl cysteine synthetase ( $\gamma$ -GCS), hemeoxygenase-1 (HO-1), and quinone oxidoreductase 1 (NQO1) decreased simultaneously, whereas the pre-administration of GSPE groups was shown to elevate these expressions. The results indicated that GSPE exerted a protective effect on ZEN-induced hepatic injury and the mechanism might be related to the activation of the Nrf2/ARE signaling pathway.

**Keywords:** grape seed proanthocyanidin extract; zearalenone; oxidative damage; Nrf2/ARE pathway; liver; mice

## 1. Introduction

The worldwide contamination of grains designated to human and animal feeding with *Fusarium* mycotoxins is a significant problem. Among *Fusarium* mycotoxins, zearalenone (ZEN) is the most prevalent mycotoxins found in cereals [1]. ZEN can cause several reproductive disorders in animals by disrupting the endocrine function via binding the estrogen receptors [2]. In addition, many studies have shown that ZEN has hepatotoxic for the liver, which is a primary target [3,4]. ZEN can induce liver lesions and alter part of enzymatic parameters of hepatic function [5–7]. Since ZEN can induce lipid peroxidation [8,9], some researchers believe that the oxidative stress may be a key mechanism of toxicity of ZEN *in vivo* and *in vitro* [10–12].

In recent years, natural products have been used to improve the health and to prevent diseases, especially those caused by oxidative stress. In this regard, many researchers have reported that the addition of nutrients especially some antioxidants such as Lycopene [13], allium sativum [14], and crocin [15] is one approach that reduces ZEN toxicity.

The natural antioxidant proanthocyanidin contains a variety of phenolic compounds and is often present in vegetables, seeds, tea, fruits, wine, and nuts [16]. Grape-seed proanthocyanidin extract (GSPE), an extract obtained from red grape seeds, which are rich in plant flavonoids, proanthocyanidin oligomers and polymerized oligomers, has been widely marketed in China, [17,18]. GSPE exhibits a wide range of biologic properties for resisting oxidative stress. They can clean off the free radicals and reduce the membrane lipid peroxidation [19,20]. Some research has revealed that GSPE can prevent drug-induced liver and kidney injury and can induce anti-tumor, anti-radiation activity [21,22]. Studies have shown that GSPE can use potent antioxidant features to improve the abnormalities and antioxidant system status of diabetic rats arising from streptozotocin [23], can protect against tissue damage induced by ischemia and/or ischemia/reperfusion in rat ovaries [24], and plays an important role as a potential candidate for the therapy of the metabolic syndrome, such as hypertension and hyperlipidemia [25]. Some research has proven that GSPE is able to conduct PKC and NF- $\kappa$ B inhibition to prevent endothelia dysfunction induced by high glucose [26] and utilize the activation of the Nrf2 pathway to improve diabetic bladder dysfunction [27]. However, there are few reports about GSPE's ability to alleviate the toxicity of mycotoxins. Although our previous study showed that GSPE could protect Aflatoxin B1-induced subchronic immune injury of mice [28], the protective effects of GSPE on ZEN *in vivo* induced hepatotoxicity, and its role of mechanism have rarely been studied.

It is known that liver injury induced by ZEN is mainly led by oxidative stress. As current existing research results indicate, Nrf2 is a transcription factor of adjusting redox status and the cellular antioxidative responses via increasing the antioxidative genes expression by using antioxidant response element (ARE) [29,30]. To date, it has been shown that ZEN can induce oxidative damage involving the Nrf2/ARE pathway, as has been demonstrated in cell culture systems [31], and that ZEN mediated toxicity found in the jejunum is generated due to one of the mechanism-signal pathways mediated by Nrf2 [32].

It is assumed that ZEN-induced toxicity in liver can be protected by GSPE through counteracting oxidative injury by regulating the Nrf2 pathway.

Therefore, this research explored the role that ZEN plays in the oxidative status of liver and investigated the Nrf2 signal pathway in mice. Moreover, the authors concluded that the activation of the Nrf2 signal pathway possibly leads to the protective effect of GSPE. The results can provide the protective effect of mechanisms of GSPE on ZEN-induced liver injury mice.

## 2. Results

### 2.1. Effects of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) on the Serum Activities

Serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) function as the biochemical signs for revealing hepatic damage. In comparison with the other groups ( $p < 0.05$ ), both ALT and AST in the ZEN group had shown a greatly increased serum activity (Table 1). However, by carrying out pre-administration of GSPE for five days, the increase in the serum activity of both ALT and AST can be significantly prevented due to the effects of ZEN. In the control group, administration of GSPE (100 mg/kg) had little effect on the alteration of the level of hepatic markers ( $p > 0.05$ ).

**Table 1.** Effect of grape-seed proanthocyanidin extract (GSPE) on zearalenone (ZEN)-induced hepatic damage by testing the serum activities of ALT and AST in mice.

Group	ALT	AST
Control	210.5 ± 12.28 <sup>a</sup>	89.27 ± 7.20 <sup>a</sup>
GSPE (100 mg/kg)	204.71 ± 10.12 <sup>a</sup>	95.38 ± 16.68 <sup>a</sup>
ZEN (40 mg/kg)	288.65 ± 34.57 <sup>b</sup>	114.19 ± 15.64 <sup>b</sup>
GSPE (100 mg/kg) + ZEN (40 mg/kg)	235.60 ± 21.25 <sup>c</sup>	97.56 ± 12.13 <sup>a</sup>

<sup>a,b,c</sup> Means within the column with different letters are significantly different,  $p < 0.05$ . AST: aspartate aminotransferase; ALT: alanine aminotransferase.

## 2.2. The Effect on Antioxidant of Liver

The hepatic level of liver malondialdehyde (MDA) is used as an index indicating the lipid peroxidation in liver damage. Compared with the control group ( $p < 0.05$ ), the administration of ZEN gave rise to an apparent rise of the MDA content in liver, while the pre-administration of GSPE greatly reduced the MDA levels (Table 2). In addition, compared with the control group ( $p < 0.05$ ), the hepatic activities of both superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) declined after ZEN treatment, and the pre-administration of GSPE remarkably reversed the reduced activities of GSH-Px and SOD ( $p < 0.05$ ) (Table 2). The findings obtained confirmed the effects of GSPE in significant improvement of the antioxidant activities in the liver, and the decrease in the serious ZEN induced oxidative damage.

**Table 2.** Effect of GSPE on liver antioxidant parameters in mice induced by ZEN.

Group	MDA (nmol/mgprot)	SOD (U/mgprot)	GSH-Px (U/mgprot)
Control	7.03 ± 1.29 <sup>a</sup>	36.32 ± 3.32 <sup>a</sup>	213.65 ± 10.12 <sup>a</sup>
GSPE (100 mg/kg)	6.21 ± 0.57 <sup>a</sup>	40.26 ± 2.21 <sup>b</sup>	236.15 ± 22.22 <sup>b</sup>
ZEN (40 mg/kg)	9.81 ± 1.55 <sup>b</sup>	32.58 ± 3.11 <sup>c</sup>	193.64 ± 8.63 <sup>c</sup>
GSPE (100 mg/kg) + ZEN (40 mg/kg)	8.07 ± 0.89 <sup>a</sup>	33.99 ± 0.99 <sup>a</sup>	207.95 ± 8.59 <sup>a</sup>

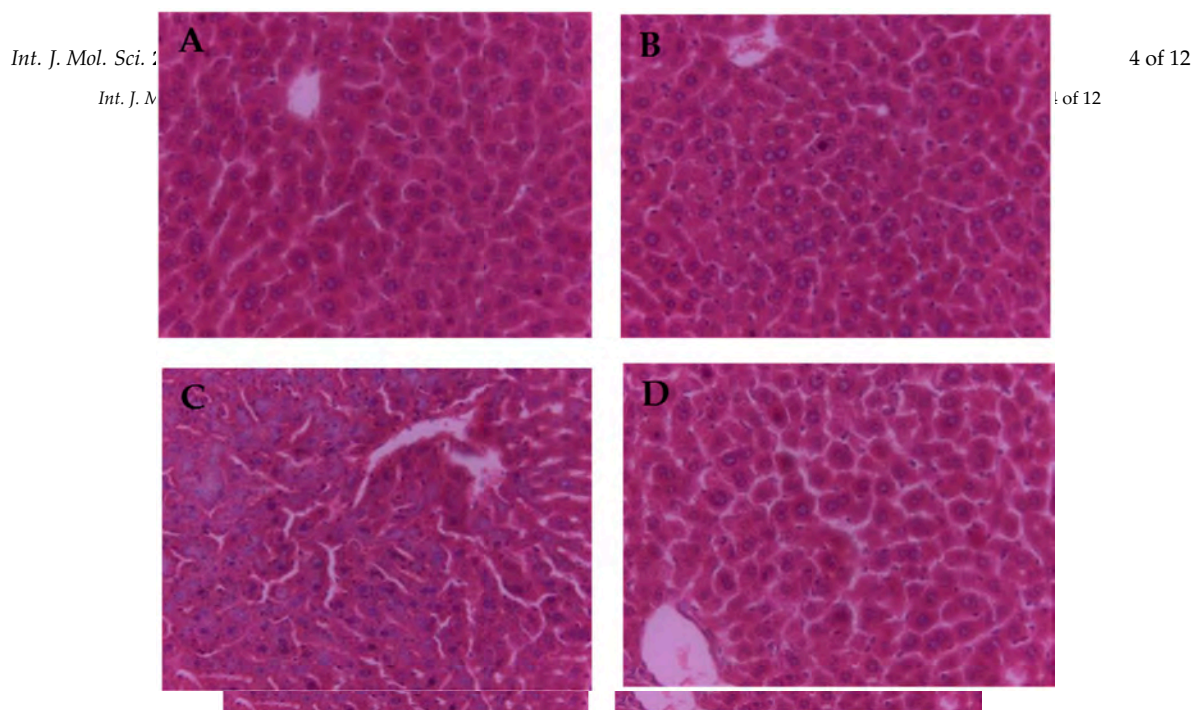
<sup>a,b,c</sup> Means within the column with different letters are significantly different,  $p < 0.05$ . MDA: malondialdehyde; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase.

## 2.3. Effect on Histopathological Variation in Liver

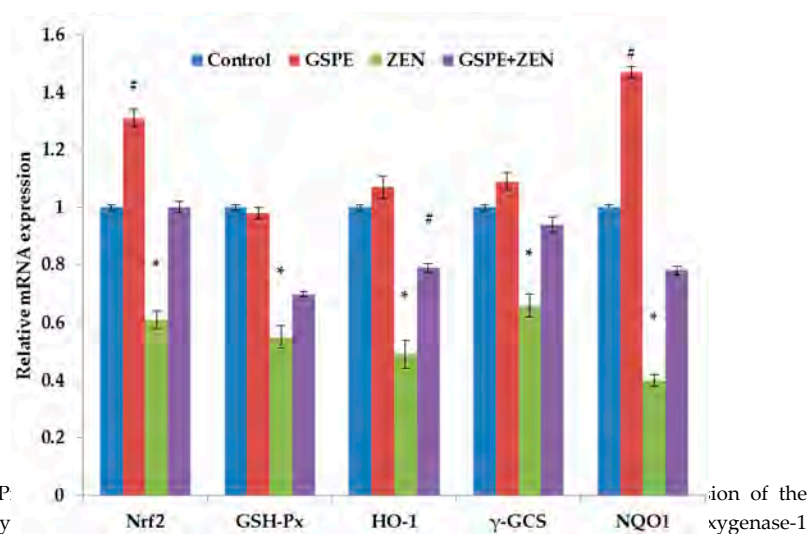
The results of liver sections with regard to the histopathological aspect were shown in Figure 1. The morphology of liver in control group and GSPE group exhibited normal hepatic cells with a clear cell nucleus and well-preserved cytoplasm (Figure 1A,B). In contrast, the ZEN group revealed the damage of liver tissues; the liver tissue displayed the disorder of the hepatic cell cords, the central venous border was not clear, the chromatin was lightly stained and dissolved, and the nuclear membrane disappeared (Figure 1C). However, the protection was observed in the pre-administration of GSPE; the liver sections of the mice from this group showed minor pathomorphological changes (Figure 1D).

## 2.4. Effect on the Gene Expression Associated with the Nrf2 Signaling Pathway

The role of GSPE that plays in genes associated with the Nrf2 signaling pathway is illustrated in Figure 1. In comparison with the control group ( $p < 0.05$ ), expression levels of Nrf2, GSH-Px, HO-1,  $\gamma$ -GCS, and NQO1 in the ZEN group were remarkably lower; in contrast, the usage of GSPE greatly rose the expression levels of Nrf2, GSH-Px, HO-1,  $\gamma$ -GCS, and NQO1. Pre-administration of GSPE groups showed a greater increase in the genes expression correlated to the Nrf2 signaling pathway than that of the ZEN group ( $p < 0.05$ ; Figure 2).



**Figure 1.** Pretreatment effects of GSPE on ZEN-induced liver histopathological changes in mice. Figure 1. Pretreatment effects of GSPE on ZEN-induced liver histopathological changes in mice. (original magnification of  $\times 400$ ). (A) control group; (B) GSPE group; (C) group administrated with ZEN at a dose of 40 mg/kg; (D) pre-administrated with GSPE at a dose of 40 mg/kg treatment group.



**Figure 2.** Pretreatment effects of GSPE on ZEN-induced the relative mRNA expression of the nuclear erythroid 2-related factor 2 (Nrf2), glutathione peroxidase (GSH-Px), hemoxylin (HO-1), γ-glutamyl cysteine synthetase (γ-GCS), and quinone oxidoreductase 1 (NQO1) in the liver.

**Figure 2.** Pretreatment effects of GSPE on ZEN-induced the relative mRNA expression of the nuclear erythroid 2-related factor 2 (Nrf2), glutathione peroxidase (GSH-Px), hemoxylin (HO-1), γ-glutamyl cysteine synthetase (γ-GCS), and quinone oxidoreductase 1 (NQO1) in the liver. Values are means  $\pm$  SEM of three mice in each group. \*  $p < 0.05$  vs. control group; #  $p < 0.05$  vs. ZEN-treated group.

by ZEN, we measured the expression of Nrf2 and the Nrf2-target proteins—GSH-Px, HO-1, NQO1, and γ-GCS in mice livers. Western blot study revealed that ZEN exerted an inhibitory effect on Nrf2 protein expression and also its down-stream target proteins GSH-Px, HO-1, γ-GCS, and NQO1. As can be seen in Figure 3A, the treatment with ZEN could greatly down-regulated the protein levels of Nrf2. To analyze whether Nrf2 activation plays a role in GSPE protection against the toxicity caused by ZEN, we measured the expression of Nrf2 and the Nrf2-target proteins GSH-Px, HO-1, NQO1, and γ-GCS in mice livers. Western blot study revealed that ZEN exerted an inhibitory effect on Nrf2 protein expression and also its down-stream target proteins GSH-Px, HO-1, γ-GCS, and NQO1. As can be seen in Figure 3A, the treatment with ZEN could greatly down-regulated the protein levels of Nrf2 in comparison with the control group elevated by the use of GSPE. Compared with the control group, the expressions of protein GSH-Px, HO-1, γ-GCS, and NQO1 showed a significant reduction in



hepatic Nrf2 in comparison with the control group elevated by the use of GSPE. Compared with the control group, the expressions of protein GSH-Px, HO-1,  $\gamma$ -GCS, and NQO1 showed a significant reduction in the ZEN group, as seen from Figure 3B–E. Although pre-administration of GSPE did not significantly reverse the decreased expression (Figure 3B–E), these protein expressions were higher than that in ZEN group.

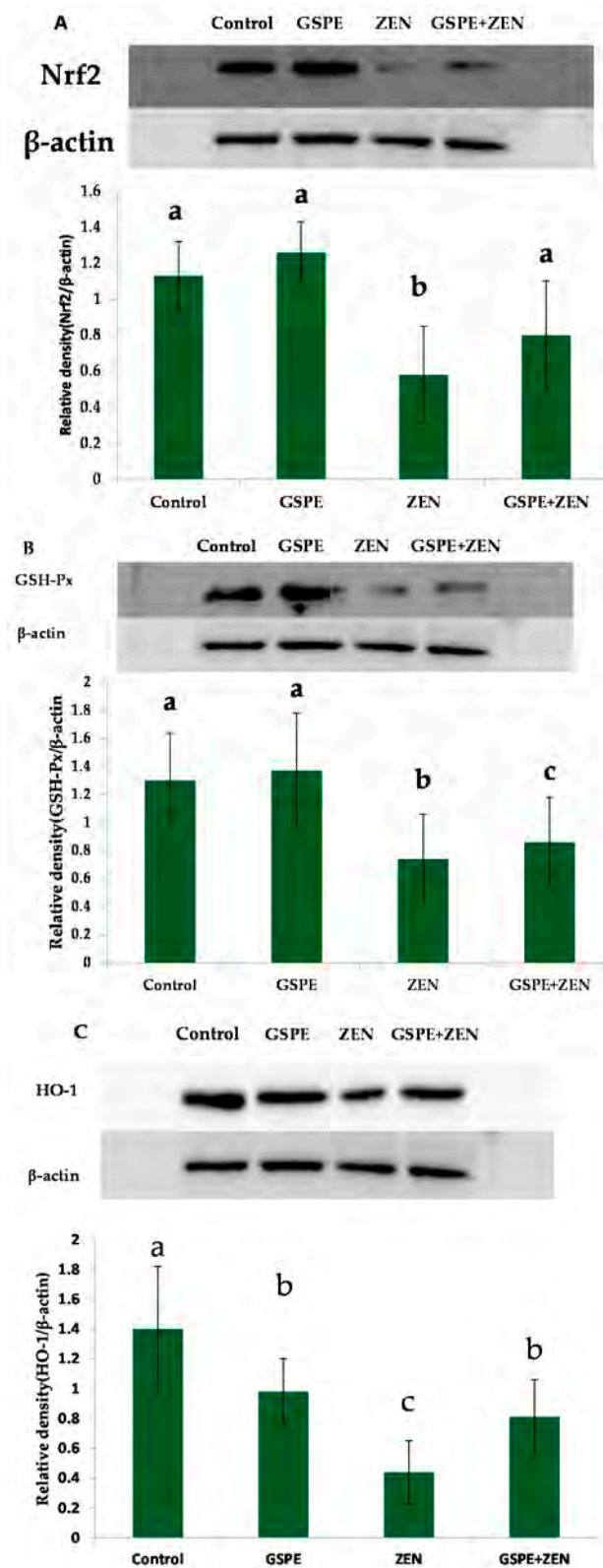
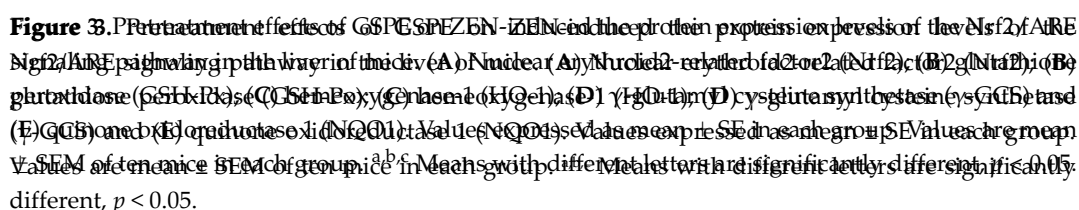


Figure 3. Cont.



### 3. Discussion

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In this work, the doses of ZEN (40 mg/kg-8% of LD<sub>50</sub>) were chosen based on preliminary experiments by both the dose [6] ZEN (40 mg/kg-8% of LD<sub>50</sub>) was chosen based on in the GSPE experiments by Boeris de la [33]. However, the dose gradient where GSPE exhibited in the current administration. This was because our previous studies had found that GSPE exhibited an excellent protective effect and was shown to be safe at 100 mg/kg of GSPE [28], which is presently being shown in this study.

On the other hand, administration of a dose of 100 mg/kg GSPE to mice also had better protective effects on liver damage.

One study indicates that ZEN shows hepato-nephrotoxicity and can affect the enzymatic and hematological parameters of mice. ZEN shows hepatotoxicity after oral administration [34]. Enzymatic and hematological parameters of ZEN within 48 h in the case of oral administration of 160 μg/kg body weight and administration of ZEN and AFB<sub>1</sub> as markers for measuring the liver health are observed in both cytoplasm and mitochondria of hepatocytes. Thus, the elevation of the two



in the serum after 48-h treatment with ZEN indicated that ZEN could change the organ pathology and induce the damage to livers. The findings were consistent with the report [34]. Pre-administration of GSPE can largely reduce the serum activities caused by ZEN in ALT and AST. The results revealed that GSPE plays a protective role in the resistance to the liver injury induced by ZEN. Furthermore, this is the first time it has been reported that GSPE can protective the liver injury of mice induced by ZEN. However, in our study, the liver of mice showed a slight histopathological variation for a short period. This may be related to the dose and counteracting time of ZEN. In general, histopathological results for the liver sections confirmed the protective effect of GSPE on the hepatic injury caused by ZEN (Figure 1D).

One study reported that ZEN induced oxidative stress mainly contributes to liver damage [10]. The parameters of oxidative stress, including GSH-Px, SOD, and MDA, are often used to measure the oxidative injury of organs especially the liver. MDA is often seen as a biomarker for oxidative stress and a key feature in liver injury, as it is an end of lipid peroxidation product and is also regarded as the cellular pathways contributing to oxidative [35]. Similar to the existing literature, the findings in our research indicated that the MDA levels in the liver were higher in the ZEN group than those in the control group [4,12]. Our results showed that pre-treated GSPE largely restrained the elevation of MDA in the mice livers in the ZEN-treated group. SOD can represent an important antioxidant defense in almost all cells that are exposed to oxygen, and GSH-Px, as a enzyme, can get rid of the excessive  $H_2O_2$  generated from the disputation of  $O^{2-}$  following the administration of MDA. In the present work, the analysis verified that SOD and GSH-Px activities showed significant reduction in the liver treated with ZEN. The results indicated that ZEN made the liver subject to the oxidative damage. The results were consistent with previous studies [4,7,10]. In the present study, GSPE largely promoted the activities of SOD and GSH-Px in the mice livers. Our results suggested that GSPE has a potent ability to ameliorate oxidative stress-induced liver damage through reducing lipid peroxide and enhancing the antioxidative enzymes activities in mice livers.

The Nrf2/ARE pathways have an anti-oxidative effect on alleviating toxicant-induced hepatotoxicity [36]. The activation in the Nrf2 signaling pathway was able to prevent the cells from oxidative stress damage. Some researchers have proposed that Nrf2 can activate the antioxidative stress system to excrete the toxic metabolites via regulating the expression of many intracellular antioxidant genes [37]. The genes of GSH-Px, HO-1,  $\gamma$ -GCS, and NQO1, belonging to the ARE, are the downstream target genes of Nrf2. These proteins have been known to be the cytoprotective effect resistant to oxidative stress [38,39].

The induction of these enzymes can be mainly realized by the bind of Nrf2 and the ARE present in the upstream regulatory region for many phase 2 genes. Our results showed that GSPE improved the decreased downstream target genes (phase 2 genes) mRNA levels of Nrf2 including GSH-Px, HO-1,  $\gamma$ -GCS, and NQO1 caused by ZEN (Figure 2). It indicated that GSPE could activate the Nrf2 expression because these phase 2 genes are shown to be Nrf2-dependent. Based on the upstream regulatory region, mRNA levels of phase 2 genes are regulated, and the ARE is controlled by Nrf2.

In the literature, it has been proposed that the declined nuclear Nrf2 may be led by the decrease in the nuclear export and Nrf2 mRNA or in Nrf2 proteins [38]. Our results showed that GSPE improved both the levels for Nrf2 mRNA and the Nrf2 protein expression decreased by ZEN. These results indicated that elevating the accumulation of proteins of Nrf2 in the nucleus might be through increasing the expression levels of Nrf2 by GSPE. Kwak *et al.* (2002) [40] reported that Nrf2 regulates its expressions by itself using an ARE-like elements present in the proximal region of its promoters. This makes Nrf2 have persistent nuclear accumulation and extend the induction for the phase 2 genes regarding toxicity. This viewpoint can explain why the levels of mRNA and the protein of Nrf2 were both increased in our results. However, through which pathway GSPE activated the Nrf2 needs to be researched. Meanwhile, in a future study, we should also study whether GSPE could inhibit Nrf2 ubiquitination and degradation, which might explain the regulative role of GSPE that results in the Nrf2 proteins accumulated in these cells.

It is known that the toxicant-induced liver injuries of animals can be prevented by activation of the Nrf2/ARE signaling pathways. However, the possibility and mechanism of ZEN-induced hepatotoxicity led by Nrf2/ARE signaling pathways is unknown. The results showed that ZEN caused oxidative damage of the liver as indicated by ALT, GSH-Px, MDA levels, AST, and SOD. Moreover, this research indicated that ZEN plays its toxic roles through inhabiting the mRNA expression and the protein levels of Nrf2, GSH-Px, HO-1,  $\gamma$ -GCS, and NQO1. However, Liu *et al.* (2014) reported that the Nrf2 expression both in mRNA and protein levels were increased in intestinal tissue of rats which were induced by ZEN [32]. The differences of the two results might be that the doses of ZEN and the challenge time were different. Although these results were different, they proved that the oxidative toxicity of ZEN was related to the Nrf2/ARE. Moreover, the results of the current study showed that GSPE was an Nrf2 activator and the activation had an essential effect on the hepatoprotection mediated by GSPE against the liver oxidative damage caused by ZEN. Hence, the findings revealed that GSPE has a hepato-protective effect on the toxicity induced by ZEN, which may have resulted from the NRF2/ARE pathways.

#### 4. Experimental Section

##### 4.1. Animals

We purchased the male Kunming mice ( $45 \pm 2$  g and 8 weeks-old) from the Experimental Animal Center of China Medical University, Shenyang, China. At firstly, the mice were bred in a room which is restricted-access, for 12-h light/dark cycles with a humidity of 40%–60% at a temperature ranging from 22 to 24 °C. By providing water and diet on the condition of minimum *ad libitum* and all stress factors, the acclimatization was conducted on the mice for 1 week prior to the experiments. These experiments were performed subject to the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the principles of good laboratory animal care. Moreover, the experiments were permitted by the ethics committee for laboratory animal care for the use of Shenyang Agricultural University, Shenyang, China.

##### 4.2. Chemicals

In this work, based on the ZEN obtained from Sigma (St. Louis, MO, USA), we prepared the stock solution of 200 mg/mL ZEN in diethyl sulfoxide, and the solution was stored at  $-20$  °C. By dispensing the stock solution into the sterilized peanut oil, we obtained the working solution. GSPE with a purity equal or greater than 95% was purchased from Nanjing Zelang Medical Technology Co., Ltd., Nanjing, China. The kits used for measuring GSH-Px, SOD, and MDA activities were obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China); SYBR green RT-PCR kit from Takara (Otsu, Japan) and DAPI from Sigma Aldrich (St. Louis, MO, USA) were also employed. The primers for Nrf2, GSH-Px, HO-1,  $\gamma$ -GCS, NQO1, and  $\beta$ -actin were synthesized and purified by Sangon Biotech (Shanghai, China); moreover, the preservation solution of RNA samples and the kits for total animal RNA extraction were obtained from Sangon Biotech (Shanghai) Co., Ltd, Shanghai, China. The Kits for Revert Aid First Strand cDNA Synthesis were purchased from MBI Fermentas (Burlington, ON, Canada); the mice anti-Nrf2,  $\gamma$ -GCS, GSH-Px antibodies were acquired from Santa cruz biotechnology (Santa, Dallas, TX, USA). The catatory number of the anti-Nrf2 antibody was sc-722, the anti- $\gamma$ -GCS antibody was sc-22755, and the anti-GSH-Px antibody was sc-30147. Anti- HO-1, NQO1, and  $\beta$ -actin antibodies were from Sangon Biotech (Shanghai, China); these antibodies were all polyclonal antibodies. We also purchased the antibodies conjugated with the secondary goat anti-mouse and goat anti-rabbit horseradish peroxidase (HRP) in Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

##### 4.3. Experimental Design and Treatment

The control group ( $n = 10$ ) was orally administrated with physiological saline every day for 7 days.

The ZEN group ( $n = 10$ ) was orally administrated with physiological saline every day for 5 days and, with a 40 mg/kg dose of ZEN (40 mg/kg-8% of LD<sub>50</sub>), was based on preliminary experiments by Boeira *et al.* (2014, 2015) [13,33] on the 6th day and 7th day.

The GSPE ( $n = 10$ ) was orally administrated with a 100 mg/kg dose of intragastric GSPE diluted with physiological saline daily, for 5 days, and then orally administrated with physiological saline for 2 days.

The GSPE+ZEN group ( $n = 10$ ) was orally administrated with a 40-mg/kg dose of ZEN on the 6th day and 7th day and with a 100-mg/kg dose of intragastric GSPE diluted with physiological saline daily for 5 days.

The mice were found to die subject to the ether anesthesia after the administration for 48 h. Afterwards, we collected the blood samples and soon separated the serum. Lastly, the liver tissues were stored at  $-80\text{ }^{\circ}\text{C}$  for further use in the experiments after being isolated from each mouse.

#### 4.4. Parameters

In the experiment, we evaluated the oxidant levels of the mice liver based on the content of the MDA and examined the enzyme levels of antioxidants in the liver by analyzing the activities of GSH-Px and SOD. SOD, MDA, and GSH-Px assay kits were used to carry out the analysis. By investigating the AST and ALT levels, we assessed the liver function. The relevant diagnostic kits were adopted in the analysis of these indexes. The details of all determination procedures followed the manufacturer's instructions for the commercial kits.

#### 4.5. Histopathology Examination of Liver Tissue

Before conducting routine processing and paraffin embedding, the liver section was set in 10% formalin. We then used hematoxylin and eosin to stain the liver sections and investigated them by using a photomicroscope.

#### 4.6. Gene Expression

TRIzol reagent was used to extract the total RNA of the livers. Then, the purity of the total RNA was measured via the quotient for OD at 260/280 nm. Moreover, the TaKaRa PrimeScript RT reagent kit was adopted to perform reverse transcription. The mRNA contents of  $\beta$ -actin, HO-1, GSH-Px, NQO1, Nrf2, and  $\gamma$ -GCS in the mice livers had been measured using a quantitative real-time PCR. To normalize the data of the gene expression,  $\beta$ -actin was employed as a housekeeping gene. Based on the PubMed database, we acquired the primer information regarding all the genes. The application software primer 5 was used to design the primers, and the Oligo 7 was used to test the specificity of the primers (Table 3). An ABI 7500 real-time PCR system and the SYBR Green PCR Kit were used to conduct real-time PCR. Each sample had been measured in triplicate. Afterwards, a method of gene expression (*i.e.*,  $2^{-\Delta\Delta C_t}$ ) was utilized to analyze the data of real-time PCR. The results revealed that there was a fold change shown in the expression of genes which were normalized to the endogenous reference genes ( $\beta$ -actin) and function as a calibrator. The system of the reaction mixtures consisted of: 2  $\mu\text{L}$  product of cDNA, 0.4  $\mu\text{L}$  reverse primers, 0.4  $\mu\text{L}$  forward, 10  $\mu\text{L}$  Taq MasterMix solution, 6.8  $\mu\text{L}$  of RNase-free water, and 0.4  $\mu\text{L}$  Rox. The conditions of conducting the PCR reaction included: at the initial stage, denaturing at  $95\text{ }^{\circ}\text{C}$  for 5 min, and then denaturing at  $95\text{ }^{\circ}\text{C}$  for 10 s, annealing at  $95\text{ }^{\circ}\text{C}$  for 5 s, and extension at  $60\text{ }^{\circ}\text{C}$  for 34 s. The amount of the template was measured based on the standard curve of quantitative analysis.

#### 4.7. Western Blot Assay

We harvested all protein extracts of the liver tissues and carried out homogenization in RIPA lysis buffer. The nuclear/cytoplasmic proteins in the liver tissues were obtained by the protein extraction kit after centrifuging the  $12,000\times g$  lysates at  $4\text{ }^{\circ}\text{C}$  for 10 min. The extraction procedure was performed strictly based on the instructions of manufacturers. The BCA protein assay kit was used to

determine the protein concentrations. The proteins of the liver tissue were separated by SDS-PAGE. After this procedure, the proteins were transferred to PVDF membranes. The blocking solution contained with 5% skimmed milk was used to block the membranes for 1 h at room temperature. Afterwards, we incubated the membranes overnight at 4 °C using primary antibodies for Nrf2, GSH-Px, HO-1,  $\gamma$ -GCS, NQO1, and  $\beta$ -actin in blocking solution. Then, the membranes were incubated with secondary antibody blocking solution for 1 h at room temperature. The improved Western blot kits of chemiluminescence detection were used to visualize the proteins. The Image-Analysis system was used to quantify the corresponding expression of target proteins.

**Table 3.** Primers for real-time PCR analyses.

Gene	Accession No.	Primer Sequences (5'-3')	Product Size/bp
Nrf2	NM_010902.3	F: TCCTATGCGTGAATCCCAAT R: GCGGCTTGAATGTTTGTCTT	103 bp
GSH-Px	X03920.1	F: GAAGTGCGAAGTGAATGG R: TGTCGATGGTACGAAAGC	224 bp
HO-1	NM_010442.2	F: GGGCTGTGAACTCTGTCCAAT R: GGTGAGGGAAGTGTGTCAGG	162 bp
$\gamma$ -GCS	U85414.1	F: TGGATGATGCCAACGAGTC R: CCTAGTGAGCAGTACCACGAATA	185 bp
NQO1	NM_008706.5	F: TTCTGTGGCTTCCAGGTCTT R: TCCAGACGTTTCTTCCATCC	104 bp
$\beta$ -actin	BC138614.1	F: CTGTCCCTGTATGCCTCTG R: TTGATGTCACGCACGATT	221 bp

#### 4.8. Statistical Analysis

Results were presented as the mean  $\pm$  standard error ( $X \pm SE$ ). Firstly, the authors used one-way ANOVA to assess the significance of differences among mean values. Afterwards, multiple pair-wise comparisons were made using a Student-Newman-Keuls (SNK) *post-hoc* test or the least significant difference (LSD). In addition, SPSS 13.0 software was used to carry out all statistical tests. Mean values were proven to be significantly different at  $p < 0.05$ .

## 5. Conclusions

In summary, this is the first study to evaluate the antioxidant activity of GSPE against ZEN-induced liver toxicity in mice. Moreover, its hepatoprotective effect might be attributed to the Nrf2/ARE signaling pathways activated.

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**Author Contributions:** Jian-Bin He, Jun Wang, and Shu-Hua Yang conceived and designed the experiments; Jian-Xin Han, Xinliang Chen, and Shuang Dong performed the experiments; Yi Zhang and Jiayi Guo analyzed the data; Peng Li contributed materials. Miao Long wrote the paper.

**Conflicts of Interest:** The authors declare no conflict of interest.

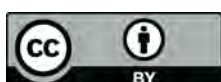
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# Grape pomace extract exerts antioxidant effects through an increase in GCS levels and GST activity in muscle and endothelial cells

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**Abstract.** In a previous study, we demonstrated that a grape pomace extract (GPE) exerted antioxidant activity in endothelial (EA.hy926) and muscle (C2C12) cells through an increase in glutathione (GSH) levels. In the present study, in order to elucidate the mechanisms responsible for the antioxidant activity of GPE, its effects on the expression of critical antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), heme oxygenase 1 (HO-1) and gamma-glutamylcysteine synthetase (GCS) were assessed in EA.hy926 and C2C12 cells. Moreover, the effects of GPE on CAT, SOD and glutathione S-transferase (GST) enzymatic activity were evaluated. For this purpose, the C2C12 and EA.hy926 cells were treated with GPE at low and non-cytotoxic concentrations (2.5 and 10  $\mu\text{g/ml}$  for the C2C12 cells; 0.068 and 0.250  $\mu\text{g/ml}$

for the EA.hy926 cells) for 3, 6, 12, 18 and 24 h. Following incubation, enzymatic expression and activity were assessed. The results revealed that treatment with GPE significantly increased GCS levels and GST activity in both the C2C12 and EA.hy926 cells. However, GPE significantly decreased CAT levels and activity, but only in the muscle cells, while it had no effect on CAT levels and activity in the endothelial cells. Moreover, treatment with GPE had no effect on HO-1 and SOD expression and activity in both cell lines. Therefore, the present results provide further evidence of the crucial role of GSH systems in the antioxidant effects exerted by GPE. Thus, GPE may prove to be effective for use as a food supplement for the treatment of oxidative stress-induced pathological conditions of the cardiovascular and skeletal muscle systems, particularly those associated with low GSH levels.

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**Abbreviations:** GPE, grape pomace extract; GSH, glutathione; CAT, catalase; SOD, superoxide dismutase; HO-1, heme oxygenase 1; GCS, gamma-glutamylcysteine synthetase; GST, glutathione S-transferase; ROS, reactive oxygen species; GAPDH, glyceraldehyde 3-phosphate dehydrogenase;  $\text{H}_2\text{O}_2$ , hydrogen peroxide;  $\text{O}_2^{\bullet-}$ , superoxide radical anion;  $\text{OH}^{\bullet}$ , hydroxyl radical;  $\text{RO}_2^{\bullet}$ , peroxy radical; CDNB, 1-chloro-2,4-dinitrobenzene; NBT, nitroblue tetrazolium salt; DETAPAC, diethylenetriaminepentaacetic acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; RIPA buffer, radio-immunoprecipitation buffer; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid

**Key words:** grape pomace extract, oxidative stress, muscle cells, endothelial cells, glutathione, antioxidants

## Introduction

Free radicals are produced in living organisms during normal metabolism (e.g., the reactions of mitochondrial respiratory chain and cytochrome P450), inflammation, phagocytosis and other physiological processes (1,2). The most important category of free radicals is constituted by reactive oxygen species (ROS), such as superoxide radical anion ( $\text{O}_2^{\bullet-}$ ), hydroxyl radical ( $\text{OH}^{\bullet}$ ), peroxy radical ( $\text{RO}_2^{\bullet}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (2). An amount of ROS is necessary for a number of functions of an organism, including phagocytosis (3), intracellular signaling (2), cell proliferation, and apoptosis (4). However, the excessive production of ROS may lead to oxidative stress, a pathophysiological condition which has been implicated in the oxidative damage of macromolecules (lipids, protein and DNA) (2,5), immune dysfunction (6), muscle damage (7) and fatigue (8).

Oxidative stress occurs frequently in muscle tissue exposed to ROS production. For example, during intense exercise there is a high rate of  $\text{O}_2$  consumption in skeletal muscle that may cause incomplete  $\text{O}_2$  reduction and electron leakage from the electron

transfer chain, as well as the extra-mitochondrial production of ROS, leading to the generation of ROS and oxidative stress. These effects in turn result in muscle fatigue and cell damage and apoptosis (9,10).

Moreover, oxidative stress-induced damage of the vascular endothelium may lead to the development of various diseases (11). A redox imbalance in endothelial cells results in the surface expression of different endothelial cell adhesion molecules, suggesting that oxidative stress induces acute and chronic phases of leukocyte adhesion to the endothelium (12,13). It has also been shown that the interaction between ROS and nitric oxide (NO) sets off a vicious circle, which results in further endothelial activation and inflammation (11). Furthermore, ROS, such as  $H_2O_2$  can diffuse throughout endothelial cells and react with cysteine groups in proteins to modify their function (14). Thus, under conditions of oxidative stress, endothelial cells can lose integrity, progress to senescence and detach into the circulation (15).

However, every living organism has antioxidant mechanisms, including both enzymatic and non-enzymatic with which to counteract the overproduction of free radicals (2). Moreover, we have previously demonstrated that the supplementation of antioxidants through diet may be used to reduce the detrimental effects of oxidative stress on human health (16-18). Some of the most well known food sources of antioxidants are grapes and wine (19). Our research group has conducted several studies on the antioxidant properties of grapes which are attributed mainly to their polyphenolic content (20-24). In another previous study of ours, we demonstrated that a grape pomace extract (GPE) rich in polyphenols derived from pomace, a by-product of the wine-making process consisting of peels, seeds and stems, reduced oxidative stress in muscle and endothelial cells mainly through an increase in glutathione (GSH) levels (25). Thus, in the present study, the effects of GPE on enzymes which are crucial for GSH metabolism, such as gamma-glutamylcysteine synthetase (GCS) and glutathione S-transferase (GST) were investigated in endothelial and muscle cells. Moreover, the effects of GPE on other critical antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and heme oxygenase 1 (HO-1) were examined in endothelial and muscle cells. The investigation of the effects of GPE on antioxidant enzymes at the cellular level may help to elucidate the molecular mechanisms through which it exerts its antioxidant effects. The understanding of these mechanisms provides valuable knowledge which may aid in the preparation of plant extracts aimed to be used as food supplements.

## Materials and methods

**Extract preparation.** The grape extract examined was obtained from Batiki Tyrnavou variety (a red grape variety grown in Central Greece) of the *Vitis vinifera* species. The isolation of the extract was carried out as previously described (26). In brief, the raw material was dried in a shady, well-ventilated environment and extracted using ethanol (96%) at 50°C for 4 h. Following filtration, the solvent was evaporated under reduced pressure, and the residue (i.e., GPE) was kept at -20°C until further use.

The polyphenolic composition of the extract identified by a liquid chromatography/high resolution mass spectrometry (LC-HRMS) method in positive and negative mode has been reported previously (26). Thus, the extract was composed

of flavan-3-ols (catechin and epicatechin), anthocyanidins, (cyanidin, malvidin, delphinidin and petunidin), anthocyanins (myrtillin, kuromanin, oenin and peonidin-3-O-glucoside) and flavonols (quercetin), phenolic acids (gallic acid and caftaric acid). Moreover, the total polyphenolic content (TPC) of the extract was evaluated and found equal to 648 mg of gallic acid per g of extract (26).

**Cell culture conditions.** The C2C12 muscle cells were a gift from Professor Koutsilieris (National and Kapodistrian University of Athens, Athens, Greece) and the EA.hy926 cells were from Professor Koukoulis (University of Thessaly, Larissa, Greece). All the cells were cultured in normal Dulbecco's modified Eagle's medium (DMEM), containing 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin (all from Gibco, Paisley, UK) in plastic disposable tissue culture flasks at 37°C in 5% carbon dioxide.

**Treatment of the cells with GPE.** The C2C12 and EA.hy926 cells were seeded in culture flasks and incubated for 24 h. The medium was then removed and replaced with serum-free medium containing GPE at non-cytotoxic concentrations (2.5 and 10 µg/ml for the C2C12 cells and 0.068 and 0.025 µg/ml for the EA.Hy926 cells) followed by incubation for 3, 6, 12, 18 and 24 h. In a previous study of ours, it was shown that these concentrations were non-cytotoxic to the C2C12 and EA.hy926 cells (25). Untreated cells were used as controls.

Following treatment, the cells were lysed in radio-immunoprecipitation buffer [RIPA buffer; 50 mM Tris-HCl, 150 mM NaCl, 0.25% SDS, 0.25% sodium deoxycholate and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] containing protease inhibitors (Complete™ mini protease inhibitors; Roche, Basel, Switzerland) for the preparation of the whole cell lysate. The cell lysates were then centrifuged at 16,250 x g for 20 min at 4°C. The supernatant was collected, and the amount of protein was then determined using Bradford reagent (Sigma-Aldrich Ltd., Munich, Germany). For the preparation of the cytosolic lysate, the cells were lysed in cytosolic lysis buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES)-potassium hydroxide (KOH) pH 7.9, 1.5 mM  $MgCl_2$ , 10 mM potassium chloride (KCl), 0.5 mM dithiothreitol (DTT) and 0.5% NP-40] to which protease inhibitors are added (Complete™ mini protease inhibitors; Roche). The samples were subsequently incubated on ice for 20 min followed by centrifugation at 16,250 x g at 4°C for 5 min. The supernatant was collected, and the amount of protein was then determined using Bradford reagent (Sigma-Aldrich Ltd.). The samples were stored at -80°C until further analysis.

**Western blot analysis for SOD, HO-1, CAT and GCS proteins.** In order to measure the expression levels of SOD, HO-1, CAT and GCS, western blot analysis was used. In particular, whole cell lysate containing 50 µg of protein was used for the determination of the SOD, HO-1 and CAT expression levels, while cytosolic lysate containing 30-50 µg of protein was used for the determination of GCS levels. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an 8% polyacrylamide gel. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes

were blocked overnight with 5% non-fat milk in 13 mM Tris/150 mM NaCl, pH 7.5, containing 0.2% Tween-20. They were then probed with polyclonal goat anti-human SOD-1 (1:1,600; Cat. no. sc-8634) or polyclonal rabbit anti-human GCS (1:1,600; Cat. no. sc-28965; both from Santa Cruz Biotechnology Inc., Dallas, TX, USA) or polyclonal goat anti-human HO-1 (1:1,400; Cat. no. AF3776) or polyclonal goat anti-human CAT (1:1,400; Cat. no. AF3398; both from R&D Systems, Minneapolis, MN, USA) primary antibodies for 1 h at room temperature. The membranes were then incubated with horseradish peroxidase-conjugated polyclonal goat anti-rabbit (1:5,000; Cat. no. 31462) or polyclonal donkey anti-goat (1:3,000; Cat. no. PA1-28659; both from Thermo Scientific, Rockford, IL, USA) secondary antibodies for 30 min at room temperature. All the membranes were re-probed with polyclonal rabbit anti-human (anti-mouse) glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1,000; Cat. no. PA1-988; Thermo Scientific) to normalize the data. The optical density of the protein bands was measured using Alpha View quantification software (Alpha Innotech, San Leandro, CA, USA). Each experiment was repeated 3 times.

**Determination of CAT activity.** The determination of CAT activity in the whole cell lysate was carried out based on the method described in the study by Aebi (27). Specifically, the reaction was carried out in a volume of 3 ml containing 150  $\mu$ l whole cell lysate and 2,845  $\mu$ l of 67 mM potassium phosphate buffer solution (pH 7.4). The measurement requires >30  $\mu$ g total amount of protein in the tested sample. The samples were incubated for 10 min at 37°C. Five microliters of 30% w/v H<sub>2</sub>O<sub>2</sub> solution were added to the samples and the change in absorbance was immediately read at 240 nm (UV) for 1.5 min. CAT activity in the cell lysates was normalized to the total cellular protein level in each sample. The results are expressed as units ( $\mu$ mol of H<sub>2</sub>O<sub>2</sub> decomposed ml/min) per mg of protein. CAT activity was examined in at least 3 different lysates (each lysate was measured in triplicate).

**Determination of GST activity.** The determination of GST activity in the cytosolic lysate was based on the method described in the study by Habig *et al* (28). More specifically, 920  $\mu$ l of phosphate buffer (100 mM, pH 7.4) were mixed with 50  $\mu$ l of GSH (1 mM) and 20  $\mu$ l of 1-chloro-2,4-dinitrobenzene (CDNB) and the samples were incubated for 5 min at 30°C. This was followed by the addition of 10  $\mu$ l of cytosolic lysate (the measurement requires >10  $\mu$ g of total amount of protein in the tested sample) and the change in absorbance was measured at 340 nm for 5 min. Upon conjugation of the thiol group of GSH to the CDNB substrate, there was an increase in the absorbance at 340 nm. The sample containing cytosolic lysate alone were used as the blank. GST activity in the cytosolic lysates was normalized to the total cellular protein level in each sample. The results are expressed as units ( $\mu$ mol of CDNB conjugate produced ml/min) per mg of protein. GST activity was examined in at least 3 different lysates (each lysate was measured in triplicate).

**Determination of SOD activity.** The determination of SOD activity in the whole cell lysate was based on the method of nitroblue tetrazolium salt (NBT) as described in the study by Oberley and Spitz (29). More specifically, this assay included a negative control which was prepared by mixing 800  $\mu$ l of SOD

buffer [1 mM diethylenetriaminepentaacetic acid (DETAPAC) in 0.05 M potassium phosphate buffer (pH 7.8), 1 unit CAT, 5.6 $\times$ 10<sup>-5</sup> M NBT and 10<sup>-4</sup> M xanthine] with 200  $\mu$ l of 0.05 M potassium phosphate buffer. Subsequently, ~60 mU of xanthine oxidase were added and the rate of increase in absorbance was measured at 560 nm for 3.5 min. In the test samples, 200  $\mu$ l of the total cell lysate (the measurement requires >10  $\mu$ g total amount of protein) were added to 800  $\mu$ l of SOD buffer followed by the addition of ~60 mU of xanthine oxidase and the rate of increase in absorbance was measured for 3.5 min at 560 nm. The calculation of SOD activity in the test samples is based on the percentage inhibition in the rate of increase in absorbance. The rate of increase in absorbance (A) per minute for the negative control and for the test samples was determined by the formula [1] and the percentage inhibition for each sample was calculated using the formula [2] as follows:

$$\Delta A_{560 \text{ nm}}/\text{min} = (A_{560 \text{ nm}} \text{ final} - A_{560 \text{ nm}} \text{ initial})/3.5 \text{ min} \quad [1]$$

$$\% \text{ Inhibition} = [(\Delta A_{560 \text{ nm}}/\text{min}_{\text{negative control}} - \Delta A_{560 \text{ nm}}/\text{min}_{\text{sample}}) / \Delta A_{560 \text{ nm}}/\text{min}_{\text{negative control}}] \times 100 \quad [2]$$

SOD activity in the whole cell lysates was normalized to the total cellular protein level in each sample. The results are expressed as units (one unit of SOD inhibits the rate of increase in absorbance at 550 nm by 50%) per mg of protein. SOD activity was examined in at least 3 different lysates (each lysate was measured in triplicate).

**Statistical analysis.** All results are expressed as the means  $\pm$  SD. For statistical analysis, one-way ANOVA was applied followed by Tukey's test for multiple pair-wise comparisons. Dose-response relationships were examined by Spearman's correlation analysis. Differences were considered statistically significant at P<0.05. All statistical analyses were performed using SPSS software (version 14.0; SPSS Inc., Chicago, IL, USA).

## Results

**Western blot analysis for SOD, HO-1, CAT and GCS protein expression.** In order to examine the effects of GPE on the expression levels of antioxidant enzymes (i.e., SOD, HO-1, CAT and GCS), the C2C12 muscle cells were treated with GPE at concentrations of 2.5 and 10  $\mu$ g/ml. The EA.hy926 endothelial cells were treated with GPE at concentrations of 0.068 and 0.250  $\mu$ g/ml.

Treatment with GPE at 2.5  $\mu$ g/ml significantly increased the GCS expression levels by 24.2 and 16.3% at 18 and 24 h, respectively compared to the control in the cytosolic lysate of C2C12 cells (Fig. 1A). However, treatment with GPE at 10  $\mu$ g/ml significantly increased the GCS levels in the C2C12 cells by 18.0, 20.3 and 26.1 at the 12, 18 and 24 h time points, respectively compared to the control (Fig. 1A). In the EA.hy926 endothelial cells, treatment with GPE at a concentration of 0.068  $\mu$ g/ml significantly increased the GCS levels by 14% at the 24 h time point, while treatment with GPE at the concentration of 0.250  $\mu$ g/ml led to a significant increase of 16.2% at 24 h time point compared to the control (Fig. 2A).

Moreover, the results revealed that treatment of the C2C12 cells with GPE at 2.5  $\mu$ g/ml significantly decreased the CAT

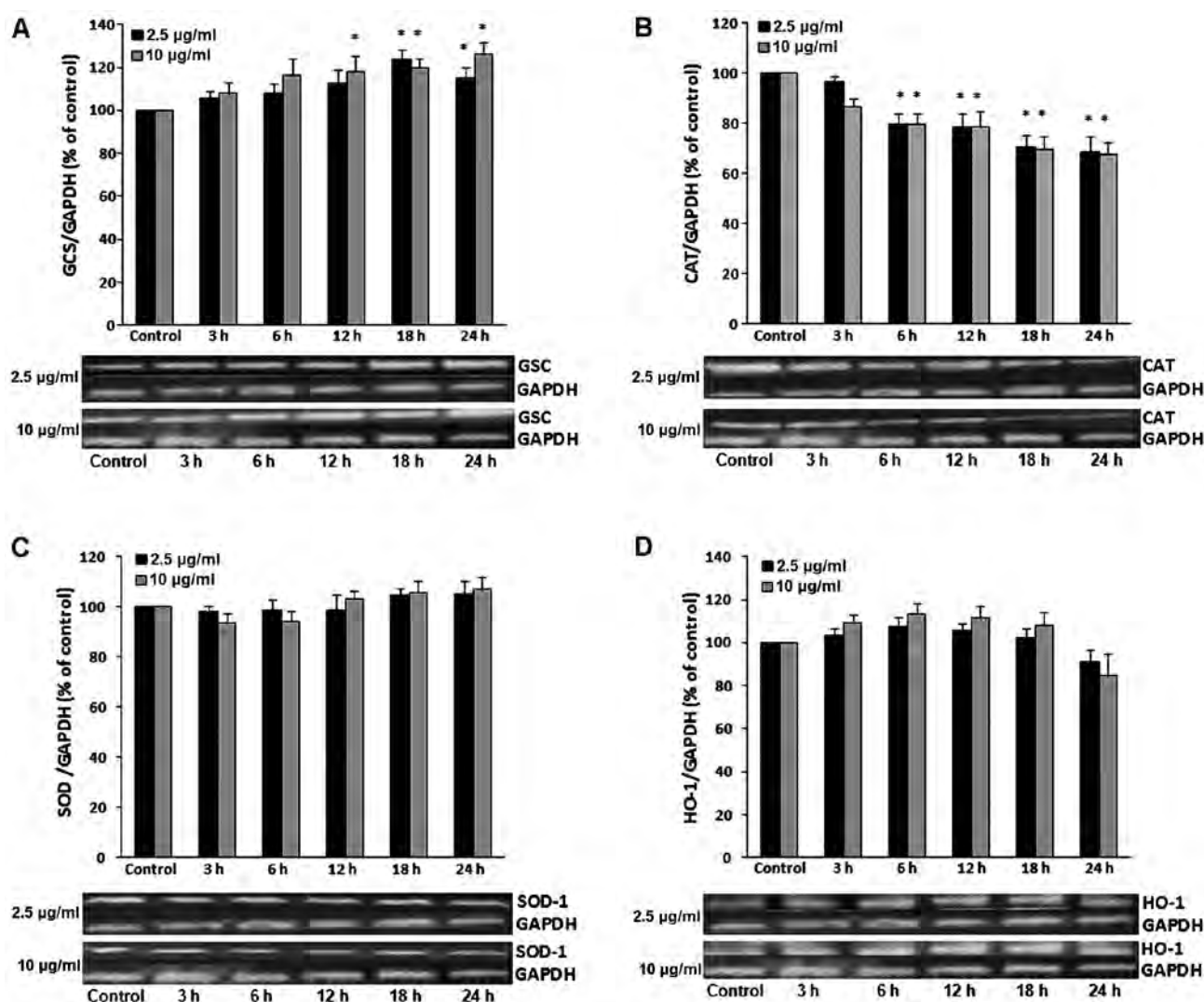


Figure 1. Representative western blots showing the effects of grape pomace extract (GPE) on the expression of (A) gamma-glutamylcysteine synthetase (GCS), (B) catalase (CAT), (C) superoxide dismutase (SOD) and (D) heme oxygenase 1 (HO-1) in C2C12 muscle cells. The results of densitometric quantification for all enzymes are also shown. The cells were incubated with GPE at 2.5 and 10 µg/ml for 3, 6, 12, 18 and 24 h. The expression of GAPDH was used as a loading control for normalization. \* $P < 0.05$ , statistically significant difference compared to the control (untreated cells). The results are presented as the mean  $\pm$  SEM.

expression levels by 20.4, 21.3, 29.2 and 31.5% at the 6, 12, 18 and 24 h time points, respectively, while treatment with GPE at 10 µg/ml decreased the CAT levels by 20.7, 22.5, 30.3 and 32.3% at the 6, 12, 18 and 24 h time points, respectively compared to control in the total lysate of C2C12 cells (Fig. 1B). However, in the EA.hy926 cells, GPE did not significantly affect CAT expression at any concentration used (Fig. 2B).

Furthermore, the results revealed that none of the GPE concentrations used significantly affected SOD expression at any time point in the total lysate of C2C12 cells compared to the control (Fig. 1C). Similar results were observed in the EA.hy926 endothelial cells (Fig. 2C).

As observed with SOD expression, treatment with GPE did not significantly alter the HO-1 expression levels at any tested concentration at any time point compared to the control in the total lysates of both the C2C12 (Fig. 1D) and EA.hy926 cells (Fig. 2D).

**Assessment of GST activity.** In the C2C12 muscle cells, treatment with GPE at the concentration of 2.5 µg/ml significantly

increased GST activity at the 18 and 24 h time points by 27.7 and 36.0%, respectively, while treatment with GPE at the concentration of 10 µg/ml increased GST activity by 37.7 and 59.0% at the 18 and 24 h time points, respectively compared to the control (Fig. 3A).

In the EA.hy926 endothelial cells, treatment with GPE at 0.068 µg/ml significantly increased GST activity at 24 h by 16.3%, while treatment with GPE at 0.250 µg/ml increased GST activity by 23.3 and 28.1% at 18 and 24 h, respectively compared to the control (Fig. 4A).

**Assessment of CAT activity.** In the C2C12 muscle cells, treatment with GPE at 2.5 µg/ml significantly decreased CAT activity by 12.7, 14.5 and 19.5% at the 12, 18 and 24 h time points, respectively compared to the control (Fig. 3B), while treatment with GPE at 10 µg/ml decreased CAT activity by 8.3, 21.0 and 26.1% at the 12, 18 and 24 h time points, respectively compared to the control (Fig. 3B).

In the EA.hy926 endothelial cells, treatment with GPE at concentrations of 0.068 and 0.250 µg/ml did not signifi-

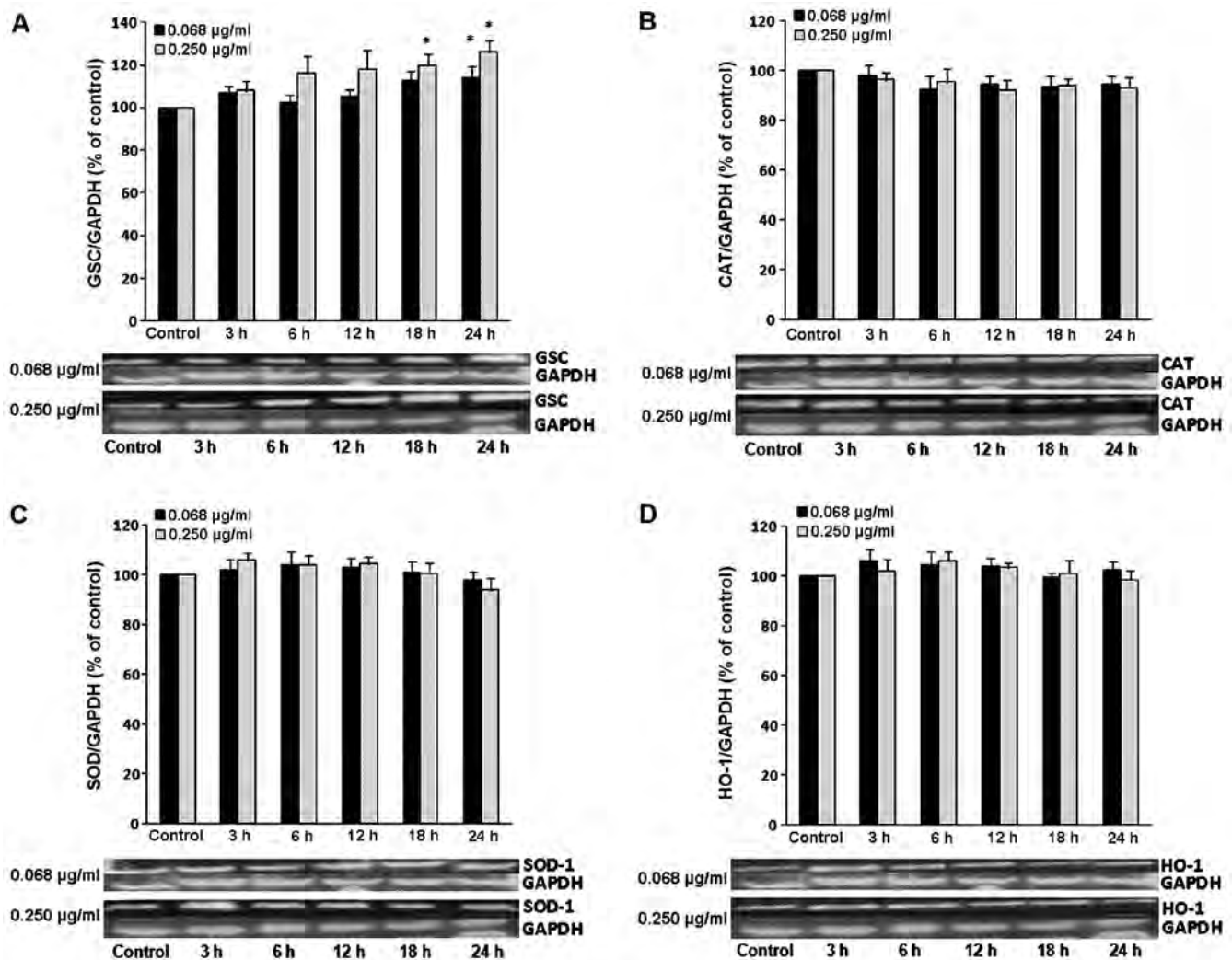


Figure 2. Representative western blots showing the effects of grape pomace extract (GPE) on the expression of (A) gamma-glutamylcysteine synthetase (GCS), (B) catalase (CAT), (C) superoxide dismutase (SOD) and (D) heme oxygenase 1 (HO-1) in EA.hy926 endothelial cells. The results of densitometric quantification for all enzymes are also shown. The cells were incubated with GPE at 0.068 and 0.250  $\mu\text{g/ml}$  for 3, 6, 12, 18 and 24 h. The expression of GAPDH was used as a loading control for normalization. \* $P < 0.05$ , statistically significant difference compared to the control (untreated cells). The results are presented as the means  $\pm$  SEM.

cantly affect CAT activity at any time point compared to the control (Fig. 4B).

**Assessment of SOD activity.** In the C2C12 muscle cells, treatment with GPE did not significantly affect SOD activity at any concentration used at any time points compared to the control (Fig. 3C). Similar results were observed in endothelial cells (Fig. 4C).

## Discussion

In a previous study, we demonstrated that GPE reduced oxidative stress in endothelial and muscle cells (25). In the present study, in order to investigate the mechanisms through which these antioxidant effects are exerted, the effects of GPE on antioxidant enzymes and molecules were assessed in the EA.hy926 endothelial and C2C12 muscle cell lines. It should be noted that the GPE concentrations used were non-cytotoxic and very low, as in several studies on antioxidant compounds, high concentrations are used which are either difficult to be achieved in a human organism or they exhibit toxicity.

GSH, a tripeptide composed of glycine, cysteine and glutamic acid, is one of the most critical antioxidant molecules in cells and is involved in the detoxification of a number of xenobiotics and ROS through either the formation of S-conjugates or by serving as an electron donor from its sulfhydryl group (-SH) (30). Conjugation with GSH can occur both enzymatically and non-enzymatically. In human organisms, there are 3 main GSH systems: the GSH/glutathione peroxidase (GPx) system, which buffers  $\text{H}_2\text{O}_2$  produced during cellular metabolism (30); the GSH/GST system, which conjugates GSH with xenobiotics for their detoxification (30); and the GSH/glutaredoxin (Grx) system which controls the cellular redox environment (31). In a previous study, we demonstrated that GPE increased GSH levels in EA.hy926 and C2C12 cells under either naive or oxidative stress conditions (25). This increase in GSH levels is explained by the GPE-induced increase in the expression of the GCS enzyme in both EA.hy926 and C2C12 cells. GCS is the first enzyme in the biosynthetic pathway of GSH, and consequently, it is critical for cell survival (32). It has also been reported that flavonoids increase intracellular GSH levels by the

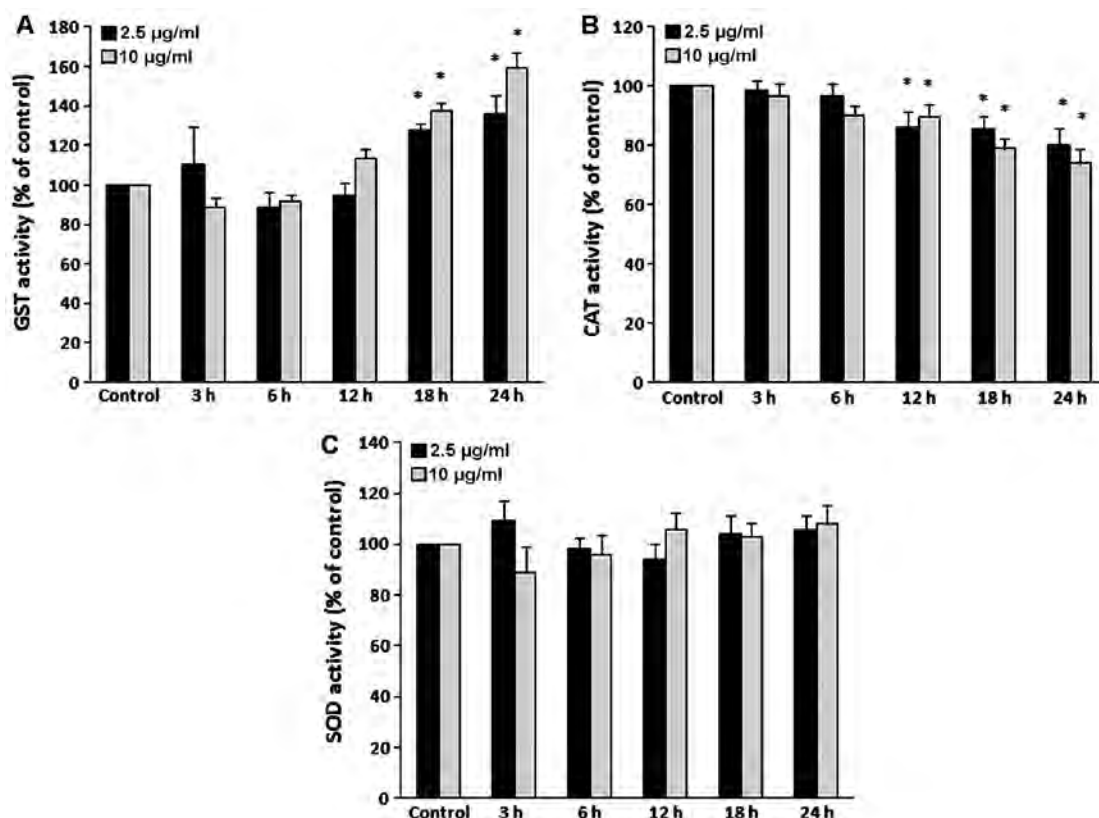


Figure 3. Effects of grape pomace extract (GPE) on the enzymatic activity of (A) glutathione S-transferase (GST), (B) catalase (CAT) and (C) superoxide dismutase (SOD) in C2C12 muscle cells. The cells were incubated with GPE at 2.5 and 10 µg/ml for 3, 6, 12, 18 and 24 h. The results are expressed as a percentage of the control values. \* $P < 0.05$ , statistically significant difference compared to the control (untreated cells). Results are presented as the means  $\pm$  SEM.

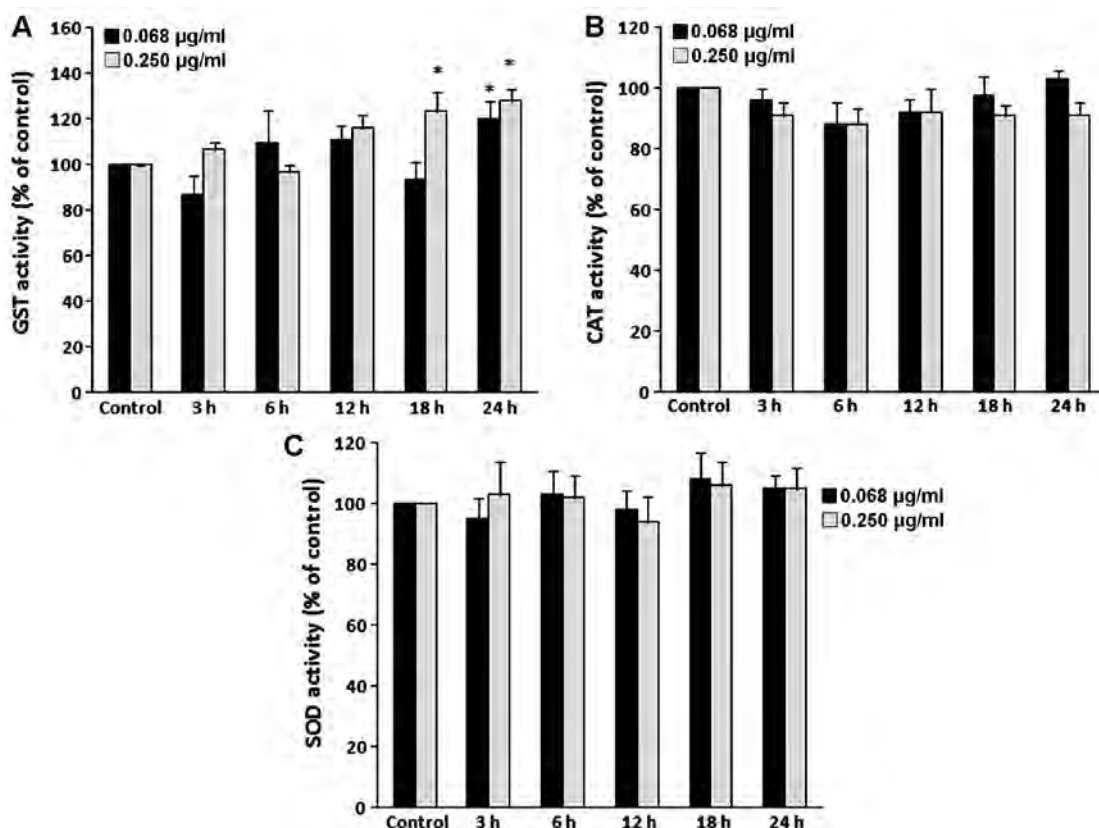


Figure 4. Effects of grape pomace extract (GPE) on the enzymatic activity of (A) glutathione S-transferase (GST), (B) catalase (CAT) and (C) superoxide dismutase (SOD) in EA.hy926 endothelial cells. The cells were incubated with GPE at 0.068 and 0.250 µg/ml for 3, 6, 12, 18 and 24 h. The results are expressed as a percentage of the control values. \* $P < 0.05$ , statistically significant difference compared to the control (untreated cells). Results are presented as the means  $\pm$  SEM.



transactivation of the GCS catalytical subunit promoter (33). The importance of GSH for the antioxidant activity of GPE is also supported by the GPE-induced increase in GST activity. GST is induced under conditions of oxidative stress and is involved in the detoxification of organic epoxides, hydroperoxides and unsaturated aldehydes formed particularly after lipid peroxidation (34). GST detoxifies these products through their conjugation with GSH. Of note, we have previously reported that GPE decreased products of lipid peroxidation in EA.hy926 and C2C12 cells (25). Consequently, this effect may be attributed to the GPE-induced increase in GST activity. The increase in GST activity may result in a decrease in GSH levels (34). However, it seems that the GPE-induced increase in GCS levels led to *de novo* GSH synthesis that hampered the decrease in GSH levels caused by GST activity.

Based on the above-mentioned findings, it can be inferred that GSH systems play a crucial role in the antioxidant effects exerted by GPE in endothelial and muscle cells. Moreover, several studies conducted *in vivo* and using cell cultures have shown that grape seed extracts exert antioxidant effects through the induction of GSH systems in a great variety of tissues and organs, such as the liver, kidneys, heart, skin, pancreas, blood, eyes and brain (34-40). Although a large number of studies have indicated that grape extracts from seeds enhanced GSH systems, there are only few studies available that have used grape pomace extracts (34,37,41-46). Our results provide further evidence that grape extracts, in general, act as antioxidants through the modulation of GSH systems.

GPE did not seem to exert its antioxidant effects through the modulation of the other tested antioxidant enzymes (i.e., CAT, SOD and HO-1 enzymes). SOD converts  $O_2^{\cdot -}$  generated during oxidative stress into  $H_2O_2$  (47).  $H_2O_2$  may be converted to harmful ROS, but it is broken down into harmless water and oxygen by CAT (48). In this study, treatment with GPE reduced both the expression and activity of the CAT enzyme in endothelial cells, while in muscle cells, it had no any effect. GPE did not affect the expression or the activity of SOD in the EA.hy926 and C2C12 cells. The fact that GPE had no any effect (or even decreased) the expression and activity of CAT and SOD enzymes may be explained by its ability to enhance other antioxidant mechanisms, such as GSH, GCS and GST. Similar to our results, other studies have reported that grape extracts did not affect CAT and SOD enzymes (34,49). However, the administration of GPE to rats has been shown to increase CAT and SOD activity in the liver and kidneys (41). Furthermore, grape seed extracts have been shown to increase CAT and SOD activity in different tissues (35,40). These discrepancies between the results of different studies may be attributed to the different tissues used, as well as to the different chemical composition of the tested extracts. In addition Yang *et al* (49) reported that the effects of grape seed extract on CAT and SOD activity in eukaryotic cells depends on the presence or absence of oxidative stress stimulus and is mediated through the extracellular-signal-regulated kinase 1/2 (ERK1/2) signaling pathway.

HO-1 is also considered an important antioxidant enzyme (50). HO-1 is an ubiquitous inducible cellular stress protein and is the rate-limiting enzyme in the catabolism of heme to biliverdin, free iron and carbon monoxide. Biliverdin is rapidly converted to the strong antioxidant, bilirubin, which

is then converted back into biliverdin through the reaction with ROS, leading to their neutralization (50). In this study, treatment with GPE did not affect HO-1 expression in the endothelial and muscle cells. Thus, the antioxidant effects of GPE do not seem to be mediated through HO-1 activity. Similar to our results, in a previous study, a grape seed extract rich in procyanidins was unable to increase HO-1 expression in liver cells (HepG2), although it induced the signaling pathway of nuclear factor E2-related factor (Nrf2)/antioxidant response element (ARE) (51). Nrf2/ARE is the main signaling pathway regulating HO-1 expression (52). In another study, the administration of grape extract to mice increased HO-1 expression levels through Nrf2 transcription factor in the testes (53). However, this grape extract was from seeds and was particularly rich in proanthocyanidins. Furthermore, resveratrol, one of the main polyphenols present in grape extracts, has been shown to increase HO-1 activity in mouse neuronal cells (54).

In conclusion, the findings of this study demonstrated that treatment with GPE exerted antioxidant effects in endothelial and muscle cells mainly through the induction of GCS and GST enzymes. These results, along with those of our previous study (25), indicate that GPE increases GSH levels in EA.hy926 and C2C12 cells, suggesting the crucial role of GSH systems in the antioxidant effects of GPE. Thus, GPE may prove to be effective for use as a food supplement for the treatment of oxidative stress-induced pathological conditions of the cardiovascular and skeletal muscle systems, particularly those associated with low GSH levels. Although there are several studies showing that grape extracts from seeds protect cardiovascular and skeletal muscle systems from ROS-induced damage, there only few studies using extracts from pomace (40,55-63). Of course, *in vivo* studies are also required to confirm these findings.

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# Grape seed proanthocyanidin extract protects the retina against early diabetic injury by activating the Nrf2 pathway

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**Abstract.** The present study aimed to investigate whether grape seed proanthocyanidin extract (GSPE) has a protective effect on diabetic retinal function. A total of 30 Wistar rats were randomly divided into three equal groups, including the control, diabetic and GSPE-treated diabetic groups. Retinal tissue was harvested and subsequently stained with hematoxylin and eosin. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and methane dicarboxylic aldehyde (MDA) levels were evaluated using respective assay kits; whereas nuclear erythroid 2-related factor 2 (Nrf2) and heme oxygenase (HO)-1 expression levels were assessed by immunohistochemical and western blot analysis. Cell apoptosis in the retina was determined using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling method. The results showed that the structure of the retina was damaged in diabetic rats, as compared with the control rats. Notably, the structure of the retina improved in the GSPE-treated diabetic group, as compared with the diabetic group. SOD and GSH-Px activities were significantly increased in the retina of rats in the GSPE-treated diabetic group, as compared with the diabetic group ( $P=0.011$  and  $P=0.001$ , respectively). Furthermore, a significant reduction in MDA was detected ( $P=0.013$ ) and the expression levels of Nrf2 and HO-1 in the bladders of rats in the GSPE-treated diabetic group were significantly increased, as compared with the diabetic group ( $P=0.038$  and  $P=0.043$ , respectively). Apoptosis of retinal cells

was significantly increased in the diabetic group, as compared with the control group ( $P<0.001$ ); a significant reduction was also detected in the GSPE-treated diabetic group, as compared with the diabetic group ( $P=0.014$ ). These results demonstrate that GSPE administration may protect the retina against hyperglycemic damage, possibly by ameliorating oxidative stress-mediated injury via the activation of the Nrf2 pathway.

## Introduction

The prevalence of diabetic retinopathy (DR) in patients with established diabetes and newly diagnosed diabetes is 27.9 and 10.5%, respectively (1), and the incidence of DR is expected to increase substantially (2). DR is the leading cause of blindness in patients of working age (3), and one of the early manifestations of DR is persistent apoptosis of vascular and neural cells in the retinal tissue (4,5). Other consequences of DR include the breakdown of the blood retinal barrier, retinal edema, neovascularization and detachment and loss of vision (5). Although the pathogenesis of DR is complicated and has yet to be fully elucidated, hyperglycemia-induced oxidative stress, an imbalance in the production of reactive oxygen species (ROS) and ROS-induced damage have been demonstrated to serve a crucial function in the pathogenesis of DR (6).

Grape seed proanthocyanidin extract (GSPE), which is a potent antioxidant derived from grape seeds, provides a concentrated source of polyphenols (7). Previous studies have demonstrated that GSPE has an important role in antioxidation, anti-inflammation, radical scavenging and antitumor activity (7,8); and that the physiological benefits of GSPE are closely associated with its antioxidative and free radical scavenging properties. Furthermore, GSPE has been demonstrated to have a protective effect in DR by reducing the production of advanced glycation end products (9).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor involved in the Nrf2-antioxidant response element signaling pathway, which protects against oxidative stress; therefore, Nrf2 is crucially involved in the attenuation of inflammation-associated pathogenesis in numerous diseases (10). Previous studies have demonstrated that Nrf2 may have a protective role in the retina (11-13). Furthermore, it has been demonstrated that Nrf2 has a cytoprotective role for neurons and vasculature in the diabetic retina (12,14).

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Scapagnini *et al* (15) found that modulation of the Nrf2 pathway was achievable using food polyphenols, which has since become a nutritional neuroprotective therapeutic strategy. To further understand the role of GSPE in the protection of DR and the mechanism of Nrf2 in the pathogenesis of DR, the present study investigated whether GSPE was capable of modulating the expression levels of Nrf2 and the downstream molecule, heme oxygenase (HO)-1, in the retina. Furthermore, whether GSPE administration could improve the structure and morphology of diabetic retinas was examined. The authors of the present study hypothesized that GSPE had a protective role in DR by modulating the Nrf2 pathway.

## Materials and methods

**Experimental design.** A total of 30 Wistar rats, aged 8-10 weeks and weighing 230-250 g, were purchased from the Animal Center of Shandong University (Shandong, China; license number, SCXX20050015) and divided into three equal groups (10 rats/group): The untreated (control); untreated diabetic (DM); and diabetic treated with GSPE (DM + GSPE) groups. Animal care and handling in the present study was approved by the Ethics Committee of Shandong University.

Diabetes was induced in the DM and DM ± GSPE rats following 18 h of fasting by a single intraperitoneal injection with 65 mg/kg streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 M citrate buffer (pH 4.5). The control rats were administered a single intraperitoneal injection of isometric citrate buffer. The rats were maintained at 25±1°C in a temperature-controlled room with a 12-h light/dark cycle and *ad libitum* access to food and water. Tail venous blood samples were harvested at 72 h after STZ treatment in order to measure blood glucose levels using a glucose monitoring system (cat. no. 1620368; Roche Diagnostics, Indianapolis, IN, USA). A total of 20 rats with serum glucose levels >300 mg/dl were included in the experiment. Following the induction of diabetes, 250 mg/kg GSPE (Tianjin Jianfeng Natural Product R&D, Co., Ltd., Tianjin, China) was administered per day in normal saline solution via oral gavage for 8 weeks.

Upon completion of the experiment, fasted rats were anesthetized with 80 mg/kg ketamine (Sigma-Aldrich), sacrificed by cervical dislocation, and their eyes were immediately removed. The right eyes were fixed in 4% paraformaldehyde (Sigma-Aldrich) for morphological analysis and apoptosis rate measurement; whereas the left eyes were harvested and stored at -80°C for the evaluation of Nrf2 expression levels and determination of redox status.

**Retinal morphology analysis.** Retinal samples were cut into 4-μm sections, placed onto glass slides, deparaffinized in xylene (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and serially treated with 100, 96 and 70% ethanol. Subsequently, the slides were stained with hematoxylin and eosin (HE; Sangon Biotech Co., Ltd., Shanghai, China) and observed at x100-400 magnification under a light microscope (BX53F; Olympus Corporation, Tokyo, Japan). Morphological analyses were performed by two independent pathologists in a blinded manner.

**Cytoplasmic and nuclear extraction.** Using a nuclear extraction kit (cat. no. P0028; Beyotime Institute of Biotechnology,

Beijing, China), each fresh isolated retina was homogenized in 200 μl ice-cold cytoplasmic extraction buffer for 15 min and centrifuged at 15,000 × g for 10 min at 4°C, according to the manufacturer's protocol. The supernatant containing the cytoplasmic protein fraction was used to determine the activity levels of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), the quantity of methane dicarboxylic aldehyde (MDA) and the expression levels of HO-1. The remaining nuclear pellet was resuspended in 50 μl ice-cold nuclear extraction buffer for 10 min and centrifuged at 15,000 × g for 10 min at 4°C. The supernatant containing the nuclear fraction was used for the quantification of Nrf2 in the nucleus. The Bicinchoninic Acid Assay kit (cat. no. P0012; Beyotime Institute of Biotechnology) was used to quantify the protein concentrations in the cytoplasmic and nuclear extracts.

**Estimation of redox status in retinas.** SOD and GSH-Px activity levels and MDA content were estimated using the Total Superoxide Dismutase Assay kit with WST-8 (S0101), the Lipid Peroxidation MDA Assay kit (S0131) and the Total Glutathione Peroxidase Assay kit (S0058), respectively (all from Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's protocols. Briefly, T-SOD activity was assessed based on the xanthine-xanthine oxidase system. GSH-Px activity was measured according the speed of enzymatic reaction, whereas MDA levels were determined by the thiobarbituric acid method.

**Western blot analysis.** Nuclear extracts were used to detect the expression levels of Nrf2, whereas cytoplasmic extracts were used to analyze HO-1 levels. A Bicinchoninic Acid Assay kit was used to determine the protein concentration in the supernatant, and the samples were subsequently stored at -80°C. Immediately prior to electrophoresis, loading buffer (Sangon Biotech Co., Ltd.) was added to the samples and heated at 95°C for 4 min. Subsequently, 40 μg protein was added to each gel well and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Sangon Biotech Co., Ltd.). Separated proteins were electroblotted onto a 0.45-μm polyvinylidene fluoride (PVDF) membrane (Roche Diagnostics) using transfer buffer (Beyotime Institute of Biotechnology). Nonspecific binding was blocked by incubating the membranes in 5% fat-free milk for 1 h. PVDF membranes were incubated overnight at 4°C with rabbit anti-rat Nrf2 polyclonal antibody (cat. no. ab31163), rabbit anti-rat Lamin B polyclonal antibody (cat. no. ab13248), mouse anti-rat HO-1 monoclonal antibody (cat. no. ab16048) and mouse anti-rat β-actin monoclonal antibody (cat. no. ab8226; all 1:1,000; all Abcam, Cambridge, UK). Subsequently, the membranes were washed three times for 10 min each with Tris-buffered saline supplemented with Tween-20 (Sangon Biotech Co., Ltd.), prior to incubation with goat anti-rabbit secondary antibody (1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 25°C for 2 h. Chemiluminescence was detected using a Kodak Image Station 2000 MM (Kodak, Rochester, NY, USA). Grayscale analysis was performed using Scion Image analysis software 4.03 (Scion Corporation, Frederick, MD, USA).

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay.** TUNEL staining of the



Table I. Body weight and blood glucose values in three groups.

Characteristic	Control	DM	DM + GSPE
Body weight (g)			
0 weeks	243.52±6.30	241.61±4.76	239.24±6.36
8 weeks	301.62±11.69	242.41±14.63 <sup>a</sup>	251.85±12.14
Blood glucose (mg/dl)	94.53±9.03	451.2±18.74 <sup>a</sup>	447.25±24.49

Data are presented as the mean ± standard deviation (n=10/group). <sup>a</sup>P<0.001 vs. the control group. DM, diabetes mellitus; GSPE, grape seed proanthocyanidin extract.

retinal sections on the glass slides was performed using a one-step TUNEL Apoptosis Assay kit (cat. no. C1089; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. In order to stain the nucleus, 4'6'-diamino-2-phenylindole dihydrochloride was added for 10 min at room temperature. Following staining, slides were observed at a 550-nm excitation wavelength under an Olympus BX53F microscope. The cells with red fluorescence were defined as apoptotic.

**Immunohistochemistry.** Immunofluorescence techniques were performed to investigate the expression levels of Nrf2. Briefly, sections were blocked with 10% normal goat serum and 0.1 M phosphate-buffered saline (both Sangon Biotech Co., Ltd.) prior to incubation with rabbit anti-Nrf2 antibody (Abcam, Cambridge, UK) at 4°C overnight. SP-9000 SP link Detection kits (cat. no. SP-9000-D; ZSGB-BIO, Beijing, China) were used according to the manufacturer's protocol. Slides were counterstained with hematoxylin for detection by light microscopy (BX53F; Olympus Corporation).

**Statistical analysis.** All data are expressed as the mean ± standard deviation (n≥6/group). Comparisons were performed using one-way analysis of variance for the different groups followed by Dunnett's post-hoc test for all pair comparisons using SPSS software, version 11.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**General characteristics.** Despite consuming an increased quantity of food and water, compared with the control rats the DM rats gradually lost weight (242.41±14.63 vs. 301.62±11.69 g; P<0.001) by eight weeks after the induction of diabetes. Furthermore, the average overnight 8-h fasting serum glucose level of the DM rats was 451.2±18.74 mg/ml, which was significantly higher compared with the control group (94.53±9.03 mg/ml; P<0.001). No significant differences in glucose levels were detected between the DM + GSPE and DM groups (447.25±24.49 vs. 451.2±18.74 mg/dl; P=0.968) (Table I).

**Retinal morphology.** Following HE staining, the retinas of the control group were highly organized with intact layers; whereas disorganized retinas with impaired layers were detected in the DM group. Retinal cells in the DM group were irregularly and loosely arranged and the nerve fiber and ganglion cell layers were narrower, as compared with the control and DM + GSPE

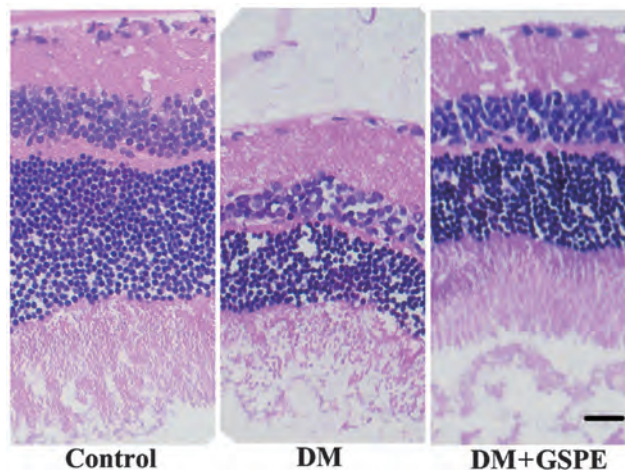


Figure 1. Retinal morphology in rats 2 months after the induction of DM and GSPE treatment. The retinas of the control and DM + GSPE groups were highly organized, with intact layers detected; whereas the DM group exhibited disorganized retinas with impaired layers (scale bar, 20 µm; stain, hematoxylin and eosin). DM, diabetes mellitus; GSPE, grape seed proanthocyanidin extract.

groups. These results suggest that GSPE is able to attenuate the disorganization and impairment of the retinal layers associated with DM (Fig. 1).

**GSPE attenuates oxidative stress in diabetic retina.** Table II presents the significant reductions in SOD (n=8; P=0.003) and GSH-Px (n=8; P=0.003) activity levels in the diabetic retina homogenates, as compared with the controls. Following GSPE administration, SOD (n=8; P=0.011) and GSH-Px (n=8; P=0.001) activity levels significantly increased in the DM + GSPE group, as compared with the DM group. Furthermore, MDA levels were significantly increased in the diabetic retina, as compared with the control group (n=8; P=0.002). MDA levels significantly decreased in the DM + GSPE group, as compared with the DM group (n=8; P=0.013) (Table II).

**GSPE activates the Nrf2 pathway.** Retinal Nrf2 expression levels were increased in the DM + GSPE group, as compared with the DM group (Fig. 2A). Nuclear Nrf2 expression levels were subsequently assessed by western blot analysis. The results demonstrated that Nrf2 protein expression levels in the nucleus were significantly increased in the retinas of the DM + GSPE group, as compared with the untreated DM group (n=6; P=0.038) (Fig. 2B). Furthermore, the expression levels of HO-1, which is the target gene of the Nrf2 pathway (14), were



Table II. Levels of the oxidative stress markers GSH-Px, SOD and MDA in the three groups (n=8 per group).

Marker	Control	DM	DM + GSPE
GSH-Px (U/mg)	18.42±3.38	12.12±2.47 <sup>a</sup>	18.03±2.69 <sup>b</sup>
SOD (U/mg)	16.63±3.27	10.80±1.54 <sup>a</sup>	14.44±2.42 <sup>b</sup>
MDA (nmol/mg)	7.09±2.03	16.86±3.97 <sup>a</sup>	11.24±1.74 <sup>b</sup>

Data are presented as the mean ± standard deviation. <sup>a</sup>P<0.05 vs. the control group; <sup>b</sup>P<0.05 vs. the DM group. GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, methane dicarboxylic aldehyde; DM, diabetes mellitus; GSPE, grape seed proanthocyanidin extract.

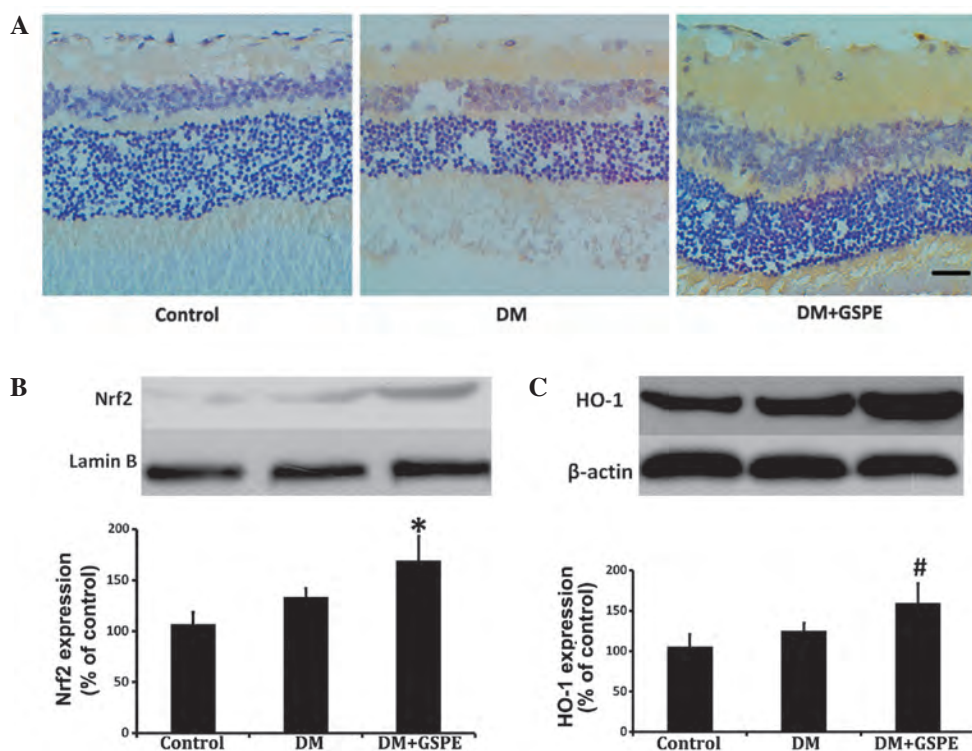


Figure 2. Expression levels of Nrf2 and HO-1 in the control, DM and DM + GSPE groups. (A) Immunohistochemical analysis demonstrated an increase in Nrf2 expression levels in the retinas of rats in the DM + GSPE group. Nrf2 was predominantly expressed in the nerve fibers, ganglion cells and inner plexiform layer of the retina (scale bar, 20  $\mu$ m; stain, hematoxylin). (B) Western blot analysis showing increased nuclear Nrf2 expression levels in the DM + GSPE group, as compared with the DM group. (C) Western blot analysis showed increased cytoplasmic HO-1 expression levels in the DM + GSPE group, as compared with the DM group. \*P=0.038 vs. the DM group; #P=0.043 vs. the DM group (n=6). DM, diabetes mellitus; GSPE, grape seed proanthocyanidin extract; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1.

significantly elevated in the DM + GSPE group, as compared with the untreated DM group (n=6; P=0.043) (Fig. 2C).

**GSPE decreases cell apoptosis.** The results of TUNEL staining demonstrated that the rate of apoptosis in retinal cells in the DM group was significantly increased, as compared with the control group (n=5; P<0.001). Apoptotic cells were predominantly detected in the nerve fiber, ganglion cell and inner plexiform layers of the retina. Treatment with GSPE significantly decreased the number of apoptotic cells (n=5; P=0.014) (Fig. 3).

## Discussion

DR remains the leading cause of blindness in adults of working age worldwide, and this condition may become a leading cause

of visual impairment (1-3). Previous studies investigating DR have predominantly been focused on the identification of pathogenic molecules (14). However, the prevention and treatment of DR has been investigated (16) which is particularly relevant to patients with long-standing diabetes (17).

The results of the present study demonstrated that GSPE, which contains natural polyphenols, has a protective effect against DR. Following treatment with GSPE, the retinal morphology of STZ-induced diabetic rats was markedly improved. In particular, retinal cells in the GSPE-treated DM group were tightly arranged in a regular manner, as compared with the DM group, and the nerve fiber and ganglion cell layers increased in thickness. Furthermore, STZ-induced diabetic rats exhibited a reduction in body weight and treatment with GSPE increased the body weight of DM rats to a certain extent,

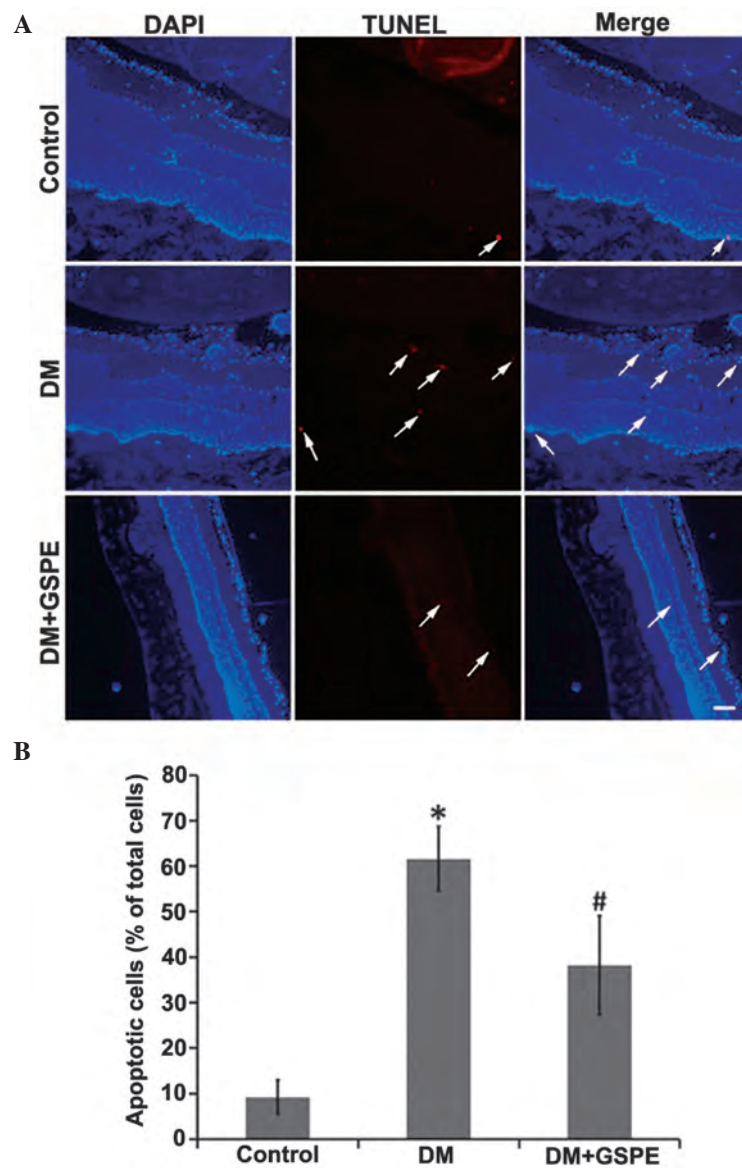


Figure 3. Apoptosis of retinal cells in the control, DM and GSPE-treated DM groups. (A) TUNEL staining demonstrated the apoptosis of retinal cells in the control, DM and GSPE-treated DM groups. DAPI (blue), arrow indicates apoptotic retinal cells (red; scale bar, 50  $\mu$ m). (B) Following TUNEL staining, slides were observed at 550-nm excitation and cells with red fluorescence were defined as apoptotic. \* $P=0<001$  vs. the control group ( $n=5$ ); # $P=0.014$  vs. the DM group ( $n=5$ ). DAPI, 4',6'-diamino-2-phenylindole dihydrochloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; DM, diabetes mellitus; GSPE, grape seed proanthocyanidin extract.

which has been controversial in previous studies (9,18,19). The results of the present study also demonstrated that GSPE was capable of attenuating oxidative stress in diabetic retinas. SOD and GSH-Px activity levels increased following GSPE administration, whereas MDA levels were decreased, which is consistent with previous findings (20).

Following this, the underlying mechanism of the protective effect of GSPE was investigated in diabetic retinas. Although GSPE has been extensively investigated due to its associations with cardiovascular system disease, nervous system disease, diabetic nephropathy, rheumatoid arthritis and human cancers (21,22), there has only been one previous study investigating the effects of GSPE in the retina (9). Li *et al* (9) found that GSPE significantly suppressed the vascular lesions of central regions and decreased capillary enlargements and neovascularization in diabetic retinas by reducing advanced

glycation end products. Diabetes-associated increases in advanced glycation end products may induce oxidative stress via various mechanisms, including enhancement of protein kinase C and hexosamine and polyol pathways fluxes (23). Nrf2 is an important protective factor which regulates the progression of DR as a part of the an important cellular pathway protecting against oxidative stress (12). Since it has previously been demonstrated that food polyphenols are capable of modulating the Nrf2 pathway (15), the present study investigated whether GSPE has a protective effect in DR by activating the Nrf2 pathway.

The results of the present study indicated that GSPE exerts protective activity in the retina via the activation of the Nrf2 pathway. The present study demonstrated that the expression levels of Nrf2 and its target gene, HO-1, were markedly increased in the retina following treatment with GSPE. Nrf2 was

predominantly expressed in the nerve fiber, ganglion cell and inner plexiform layers (Fig. 2A). It is well established that, as an antioxidation transcription factor, Nrf2 functions exclusively in the nucleus (14). Furthermore, treatment with GSPE significantly attenuated the apoptosis of retinal cells in the present study. These results suggested that GSPE may be capable of activating the Nrf2 pathway, which may protect diabetic retinal cells against apoptosis.

However, the precise mechanism underlying the anti-apoptotic effect of GSPE and the Nrf2 pathway remain unclear. A previous study has demonstrated that the protective effects of GSPE may be partially attributed to its ability to inhibit anti-death signaling mediated via proapoptotic transcription factors and genes, including c-Jun N-terminal kinase (JNK)-1 and c-Jun (24). Zou *et al* (25) have previously demonstrated that the activation of Nrf2 was capable of preventing oxidative stress-induced apoptosis by hydroxytyrosol in human retinal pigment epithelial cells via the JNK-p62/SQSTM1 pathways. Furthermore, Pehar *et al* (26) demonstrated that decreased Nrf2 expression and the downregulation of the enzymes associated with oxidative stress induces p75 neurotrophin receptor-induced motor neuron apoptosis (26). Furthermore, previous studies have indicated that activation of HO-1, which is the target gene of Nrf2, may protect diabetic retinal cells against apoptosis (27,28). Further studies are required in order to fully elucidate the anti-apoptotic effect of GSPE, and the underlying mechanisms.

In conclusion, the results of the present study suggested that early treatment with GSPE may protect diabetic retinal cells against diabetic retinopathy by attenuating oxidative stress-mediated cellular apoptosis, which may be associated with the activation of the Nrf2 pathway.

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## Research Article

# Neuroprotection of Grape Seed Extract and Pyridoxine against Triton-Induced Neurotoxicity

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Triton WR-1339 administration causes neurotoxicity. Natural products and herbal extracts can attenuate cerebral injury. In the present study, we investigated the neuroprotective role of grape seed extract and/or vitamin B6 against triton-induced neurotoxicity. Fifty-five adult male albino rats of the Sprague-Dawley strain, weighing 140–145 g, were divided into five groups: control, triton, grape seed extract + triton, grape seed extract + triton + vitamin B6, and vitamin B6 + triton. The hematological and biochemical analyses were carried out. Alteration in iNOS mRNA gene expression was determined using reverse-transcriptase PCR analysis. In addition, qualitative DNA fragmentation was examined using agarose gel electrophoresis. Triton-treatment caused significant disturbances in the hematological parameters, the neurological functions, and the antioxidant profile. Also, triton significantly increased the iNOS mRNA expression and DNA damage. Our results showed that grape seed extract and/or vitamin B6 could attenuate all the examined parameters. These natural substances could exhibit protective effects against triton-induced neurological damage because of their antioxidative and antiapoptotic capacities.

## 1. Introduction

The redox imbalance, inflammation, and apoptosis are the major mechanisms of cerebral injury [1, 2]. The increase of oxidative metabolic activity in addition to the low concentrations of endogenous antioxidants causes brain oxidative insults [3]. The administration of the nonionic detergent, triton WR1339 (Tyloxapol), was able to increase the oxidative markers in animals. This oxidation plays a crucial role in the pathologic processes [4]. Experimentally, natural products and herbal extracts can attenuate cerebral injury and showed protective effects against brain damage [5, 6].

Grapes (*Vitis vinifera*) are among the fruits that have the highest phenolic compound contents. It has been associated with the antioxidant, anti-inflammatory, anticarcinogenic, and antibacterial activities [7]. Grape seed extract (GSE) contains plant flavonoids such as proanthocyanidins which are potent antioxidants and exert many health-promoting effects [8]. The antioxidant activities of proanthocyanidins are

exerted directly by scavenging reactive oxygen species (ROS) as well as by chelating redox-active transition metals, such as iron and copper [9]. Previous studies have suggested that wild grape root and stem have antiangiogenic, antioxidant, anti-inflammatory, and neuroprotective effects [10].

Vitamin B6 is a generic term that refers to the six interconvertible pyridine compounds (vitamers): pyridoxine (PN, commonly known as vitamin B6), pyridoxamine (PM), pyridoxal (PL), and their 5'-phosphorylated forms (PNP, PMP, and PLP, resp.) [11]. An additional function of B6 vitamers is to act as reactive oxygen species (ROS) scavengers and as factors able to increase resistance to biotic and abiotic stress. PLP and PN may also function as regulators of membrane ion transporters and have been found to bind to steroid receptors to modulate transcription factors [12]. Pyridoxine (vitamin B6) is a water-soluble vitamin and is present in whole grains, legumes, potatoes, nuts, fish and poultry. It participates in protein metabolism, amino acid, and monoamine neurotransmitter synthesis [13]. Also, it is involved in the

methionine/glutathione transsulfuration pathway to form the natural antioxidant, glutathione. Pyridoxine is converted by all organs of the body to pyridoxal 5-phosphate (PLP) and pyridoxamine, which serve as coenzymes for transaminase reaction [13]. PLP has been reported to reduce the complications associated with coronary artery disease, diabetes, hypertension, aging, and neurodegenerative disorders [14]. In this study, we aimed to investigate the neuroprotective role of GSE, the combination between GSE and B6, and B6 alone against triton (WR-1339)-induced neurotoxicity. This was carried out through the examination of important biomarkers that imply neurological, biochemical, and molecular changes.

## 2. Materials and Methods

**2.1. Animals.** Thirty-five adult male albino rats of the Sprague-Dawley strain, weighing 140–145 g, were purchased from the Faculty of Medicine, Alexandria University, Alexandria, Egypt. Animals were housed 7 per cage and were fed a rodent laboratory chow and water *ad libitum*, kept on 12 h light-dark cycle periods, and acclimatized for at least one week prior to the experiment. The local committee approved the design of the experiments, and the protocol conforms to the guidelines of the National Institutes of Health (NIH).

**2.2. Experimental Design and Sample Collection.** After one week of acclimation, animals were divided into five equal groups, 7 per each. The first group was the control. The second was triton group; rats were injected intraperitoneally (IP) with triton (50 mg/kg BW) day after day for 4 weeks according to Bhuvaneshwari and Sasikuma [15]. In GSE + triton-treated group, rats were orally given GSE (300 mg/kg BW) according to Sreemantula et al. [16] daily for 4 weeks plus triton. In the GSE + triton + B6-treated group, rats were orally given triton, GSE, and B6 (300 mg/kg BW, 12 mg/kg BW, and 50 mg/kg BW, resp.). In the B6 + triton-treated group, rats of this group were orally given B6 (12 mg/kg BW) according to Basu and Mann [17] daily for 4 weeks plus triton (50 mg/100 g BW). At the end of the experiment, rats were starved for 12 h and then sacrificed by decapitation under diethyl ether anesthesia. Blood samples were collected in tubes containing heparin for hematological analysis. The brain tissues were immediately removed and kept at  $-80^{\circ}\text{C}$  till analysis. The brain tissues were homogenized (10%, w/v), separately, in ice-cold phosphate buffer (0.01 M, pH 7.4) containing 1.15% KCl in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at  $10,000 \times g$  for 20 min at  $4^{\circ}\text{C}$  and the resultant supernatant was used for the biochemical analysis.

**2.3. Hematological Examination.** The noncoagulated blood samples were tested, shortly after collection, for hemoglobin (Hb), total erythrocyte count (TEC), packed cells volume (PCV), total leukocyte count (TLC), and platelets count by Particle Counter (ERMA Inc., Tokyo; Model PCE-210).

**2.4. Biochemical Parameters.** The level of serotonin in the brain was determined by the spectrofluorimetry according to Schlumpf et al. [18]. Acetyl cholinesterase (AChE; EC 3.1.1.7)

activity in the brain was estimated using acetylcholine iodide as a substrate according to the method of Ellman et al. [19]. Tissue supernatant thiobarbituric acid-reactive substances (TBARS) were measured at 532 nm using 2-thiobarbituric acid (2,6-dihydroxypyrimidine-2-thiol, TBA) and extinction coefficient of  $156,000 \text{ M}^{-1} \text{ cm}^{-1}$  was used for calculation [20]. Reduced glutathione (GSH) content was assayed using 5,5-dithiobis(2-nitrobenzoic acid, DTNB) for color development and its density was measured at 412 nm [21]. The catalase (CAT; EC 1.11.1.6) converts  $\text{H}_2\text{O}_2$  into water and its activity in tissue supernatant was measured spectrophotometrically at 240 nm [22].

Superoxide dismutase (SOD; EC 1.15.1.1) activity was estimated according to Misra and Fridovich [23]. The activity of glutathione peroxidase (GPX) was determined using the method of Chiu et al. [24] in the brain extracts. The activity of lactate dehydrogenase (LDH) was determined in the tissue supernatant according to Moss et al. [25]. The protein content of the brain tissue was determined by the method of Lowry et al. [26] using bovine serum albumin as a standard.

**2.5. RT-PCR for iNOS Messenger RNA Gene Expression.** Brain tissues were kept in RNA later, a reagent for immediate stabilization of the gene expression profile in harvested animal tissues. Then, total RNA was extracted from brain tissues according to Chomczynski and Sacchi [27] using Biozol RNA Isolation Kit. The total RNA extract was resuspended in 50–100  $\mu\text{L}$  RNase-free  $\text{H}_2\text{O}$ . RNA concentration was determined using the following equation: 1 absorbance unit at 260 nm corresponds to approximate concentration of 40  $\mu\text{g}/\text{mL}$  of single-stranded RNA. The purity of the RNA preparation was estimated to be 1.8–2.0 according to the ratio of absorbance readings at 260 nm and 280 nm [Abs 260/Abs 280] [27]. Alteration in iNOS mRNA gene expression level was determined using reverse-transcriptase PCR analysis. One-step RT-PCR (RT/PCR Master Mix Gold Beads, BIORON) reaction was used for the cDNA synthesis and for amplification of target gene using specific primer sets as follows.

Primers for iNOS (GCTGCCAGGGTCACAACCTTT and CCAGTGACACTGTGTCCCGT) and for beta actin (GCTTCTTTGCAGCTCCTTCGT and CGTCATCCATGGCGA-ACTG) yielded PCR products of 71 and 59 bp, respectively. Specificity of PCR was checked by analyzing the melting curve. Relative mRNA levels were determined by comparing (a) the PCR cycle threshold between cDNA of iNOS and beta actin and (b) values between treated and untreated conditions as described previously [28]. The amplified RT-PCR product (10  $\mu\text{L}$ ) was mixed with 2  $\mu\text{L}$  of sample loading dye, electrophoresed on 1.5% agarose gel (1.5 g/100 mL 0.5x TBE) containing 10  $\mu\text{g}/\text{mL}$  ethidium bromide (EtBr) dye, and visualized with gel documentation system [29].

**2.6. Qualitative DNA Fragmentation Assay by Agarose Gel Electrophoresis.** DNA was extracted using the genomic DNA purification kit purchased from Bio Basic Inc., Canada. Afterwards, DNA was quantified spectrophotometrically and then loaded onto agarose gel (15  $\mu\text{g}/\text{lane}$ ). DNA laddering

Table 1: Changes in the hematological parameters in the blood samples of the different experimental groups.

Source	Parameters	Experimental groups				
		Control	Triton	GSE + triton	GSE + triton + B6	B6 + triton
Blood	HB	12.68 ± 0.22	6.74 <sup>a</sup> ± 0.28 −46.8%	10.42 <sup>ab</sup> ± 0.14 −17.9%	11.66 <sup>ab</sup> ± 0.07 −8%	9.84 <sup>ab</sup> ± 0.093 −22%
	PCV	37.99 ± 0.59	22.06 <sup>a</sup> ± 0.41 −41.9%	32.71 <sup>ab</sup> ± 0.50 −13.9%	35.61 <sup>ab</sup> ± 0.22 −6%	28.48 <sup>ab</sup> ± 0.51 −25%
	TEC	4.70 ± 0.095	2.58 <sup>a</sup> ± 0.06 −45%	3.80 <sup>ab</sup> ± 0.05 −19%	4.22 <sup>ab</sup> ± 0.12 −10%	3.06 <sup>ab</sup> ± 0.07 −34.9%
	TLC	8.20 ± 0.09	4.94 <sup>a</sup> ± 0.07 −39.7%	5.90 <sup>ab</sup> ± 0.095 −28%	8.12 <sup>b</sup> ± 0.10 −0.97%	6.56 <sup>ab</sup> ± 0.17 −20%
	PLT	309.60 ± 0.51	552.40 <sup>a</sup> ± 1.36 78.4%	299.60 <sup>ab</sup> ± 2.23 −3%	309.00 <sup>b</sup> ± 0.55 −0.19%	283.00 <sup>ab</sup> ± 3.21 −8.6%

Values are expressed as means ± SE.

<sup>a</sup>Th mean values are significantly different in comparison with the control group at  $p \leq 0.05$ .

<sup>b</sup>Th mean values are significantly different in comparison with the triton-intoxicated group at  $p \leq 0.05$ .

Hb: hemoglobin (g/dL); PCV: packed cells volume (%); TEC: erythrocyte count ( $\times 10^{12} \text{ L}^{-1}$ ); TLC: total leukocyte counts ( $\times 10^9 \text{ L}^{-1}$ ); PLT: platelets ( $\times 10^{12} \text{ L}^{-1}$ ).

was determined by constant voltage mode electrophoresis at 80 V, for 45 min on a 1.2% agarose gel containing 0.5  $\mu\text{g/mL}$  ethidium bromides. A 1kbp ladder (Bioron) served as DNA base pair marker. Gels were illuminated with 300 nm UV light and a photographic record was made [30].

**2.7. Statistical Analysis.** Data were analyzed according to Steel and Torrie [31]. Statistical significance of the difference in values of control and treated animals was calculated by ( $F$ ) test at 5% significance level. Data of the present study were statistically analyzed by using LSD Multiple Range Test [32].

### 3. Results

**3.1. Hematological Examination.** The results of triton-treated group showed significant ( $p < 0.05$ ) decrease in the total leukocyte count (TLC), total erythrocyte count (TEC), hemoglobin (Hb) concentration, and the packed cells volume (PCV) while there was significant ( $p < 0.05$ ) increase in the platelet count (PLT) compared to the controls. On the other hand, the hematological parameters in triton + GSE- and/or triton-treated groups were significantly ( $p < 0.05$ ) improved compared to the triton-treated one (Table 1).

**3.2. Biochemical Parameters.** The data represented in Table 2 summarized the biochemical parameters of the experimental groups. The results indicated that the brain serotonin level was significantly ( $p < 0.05$ ) decreased in rats of triton-treated group compared to control. Meanwhile, serotonin level was significantly ( $p < 0.05$ ) increased in groups treated with GSE + triton, GSE + triton + B6, or B6 + triton compared to those treated with triton only. Also, the brain acetyl cholinesterase activity was significantly ( $p < 0.05$ ) increased in triton-treated group compared to that of the control. Meanwhile, treatment with GSE + triton, GSE + triton + B6, or B6 + triton exhibited significant ( $p < 0.05$ ) decrease in acetyl cholinesterase activity when compared to triton-treated group.

Moreover, the brain glutathione (GSH) level as well as the activities of brain glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT), and lactate dehydrogenase (LDH) was significantly ( $p < 0.05$ ) decreased in triton-treated group compared to the control one (Table 2). Interestingly, all the above parameters showed significant ( $p < 0.05$ ) increases in groups treated with GSE + triton, GSE + triton + B6, or B6 + triton as compared to triton-treated one. Conversely, the level of brain malondialdehyde (MDA) was significantly ( $p < 0.05$ ) increased in triton-treated group compared to that of control. Meanwhile, the MDA levels of GSE + triton-, GSE + triton + B6-, and B6 + triton-treated groups were significantly ( $p < 0.05$ ) decreased in comparison to triton-treated one.

**3.3. RT-PCR for iNOS Messenger RNA Gene Expression.** The levels of iNOS mRNA expression in brain tissue of control and treated rats were measured by RT-PCR relative to  $\beta$ -actin as represented in Figures 1 and 2. Our results indicated that treatment with triton or B6 + triton significantly ( $***p < 0.001$ ) induced iNOS mRNA expression in brain tissue (lanes 2 and 5) as compared to control (lane 1). However, B6 + triton-treatment caused nonsignificant downregulation in iNOS mRNA expression level (lane 5) compared to triton-treatment (lane 2). On the other hand, the iNOS mRNA expression was barely detectable in both GSE + triton- and the GSE + triton + B6-treated groups (lanes 3 and 4, resp.), the same as the control (lane 1). The treatment with GSE + triton and the GSE + triton + B6 significantly downregulated the iNOS mRNA expression level in comparison to triton-treatment.

**3.4. Qualitative DNA Fragmentation Assay by Agarose Gel Electrophoresis.** The DNA fragmentation of rat brain tissue was detected on agarose gel electrophoresis as shown in Figure 3. The DNA fragmentation in triton-treated rats (lane 2) showed smear formation, which may indicate a typical feature of necrosis, compared to the control (lane 1). Interestingly, GSE + triton-treatment (lane 3) prevents the



Table 2: Changes in the levels of serotonin, GSH, and MDA and the activities of AchE, GPX, SOD, CAT, and LDH in brain extract of different experimental groups.

Parameters	Experimental groups				
	Control	Triton	GSE + triton	GSE + triton + B6	B6 + triton
AchE <sup>A</sup>	21.34 ± 0.33	35.38 <sup>a</sup> ± 0.22 65.8%	22.91 <sup>ab</sup> ± 0.21 7%	21.28 <sup>b</sup> ± 0.39 -0.28%	23.90 <sup>ab</sup> ± 0.28 12%
Serotonin <sup>B</sup>	350.70 ± 0.61	132.56 <sup>a</sup> ± 0.26 -62%	280.88 <sup>ab</sup> ± 2.23 -20%	252.52 <sup>ab</sup> ± 0.40 -28%	232.22 <sup>ab</sup> ± 0.33 -33.8%
GSH <sup>C</sup>	50.26 ± 0.24	23.84 <sup>a</sup> ± 0.38 -52.6%	42.14 <sup>ab</sup> ± 0.17 -16%	49.76 <sup>b</sup> ± 0.14 -1%	43.23 <sup>ab</sup> ± 0.17 -14%
GPX <sup>D</sup>	53.99 ± 0.18	19.24 <sup>a</sup> ± 0.29 -64%	41.08 <sup>ab</sup> ± 0.27 -24%	56.08 <sup>ab</sup> ± 0.27 3.9%	45.85 <sup>ab</sup> ± 0.28 -15%
SOD <sup>D</sup>	65.64 ± 0.28	26.75 <sup>a</sup> ± 0.13 -59%	51.08 <sup>ab</sup> ± 0.26 -22%	60.16 <sup>ab</sup> ± 0.32 -0.1%	52.60 <sup>ab</sup> ± 0.25 -20%
CAT <sup>D</sup>	60.55 ± 0.26	16.14 <sup>a</sup> ± 0.41 -89.9%	52.90 <sup>ab</sup> ± 0.19 -12.6%	58.15 <sup>ab</sup> ± 0.35 -4%	47.63 <sup>ab</sup> ± 0.37 -21%
MDA <sup>E</sup>	24.84 ± 0.11	76.24 <sup>a</sup> ± 0.21 206.9%	34.60 <sup>ab</sup> ± 0.39 39%	28.44 <sup>ab</sup> ± 0.25 14%	36.86 <sup>ab</sup> ± 0.17 48%
LDH <sup>F</sup>	113.20 ± 0.42	37.69 <sup>a</sup> ± 0.30 -66.7%	95.94 <sup>ab</sup> ± 0.11 -15%	101.46 <sup>ab</sup> ± 0.15 -10%	93.44 <sup>ab</sup> ± 0.28 -17%

Values are expressed as means ± SE.

<sup>a</sup>Th mean values are significantly different in comparison with the control group at  $p \leq 0.01$ .

<sup>b</sup>Th mean values are significantly different in comparison with the triton-intoxicated group at  $p \leq 0.01$ .

A:  $\mu\text{mol}$  substrate hydrolyzed/min/mg protein, B: ng/g tissue, C:  $\mu\text{mol/g}$  tissue, D: U/mg protein, E: nmol/g tissue, and F: mg/g tissue.

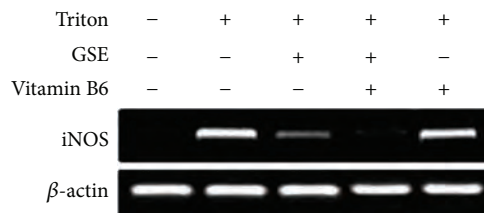


Figure 1: Reverse-transcriptase PCR (RT-PCR) analysis of iNOS mRNA expression in the different experimental groups.

necrotic effect of triton as indicated by the complete absence of smear formation. Also, the treatment with GSE + triton + B6 (lane 4) or B6 + triton (lane 5) could ameliorate the necrotic effect of triton.

## 4. Discussion

The present study was designed to examine the protective effect of grape seed extract or/and vitamin B6 against triton (WR-1339)-induced neurotoxicity.

**4.1. Hematological Examination.** The results represented in Table 1 indicated that the triton-treatment caused significant decreases in the total leukocyte, the total erythrocyte count, the hemoglobin level, and the packed cells volume as compared to the controls. Meanwhile, triton-treatment caused significant increase in platelet count compared to control. The hematological parameters in GSE and/or B6-treated groups

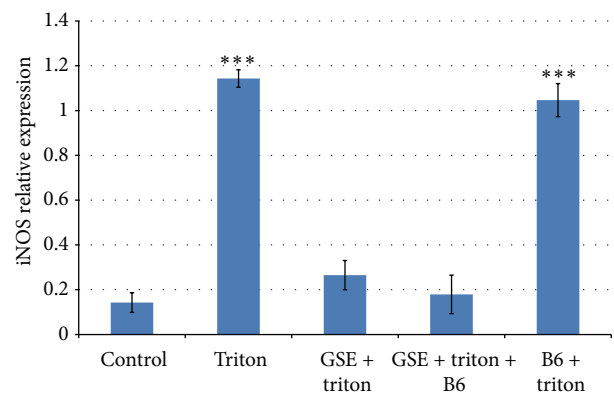


Figure 2: iNOS mRNA expression in the different experimental groups relative to  $\beta$  actin level as internal control. \*\*\* $p < 0.001$  calculated with reference to control sample.

were significantly improved compared to the triton-treated one.

**4.2. Biochemical Parameters.** Our results indicated that brain serotonin level was significantly decreased in triton-treated group while the acetyl cholinesterase activity was increased compared to controls. These biochemical parameters were improved in rats treated with GSE + triton, GSE + triton + B6, and B6 + triton compared to triton-treated group (Table 2). Many studies have provided evidence that GSE contains proanthocyanidin which has potent radical scavenging ability and antioxidant properties and thus provides significant

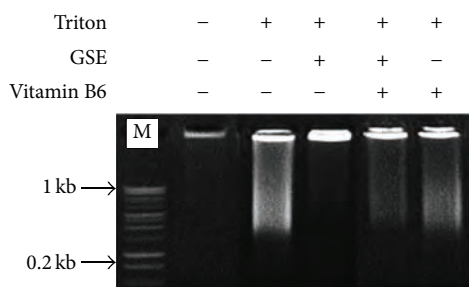


Figure 3: Detection of brain DNA fragmentation on agarose gel electrophoresis of the different experimental groups. The first lane, control; the second lane, triton-treated rats; the third lane, GSE + triton-treated rats; the fourth lane, the GSE + triton + B6-treated rats; the fifth lane, B6 + triton-treated rats.

neuroprotective effect [33, 34]. Also, Soltaninejad et al. [35] reported that GSE treatment restored AChE activity near to the control value indicating their ameliorating effect. The phenolic dietary antioxidant supplements of grape seeds have been also shown to enhance hippocampal neurogenesis [36]. In addition, Choi et al. [37] found that administration of GSE caused the regulation of brain epinephrine, noradrenaline, serotonin, and dopamine. Plecko et al. [38] indicated that pyridoxal phosphate-dependent enzymes play a role in the biosynthesis of five important neurotransmitters (serotonin, dopamine, adrenaline, noradrenaline, and  $\gamma$ -amino butyric acid). Tryptophan is converted to 5-hydroxy tryptophan by an enzyme, tryptophan hydroxylase, and 5-hydroxy tryptophan is converted to 5-hydroxy tryptamine (5-HT) in the presence of tryptophan decarboxylase and the coenzyme pyridoxal phosphate (PLP). So, vitamin B6 supplementation and a tryptophan-rich diet can alleviate major depressive symptoms among patients with multiple drug addiction [39]. Specifically, vitamins B2, B6, and B12 are important for the metabolism of dopamine and noradrenaline within the central nervous system [40].

The brain GSH level as well as GPX, SOD, and CAT activities showed significant decrease, while MDA level significantly increased in triton-treated group when compared to control (Table 2). On the other hand, the treatment with triton in combination with either GSE and/or B6 significantly increased the levels of GSH and the activities of GPX, SOD, and CAT in comparison to triton-treatment only. Meanwhile, the treatment with triton in combination with GSE and/or B6 significantly decreased the MDA level compared to triton-treated group. In accordance with our results, Oh and Lim [41] showed that the level of plasma TBARS was increased 18 h after triton WR-1339 administration to mice and the activity of CAT and GPx was decreased compared with the control group. Alía et al. [42] found that the cellular antioxidant enzyme system, including GR, GPx, CAT, and SOD, plays an essential role in the defense against oxidative stress and can be used as biomarkers for the antioxidant response. Polyphenolic compounds that are present in grape seeds have powerful antioxidant properties and GSE may inhibit lipid peroxidation by scavenging free radicals and increasing intracellular concentration of glutathione [43]. Also, GSE inhibits

the enzyme systems that are responsible for the production of free radicals [44]. The administration of grape seed proanthocyanidin treatment could alleviate the brain injury caused by hypoxia from sleep breathing disorder [34]. The chronic hypoxia increased the levels of reactive oxygen species and therefore overloads the endogenous clearing system, like SOD [45]. In addition, B6 is cofactor for cystathionine-synthase (CBS), catalyzing the transsulfuration pathway that is essential for GSH synthesis [46]. Moreover, the SOD activities and the antioxidant potential in kidney tissue of vitamin B6 deficient rats were significantly lower than those of the control ones [47]. In oxidative stress, SOD converts reactive superoxide into less harmful hydrogen peroxide, which is broken down into water and oxygen by CAT and GPx. Also, GPx catalyzes the reduction of lipid hydroperoxides to hydroxides by GSH and GR recycles the oxidized glutathione back to GSH [48]. It is noteworthy that the brain LDH activity exhibited significant decrease in triton-treated group compared to control (Table 2). Meanwhile, treatment with GSE and/or vitamin B6 plus triton caused significant increase in its activity in the brain extract (Table 2). In accordance with our results, Singh et al. [49] found that flavonoids in GSE exert many health-promoting effects, including the ability to increase intercellular antioxidant levels, decrease capillary permeability and fragility, and scavenge oxidants and free radicals. Coinciding with our results, Saada et al. [50] reported that GSE attenuates the ionizing radiation-induced oxidative stress in heart tissues by a significant amelioration of serum lactate dehydrogenase, creatine phosphokinase, and aspartate aminotransferase enzymes activity. They also found that GSE attenuates the oxidative stress in pancreas tissues in rats by significant improvement in hyperglycemia and hyperinsulinemia.

**4.3. Molecular Analysis.** Our results represented in Figure 1 indicated that the treatment with GSE + triton or the GSE + triton + B6 (lanes 3 and 4) significantly downregulated the iNOS mRNA expression and maintains the control level (lane 1). Meanwhile, B6 + triton (lane 5) could downregulate the iNOS mRNA expression level, but in a nonsignificant manner in comparison to triton-treatment. The study of Olivenza et al. [51] indicated that the long-term exposure to stress leads to neurodegenerative changes in many species, including humans. This phenomenon was investigated through the role of endogenously released nitric oxide (NO) and the possible induction of the inducible NO synthase (iNOS) isoform. Ponnuswamy et al. [52] observed that immobilization of adult male rats for 6 h during 21 days (stress condition) increases the activity of a calcium-independent NO synthase and induces the expression of iNOS in cortical neurons as seen by immunohistochemical and western blot analysis. Potential proatherogenic effects of iNOS include iNOS mediated oxidative stress and iNOS expression in different cellular compartments. On the other hand, Terra et al. [53] reported that GSE reduced nitric oxide (NO) overproduction in stimulated macrophages via modulation of iNOS expression. Terra et al. [53] found that the IC<sub>50</sub> value of GSE (50  $\mu$ g/mL) was more potent than that of aspirin (3  $\mu$ M), indomethacin (20  $\mu$ M), and dexamethasone (9 nM) as regards suppressing

nitric oxide synthesis. Vitamin B6 (pyridoxal) pretreatment of RAW cells inhibited LPS-induced expression of iNOS and COX-2 at the mRNA and protein levels. It inhibited LPS-induced nuclear translocation of the NF- $\kappa$ B, the proinflammatory transcription factor. Furthermore, elevating dietary vitamin B6 suppressed NO production *in vivo* in response to LPS administration. Therefore, the anti-inflammatory effect of vitamin B6 is mediated by suppression of NF- $\kappa$ B activation [54]. The DNA degradation on agarose gel electrophoresis was represented in Figure 3. The observed smear pattern of DNA degradation could indicate a necrotic effect in triton-treated group (lane 2) as compared to the control (lane 1). Otherwise, GSE-treated group (lane 3) showed no evidence of DNA degradation compared to the triton-treated group (lane 2). Also, the treatment with GSE + triton + B6 or B6 + triton (lanes 4 and 5) could reduce the necrotic effect induced by triton (lane 2) but in lesser extent than the treatment with GSE + triton. In further support of our results, the previous studies indicated that triton X-100 efficiently induced the apoptotic cell death in hepatoma cell line [55]. Meanwhile, Mahmood [56] found that GSE is rich in polyphenols and flavonoids scavenging peroxynitrite-induced DNA damage in isolated human lymphocytes. Thereby, it protected DNA from nitrogen species-induced damage. Similarly, GSE demonstrated significant protective ability against oxidative damage in rat leukocytes [57]. GSE (60 mg/kg) also showed neuroprotective effects on neuronal injury induced by transient forebrain ischemia in gerbil achieved by inhibiting DNA damage in the gerbil hippocampus [58]. Furthermore, GSE (100 mg/kg, 30 days) could inhibit the accumulation of age-related oxidative DNA damage in the spinal cord and in various brain regions [59]. The protective role of anthocyanins and their derivatives against oxidative stress, apoptosis, and DNA damage in rat smooth muscle and hepatoma cells induced by tertiary-butyl hydroperoxide was confirmed by the study of Lazzé et al. [60]. Polyphenols are important metabolic modulators by virtue of their ability to moderate and influence several cellular processes such as signaling, proliferation, apoptosis, and redox balance [61]. Charvet et al. [62] suggested that pyridoxamine treatment scavenges lipid peroxides in mouse retina, as exemplified by isolevuglandins, and improves retinal mitochondrial morphology after animal exposure to bright light. Pyridoxamine supplementation should be considered for inclusion in antioxidant vitamin formulations. Furthermore, Depeint et al. [63] found that the protective effect of pyridoxal against protein carbonylation and DNA damage was maintained over time, and, in the case of DNA oxidation, pyridoxal exhibited an antidotal or rescue effect.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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RESEARCH ARTICLE

# Grape Seed Proanthocyanidin Extract Ameliorates Diabetic Bladder Dysfunction via the Activation of the Nrf2 Pathway

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## Abstract

Diabetes Mellitus (DM)-induced bladder dysfunction is predominantly due to the long-term oxidative stress caused by hyperglycemia. Grape seed proanthocyanidin extract (GSPE) has been reported to possess a broad spectrum of pharmacological and therapeutic properties against oxidative stress. However, its protective effects against diabetic bladder dysfunction have not been clarified. This study focuses on the effects of GSPE on bladder dysfunction in diabetic rats induced by streptozotocin. After 8 weeks of GSPE administration, the bladder function of the diabetic rats was improved significantly, as indicated by both urodynamics analysis and histopathological manifestation. Moreover, the disordered activities of antioxidant enzymes (SOD and GSH-Px) and abnormal oxidative stress levels were partly reversed by treatment with GSPE. Furthermore, the level of apoptosis in the bladder caused by DM was decreased following the administration of GSPE according to the Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) assay. Additionally, GSPE affected the expression of apoptosis-related proteins such as Bax, Bcl-2 and cleaved caspase-3. Furthermore, GSPE showed neuroprotective effects on the bladder of diabetic rats, as shown by the increased expression of nerve growth factor (NGF) and decreased expression of the precursor of nerve growth factor (proNGF). GSPE also activated nuclear erythroid2-related factor2 (Nrf2), which is a key antioxidative transcription factor, with the concomitant elevation of downstream hemeoxygenase-1 (HO-1). These findings suggested that GSPE could ameliorate diabetic bladder dysfunction and decrease the apoptosis of the bladder in diabetic rats, a finding that may be associated with its antioxidant activity and ability to activate the Nrf2 defense pathway.



**Competing Interests:** The authors have declared that no competing interests exist.

## Introduction

Grape seed proanthocyanidin extract (GSPE) is chemically composed of a mixture of pycnogenol and flavonoid including oligomeric proanthocyanidins[1], which are potent antioxidants extracted from grape seeds and skins. GSPE has been reported to demonstrate a remarkable spectrum of biological, pharmacological and therapeutic properties against oxidative stress[2, 3]. The antioxidative activities of GSPE were found to be much stronger than those of vitamins C and E[4]. Previous studies have indicated that GSPE showed a protective effect on cardiovascular disease[5], nephropathy[6, 7], atherosclerosis[8], and neuropathy[2, 9], among other conditions. Despite these pharmacological benefits, whether GSPE exerts protective effects on diabetic bladder function and the underlying mechanism remain obscure.

Diabetic bladder dysfunction (DBD), a common complication of DM[10], manifests with an overactive bladder (OAB), urgency, urinary retention, dysuria and other phenotypes such as decreased sensation, which severely affects the quality of life[11]. The mechanism of DBD is multifactorial, and both the changes in detrusor smooth muscle cells and innervation or function of the neuronal component have been implicated in diabetic cystopathy[12]. Although the etiology and pathogenesis of DBD are complicated, a growing number of studies has shown that oxidative stress plays a significant role in the development of DBD[13]. Some studies have indicated that the apoptosis of smooth muscle cells caused by oxidative stress possibly contributes to DBD[14, 15]. In addition, our previous results demonstrated that the impaired neuronal function and decreased expression of nerve growth factor (NGF) might be another potential mechanism of DBD[16, 17].

Oxidative stress plays crucial role in the injury of both smooth muscle and innervation in the bladder, a phenomenon that leads to DBD. Nuclear erythroid related factor2 (Nrf2) is a transcription factor involved in regulating the cellular antioxidative responses and redox status by promoting the expression of antioxidative genes (phase II genes) through the antioxidant response element (ARE)[18, 19]. The Nrf2 signaling pathway can be activated by many types of phytochemicals as well as food polyphenols[20, 21]. The activation of the Nrf2 signaling pathway that leads to the up-regulation of antioxidative genes protects neural functions and demonstrates large effects on neurodegenerative diseases[20, 22]. In addition, some studies have suggested that the activation of the Nrf2 pathway plays roles in myocyte differentiation as well as muscular contractile and metabolic properties in a diabetic model of muscle atrophy[23]. In some other studies, the activation of the Nrf2 pathway could protect various cells against apoptosis [24].

In the present study, we used the streptozotocin (STZ)-induced type 1 diabetic mouse model to explore whether GSPE could improve diabetic bladder dysfunction. In addition, whether the protective effect of GSPE was associated with the activation of the Nrf2 signal pathway was also investigated.

## Materials and Methods

### Materials and animals

GSPE (comprising 56% dimeric proanthocyanidins, 12% trimeric proanthocyanidins, 6.6% tetrameric proanthocyanidins and small amounts of monomeric and highmolecular-weight oligomeric proanthocyanidins and flavanols) was provided by Jianfeng Inc. (Tianjin, China). The components of GSPE were analyzed using high-performance liquid chromatography with gas chromatography-mass spectrometry detection. Rabbit anti-Nrf2 antibody, mouse anti-HO-1 antibody, rabbit anti-NGF antibody, rabbit anti-histone H2A.X antibody, and rabbit anti-Bax antibody were obtained from Abcam (Cambridge, UK); rabbit anti-bcl-2 antibody, mouse anti-actin antibody, goat-anti-rabbit IgG-HRP, and goat-anti-mouse IgG-HRP were obtained from

Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-Cleaved-Caspase-3 was obtained from Cell Signaling Technology (Beverly, MA, USA); rabbit anti-pro-NGF was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The total Superoxide Dismutase (SOD) assay kit, Glutathione Peroxidase (GSH-Px) assay kit, and Malondialdehyde (MDA) assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The BCA assay kit, Nuclear and Cytoplasmic Protein Extraction Kit, and TdT-mediated dUTP Nick-End Labeling (TUNEL) Apoptosis Assay Kit were from Beyotime Institute of Biotechnology (Beijing, China).

Wistar rats (Female, 8 weeks old, weighing approximately 250 g) were purchased from the Animal Center of Shandong University (license number: SCXX20090011). The female rats were suitable for the cystometrography. The rats were fed under a 12 h light/dark cycle and had free access to standard rat chow and tap water under the condition of 24°C and 60% humidity. Animal care and management were approved by the Ethics Committee of Qilu Hospital of Shandong University.

## Induction of DM

The rats were fasted for 18 h ahead of the induction of diabetes. Intraperitoneal injection of streptozotocin (50 mg/kg body weight; Sigma-Aldrich), which was freshly dissolved in citrate buffer (0.1 M, pH 4.5), was used to induce DM. The control rats were injected with citrate buffer equally. At 72 h after STZ injection, tail venous blood samples were used for the measurement of blood glucose via a glucose strip test in a glucometer (Roche Diagnostics Corporation, Indianapolis, IN, USA). Rats with fasting serum glucose levels above 300 mg/dl were included in the diabetic animal group.

## Experimental design

After adaptation to the laboratory environment, thirty rats were randomly divided into three groups ( $n = 10$ , each) as follows: group I, the control group (Control); group II, the untreated diabetic group (DM); group III, the GSPE-treated group (DM/GSPE). The GSPE-treated group was given 250 mg/kg of GSPE, which was in powder form and dissolved in normal saline solution, by oral gavage once daily for 8 weeks. The rats in groups I and II were treated using isometric normal saline. After 8 weeks of feeding, the intravesical pressure was measured, and then the bladder tissue was removed for subsequent analysis.

## Cystometrography

The procedure for recording the intravesical pressure under isovolumetric conditions has been applied in our previous study[25]. Briefly, under successful anesthesia by urethane (1.2 g/kg subcutaneously), a PE-240 catheter was used for tracheal intubation. The bladder was exposed by lower-midline abdominal incision, and then the ureters were cut and tied. A PE-50 catheter was inserted through the bladder dome and was connected with a pressure transducer (Laborie Medical Technologies Inc., Beijing, China) via a three-way stopcock for recording the intravesical pressure and a micromedicine infusion-pump (Hangzhou Zeda Instruments Co., Ltd., Hangzhou, China) for infusing saline into the bladder. Thereafter, saline was slowly pumped into the bladder at 0.05 ml per minute until rhythmic bladder contractions appeared. Finally, the intravesical pressure was recorded in half an hour.

## Bladder tissue preparation

Following cystometrography, rats were sacrificed using the cervical dislocation method. Bladder tissues were removed and divided into four parts immediately. The first part of the bladder

tissues in each group was used for paraffin sections. After fixation in 10% formalin solution, the bladder tissues were dehydrated by 70% and 100% alcohol solutions and then embedded in paraffin. Next, 4- $\mu$ m-thick sections were cut using a microtome and placed on glass slides. Thereafter, hematoxylin and eosin (HE) staining, immunohistochemistry and Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) staining were performed utilizing these sections.

The second part of the tissue was homogenized in ice-cold PBS for the estimation of oxidative stress in the bladder. The third part was homogenized in ice-cold RIPA buffer (Beyotime Institute of Biotechnology) containing 1 mM PMSF (Beyotime Institute of Biotechnology) to extract the total protein of the tissue. The last portion was used for nucleoprotein extraction using a nuclear extraction kit according to the manufacturer's instructions. The protein concentrations were detected using a BCA assay kit. The prepared tissue homogenate and extracted proteins were stored separately at -80°C for further analyses.

### Bladder histopathological examination and immunohistochemical staining

After regular deparaffinization, the sections were stained with hematoxylin and eosin (HE) and observed from  $\times 100$  to  $\times 400$  magnifications.

Regular immunohistochemical staining was conducted as previously described[17]. Briefly, SP-9000 SP link Detection Kit (ZSGB-BIO, Beijing, China) and DAB detection kit (ZSGB-BIO) were used for the immunohistochemical studies according to the manufacturer's instructions. Rabbit anti-rabbit Nrf2 antibody was utilized at a dilution of 1/200. Two pathologists conducted the histopathological observations in a blinded manner.

### Estimation of oxidative stress in the bladder

Oxidative stress in the bladder was measured as described previously[17]. GSH-Px, T-SOD, and MDA assays in bladder tissues were estimated by spectrophotometry according to the manufacturer's protocols. Briefly, GSH-Px activity was measured using the enzyme-catalyzed reaction product (reduced glutathione) and the absorbance was recorded at 412 nm. The measurement of T-SOD activity was based on the combination of xanthine and xanthine oxidase, and the absorbance was read at 550 nm. The MDA level was detected using the thiobarbituric acid (TAB) method and the maximum absorbance was at 532 nm. The activities of GSH-Px and T-SOD are expressed as U/mg pro, while the level of MDA is expressed as nmol/mg pro.

### (TUNEL) Assay to detect the level of apoptosis

The one-step TUNEL apoptosis assay kit was used to detect the apoptosis level in the bladder according to the manufacturer's instructions. Briefly, the sections were regularly hydrated and incubated with a 3% hydrogen peroxidase solution to remove the endogenous peroxidase. Next, the sections were incubated with the TUNEL mixture in a humidified and dark chamber for 30 minutes. Finally, the sections were stained with DAPI (Sigma-Aldrich) for 10 minute at room temperature to stain cell nuclei. The stained sections were observed under a fluorescence microscope. The apoptotic cells showed red fluorescence excited by light with a wavelength of 550 nm, while the cell nuclei demonstrated blue fluorescence excited by light with a wavelength of 358 nm. The percentage of TUNEL-positive cells was recorded.

### Western blot analysis

Western blot analysis was performed as described previously[25]. Protein (60  $\mu$ g) was separated by denaturing SDS—PAGE and transferred onto a PVDF Membrane (Millipore, Bedford,

MA, USA), utilizing electrophoretic transfer (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% non-fat milk for 1 h at room temperature and incubated overnight at 4°C with the primary antibody (in TBST with 5% BSA). Next, the membrane was washed with TBST three times and then incubated with secondary antibodies conjugated with HRP for 2 h. Finally, the bands were revealed using the super ECL plus detection reagent (Applygen Technologies Inc., Beijing, China). Relative protein quantification was measured using Quantity One software (Bio-Rad, Hercules, CA, USA).

## Statistical analysis

All of the data were presented as the mean  $\pm$  standard error of mean (SEM) ( $n = 5$  at least per group). Comparisons were performed using one-way ANOVA for the different groups followed by Dunnett's test for comparisons between two groups. A  $P$  value less than 0.05 was considered to indicate statistical significance. All of the data were analyzed using the Statistical Package for Social Sciences (Version 19.0; SPSS, Chicago, IL, USA).

## Results

### Effects of GSPE on general characteristics of diabetic rats

As shown in Table 1, diabetic rats displayed a statistical weight loss despite taking in more food and water than control rats ( $241.41 \pm 7.38$  g versus  $311.92 \pm 4.69$  g, respectively;  $P = 0.000$ ) at the end of 8 weeks. GSPE obviously increased the body weight of the diabetic rats ( $272.86 \pm 6.89$  g versus  $241.41 \pm 7.38$  g, respectively;  $P = 0.038$ ). However, GSPE had no obvious effects on the blood glucose level at 8 weeks ( $444.27 \pm 8.19$  mg/dl versus  $455.39 \pm 8.30$  mg/dl, respectively;  $P = 0.717$ ). In addition, the urine volume per day was significantly increased in diabetic rats than in control animals ( $65.32 \pm 1.74$  ml versus  $16.95 \pm 0.89$  ml, respectively;  $P = 0.000$ ), a situation that aggravated the burden of the bladder. Nevertheless, GSPE did not influence the urine volume of the diabetic rats ( $60.53 \pm 2.92$  ml versus  $65.32 \pm 1.74$  ml, respectively;  $P = 0.433$ ).

### GSPE protected against diabetic bladder dysfunction

Cystometrography was performed to show the change in bladder function in different groups as previously described [15, 26]. We mainly focused on the change in the maximal detrusor pressure and resting pressure, which represented the contractile function of the bladder. We found that the maximal detrusor pressure was significantly decreased in diabetic rats compared to control animals ( $P = 0.003$ ) (Fig 1A and 1B). Application of GSPE had a statistical influence

**Table 1. General characteristics of the three groups.**

General characteristics	Control	DM	DM+GSPE
Body weight at 0 weeks (g)	242.42 $\pm$ 1.40	242.61 $\pm$ 1.36	242.84 $\pm$ 1.54
Body weight at 8 weeks (g)	311.92 $\pm$ 4.69	241.41 $\pm$ 7.38 <sup>a</sup>	272.86 $\pm$ 6.89 <sup>b</sup>
Blood glucose at 8 weeks (mg/dl)	95.17 $\pm$ 3.31	455.39 $\pm$ 8.30 <sup>c</sup>	444.27 $\pm$ 8.19
Urine volume per day (ml)	16.95 $\pm$ 0.89	65.32 $\pm$ 1.74 <sup>d</sup>	60.53 $\pm$ 2.92

The values represent the mean  $\pm$  SEM of 10 animals per group;

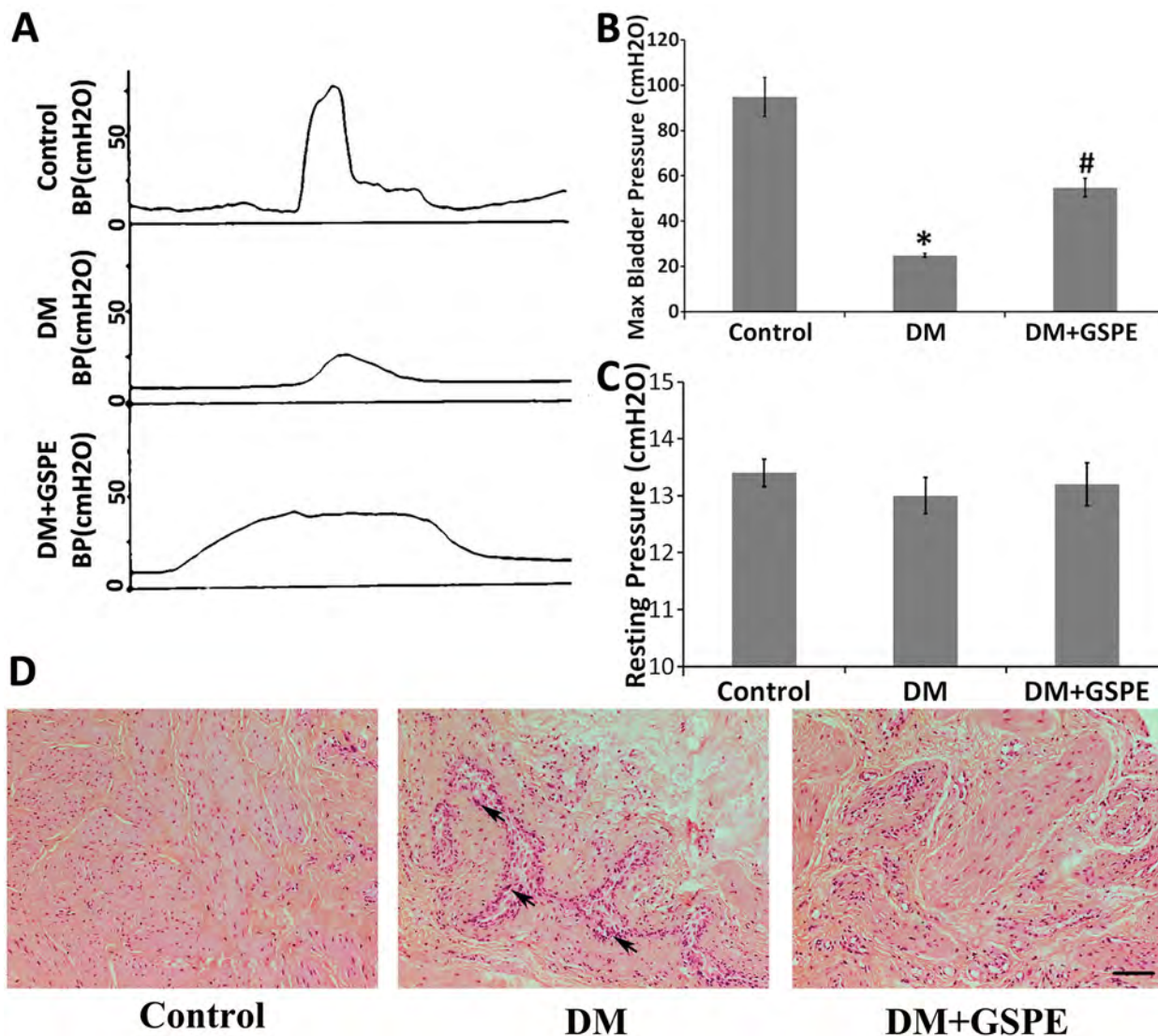
<sup>a</sup>,  $n = 10$ ,  $P = 0.000$  vs. Control

<sup>b</sup>,  $n = 10$ ,  $P = 0.038$  vs. DM

<sup>c</sup>,  $n = 10$ ,  $P = 0.000$  vs. Control

<sup>d</sup>,  $n = 10$ ,  $P = 0.000$  vs. Control

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**Fig 1. Functional and morphological evaluation of the bladder in the control, DM, DM/GSPE group.** A, cystometrograms of the three groups; B, the statistical results of maximal bladder pressure. \*  $n = 5$ ,  $P = 0.003$  vs. the Control group. #  $n = 5$ ,  $P = 0.004$  vs. the DM group; C, the statistical results of resting pressure; D, histological sections of the bladder via HE staining showed that there was structural damage and inflammatory cell infiltration in the DM group while GSPE could improve the morphological changes. The arrow shows inflammatory cells infiltration. Scale bar, 20  $\mu$ m.

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on maximal detrusor pressure compared to the DM group ( $P = 0.004$ ) (Fig 1A and 1B). However, there were no significant changes about the resting pressure in different groups (Fig 1A).

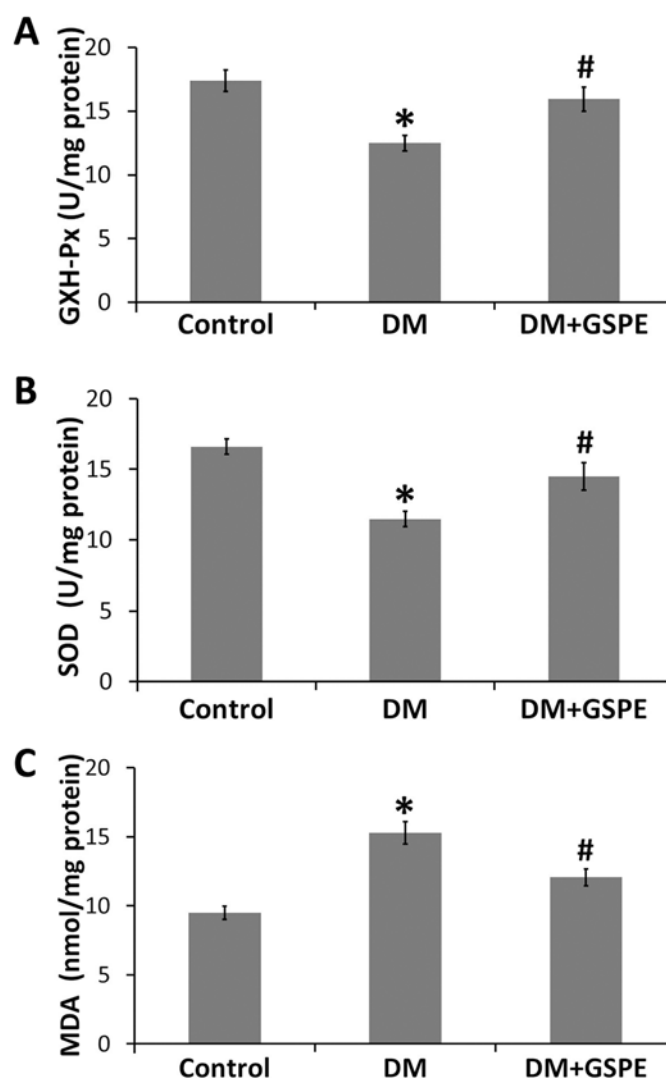
### GSPE improved DM induced histological changes in bladder

As displayed in Fig 1C, the results of HE staining showed that GSPE possessed the protective effect on diabetic bladder. At the end of 8 weeks, the bladder tissues in DM group were thinner than control group. DM caused obvious histological changes including structure damage of the detrusor smooth muscle and inflammatory cells infiltration in rat bladder tissues. Treatment with GSPE significantly alleviated the histological damage in bladder of diabetic rats (Fig 1C).



## GSPE attenuated the oxidative stress in diabetic bladder

Many studies demonstrated that the activity of SOD, GXH-Px and the level of MDA was the indicators of oxidative stress status [17, 27]. Our studies showed that the activities of GXH-Px and SOD in bladder were significantly decreased in diabetic rats by 28% and 34%, respectively, compared with the controls ( $P < 0.01$ ). However, treatment with GSPE could restore the GXH-Px and T-SOD activities significantly ( $P = 0.025$  and  $P = 0.023$ , respectively) (Fig 2A and 2B). The level of MDA in the bladder compared with the controls was elevated markedly due to the influence of DM ( $P = 0.000$ ). However, the results also revealed that the level of MDA was decreased significantly via the administration of GSPE ( $P = 0.013$ ) (Fig 2C).



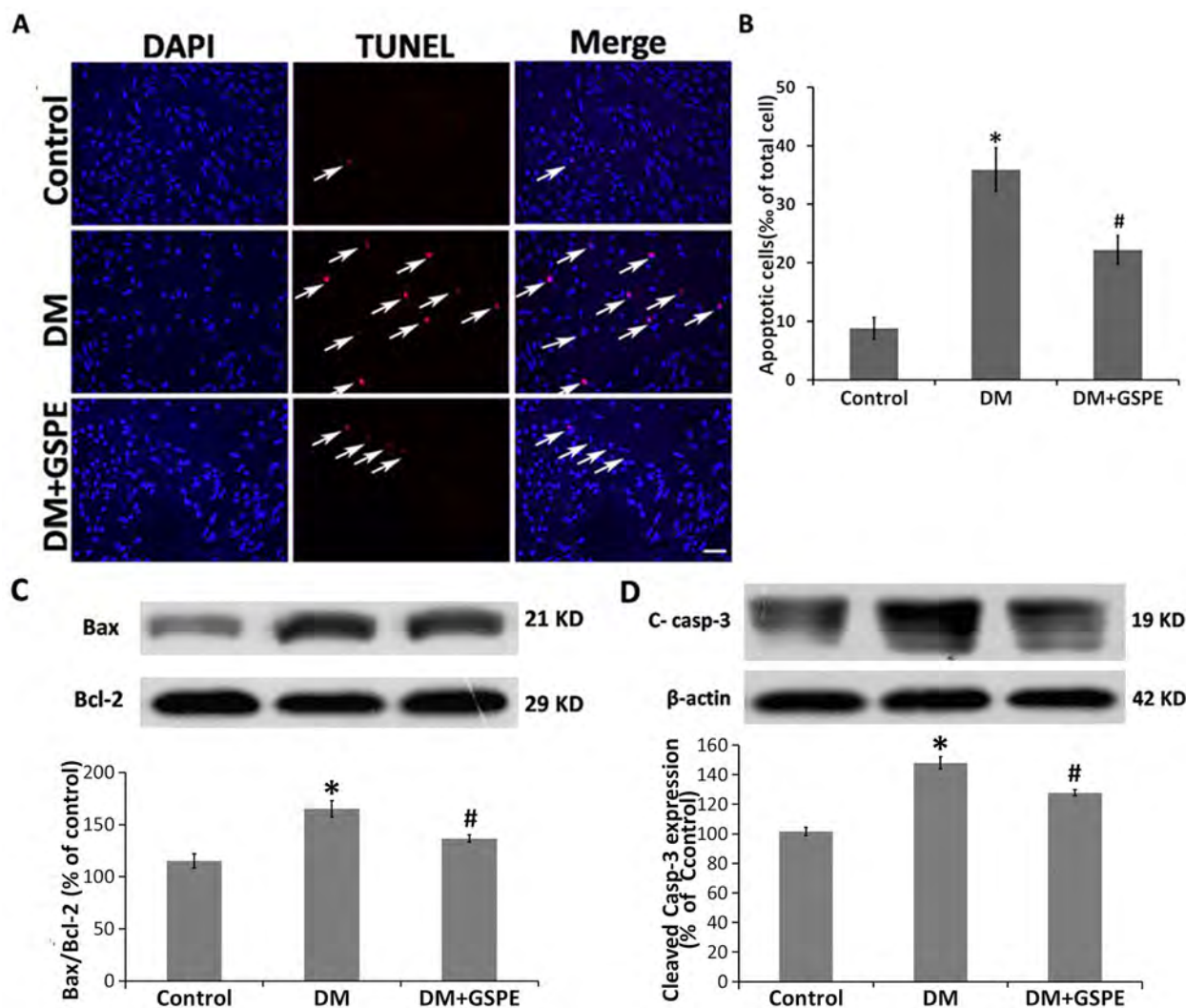
**Fig 2. Effect of GSPE on oxidative stress in the bladder.** A, GXH-Px activity in the bladder of the three groups, \*  $n = 9$ ,  $P = 0.001$  vs. the Control group. #  $n = 9$ ,  $P = 0.025$  vs. the DM group; B, T-SOD activity in the bladder of the three groups, \*  $n = 9$ ,  $P = 0.000$  vs. the Control group. #  $n = 9$ ,  $P = 0.023$  vs. the DM group; C, MDA level in the bladder of the three groups, \*  $n = 9$ ,  $P = 0.000$  vs. the Control group. #  $n = 9$ ,  $P = 0.013$  vs. the DM group.

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## Protective effects of GSPE on apoptosis in the diabetic bladder

Because the diabetic bladder dysfunction was associated with the apoptosis level of the bladder caused by oxidative stress[14, 28], we next examined whether GSPE had any protective effects on the apoptosis level of the diabetic bladder by TUNEL staining. The number of TUNEL-positive cells in the bladder of diabetic rats was markedly increased compared with the control animals ( $P = 0.002$ ), whereas GSPE statistically decreased the bladder TUNEL-positive cells of diabetic rats ( $P = 0.049$ ) (Fig 3A and 3B). In accordance with TUNEL results, Western blotting demonstrated a significant increase in the expression of cleaved-caspase-3 in the bladder of DM rats ( $P = 0.000$ ), while treatment with GSPE could significantly decrease the expression of cleaved-caspase-3 (Fig 3D) ( $P = 0.036$ ).



**Fig 3. Effect of GSPE on apoptosis in the bladder.** A. TUNEL staining showing the cell apoptosis of the bladder in the control, DM and DM/GSPE groups. A nucleus stained with DAPI (blue); the arrow shows TUNEL staining (red). Scale bar, 50  $\mu$ m. B. The statistical results of TUNEL staining, \*  $n = 5$ ,  $P = 0.002$  vs. the Control group. #  $n = 5$ ,  $P = 0.049$  vs. the DM group. C. The protein expression of Bax to Bcl2 ratio in the bladder of the three groups, \*  $n = 7$ ,  $P = 0.001$  vs. the Control group. #  $n = 7$ ,  $P = 0.028$  vs. the DM group. D. The protein expression of cleaved caspase-3 in the bladder of the three groups, \*  $n = 5$ ,  $P = 0.000$  vs. the Control group. #  $n = 5$ ,  $P = 0.036$  vs. the DM group.

doi:10.1371/journal.pone.0126457.g003

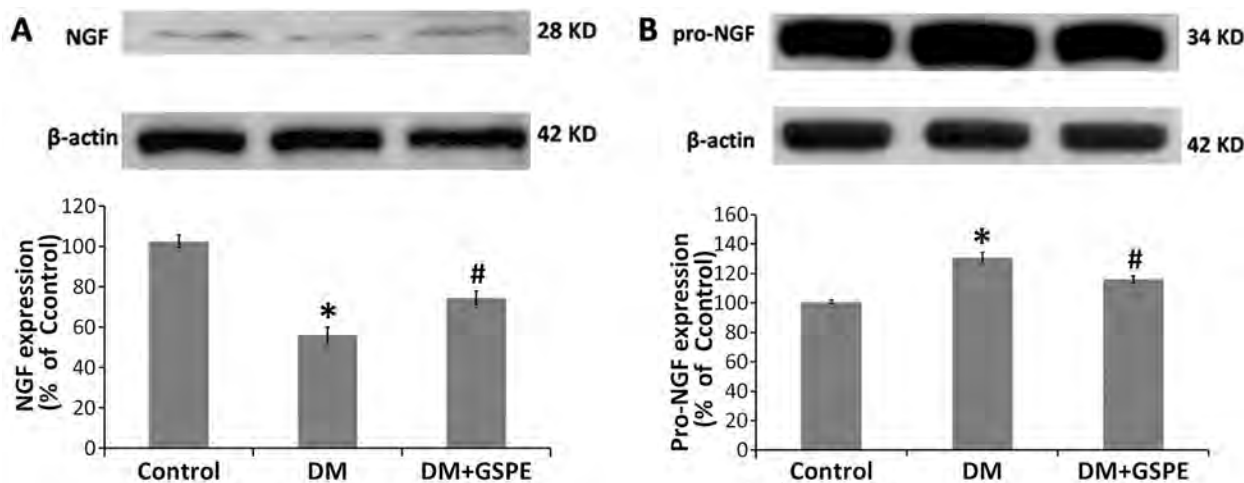
Because previous studies have indicated that diabetic cystopathy was closely related to mitochondrial apoptotic pathway-mediated bladder apoptosis [28], we next investigated whether GSPE could avert the activation of the mitochondrial apoptotic pathway in the diabetic bladder. The Bax to Bcl2 expression ratio was significantly increased in the bladder of DM rats compared with the control animals according to Western blot analysis ( $P = 0.001$ ). GSPE significantly averted the increase in the Bax to Bcl2 expression ratio in the bladder of DM rats ( $P = 0.028$ ) (Fig 3C).

### GSPE restored the balance of the expression of NGF and proNGF in the diabetic bladder

DBD was partly caused by DM-induced peripheral neuropathy with the reduced expression of NGF [29]. Because the changes in the levels of NGF and proNGF were associated with DM-induced neural injury [30], we next examined the expression of NGF and proNGF in the bladder of different groups. As shown in Fig 4, the expression of NGF was significantly decreased in the DM group compared with the controls ( $P = 0.000$ ), while the expression of proNGF was increased ( $P = 0.001$ ). After the administration of GSPE, the balance of proNGF/NGF was restored. The expression of NGF was increased in the DM/GSPE group compared with the DM group ( $P = 0.025$ ), while the expression of proNGF was statistically reduced ( $P = 0.031$ ).

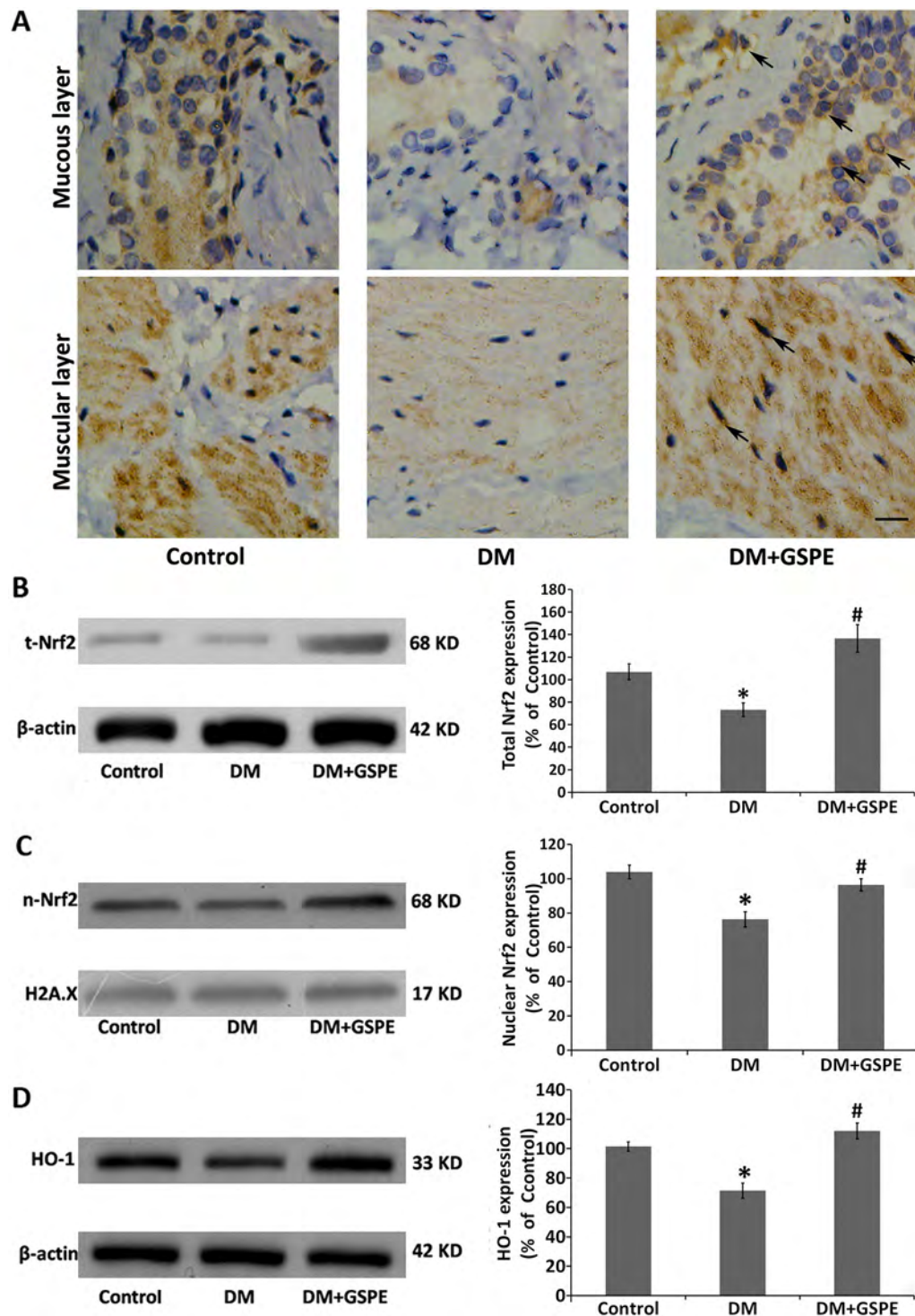
### The protective effects of GSPE involved the activation of Nrf2 pathway

The transcription factor Nrf2 is a vital mediator involved in regulating cellular antioxidative responses. Activation of the Nrf2 pathway leads to the inhibition of apoptosis [24] and neuroprotective effects [20, 22]. We next investigated the effects of GSPE on the expression of Nrf2. As shown in Fig 5A, immunohistochemical results demonstrated that the expression of Nrf2 was qualitatively increased both in the mucous and muscular layers of the bladder by the treatment with GSPE compared with the DM group. However, the expression of Nrf2 was declined in the bladder of the DM group compared with the controls. More importantly, the expression of Nrf2 was mainly located in the nucleus of the bladder cells. Western blotting also showed that the expression of Nrf2 was significantly increased in the bladder of the DM/GSPE group



**Fig 4. Effect of GSPE on the expression of NGF and proNGF in the bladder.** A. The protein expression of NGF in the bladder of the three groups, \*  $n = 5$ ,  $P = 0.000$  vs. the Control group. #  $n = 5$ ,  $P = 0.025$  vs. the DM group. B. The protein expression of proNGF in the bladder of the three groups, \*  $n = 5$ ,  $P = 0.001$  vs. the Control group. #  $n = 5$ ,  $P = 0.031$  vs. the DM group.

doi:10.1371/journal.pone.0126457.g004



**Fig 5. Effect of GSPE on the Nrf2 pathway in the bladder.** A. IHC staining results of Nrf2 in the mucous and muscular layers of bladder tissues. The arrow shows Nrf2 positive staining is mainly located in the nucleus of the bladder cells. Scale bar, 50  $\mu$ m. B. The protein expression of total Nrf2 in the bladder of the three groups, \*  $n = 7$ ,  $P = 0.011$  vs. the Control group. #  $n = 7$ ,  $P = 0.004$  vs. the DM group. C. The protein expression of nuclear Nrf2 in the bladder of the three groups, \*  $n = 7$ ,  $P = 0.003$  vs. the Control group. #  $n = 7$ ,  $P = 0.018$  vs. the DM group. D. The protein expression of HO-1 in the bladder of the three groups, \*  $n = 5$ ,  $P = 0.005$  vs. the Control group. #  $n = 5$ ,  $P = 0.002$  vs. the DM group.

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compared with the DM group ( $P = 0.004$ ), while Nrf2 was decreased in the bladder of the DM group compared with the controls ( $P = 0.011$ ) (Fig 5B).

Previous studies have shown that Nrf2, as an antioxidative transcription factor, plays a role only by entering the nucleus[21]; thus, we measured the expression of Nrf2 in the nucleus. Consistent with previous studies, the level of Nrf2 in the nucleus was also markedly increased in the DM/GSPE group compared with the DM group ( $P = 0.018$ ), and the level of Nrf2 in the nucleus was significantly decreased in the DM group compared with the controls ( $P = 0.003$ ) (Fig 5C).

We next investigated the antioxidative function of Nrf2 by measuring the expression of its downstream target gene HO-1. As shown in Fig 4D, the expression of HO-1 was increased in the DM/GSPE group compared with the DM group ( $P = 0.002$ ), a finding that was consistent with the expression of Nrf2 in the nucleus. However, our results showed that no significant difference in the expression of total Nrf2, nuclear Nrf2 and total HO-1 could be observed in the bladder from the DM/GSPE group compared with that from the controls.

## Discussion

The number of diabetic patients is increasing greatly, and diabetics are subjected to various costly complications. Diabetic bladder dysfunction (DBD), which is also called diabetic cystopathy[31], is one of the most common complications of DM[11, 32]. The clinical DBD manifestations consist of storage and voiding problems, which substantially affect the quality of life [33, 34]. Previous studies have shown that the pathogenesis of DBD was associated with the myogenic and neurogenic alterations of the bladder[11, 16, 35, 36]. In our studies, we demonstrated that the contractile function of the bladder was damaged significantly in diabetic groups along with the morphological changes (Fig 1). However, we proved here for the first time that the administration of GSPE for 8 weeks could significantly increase the pressure of bladder detrusor and ameliorate the histopathologic changes of the bladder (Fig 1). These results indicated that GSPE could protect rats against DM-induced dysfunction and morphological damage of the bladder.

Previous studies have shown that oxidative stress caused by hyperglycemia plays important roles in the pathogenesis of DBD[11–15]. For instance, the decrease in detrusor smooth muscle force induced by DM was related to the high level of lipid peroxidation products, high expression of aldose reductase and activation of the polyol pathway, all of which were ascribed to oxidative stress[37]. Oxidative stress could also induce apoptosis of the detrusor smooth muscle, which could lead to caused DBD[14, 15, 28]. Oxidative damage might cause neurodegeneration in the bladder by interfering with neurotrophins, such as NGF, for neuron survival[16, 29, 38]. Consistent with previous studies, our results showed that the level of MDA, as an indicator of lipid peroxidation, was higher in the bladder of the DM group than in controls. The activities of GSH-Px and SOD, as markers of redox status, were significantly decreased in the bladder of the DM group than in the controls (Fig 2). GSPE has been reported to possess the prominent properties against oxidative stress[2, 3]. In the present study, GSPE decreased MDA production in the bladder of the DM group (Fig 2C), a finding that may be ascribed to the potent antioxidant activities of GSPE. We also found that GSPE significantly enhanced the activities of GSH-Px and SOD in the bladder of diabetic rats (Fig 2A and 2B). Our results suggested that GSPE could partly alleviate oxidative stress by decreasing lipid peroxide and increasing the activities of antioxidative enzymes in the bladder of diabetic rats.

Cell apoptosis induced by oxidative stress is an important mechanism of diabetic bladder dysfunction[14, 28, 39]. A previous study showed that oxidative stress induced apoptosis in the bladder via the activation of the mitochondrial apoptotic pathway[28]. In accordance with the



previous studies, our results showed that the apoptotic level of the bladder was significantly increased in the DM group compared with that in the controls via the TUNEL staining (Fig 3A and 3B). The increased expression of the ratio of Bax to Bcl2 content in the bladder of diabetic rats showed the activation of the mitochondrial apoptotic pathway (Fig 3C). Cleaved caspase-3 was the essential apoptotic initiator and was also activated in the bladder of diabetic rats (Fig 3D). Many studies have shown that GSPE possessed the protective effect on apoptosis induced by oxidative stress[40–43]. Consistently, we found that administration of GSPE could significantly decrease the apoptotic level in the bladder of the DM group by down-regulating the expression of the ratio of Bax to Bcl2 (Fig 3). Thus, our findings suggested that GSPE might protect the bladder against apoptosis induced by oxidative stress via decreasing the Bax/Bcl2 ratio.

As previously described, the changes in the NGF level, which led to neurodegeneration in the bladder, is a possible mechanism of DBD. We next discussed the changes in the expression of NGF in the bladder of diabetic rats. Numerous studies have demonstrated that the balance between the proNGF and NGF levels played important roles in homeostasis. The disruption of the balance possibly led to neurodegeneration, which might further lead to many diseases such as Alzheimer's disease[30, 44]. ProNGF, as the precursor of NGF, has distinct functions from NGF. Specifically, proNGF with a higher affinity for p75<sup>NTR</sup> determined apoptotic signaling, while NGF with a higher affinity for TrkA<sup>NTR</sup> activated the trophic signaling pathway[45, 46]. Our previous studies also indicated that the changes in the proNGF/NGF balance might be a possible mechanism of diabetic urethral dysfunction, leading to the changes in the expression of  $\alpha$  receptor[25]. In the present study, we found that the balance of the proNGF and NGF level was disrupted in the bladder of diabetic rats. The expression of proNGF was increased significantly in the bladder of DM group compared with that in the controls, while the expression of NGF was decreased (Fig 4). GSPE had a protective effect on peripheral nerves in STZ-induced diabetic rats[9]. Another study showed that the intervention of GSPE was possibly helpful in the treatment of Alzheimer's disease by decreasing the levels of a memory-impairing A $\beta$  oligomer[47]. In the present study, GSPE could significantly increase the expression of NGF and decrease the level of proNGF in the bladder of DM rats(Fig 4). Thus, in the present study, we demonstrated that GSPE could restore the balance of the proNGF/NGF level in the bladder of diabetic rats for the first time.

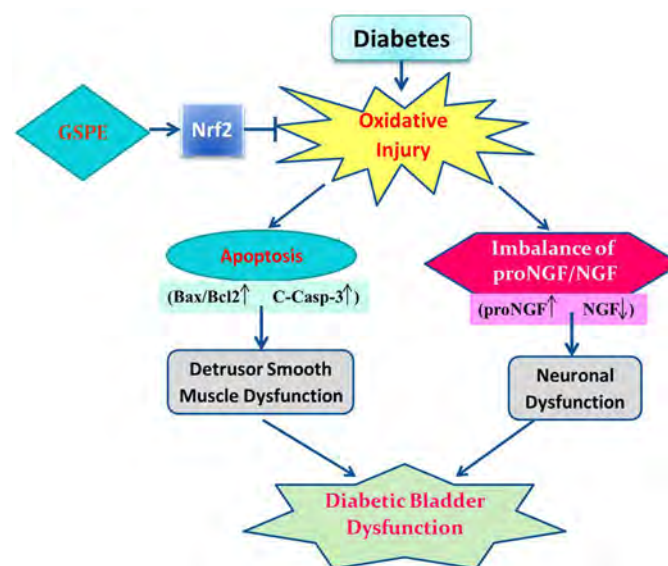
The Nrf2 signaling pathway is known to exert antioxidative effects on various cells[18, 19]. Activation of the Nrf2 signaling pathway was reported to protect various cells against apoptosis[24, 48]. Nrf2 was reported to regulate NGF mRNA expression in glioblastoma cells and normal human astrocytes[49]. Some studies have shown that the activation of the Nrf2 pathway could increase the expression of NGF to promote neurite outgrowth in high glucose-treated neurons.[50]. In addition, food polyphenols could activate the Nrf2 signal pathway[20, 21]. In the present study, we first found that the administration of GSPE could enhance the activity of the Nrf2 pathway. After the administration of GSPE, the expression of Nrf2 in the bladder was increased significantly compared with the DM groups (Fig 5A and 5B). Because Nrf2, as a transcription factor, plays a role in the nucleus, we next found that the expression of nuclear Nrf2 was also markedly increased in the bladder of the DM/GSPE group compared with the DM group (Fig 5C). The expression of HO-1, the downstream gene of the Nrf2 pathway, was consistent with the expression of Nrf2 (Fig 5D). In addition, the changes in the activity of SOD and GPH-Px also agreed with the changes in the Nrf2 pathway (Fig 2A and 2B). Thus, our findings suggested that the protective effects of GSPE on the bladder of diabetic rats might be due to the activation of the Nrf2 signaling pathway.

However, there are still a few restrictions in the present study. We used the STZ-induced type 1 DM model to investigate DBD, which was consistent with most previous studies[12, 35],



while most of the patients with DBD have type 2 DM clinically. There are some differences between type 1 and type 2 DM such as the insulin level, blood glucose level and metabolic properties[51]. However, the type 1 DM model is widely used to simulate the pathogenesis of DBD because the damage induced by hyperglycemia is the primary cause of DBD. In this study, we did not provide insulin treatment for DM group. It is known that insulin is able to interact with NGF receptor TrkA in PC12 cells[52] and NGF plays an important role in the neurological function of the bladder. So insulin may have influence on the neurological function of the bladder. In addition, in the present study, we did not use the dose gradients in the administration of GSPE, partly because we found that the protective effect of GSPE on diabetic rats was excellent and safe at the dose of 250 mg/kg in our previous studies[53]. Although GSPE is extracted from natural plants and is relatively safe, the side effects and pharmacokinetics of GSPE should be further investigated in the future.

In summary, our study demonstrated for the first time that GSPE has significant protective effects against diabetic bladder dysfunction, a finding that may be associated with the activation of the Nrf2 pathway in the bladder of diabetic rats. As illustrated in Fig 6, DM-induced oxidative stress triggers apoptosis of the bladder by activating mitochondrial pathways, leading to detrusor smooth muscle dysfunction. At the same time, oxidative stress disrupts the balance of the proNGF and NGF level, leading to neuronal dysfunction of bladder. Detrusor smooth muscle dysfunction and neuronal dysfunction are two important mechanisms of DBD. GSPE treatment attenuates DM induced oxidative damage by enhancing and activating Nrf2. Next, GSPE diminished the apoptotic level of the bladder and restored the balance of the proNGF and NGF levels. Thus, the administration of GSPE ameliorates DM-induced bladder dysfunction and



**Fig 6. Schematic diagram of the protective mechanism for GSPE in DBD.** “→” indicates activation or induction, and “|” indicates inhibition or blockade. DM induces oxidative stress in the bladder. The enhanced oxidative stress induces apoptosis of the bladder by activating the mitochondrial pathways, leading to the detrusor smooth muscle dysfunction. At the same time, oxidative stress also disrupts the balance of the proNGF and NGF level, leading to neuronal dysfunction of bladder. Detrusor smooth muscle dysfunction and neuronal dysfunction are two important mechanisms of DBD. GSPE treatment attenuates DM-induced oxidative damage most likely by enhancing and activating Nrf2. Additionally, the use of GSPE decreases the level of apoptosis and retains the balance of the proNGF/NGF level. Finally, the use of GSPE improves detrusor smooth muscle dysfunction and neuronal dysfunction. Therefore, the use of GSPE ameliorates diabetic bladder dysfunction.

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histopathologic changes. Therefore, the administration of GSPE in diabetic patients is promising and, in the future, may be recommended to protect against diabetic bladder dysfunction.

## Author Contributions

Conceived and designed the experiments: BS SC YZ. Performed the experiments: SC YZ Zhi-feng Liu ZG BL ZZ XJ Zhengfang Liu LM YY. Analyzed the data: BS SC YZ DZ. Contributed reagents/materials/analysis tools: BS YZ BL. Wrote the paper: BS SC YZ.

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# AUSTRALIAN FUNCTIONAL NUTRACEUTICAL FLAVOURS, FRAGRANCES & INGREDIENTS

Plant Extracts-Naturally Fermented Fruits and Vinegars  
Cold Pressed Oils-Essential Oils-Phenolic Rich Powders

## GRAPE EXTRACTS

### CONCENTRATED PHENOLIC RICH PHYTONUTRIENTS

Create-Innovate-Differentiate your  
products and formulations  
with a new class of shelf stable  
Grape Extracts.



#### RED GRAPE SKIN EXTRACT

Concentrated Red Grape Skin Extract with  
Resversatrol and Anthocyanins.

Total Phenolic content: 30,000-70,000 mg/L

#### RED GRAPE SEED EXTRACT

Concentrated Red Grape Seed Extract with  
Anthocyanins, Phenols and Antioxidants.

Total Phenolic content: 30,000-70,000 mg/L

#### WHITE GRAPE SKIN EXTRACT

Concentrated Red Grape Skin Extract with  
Resversatrol and Anthocyanins.

Total Phenolic content: 30,000-70,000 mg/L

#### WHITE GRAPE SEED EXTRACT

Concentrated Red Grape Seed Extract with  
Anthocyanins, Phenols and Antioxidants.

Total Phenolic content: 30,000-70,000 mg/L

**It takes 100kg of grapes  
to make 50g of grape extract**

**Applications**  
**Functional Foods & Beverages**  
**Natural Healthcare**  
**Cosmeceutical**

#### FERMENTED RED GRAPE SEED EXTRACT

Shelf stable preservative free slightly  
acidic red grape seed extract with  
concentrated Phenolics, Antioxidants.  
& Anthocyanins. Total Phenolic content  
10,000-50,000mg/L

#### FERMENTED WHITE GRAPE SEED EXTRACT

Shelf stable preservative free slightly  
acidic white grape seed extract with  
concentrated Phenolics, Antioxidants..  
Total Phenolic content 10,000-50,000mg/L

#### FERMENTED RED GRAPE SKIN EXTRACT

Shelf stable preservative free slightly  
acidic red grape skin extract with  
concentrated Phenolics, Antioxidants..  
Anthocyanins & Resveratrol.Total Phenolic  
content 10,000-50,000mg/L

#### FERMENTED WHITE GRAPE SKIN EXTRACT

Shelf stable preservative free slightly  
acidic white grape skin extract with  
concentrated Phenolics, Antioxidants..  
Anthocyanins & Resveratrol.Total Phenolic  
content 10,000-50,000mg/L



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# AUSTRALIAN FUNCTIONAL NUTRACEUTICAL FLAVOURS, FRAGRANCES & INGREDIENTS

**Plant Extracts-Naturally Fermented Fruits and Vinegars  
Cold Pressed Oils-Essential Oils-Phenolic Rich Powders**



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