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## Biological significance of grape seed extract against lung injury induced by formaldehyde inhalation in rats

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### ABSTRACT

This study aimed to elucidate the protective role of grape seed extract (GSE) in mitigating the oxidative stress, inflammatory and apoptotic insults on lung tissue induced by formaldehyde (FA) inhalation in rats. Fifty adult albino rats were divided into 5 groups; (1) was negative control, (2) was FA-challenged group exposed to 10 ppm FA, (3) was FA-challenged group exposed to 20 ppm FA, (4) was orally administered with GSE (150 mg/kg b. wt.) prior exposure to 10 ppm FA and (5) was orally administered with GSE (150 mg/kg b. wt.) prior exposure to 20 ppm FA. Pro-oxidants (NO, MDA and H<sub>2</sub>O<sub>2</sub>), antioxidant enzymes (GSH-Px, SOD and CAT) and apoptotic markers (Bcl-2 and P53) were quantified in lung tissue. The inflammatory cytokines (IL-6, IL-8 and IL-10) were estimated in serum. Histological examination of lung tissue was performed. In comparison with the negative control, FA-challenged groups recorded significant increase in lung NO, H<sub>2</sub>O<sub>2</sub>, MDA and P53 as well as serum IL-6 and IL-8 levels accompanied with significant decrease in lung GSH-Px, SOD, CAT, Bcl-2 and serum IL-10 in a dose dependent manner. Histological examination of lung tissue of rats in FA-challenged groups showed peribronchiolar lymphoid hyperplasia with severe congestion in the pulmonary blood vessels. Pre-treatment with GSE elicited significant modulation in the pro-oxidants, inflammatory cytokines, antioxidant enzymes and apoptotic markers. These findings were documented by the histological examination of the lung tissue. In conclusion, the present work provided a clear evidence for the protective role of GSE against lung injury induced by FA inhalation in rats.

**Key words:** Lung injury, formaldehyde, grape seed extract, oxidative stress, inflammation, apoptosis.

### INTRODUCTION

Formaldehyde is a colorless, flammable reactive gas that is readily polymerized at room temperature with a pungent odor [1]. It is commercially available as a solution called formalin and according to Occupational Safety and Health Administration (OSHA), it is formed from various proportions of formaldehyde, water and alcohol [2]. Formaldehyde is used as sterilizing agent and disinfectant in medical work setting such as hospitals and laboratories. It is an excellent tissue fixative and commonly used for the preservation of tissues [3]. Also, formaldehyde is used in occupational environments (textiles, paper, resins, wood composites) and house indoor environments (insulating materials, fabrics, chipboard, cooking emissions) [4]. As well motor vehicle exhaust, the burning of gas, oil, coal, wood, rubbish and photochemical smog are some environmental sources for formaldehyde [5]. It is considered as one of the major components responsible for sick building syndrome as it is recognized as toxic at certain doses and the chances of its harmful effects are increased at room temperature due to its volatility [6]. The toxicity of formaldehyde is of concern to all who work closely with it. Embalmers, anatomists, technicians and medical, dental

or veterinary students are among the people who have high exposure to formaldehyde [7]. Respiratory system is the major target of formaldehyde, particularly the respiratory nasal epithelium which is considered as the primary target for formaldehyde induced toxicity [8]. Long-term formaldehyde inhalation at a dose of 15 ppm induced squamous cell carcinomas in the nasal cavities of rats and mice [9]. Moreover, it has been demonstrated that after formaldehyde inhalation in rats, the volume of formaldehyde is higher in the lung than in the blood, brain, liver and kidney [10].

Inflammatory changes are observed in the upper airways after acute low-level exposure to formaldehyde, and damage to the lower airways is reported after exposure to high levels (5 to 30 ppm) [11]. Furthermore, formaldehyde can react with monoamines or amides to form methylene bridges and produces covalently cross-linked complexes with proteins and DNA [12]. In addition to DNA–protein cross-links, it has been reported that formaldehyde could modulate the cellular glutathione (GSH) status [13] and hence, oxidative stress represented a potential mechanism of formaldehyde toxicity.

Antioxidants are important candidates for protection against oxidative stress due to their ability to detoxify free radicals, such as reactive oxygen species (ROS) [14]. Grape seed extract exhibits chemoprotective properties against ROS [15], anti-inflammatory [16], anti-cancer [17], anti-ulcer [18] and anti-diabetic potentials [19]. Recently, it has been demonstrated that GSE mitigates amiodarone (AM) induced lung injury *via* its anti-inflammatory and antioxidant activity [20]. Grape seed extract has been found to contain phenolic compounds which have many protective properties due to their powerful antioxidant activity [21]. Among the most abundant phenolic compounds present in grape seeds are proanthocyanidins. These compounds are high-molecular-weight polymers comprised of dimers or trimers of (+)-catechin and (–)-epicatechin [22]. Grape seed proanthocyanidin exhibits more powerful antioxidant effects than other well-known antioxidants such as vitamins C and E as well as gallic acid [23]. In addition to free radical scavenging and antioxidant activity, proanthocyanidins have also been shown to inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility [24].

Proanthocyanidins has anti-inflammatory effect on experimental inflammation in rats and mice. Its mechanisms of anti-inflammatory action relevant to oxygen free radical scavenging and inhibition of the formation of inflammatory cytokines [25].

The focus of our interest was to investigate the potent protective role of grape seed extract in ameliorating oxidative damage, inflammatory cascade and apoptotic insult on lung tissue induced by inhalation of formaldehyde in rats.

## MATERIALS AND METHODS

### Chemicals and Drugs

**1- Formaldehyde** was supplied from LOBA CHEMIE PVT. LTO, India.

**2- Grape Seed Extract:** Form: powder, Part: seed extraction, Type: solvent extraction; grade 95%, Model Number: grape seed extract, Assay: proanthocyanidins by UV  $\geq$  95%. The used extract solvent was water and methyl alcohol 70% sieve analysis was 100% pass 80 mesh. This product was purchased from Sigma Company (St. Louis, Missouri, USA).

### Experimental Animals

Fifty adult male albino rats of Wistar strain weighing 150-170 g were obtained from the Animal House Colony of the National Research Centre, Giza, Egypt and acclimatized for one week in a specific pathogen free (SPF) barrier area where the temperature ( $25\pm 1$ ) and humidity (55%). Rats were controlled constantly with a 12 h light/dark cycle at National Research Centre Animal Facility Breeding Colony. Rats were allowed *ad libitum* access to a water and standard pellet diet consisting of casein 10%, salts mixture 4 %, vitamins mixture 1%, corn oil 10 % and cellulose 5% completed to 100 g with corn starch [26]. Animal cared for according to the guidelines for animal experiments which were approved by the Ethical Committee of Medical Research of the National Research Centre, Giza, Egypt.

### Experimental Set-Up

After the acclimatization period, the rats were classified into five groups (10 rats /group); **Group (1):** Healthy animals served as negative control group, **Group (2)** The animals in this group were exposed to 10 ppm FA by inhalation (6 h/day, 5 days/week) for 5 weeks [27] (**FA 10 ppm**), **Group (3)** The animal in this group were exposed to 20 ppm FA for (6 h/day, 5 days/week) for 5 weeks [27] (**FA 20 ppm**), **Group (4)** The animals in this group were orally administered with GSE in a dose of 150 mg/ kg/day [28] prior exposure to 10 ppm FA by inhalation for 5 weeks (**FA 10 ppm+ GSE**), **Group (5)** The animals in this group were orally administered with GSE in a dose of 150 mg/kg/day prior exposure to 20 ppm FA by inhalation for 5 weeks (**FA 20 ppm+ GSE**).

### Inhalation Protocol

The rats had free access to food and water in their home cages but not for the brief periods in the inhalation chambers. For each daily inhalation, the rats were transported from the vivarium to the lab, in their home cages, and placed into an inhalation chamber. Each dose of formaldehyde was inhaled once a day for 6 hours to mimic the low (10 ppm) and high (20 ppm) doses exposure in human. Vapors inhalation was given in sealed 36-l cylindrical glass jars with acrylic lids, similar to description in Bowen and Balster [29]. The lids were equipped with injection ports, a fan and a stainless steel mesh box holding filter paper. During formaldehyde inhalation, one dam was placed onto a grid floor 20 cm from the bottom and 30 cm from the filter paper in the lid of the chamber. The lid was replaced and a calculated amount of solvent was injected onto filter paper from which the fan volatilized the solvent. At the end of the inhalation period, rats were removed immediately and returned to their home cages to wait the next inhalation period with the same procedure which was repeated daily for 5 weeks [30].

The inhalation dose of formaldehyde was calculated as follow:

$$\text{ppm} = \frac{\text{mg solute}}{10^6 \text{ mg water}} = \frac{\text{mg solute}}{\text{liter solution}}$$

$$\text{volume ml} = \frac{\text{weight of solute}}{\text{density of solution}}$$

At the end of the experimental period, the rats were fasted overnight, subjected to diethyl anaesthesia. The blood samples were immediately collected from the retro orbital venous plexus in the tubes free from any anticoagulant agent for separation of serum samples for biochemical analyses (IL-6, IL-8 and IL-10). Then, the rats were sacrificed by decapitation and the whole lung of each rat was rapidly and carefully dissected, thoroughly washed with isotonic saline and plotted dry and weighed. The whole lung from each animal was divided into two portion, the first portion was homogenized in a four volumes of ice-cold Tris-HCl buffer (50 millimolar, pH 7.4) containing 0.50 ml/L Triton X-100 with a homogenizer (IKA Ultra-Turrax T 25 Basic, Germany) for 2 minutes at 13000 rpm. The homogenate was then centrifuged at 5000 x g for 20 minutes to remove debris. The supernatant was separated for conducting further biochemical analyses (NO, H<sub>2</sub>O<sub>2</sub>, MDA, GSH-PX, CAT, Bcl-2 and P53). Clear supernatant was taken for a further extraction procedure for analysis of SOD and protein. It was extracted in ethanol/chloroform mixture (5/3, v/v) and after second centrifugation at 5000 x g for 20 min, the clear upper layer (the ethanol phase) was taken and used in the SOD and protein assay determination [31] using spectrophotometric methods. The second portion of lung was fixed in formalin saline (10%) for histopathological examination.

### Biochemical analyses

Lung nitric oxide (NO) level was determined by colorimetric method using nitric oxide assay kit purchased from Biodiagnostic Co., Egypt, according to the method of Montgomery and Dymock [32]. Lung hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) level was assayed by colorimetric method using hydrogen peroxide assay kit purchased from Biodiagnostic Co., Egypt, according to the method of Aebi [33]. Lung malondialdehyde (MDA) level was quantified by colorimetric method using lipid peroxide (MDA) assay kit purchased from Biodiagnostic Co., Egypt, according to the method described by Satoh [34]. Lung superoxide dismutase (SOD) activity was estimated by colorimetric method using superoxide dismutase assay kit purchased from Biodiagnostic Co., Egypt, following to the method described by Nishikimi *et al.* [35]. Lung catalase (CAT) activity was determined by colorimetric method using catalase assay kit purchased from Biodiagnostic Co., Egypt, according to the method described by Aebi [33]. Lung glutathione peroxidase (GSH-Px) activity was assayed by spectrophotometric method using glutathione peroxidase assay kit purchased from Biodiagnostic Co., Egypt, according to the method of Paglia and Valentine [36].

Measurement of serum interleukins (IL-6, IL-8 and IL-10) were accomplished by enzyme linked immunosorbent assay (ELISA) procedure. IL-6 assay kit purchased from Diaclone Co., Besancon cedex, France, according to the method described by Bowcock *et al.* [37], IL-8 and IL-10 assay kits purchased from Orgenium Co., Vantaa, Finland, following to the methods described by Baggiolini *et al.* [38] and Greig *et al.* [39].

Lung Bcl-2 level was detected by ELISA technique using Bcl-2 assay kit purchased from Bender Med Systems Co., Vienna, Europe, according to the method of Barbareschi *et al.* [40]. Lung P53 level was assayed by ELISA technique using P53 assay kit purchased from Diaclone Co., Besancon cedex, France, following the method described by EL-Far *et al.* [41]. Lung protein level was measured by colorimetric method according to Lowery *et al.* [42] procedure.

### Histopathological examination

After fixation of lung samples from rats in the different studies groups in 10% formaline saline for twenty four hours, washing was done with running tap water. Then series of alcohols (methyl, ethyl and absolute ethyl alcohol)

were used for dehydration. The specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin wax tissue blocks were prepared for sectioning at 4 microns thickness by slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained with hematoxylin and eosin stain. Then, examination was done through the light electric microscope [43].

### Statistical analysis

In the present study, all results were expressed as Mean  $\pm$  S.E of the mean. Data were analyzed by using a commercially available statistics software package (SPSS Inc. for windows, version 11.0 Chicago, IL, USA). All groups showed normal distribution so parametric statistical methods were used to analyze the data. A one-way ANOVA test followed by least significant difference (LSD) were used to compare significance between the groups [44]. The difference was considered significant when *P* value was  $<0.05$ .

Percentage difference representing the percent of the variation with respect to corresponding control group was also calculated using the following formula:

$$\% \text{ Difference} = \frac{\text{Treated value} - \text{Control value}}{\text{Control value}} \times 100$$

## RESULTS

### Biochemical Analyses

The data in **Table (1)** illustrated the effect of pre-treatment with grape seed extract on lung level of pro-oxidant markers and antioxidant enzymes in FA-challenged rats. The present data revealed that inhalation of FA in a dose 10 ppm and 20 ppm displayed significant increase ( $P < 0.05$ ) in NO (73.1% for 10 ppm and 114.7% for 20 ppm), H<sub>2</sub>O<sub>2</sub> (73.5% for 10 ppm and 111.7% for 20 ppm) and MDA (72.4% for 10 ppm and 116.6% for 20 ppm) levels respectively. While, it produced significant decrease ( $P < 0.05$ ) in SOD (33.3% for 10 ppm and 43.5 % for 20 ppm), CAT (41.4% for 10 ppm and 52.4% for 20 ppm) and GSH-Px (33.2% for 10 ppm and 49.4% for 20 ppm) activities in lung tissue, compared to those in the negative control group. However, the groups of rats treated with grape seed extract prior FA inhalation exhibited significant decrease ( $P < 0.05$ ) in lung pro-oxidant levels; NO (22.8% for 10 ppm and 21.2% for 20 ppm), H<sub>2</sub>O<sub>2</sub> (22% for 10 ppm and 25% for 20 ppm) and MDA (22.1% for 10 ppm and 24% for 20 ppm) relative to the corresponding untreated FA-challenged groups. on the other side, the groups of rats treated with grape seed extract prior FA inhalation displayed significant increase ( $P < 0.05$ ) in lung antioxidant enzymes activity; SOD (23.0% for 10 ppm and 29.5% for 20 ppm), CAT (29.2% for 10 ppm and 46.7% for 20 ppm) and GSH-Px (23.5% for 10 ppm and 44.8% for 20 ppm) with respect to the corresponding untreated FA-challenged groups.

**Table (1): Effect of grape seed extract (GSE) pre-treatment on pro-oxidant markers and antioxidant enzymes levels in lung of FA-challenged rats**

Parameters Groups	NO (nmol/mg protein)	H <sub>2</sub> O <sub>2</sub> (mM/mg protein)	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GSH-Px (U/mg protein)
Negative control	1.49 $\pm$ 0.08	0.0034 $\pm$ 0.0001	2.43 $\pm$ 0.11	0.78 $\pm$ 0.02	8.18 $\pm$ 0.29	80.15 $\pm$ 1.49
FA 10 ppm	2.58 $\pm$ 0.04 <sup>a</sup> 73.1%	0.0059 $\pm$ 0.0001 <sup>a</sup> 73.5%	4.19 $\pm$ 0.09 <sup>a</sup> 72.4%	0.52 $\pm$ 0.01 <sup>a</sup> -33.3%	4.79 $\pm$ 0.15 <sup>a</sup> -41.4%	53.53 $\pm$ 0.22 <sup>a</sup> -33.2%
FA 20 ppm	3.20 $\pm$ 0.09 <sup>a</sup> 114.7%	0.0072 $\pm$ 0.0002 <sup>a</sup> 111.7%	5.27 $\pm$ 0.32 <sup>a</sup> 116.8%	0.44 $\pm$ 0.02 <sup>a</sup> -43.5%	3.89 $\pm$ 0.26 <sup>a</sup> -52.4%	40.52 $\pm$ 0.18 <sup>a</sup> -49.4%
GSE+ FA 10 ppm	1.99 $\pm$ 0.11 <sup>b</sup> -22.8%	0.0046 $\pm$ 0.00003 <sup>b</sup> -22%	3.26 $\pm$ 0.11 <sup>b</sup> -22.1%	0.64 $\pm$ 0.06 <sup>b</sup> 23%	6.19 $\pm$ 0.18 <sup>b</sup> 29.2%	66.12 $\pm$ 4.19 <sup>b</sup> 23.5%
GSE+ FA 20 ppm	2.52 $\pm$ 0.09 <sup>c</sup> -21.2%	0.0054 $\pm$ 0.0001 <sup>c</sup> -25%	4.01 $\pm$ 0.14 <sup>c</sup> -24%	0.57 $\pm$ 0.03 <sup>c</sup> 29.5%	5.71 $\pm$ 0.45 <sup>c</sup> 46.7%	58.69 $\pm$ 2.15 <sup>c</sup> 44.8%

Data are presented as: mean  $\pm$  S.E. for 10 rats/group

The mean difference is significant at  $P < 0.05$

a: Significant change at  $P < 0.05$  in comparison with -ve control group.

b: Significant change at  $P < 0.05$  in comparison with the untreated FA 10 ppm group.

c: Significant change at  $P < 0.05$  in comparison with the untreated FA 20 ppm group.

% : percent of change from the corresponding control group.

The effect of pre-treatment with grape seed extract on serum level of inflammatory cytokines in FA-challenged rats is illustrated in **Table (2)**. The present results indicated that inhalation of FA in a dose 10 ppm (low dose) and 20 ppm (high dose) experienced significant increase ( $P < 0.05$ ) in the pro-inflammatory cytokines IL-6 (66.9% for 10 ppm and 107.9% for 20 ppm) and IL-8 (31.5% for 10 ppm and 50.4% for 20 ppm) in concomitant with significant decrease ( $P < 0.05$ ) in the anti-inflammatory cytokines IL-10 (23.6% for 10 ppm and 41.5% for 20 ppm) serum levels with respect to those in the negative control group. Meanwhile, the group of rats treated with grape seed extract prior FA inhalation (10 ppm) revealed significant decrease ( $P < 0.05$ ) in the inflammatory cytokines IL-6

(18.7%) and IL-8 (14.4%) and insignificant change ( $P > 0.05$ ) in the anti-inflammatory cytokine IL-10 (8.9%) serum level versus the untreated FA-challenged (10 ppm) group. Significant decrease ( $P < 0.05$ ) in the pro-inflammatory cytokines IL-6 (31.5%) and IL-8 (20%) accompanied with significant increase in the anti-inflammatory cytokine IL-10 (36.1%) serum levels were detected in the group of rats treated with grape seed extract prior to FA inhalation (20 ppm) compared with the untreated FA-challenged (20 ppm) group.

**Table (2): Effect of pre-treatment with grape seed extract (GSE) on serum inflammatory markers of FA-challenged rats**

Groups	Parameters	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-10 (pg/ml)
Negative control		14.65 ± 0.11	108.87 ± 0.64	4.69 ± 0.22
FA 10 ppm		24.46 ± 1.92 <sup>a</sup> 66.9%	143.24 ± 3.60 <sup>a</sup> 31.5%	3.58 ± 0.19 <sup>a</sup> -23.6%
FA 20 ppm		30.47 ± 2.17 <sup>a</sup> 107.9%	163.82 ± 11.65 <sup>a</sup> 50.4%	2.74 ± 0.14 <sup>a</sup> -41.5%
GSE + FA 10 ppm		19.87 ± 0.34 <sup>b</sup> -18.7%	122.94 ± 1.88 <sup>b</sup> -14.1%	3.90 ± 0.29 8.9%
GSE + FA 20 ppm		20.86 ± 0.23 <sup>c</sup> -31.5%	130.98 ± 1.28 <sup>c</sup> -20%	3.73 ± 0.25 <sup>c</sup> 36.1%

Data are presented as: mean ± S.E. for 10 rats/group  
The mean difference is significant at  $P < 0.05$

a: Significant change at  $P < 0.05$  in comparison with -ve control group.

b: Significant change at  $P < 0.05$  in comparison with the untreated FA 10 ppm group.

c: Significant change at  $P < 0.05$  in comparison with the untreated FA 20 ppm group.

% : percent of change from the corresponding control group.

The data depicted in **Table (3)** represented the effect of pre-treatment with grape seed extract on lung level of apoptotic markers in FA-challenged rats. The present results showed that inhalation of FA in a dose 10 ppm (low dose) and 20 ppm (high dose) exhibited significant increase ( $P < 0.05$ ) in the level of pro-apoptotic markers P53 (57.5% for 10 ppm and 109% for 20 ppm) and significant decrease ( $P < 0.05$ ) in the level of anti-apoptotic marker Bcl-2 (34.4% for 10 ppm and 41.8% for 20 ppm) in lung compared to those in the negative control group. On the other hand, the groups of rats treated with grape seed extract prior FA inhalation showed significant decrease ( $P < 0.05$ ) in lung pro-apoptotic marker P53 (26.9% for 10 ppm and 34.7% for 20 ppm) accompanied with significant increase in lung anti-apoptotic marker Bcl-2 (22.8% for 10 ppm and 29% for 20 ppm) relative to the corresponding untreated FA-challenged groups.

**Table (3): Effect of pre-treatment with grape seed extract (GSE) on apoptotic markers level in lung tissue of FA-challenged rats**

Groups	Parameters	P53 (U/mg protein)	Bcl-2 (ng/mg protein)
Negative control		0.33 ± 0.01	0.160 ± 0.006
FA 10 ppm		0.52 ± 0.02 <sup>a</sup> 57.5%	0.105 ± 0.001 <sup>a</sup> -34.4%
FA 20 ppm		0.69 ± 0.08 <sup>a</sup> 109%	0.093 ± 0.004 <sup>a</sup> -41.8%
GSE + FA 10 ppm		0.38 ± 0.03 <sup>b</sup> -26.9%	0.129 ± 0.008 <sup>b</sup> 22.8%
GSE + FA 20 ppm		0.45 ± 0.03 <sup>c</sup> -34.7%	0.120 ± 0.005 <sup>c</sup> 29%

Data are presented as: mean ± S.E. for 10 rats/group  
The mean difference is significant at  $P < 0.05$

a: Significant change at  $P < 0.05$  in comparison with -ve control group.

b: Significant change at  $P < 0.05$  in comparison with the untreated FA 10 ppm group.

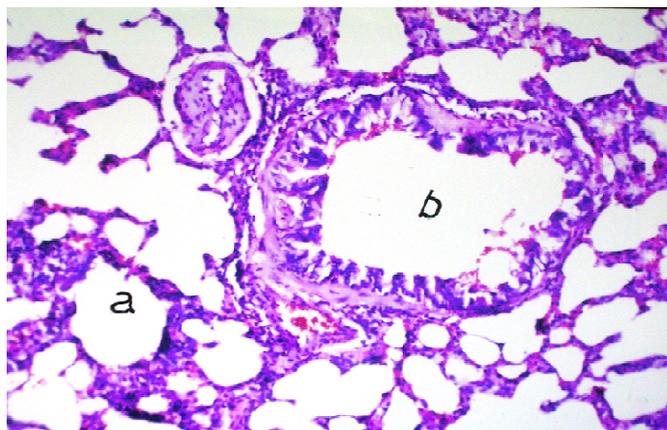
c: Significant change at  $P < 0.05$  in comparison with the untreated FA 20 ppm group.

% : percent of change from the corresponding control group.

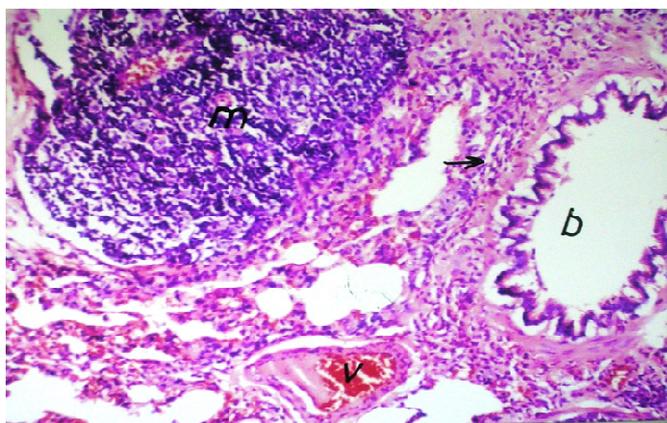
### Histopathological Examination

Histological investigation of lung tissue sections of rats in the control group showed no histopathological alteration and the normal histological structure of bronchioles and air alveoli was observed (**Fig. 1**). While, lung tissue sections of rats in the group exposed to FA 10 ppm showed peribronchiolar lymphoid hyperplasia associated with sever congestion in the blood vessels as well as peribronchoilar inflammatory cells infiltration (**Fig. 2**). Microscopic investigation of lung tissue sections of rats in the group exposed to FA 20 ppm showed sever peribronchiolar lymphoid hyperplasia surrounding the hyperplastic bronchioles in association with sever congestion in the pulmonary blood vessels as well as alveolar collapse and emphysema (**Fig. 3**). Histological examination of lung tissue sections of rats in the group treated with grape seed prior FA (10 ppm) inhalation showed peribronchiolar inflammatory cells infiltration (**Fig. 4**). Meanwhile, oedema in the perivascular and interstitial tissue associated with

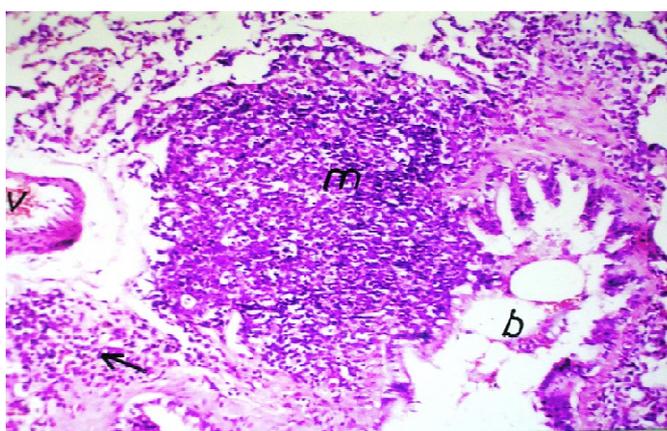
inflammatory cells infiltration and congestion in the blood vessels were observed in the microscopic investigation of lung tissue section of rats in the group treated with grape seed extract prior FA (20 ppm) inhalation (**Fig. 5**).



**Fig. (1):** Photomicrograph of lung of control rat showed the normal histological structure and bronchioles (b) and air alveoli (a). (H&E x40)



**Fig. (2):** Photomicrograph of rat lung after inhalation formaldehyde (10 ppm) showed the peribronchiolar lymphoid hyperplasia (m) and severe vascular congestion (v) as well as inflammatory cells infiltration (arrow). (H&E x40)



**Fig. (3):** Photomicrograph of rat lung after inhalation formaldehyde (20 ppm) peribronchiolar lymphoid hyperplasia (m), severe congestion in the pulmonary blood vessels (v), bronchiolar hyperplasia (b) and inflammatory cells infiltration (arrow). (H&E x40)

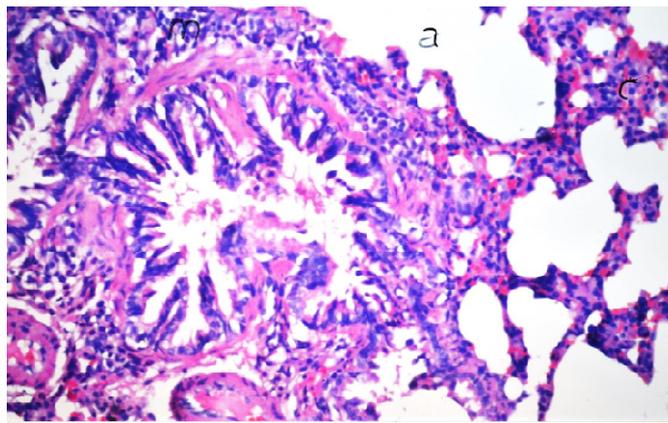


Fig. (4): Photomicrograph of rat in the group treated with grape seed prior FA (10 ppm) inhalation showed peribronchiolar inflammatory cells infiltration (m) with collapse (c) and emphysema in air alveoli (a). (H&E x40)

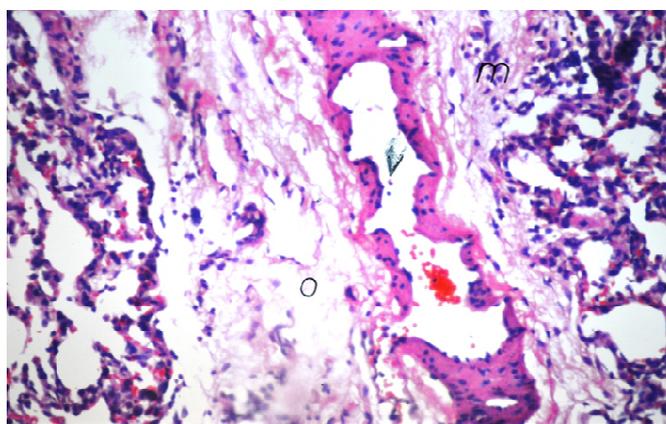


Fig. (5): Photomicrograph of rat in the group treated with grape seed prior FA (20 ppm) inhalation showed edema (O) with inflammatory cells infiltration (m) in the interstitial connective tissue with edema only (O) surrounding the dilated blood vessels (v). (H &E x40)

## DISCUSSION

The present results showed that exposure of rats to FA either with low (10 ppm) or high (20 ppm) dose resulted in significant increase in NO level in the lung in comparison with the negative control rats. This result is in agreement with Lino-dos-Santos-Franco *et al.* [45] who reported that increased NO level after FA exposure could be attributed to the increased generation of ROS and reactive nitrogen species (RNS) during FA exposure. FA can cause severe tissue injury by producing ROS. In several experimental studies on animal models, it has been found to cause an increase in the amount of NO in tissue and serum. This finding was explained by Gulec *et al.* [46] who clarified that NO appears to be intimately involved in superoxide mediated tissue injury. The presence of free radicals potentiates systems in the cell that may lead to increased production and release of NO. In addition, Sögüt *et al.* [27] reported that the concurrent production of mitochondrial superoxide and cytoplasmic NO leads to rapid formation of peroxynitrite which lead to increasing NO level. As well, the results of the present study are in conformity with those reported by Franklin *et al.* [11] who demonstrated an association between inhaled FA concentrations and exhaled NO levels in their study performed on children with no previous airway damage and atopy. Furthermore, our result might be explained by the augmentation of the activity and/or gene expression of the oxidant enzymes such as cyclooxygenase (COX) and nitric oxide synthase (NOS) as consequence of FA exposure. This suggestion was supported by the previous studies of Uçmaklı *et al.* [47] who reported that FA exposure in Wistar albino rats increased gene expression of inducible nitric oxide synthase (iNOS), and the amount of NO in liver tissue in comparison with the control rats. These authors assumed that this effect can occur in one of two ways, the first is that FA or its metabolites act like a stimulator/activator that induces iNOS protein synthesis directly. The fact that FA is a water-soluble molecule which easily diffuses into membranes and directly cross reacts with DNA-protein chains, supports this approach [48]. The second way may be through cytokines [49], as accumulating evidences have suggested that FA has a stimulating impact on cytokines, which affect iNOS metabolism [50].

The present findings showed that exposure of rats to FA either with low (10 ppm) or high (20 ppm) concentration experienced significant increase in H<sub>2</sub>O<sub>2</sub> level in lung tissue when compared with the negative control rats. An increased formation of H<sub>2</sub>O<sub>2</sub> can occur directly or as a consequence of any situation where there is an increased formation of O<sub>2</sub><sup>-</sup>. The only exception to the central role of hydrogen peroxide in the toxicity of activated oxygen species is the direct formation of the -OH as a result of the radiolysis of water. Glutathione peroxidase, needs glutathione to detoxify H<sub>2</sub>O<sub>2</sub>. Glutathione repairs oxidized and damaged molecules and helps to regulate a variety of cellular functions. In lung tissue, GSH is essential for defensive responses to oxidants and inflammatory agents [51]. Moreover, oxidation of FA to formic acid is catalyzed by several enzymes including NAD dependent formaldehyde dehydrogenase, which requires reduced glutathione as a cofactor, catalase and peroxidase [52]. Indeed, the induction of H<sub>2</sub>O<sub>2</sub> in the lung tissue due to FA inhalation could be attributed to the suppression of the antioxidant enzymes activities, particularly catalase [53]. This explanation is clearly supported by our results in the present study.

The data of the current study revealed that exposure of rats to FA either with low (10 ppm) or high (20 ppm) dose led to significant increase in MDA level in lung tissue relative to the negative control rats. This result is in accordance with that of Gulec *et al.* [46] who stated that the increased MDA level due to breathing FA caused severe tissue injury by producing ROS [54]. ROS formation causes damage to an array of biomolecules found in tissues, including membrane lipids, proteins and nucleic acids. Membrane-associated polyunsaturated fatty acids are readily attacked by ROS in a process that results in the peroxidation of lipids. Peroxidation of membrane lipids can disrupt membrane fluidity and cell compartmentation, which can result in cell lysis. Thus, ROS-initiated lipid peroxidation and protein oxidation may contribute to the impaired cellular function and necrosis associated with toxicity of FA or its derivatives [55]. Furthermore, Sögüt *et al.* [27] reported that GSH improves endurance performance and prevents liver lipid peroxidation during FA inhalation in rats. GSH-related cellular defensive mechanisms have been shown to be depressed in liver tissues and therefore susceptibility to oxidative damage may increase in rats exposed to FA. In the present study, SOD, CAT and GSH-Px activities were significantly decreased in the lung tissues after FA inhalation in rats with respect to control rats. This indicates that FA inhalation disrupted the enzymatic antioxidant defense mechanism of the lung cells causing oxidative damage. These results are in agreement with the previous reports, which suggested that FA exposure in experimental animals caused depression of their antioxidant status due to increased lipid peroxidation and formation of free radicals [55]. The impaired antioxidant enzyme activities in the tissues may cause an enhancement of ROS-induced membrane lipid peroxidation and protein oxidation leading to apoptotic/necrotic cell death.

Sögüt *et al.* [27] reported significant decrease in glutathione level of liver tissue. This decrease might probably be in response to the extra production of ROS as a result of excessive exposure to FA [56]. Lung glutathione peroxidase, which is present widely in tissue, needs GSH to detoxify hydrogen peroxide. If the enzymatic antioxidant system cannot work properly in tissues, there will be an extra free oxygen radical load. At the end of the process the exhaustion of glutathione and consequently GSH-Px activity will occur.

Parallels to other studies, the biochemical findings presented in our study concerning the antioxidant enzymes (SOD, CAT and GSH-Px) are in agreement with the results of Ozen *et al.* [57]; Tang *et al.* [58] and Zhou *et al.* [59], as these reports indicated that there are significant increase in MDA level while both SOD and GSH-PX enzymes activities are significantly reduced in the testicular tissue of the animals exposed to FA. As well, Zararsiz *et al.* [60, 61] demonstrated the same effect in the brain tissue of the exposed rats.

Our results indicated that FA caused a disruption of the physiological balance of oxidant and antioxidant homeostasis in lung tissue, most likely favoring the oxidant pathways and, therefore, positively promoting lung inflammation.

Inflammation is a recognized FA induced response, because FA is known to irritate the respiratory system [62] and increase asthmatic response [63]. In the current study, the effect of FA inhalation on the production of the pro-inflammatory cytokine (IL-6) and the neutrophil chemoattractant (IL-8) has been investigated. In view of our results, it has been demonstrated that FA exposure significantly enhanced the serum levels of IL-6. Lino-dos-Santos-Franco *et al.* [45] speculated that the oxidative stress plays an important role in the inflammatory effects triggered by FA inhalation. FA has been found to induce oxidative stress in a mechanism by which it could elevate levels of NO and H<sub>2</sub>O<sub>2</sub> in lung tissue of rats inhaled FA, as shown in the present study, thereby supporting the hypothesis of releasing ROS and RNS during FA exposure which trigger lung inflammation. ROS has been shown to be implicated in initiating inflammatory responses in the lungs *via* the activation of the transcription factors such as nuclear factor kappa B (NF-κB), leading to enhanced gene expression of pro-inflammatory mediators such as IL-1β and IL-6 [45].

The current study also recorded significant increase in IL-8 serum levels of rats after inhalation of FA. This finding is greatly supported by Rager *et al.* [64] who observed significant increase in protein expression of IL-8 in the FA

exposed lung cell versus control cells. These findings are in the same line with those previously reported by Sexton *et al.* [65] who indicated that, the release of IL-8 in lung cells represented inflammatory response after exposure to air pollutants. In addition, previous investigators recorded increased IL-8 level in lungs of patients with diseases such as acute lung injury [66], adult respiratory distress syndrome [67] and asthma [68]. IL-8 related signaling molecules have been found to present in the miRNA target networks. The findings of Rager *et al.* [64] suggested that the canonical pathways associated with FA-induced miRNA alterations may affect the regulation of biological pathways associated with various disease states, including cancer and inflammation. The results of these authors suggested that cytokine signaling may be altered through changes in miRNA expression levels. In accordance with this hypothesis, there is another study showing that the modifications in the miRNAs may influence the expression of cytokines, including IL-6 and IL-8 [69].

The present data revealed that serum IL-10 level showed significant reduction in FA-challenged rats. In consistent, Sasaki *et al.* [70] found that FA selectively suppressed IL-10 mRNA expression and protein production in stimulated T cells. IL-10 is produced mainly from T helper-2 (Th-2) cells, certain B cells subsets, dendritic cells, monocytes and mast cells. IL-10 inhibits synthesis of pro-inflammatory cytokines as IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IFN- $\gamma$ , IL-2, IL-3, TNF- $\alpha$ , reduces expression of class II major histocompatibility complex (MHC) following activation of monocytes with lipopolysaccharide [71] and suppresses the activity of macrophages, dendritic cells, neutrophils, eosinophils and Th-1 cells [72]. In chronic inflammatory conditions, defective IL-10 synthesis contributes to increased pro-inflammatory cytokine levels [73]. Here in, the significant elevation in the pro-inflammatory cytokines namely (IL-6 and IL-8) in serum of FA-challenged rats indicated the chronic inflammation of the lung tissue which may elicit the observed reduction in the IL-10 serum level. This explanation represents the suggestive mechanism for the reduction of IL-10 serum level as a consequence of FA inhalation.

The current data revealed that exposure of rats to FA produced significant increase in p53 level in lung tissue in concomitant with a significant decrease in Bcl-2 level versus the negative control rats. The cytotoxic mechanisms of FA have been studied in several experimental models. FA is extremely reactive, cross-linking with proteins and with single stranded DNA, causing cellular dysfunction and even apoptosis [74].

The present results recorded significant elevation in the P53 levels in the lung tissue of rats due to FA inhalation which indicated the triggering of apoptosis. In conformity of our results Sandikic *et al.* [75] recorded that the number of apoptotic cells in the lung tissue was significantly high in the young and adult rats exposed to FA. Moreover, Sul *et al.* [76] stated that the exposure of rats to FA leads to aberrant expression of several genes involved in apoptosis, immunity, metabolism, signal transduction, transportation. Furthermore, we could suggest that the increased rate of apoptosis in lung tissue of rats exposed to FA might be associated with the increased levels of free radicals. This explanation is greatly supported by our finding as both reactive oxygen (H<sub>2</sub>O<sub>2</sub>) and nitrogen species (NO) were increased in lung tissue as a consequence of FA inhalation. As well, previous study by Sul *et al.* [76] indicated that exposure of rats to different concentrations of FA is associated with an increase in the level of MDA, carbonyl insertion, and DNA damage in the lung tissue. It has been reported that oxidative stress is one of the main mechanisms for inducing apoptotic cell death, in which levels of ROS are up regulated [77]. Cells are susceptible to oxidative stress-induced apoptosis when levels of intracellular antioxidants are down regulated. In the present study, the activity of SOD and GSH-Px and CAT decreased significantly in lung tissue of FA-challenged rats. Therefore, this may be the underlying mechanism by which FA can cause apoptosis.

Lim *et al.* [78] suggested various mechanisms for FA-induced apoptosis, including the formation of toxic metabolites, damaging of mitochondria, changing in the ratio of Bax/Bcl-2 expression, and activation of the apoptosis mitochondrial pathway. Changes in the expression of Bcl-2 family proteins are usually thought to lead to apoptosis through the intrinsic route. In the present study, Bcl-2 protein level decreased in lung tissue of rats in response to FA. Consistent with our results, Kanter [79] and Turkoglu *et al.* [80] reported similar results as they recorded DNA damage after FA exposure, thereby leading to apoptosis. Moreover, Tsukahara *et al.* [81] reported that inhaling different levels of FA increased Bax/Bcl-2 expression in the hippocampus leading to the apoptotic cell death in this area of the brain.

Few studies have been designed to investigate the histopathologic effect of inhalation of FA on the lower respiratory tract, in particularly the lung. In such reports, the exposure dosage, as well as period of exposure is at variant with our study and observations [82, 83]. The histopathological examination in our study showed peribronchiolar lymphoid hyperplasia associated with sever congestion in the blood vessels as well as peribronchiolar inflammatory cells infiltration in the group of rats exposed to FA 10 ppm. Also, the current study showed sever peribronchiolar lymphoid hyperplasia surrounding the hyperplastic bronchioles in association with sever congestion in the pulmonary blood vessels as well as alveolar collapse and emphysema in the group of rats exposed to FA 20 ppm. These findings are in agreement with Ohtsuka *et al.* [84] who observed that after F344 rats inhaled FA solution

aerosol for 3 hours per day, for 10 days, changes such as degeneration, necrosis stratification and squamous metaplasia are observed in bronchi of the lungs. The mechanism of polymorphonuclear leukocytes inflammatory cells invasion induced by FA inhalation has been explained by Ryoko *et al.* [85] who reported that inhaled FA rapidly increases vascular permeability in rat airway and produces microvascular leakage in the airway through stimulation of tachykinin NK1 receptors by tachykinins released from sensory nerves. Furthermore, Njoya *et al.* [86] reported that FA exposure brought about the ulceration of the alveoli by excavation and desquamation of the surface epithelium and derangement with distorted supporting tissues of alveolar wall. In addition to, massive cellular proliferation of bronchiolar epithelium. Also, the epithelial lining of bronchioles showed loss of mucosal folds and the cellular proliferation resulted in conversion of the epithelial lining of bronchi from pseudostratified columnar ciliated epithelium into thickened hyperplastic bronchiolar epithelium formed of many layers of cells. Roemer *et al.* [87] reported that exposure to FA by inhalation for 6 hours per day for one or three consecutive days could induce lung cell proliferation of rats, indicating a carcinogenic potential of this aldehyde. Also, Monticello *et al.* [88] observed an increase in cell proliferation in the respiratory tract and hyperplastic epithelial changes following repeated exposure to FA.

The present investigation was directed to elucidate the possible protective role of GSE against FA inhalation-induced lung injury in rats. The present data showed that the treatment with GSE prior FA inhalation elicited significant reduction in NO, H<sub>2</sub>O<sub>2</sub> and MDA levels in the lung tissue relative to untreated FA-challenged group. In accordance with these findings, experimental studies have shown that oral administration of GSE lowered ROS generation and plasma protein carbonyl groups, while it enhanced the activity of the endogenous antioxidant system [89]. In particular, proanthocyanidins (PC) the active constituent of GSE have been reported to be able to scavenge free radicals and NO and reduce their levels [90]. This effect could be attributed to the capability of PC to suppress nitric oxide synthases activity in the pleural fluid [91]. It is well known that NO is synthesized by inducible nitric oxide synthase, and it has been found that IL-1 $\beta$  induced iNOS protein expression and overproduction of NO [92]. Kim *et al.* [93] demonstrated that the pre-treatment of rats with epicatechin (EC), the second active component of GSE, inhibited both IL-1 $\beta$  induced nitrite production and iNOS gene expression *via* the inhibition of nuclear factor  $\kappa$ B inhibitor protein, I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B activation in the pancreatic  $\beta$ -cells.

Several studies have shown that ROS and RNS, produced by the inflammatory and immune cells [94], have specific role in tissue destruction associated with inflammatory diseases [95]. Activated macrophages are a rich source of NO production [96]. Govindaraj *et al.* [97] demonstrated that proanthocyanidin was shown to decrease the levels of ROS, RNS, myeloperoxidase and lysosomal enzymes in the experimental periodontitis in rats. Therefore, PC is considered as a potent antioxidant attenuated oxidative stress by neutralizing the free radicals. This finding is greatly supported by the earlier report of Li *et al.* [25] who found that proanthocyanidin from grape seeds significantly decreased the levels of ROS and markedly lowers the activity of nitric oxide synthase as well as its product NO in carrageenan-induced paw edema in rats. These data provided a clear evidence for the conclusion that the inhibition of lipid peroxidation and NO formation was an anti-inflammatory mechanism of proanthocyanidins.

Mantena and Katiyar [98] reported that the antioxidant property of GSE contributed to the inhibition of phosphorylation of mitogen-activated protein kinase (MAPK) through: (i) inhibition of H<sub>2</sub>O<sub>2</sub> production and (ii) inhibition of depletion of antioxidant defense enzymes. Thus, the inhibition of the H<sub>2</sub>O<sub>2</sub>-mediated phosphorylation of MAPK in NHEK by GSE indicates that GSEs have the ability to neutralize the effect of H<sub>2</sub>O<sub>2</sub>.

In the current work, MDA levels in the lung tissue was significantly decreased in the groups of rats treated with GSE prior FA inhalation. This means that GSE could attenuate FA-induced oxidative stress in rats. This result comes in line with the previously reported data of Madkour and Ahmed [20] who stated that the possible reason for the inhibitory influence of GSE on MDA level might be due to the ability of GSE to remove toxic oxygen radicals and therefore reduce oxidative damage caused by ROS. Also, GSE has been demonstrated to prevent DNA oxidative damage induced by many agents in various tissues [99]. This activity could be due to the detoxification of cytotoxic radicals and presumed contribution to DNA repair [100] along with its ability to protect against both water- and fat-soluble free radicals providing incredible protection to the cells [101]. Also, Yucel *et al.* [102] reported that the treatment with PC could improve the decreased SOD and GPx activities, and decrease MDA levels in the lung tissue of rats. In general, the antioxidant activity of phenolic compounds is due to their redox properties that allow them to act as reducing agents by donating hydrogen, quenching singlet oxygen or acting as metal chelators [103].

The present findings demonstrated that the lung SOD, CAT and GSH-Px were significantly increased in FA-challenged rats pre-treated with GSE. Shan *et al.* [104] suggested that GSE could enhance the activity of the antioxidant enzymes (SOD and GSH-Px) in liver and decrease the liver content of MDA. This indicates that the antioxidant function of GSE may be work by increasing the activity of body's antioxidant enzymes. Furthermore, Chis *et al.* [105] reported that the GSE treated rats revealed a significant reduction in lipid peroxidation and protein

oxidation accompanied with significant increase in the activity of SOD and CAT in plasma and hepatic tissue. Proanthocyanidins as phenolic compounds were suggested to be more potent when compared to flavanols in their antioxidant capacity due to the fact that oxidation of proanthocyanidins predominantly produced semiquinone radicals that coupled to produce oligomeric compounds through nucleophilic addition [106]. One of the most advantageous features of proanthocyanidins oligomers free radical scavenging activity is that, because of its chemical structure, it is incorporated within cell membranes. This physical characteristic along with its ability to protect against both water- and fat-soluble free radicals provides incredible protection to the cells against free radical changes [107] leading to the maintenance of the antioxidant enzymes activity.

Lago *et al.* [108] reported that the anti-inflammatory effects of phenolic compounds are related to their ability to modulate the expression of pro-inflammatory genes, such as NOS, cyclooxygenase, lipoxygenase and also by acting throughout NF- $\kappa$ B signaling and MAPK [109]. Previous studies have confirmed that flavonoids exert their anti-inflammatory effects by modulating the inflammatory cells, inhibiting the T lymphocyte proliferation, inhibiting pro-inflammatory cytokines (TNF- $\alpha$  and IL-1), or controlling enzymes derived from the arachidonic acid pathway [110].

In view of the present data, the pre-treatment of FA-challenged rats with GSE resulted in significant reduction in serum levels of IL-6 and IL-8 while, it produced significant elevation in serum level of IL-10. These findings are in conformity with AL-Hanbali *et al.* [111] who demonstrated that epicatechin suppressed pro-inflammatory cytokines IL-6 and IL-8 and enhanced the production of the anti-inflammatory cytokine IL-10 in whole blood cultures stimulated with phytohemagglutinin (PHA) plus lipopolysaccharide (LPS). This indicates that EC possesses anti-inflammatory action and coincides with what is known traditionally or experimentally about grape as anti-inflammatory plant [16]. Moreover, Kim *et al.* [112] stated that catechins suppressed the production of some pro-inflammatory cytokines including IL-8 in microvascular endothelial cells in a concentration dependent manner.

Models of LPS-induced lung inflammation are also used to study the anti-inflammatory effects of flavonoids because LPS is present in the membrane of gram-negative bacteria and LPS is considered as one of the main risk factors for acute respiratory distress syndrome (ARDS) [113]. Earlier studies on EC have shown its inhibitory impact on the inflammatory cytokines and NF- $\kappa$ B from LPS or IL-1-stimulated monocytes or macrophages or non-immune cells. Some of these earlier studies have shown that EC suppressed NF- $\kappa$ B nuclear translocation [114].

NF- $\kappa$ B has been reported as one of the most notable pro-inflammatory gene expression regulators that mediates several cytokines synthesis, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 as well as COX-2 [115]. NF- $\kappa$ B is a dimer protein composed of p50 and p65 and in a resting state, NF- $\kappa$ B is sequestered in the cytosol where it is bound to inhibitory protein I  $\kappa$ B. Upon its activation, I  $\kappa$ B becomes phosphorylated by I  $\kappa$ B kinase and then degraded leading to the releasing NF- $\kappa$ B. Then, NF- $\kappa$ B translocates to the nucleus, where it binds to the  $\kappa$ B binding sites in the promoter region, leading to the increased its gene transcription [116]. The increased gene transcription of NF- $\kappa$ B promote the pro-inflammatory gene transcription of the pro-inflammatory cytokines including IL-6 and IL-8 [111]. Mantena and Katiyar [98] suggested that the inhibitory effect of GSE on NF- $\kappa$ B/p65 activation may be mediated through the inhibition of proteolysis of I $\kappa$ B $\alpha$  protein. It is well documented that through a protein-protein interaction, I $\kappa$ B $\alpha$  is bound to NF- $\kappa$ B/p65 preventing migration of NF- $\kappa$ B/p65 into the nucleus [117]. Kim *et al.* [93] demonstrated that the pre-treatment with epicatechin inhibited IL-1 $\beta$ -induced I $\kappa$ B $\alpha$  protein degradation. Thus, epicatechin inhibits IL-1 $\beta$ -induced nuclear translocation of the p65 NF- $\kappa$ B subunit with consequent inhibition to NF- $\kappa$ B DNA binding activity in rats pancreatic islets. Thus, the action of epicatechin appears to be associated with the inhibition of NF- $\kappa$ B activation and its nuclear localization, and this might be done *via* inhibition of I $\kappa$ B $\alpha$  protein degradation. By this way GSE could inhibit the production of the pro-inflammatory cytokines (IL-6 and IL-8) serum levels in the current study.

Another mechanism for producing pro-inflammatory cytokines involved the induction of pro-inflammatory gene transcription is *via* the activation of MAPK subgroups such as P38 and JNK. MAPK family, such as ERK1/2, JNK, and p38, are mediators of signal transduction from the cell surface to the nucleus and they play a major role in triggering and coordinating gene responses [118]. JNK and p38 are primarily activated by environmental stresses such as UV radiation, inflammatory cytokines, heat shock, and DNA-damaging agents [119]. Phosphorylation of JNK and p38 has a role in cellular differentiation and inflammatory responses [120]. Mantena and Katiyar [98] demonstrated that the treatment with GSE inhibited the activation of MAPK family. More in details, AL-Hanbali *et al.* [111] reported that EC induced its suppressive effect on IL-8 and IL-6 through enhancing MAPK phosphatase 1 which dephosphorylates and deactivates JNK.

IL-10 production requires at least two signals; the first is provided by LPS (or its physiologic equivalent), and the second by endogenous TNF- $\alpha$  and/or IL-1 [121]. These signals induce IL-10 transcription through cAMP-response

elements (CRE) [122]. These authors suggested that EC induced its suppressive effect in the whole blood polyclonal activation system through inducing IL-10 which could be achieved through cAMP-response elements.

The present results showed that the pre-treatment of FA-challenged rats with GSE induced significant decrease in P53 level accompanied with significant increase in Bcl-2 level in lung tissue. Sato *et al.* [123] demonstrated that p53, JNK, and c-Jun worked as pro-apoptotic factors while, Bcl-2 worked as anti-death gene [124]. Moreover, it has been reported that JNK activated the tumor suppressor p53 [125], a pro-apoptotic transcription factor that suppresses the anti-death gene Bcl-2 and enhances Bax induction [126]. JNK could also antagonize the function of the anti-apoptotic protection of Bcl-2 through phosphorylation [127]. GSE has been found to reduce apoptosis *via* inhibition of JNK and c-Jun [128] with consequent reduction in P53.

In accordance with our results, it has been reported that GSE is bioavailable and may protect multiple target organs (liver, lung, spleen and kidney) from structurally diverse drug- and chemical-induced toxicity and adverse effects [129]. Joshi *et al.* [130] recorded an increase in the expression of anti-apoptotic protein Bcl-2 in GSE-treated cells *in vitro*. Thus, GSE is considered as a potential candidate to ameliorate the toxic effects associated with drugs/chemicals and hence one of the mechanisms involved in the cytoprotection of GSE may include upregulation of Bcl-2 expression. Previous *in vivo* studies have linked the protective ability of GSE with the modulation of anti-apoptotic gene Bcl-XL' and modification of several other critical molecular targets such as DNA damage/DNA repair and lipid peroxidation [131].

Additional mechanism by which GSE could increase Bcl-2 level in lung tissue is related to its ability to inhibit cytochrome P450 2E1. It has reported that increased oxidative stress associated with high apoptosis could, at least partially, result from the induction of P450 2E1 [132]. The capability of GSE to suppress P450 2E1 spoke for the importance of the anti-apoptotic mechanism of GSE to be a cytoprotective agent in conjunction with its antioxidative, detoxifying, and anti-endonucleolytic potential.

The histopathological observation in our study revealed that the air alveoli showed collapse and compensatory emphysema while, the bronchiol showed peribronchiolar inflammatory cells infiltration in the group of rats treated with GSE prior exposure to FA 10 ppm. These findings are in agreement with Hemmati *et al.* [133] who attributed the protective effect of grape seed to its ability to inhibit the formation of the inflammatory cytokines. However, oedema was noticed in the perivascular and interstitial tissue associated with inflammatory cells infiltration in the later and congestion in the blood vessels in group of rats treated with GSE prior exposure to FA 20 ppm. These results are supported by Hasseeb *et al.* [134] who reported that the lungs appeared with less or delayed protective effect where the pulmonary alveoli still in the stage of resolution and appeared to contain some mononuclear cells. Here in, it could be suggested that GSE could inhibit apoptotic cell death induced FA inhalation through increasing the anti-apoptotic capacity in concomitant with decreasing the pro-apoptotic activity of the lung cells.

In conclusion, the present study provided experimental evidences for the protective role of GSE against lung injury induced by FA inhalation. The mechanistic pathways of the protective action exerted by GSE include (i) powerful free radical scavenging property, (ii) strong antioxidant activity, (iii) potent anti-inflammatory capacity and (iv) effective anti-apoptotic potential.

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