Anti-inflammatory Effect of Grape Seed Extract on Bronchial Asthma Induced in Mice

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Abstract: Bronchial asthma is a growing major public health problem worldwide. Anti-inflammatory agents have been suggested to alleviate asthma. Grape seed extract (GSE) has been reported to have a powerful antioxidant and anti-inflammatory properties. The present study aims to determine whether GSE has a therapeutic effect on allergic airway inflammation in mice. Airway inflammation was induced *via* intraperitoneal (i.p) injected with 10 ug ovalbumin plus 2 mg aluminum hydroxide in 200 uL phosphate buffered saline (PBS). Male mice (n=50) were divided to five equal groups. The first group; negative control received i.p. injection of PBS, the second group; positive control**;** asthma-model (OVA), the third group; asthma mice treated orally with montelukast (30 mg/kg),the fourth group; asthma treated orally with GSE at a dose level of (150 mg/kg) and the fifth group; asthma mice treated with both GSE+ montelukast. Montelukast and GSE were given on day 27 and from day 28 to 30 1hr before each challenge. The inflammatory cytokines interleukin-5 &13 (IL-5& IL-13) were estimated in bronchoalveolar lavage fluid*(*BALF) and immunoglobulin E(IGE) was determined in plasma. Lung levels of pro-oxidants; malondialdehyde (MDA), nitric oxide (NO) and superoxide dismutase (SOD) were determined. Lung samples from mice in different groups examined microscopically. There was a significant increase (p<0.001) in BALF levels of IL-5 and IL-13, plasma IGE, as well asa significant increase (p<0.001) in the lung levels of NO and MDA with a significant decrease (p<0.001) in SOD levels when compared with control group. Histopathological studies of lung confirmed these results, bronchiolar and perivascular infiltrates, as well as perivascular oedema were shown in the lung tissues of OVA mice. While mice administrated GSE either alone or with montelukast showed a significant improvement in all the tested parameters. The treatment afforded by co-administration of GSE and montelukastwas found to be better than that of montelukast alone. Results of the present study suggest that montelukast should be used along with GSE for better immune-regulatory effect, as well as, to reduce drugs adverse effects. Therefore, GSE seems to be a potential new drug to modulate inflammatory response in asthma.

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Key words: Asthma, Grape Seed Extract, Anti-inflammatory, Mice.

1. Introduction

Bronchial asthma, commonly called "asthma", it is one of the most common chronic lung diseases in children and adults. Unfortunately it is a disease that currently unable to prevent or cure or method of prevention**(Chunhua *et al*., 2013).**Asthma is a disorder of the conducting airways, which contract too much and too easily both spontaneously and in response to a wide range of exogenous and endogenous stimuli. This airway hyper-responsiveness is accompanied by enhanced sensory irritability of airways and increased mucus secretion and an increase of T-helper cell 2 levels- (Th2) related cytokines.The different clinical expressions of asthma involve a variety of environmental factors that interact with the airways to cause acute and chronic inflammation. Several other factors contribute to this phenomenon, including smooth muscle contraction, edema and remodeling of airways **(Grainge *et al*., 2011).**

Pathogenesis of chronic bronchial asthma (CBA) is complex. Reactive oxygen species (ROS) have been shown to be directly associated with asthma and an oxidant-antioxidant imbalance.Many factors contribute to the magnitude of this burden, such as air pollutants, obesity, mold species and some medications such as non-steroidal anti-inflammatory drugs **(Al-Jawad *et al*., 2012)**. Epidemiological studies in Saudi Arabia revealed an increasing prevalence of asthma in the past three decades, which may be attributed to the rapid lifestyle changes related to the modernization of the Saudi society. As well as, changes in dietary habits and exposure to an environmental factor such as indoor allergens, dust, sand storms, tobacco smoke and indoor animals **(Al-Moamary *et al*., 2009)**. In Saudi Arabia, asthma is one of the most common chronic diseases, affecting more than 2 million **(Al Frayh, 2006)**. Potent anti-inflammatory corticosteroids are the most effective medications available for asthma treatment **(Derendorf *et al*., 2006)**. However, medications for asthma are not completely satisfactory. Therefore, there are concerns regarding the known side effects of corticosteroids, such as disturbance of adrenal functions and overall immune suppression.The chronic nature of this disease and the lack of definitive, preventive and curative therapies lead up to 60% of patients to seek alternative medical

treatments **(Bolleddula *et al*., 2007)**. Antioxidants are important for their protection against oxidative stress due to their ability to detoxify free radicals, such as reactive oxygen species (ROS) **(Angaji *et al*., 2012).**

Grape seed extract (GSE) is known to have a greater antioxidant activity **(Ariga, 2004)**. Itsfunction to prevent the development of many diseases such as diabetic nephropathy, drug-induced renal toxicity, cancer metastasis, fungal infection and ischemic cardiomyopathy has been shown in animal models**(Mantena *et al*., 2006, Han, 2007, Li *et al*., 2008, Guler *et al*., 2011 and Ulusoy, 2012)**. Moreover, GSE was shown to attenuate inflammation in collagen-induced arthritis in mice suggesting its therapeutic role in inflammatory disorders **(Cho, 2009)**. Many of these effects are believed to be the result of strong free radicals scavenging activity of GSE **(Nassiri-Asl and Hosseinzadeh, 2009 and Xia, 2010)**. Grape seed extract also, contains many phenolic compounds, the most bioactive compounds present in grape seeds are proanthocyanidins. Grape seed proanthocyanidin exhibits more powerful antioxidant effects. It has anti-inflammatory effect on experimental inflammation in mice **(Al-Jahdali *et al*., 2008)**. Therefore, the present study aims to evaluate the effect of GSE as curative immune-regulatory against ovalbumin-induced bronchial asthma in male mice.

2. Material and Methods:

Chemical, drugs and kits:

Ova-albumin in the form of white powder was purchased from Rockford, USA. Montelukast was purchased from a pharmacy. Grape seeds extract by Nature`s Way prepared from grape seeds (95% polyphenols) in capsules was obtained from General Nutrition Centers (GNC) in Saudi Arabia. Immunoglobulin E (IgE) ELISA, Interleukin (IL-5) and (IL-13) ELISA kits were purchased from Cusabio Biotech Co., LTD. China. Nitric oxide (NO) as total nitrites/nitrates colorimetric assay kits was purchased from IBL International (Hamburg, Germany). Superoxide dismutase (SOD) and lipid peroxides measured as malondialdehyde (MDA) were purchased from Cayman Chemical Company USA. All chemicals with high analytical grade were purchased from Sigma-Aldrich (St. Louis, MO) Chemical Co.

Sensitization and airway challenge:

Airway inflammation was induced by using the methods described by **Oh *et al*. (2006)**.Mice were immunized via intraperitoneal (i.p.) injection with 10 ug ovalbumin plus 2 mg aluminum hydroxide in 200 uL phosphate buffered saline (PBS)/ mouse, on day 0 and 14. Control mice received i.p. injection of phosphate-buffered saline (PBS) with aluminum hydroxide; then mice were challenged by inhalation of 1% (w/v) OVA solution in PBS using ultrasonic nebulizer with a regulated flow rate for 1 h/day from day 28 to day 30 **(Chunhua *et al*., 2013)**.

Experimental design:

Male mice (n=50) were divided equally to five groups. The first group; negative control received i.p. injection of phosphate-buffered saline (PBS) with aluminum hydroxide. The second group; positive control; asthma-model (OVA). The third group; asthma treated orally with montelukast (30 mg/kg).The fourth group: asthma treated with GSE at a dose level of (150, mg/kg/day orally). The fifth group: asthma group treated with montelukast+ GSE at the same used doses. Montelukastand GSE were given on day 27 and from day 28 to 30 1hr before each challenge.

Blood collection and plasma separation:

Mice were scarified 48 h after the last challenge (on day 32).Blood samples were collected and plasma samples were stored at -20° C until biochemical analysis.

Bronchoalveolar Lavage Fluid (BALF):

On day 32 after blood collection, the BALF was collected by placing polyethylene catheter into the trachea. BALF was collected by washing it with aliquots of 1mL of Hank's balanced Salt Solution through the trachea, then centrifuged and the BALF supernatant stored for determination of cytokines.

Preparation of lung homogenates:

After the BALF fluid had been collected, the lungs were removed and immediately one lung from each mouse was homogenized in 3 mL of ice-cold PBS buffer, then lung supernatant were utilized for subsequent measurement of enzymatic and non-enzymatic antioxidant levels.

Measurement of immunoglobulin E (IgE):

Plasma total immunoglobulin E(IgE) level was determined by enzyme-linked immunosorbent assay (ELISA) kit according to **Burrows *et al.*(1980).**

Measurement of cytokines IL-5, IL-13 in BLAF:

Cytokines concentration including interleukin-5 (IL-5) and interleukin-13 (IL-13) were measured by enzyme-linked immunosorbent assay (ELISA) using reagent kits in mice bronchoalveolar lavage fluid (BALF) according to the manufacture's protocol.

Determination of antioxidant status in lung:

Nitric oxide (NO) as total nitrites/nitrates, malondialdehyde (MDA) and superoxide dismutase (SOD) were determined in lung tissues according to **Johnston *et al.*(2003)**, **Yoshioka *et al*. (1979)**and **Wheeler *et al*. (1990)**, respectively.

Lung tissues histopathology:

After the BALF were obtained, lung tissue was removed, fixed with 10% (v/v) neutral buffered formalin. Tissues were embedded in paraffin,

sectioned at 4 µm thickness, and stained with hematoxylin and eosin (H&E) (Sigma, St. Louis, MO) according to **Bancroft *et al*. (1996).**

Statistical analysis:

The obtained results were analyzed statistically by analysis of variance, for statistical significance ($p \leq 0.05$) using L.S.D. test, one way ANOVA and post hoc multiple comparisons. An IBM computer with a software system SPSS version 22was used for these calculations.

3. Results

Effect of GSE and/or montelukast on cytokine in BLAE and IGE in plasma in asthmatic mice:

In the present study, after the last challenge, there was a significant increase (p<0.001) in BALF IL-5 and IL-13 as well as, plasma IGE of OVA group as compared to control group. Treatment with montelukast was significantly inhibited (p<0.001) OVA-induced inflammation, montelukast group showed a significant decrease (p<0.001) in BALF IL-5 and IL-13 as well as, plasma IGE when compared to OVA untreated group. Montelukast treatment showed a significantly decreased in IL-5, IL-13 and IGE as compared with OVA untreated group. Administration of GSE was significantly decreased (p<0.001) the level of BALF IL-5 and IL-13, as well as plasma level of IGE as compared with OVA untreated group. Co-administration of GSE+ montelukast significantly normalized the levels of all analyzed cytokines, there were a significant decrease in BALF IL-5 and IL-13 as well as plasma IGE as compared with OVA untreated group, at the same time the levels of tested cytokines were significantly decreased (p<0.05) as compared with montelukast treated group.

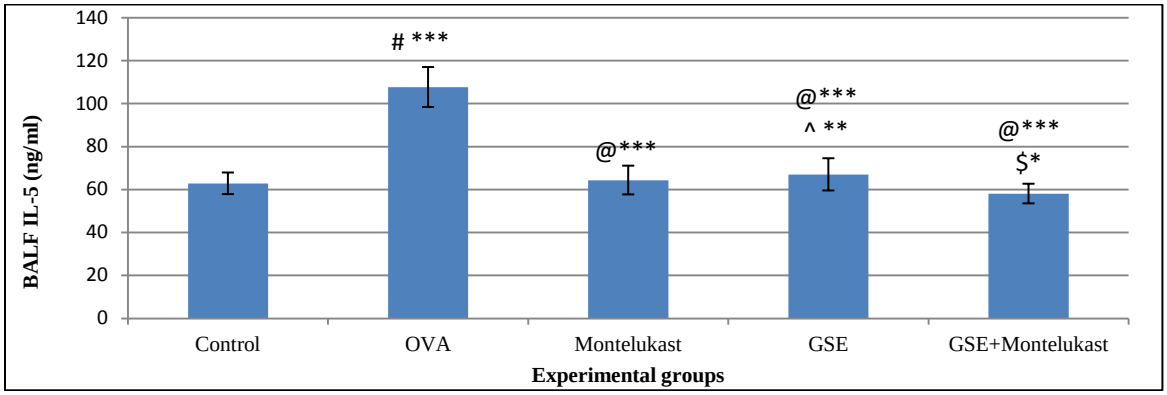

Figure 1: Effect of GSE and/or montelukast on BALF IL-5 concentration in asthmatic mice

Each value represents the mean of 10 mice ± SD. [a] Significant difference between control and OVA group. [b] Significant difference between OVA and OVA treated groups. [c] Significant difference between OVA treated with montelukast and other treated OVA groups. [d] Significant difference between OVA treated with GSE and treated GSE+ montelukast groups. *p< 0.05, **p< 0.01, and ***p< 0.001.

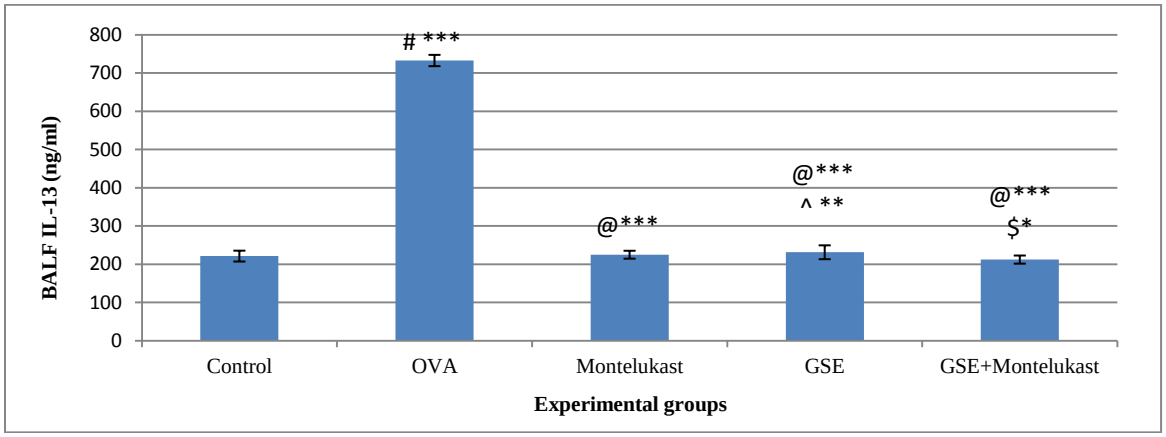

Figure 2: Effect of GSE and/or montelukaston BALFIL-13 concentration in asthmatic mice

Each value represents the mean of 10 mice ± SD. [a] Significant difference between control and OVA group. [b] Significant difference between OVA and OVA treated groups. [c] Significant difference between OVA treated with montelukast and other treated OVA groups. [d] Significant difference between OVA treated with GSE and treated GSE+ montelukast groups. *p< 0.05, **p< 0.01, and ***p< 0.001.

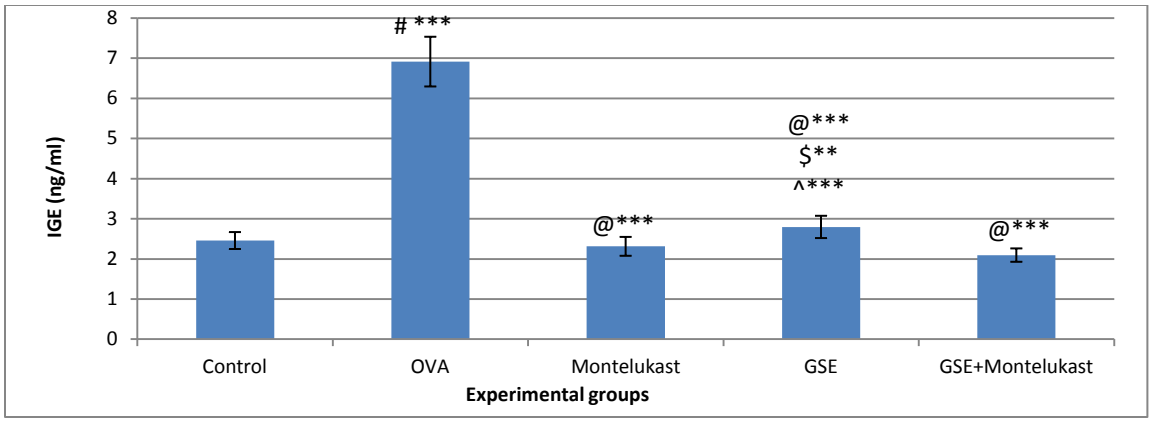

Figure 3: Effect of GSE and/or montelukast on plasma IGE concentration in asthmatic mice

Each value represents the mean of 10 mice ± SD. [a] Significant difference between control and OVA group. [b]Significant difference between OVA and OVA treated groups. [c]Significant difference between OVA treated with montelukast and other treated OVA groups. [d] Significant difference between OVA treated with GSE and treated GSE+ montelukast groups. *p< 0.05, **p< 0.01, and ***p< 0.001.

Effect of GSE and/or montelukast on antioxidant status in lungs of asthmatic mice:

The effect of GSE and /or montelukaston lung levels of pro-oxidant markers and antioxidant enzyme (No, MDA and SOD) in asthmatic mice shown in Table (1). When compared with normal group, OVA group showed a significant increase (p<0.001) in NO and MDA, as well as significant decreased (p<0.001) in SOD levels.Montelukast treatment showed a significant decrease in lung levels of NO and MDA with a significant increase in SOD as compared with OVA untreated group.

Table (1): Effect of GSE and/or montelukastin the lung levels of NO, MDA and SODof asthmatic mice

Experimental groups	NO (µg/ml)	MDA (nM/ mg of protein)	SOD (U/mg of protein)
Control (-ve)	57.27 ± 5.84	4.30 ± 0.38	7.15 ± 0.58
OVA	184.82 ± 6.41 [a] ***	8.74 ± 0.50 [a] ***	3.15 ± 0.28 [a] ***
Montelukast	61.14 ± 5.98 [b] ***	4.76 ± 0.54 [a] *[b] ***	6.76 ± 0.65 [b] ***
GSE	59.28 ± 5.81 [b] ***	4.63 ± 0.45 [b] ***	7.13 ± 0.36 [b] ***
GSE+ Montelukast	55.13 ± 3.88 [b] *** [c]*	4.24 ± 0.36 [b] *** [c]*	7.38 ± 0.62 [b] *** [c]*

Each value represents the mean of 10 mice ± SD. [a] Significant difference between control and OVA group. [b]Significant difference between OVA and OVA treated groups. [c]Significant difference between OVA treated with montelukast and other treated OVA groups. [d] Significant difference between OVA treated with GSE and treated GSE+ montelukast groups. *p< 0.05, **p< 0.01, and ***p< 0.001.

Administration of GSE showed a significant decrease (p<0.001) in the lung NO and MDA levels, accompanied with a significant increase (p<0.001) in lung SOD level as compared with OVA untreated group. The most effective treatment was the group treated with GSE+ montelukast was as compared with the other treated groups.

Effect of GSE and/or montelukast on OV- induced pathological alteration in mice lungs:

The inflammation degree and pathological change in the lung of mice were observed. There was no pulmonary inflammation in control mice (Figure 4&5), but widespread per- bronchiolar and perivascular infiltrates, as well as perivascular oedema (Figure 6-8) were shown in the lung of OVA mice. Treatment with montelukast inhibited the inflammatory cell infiltration as shown in Figure (9&10), the same improvement were shown in GSE treated group. In the group treated with GSE+ montelukast the lung sections appeared to be normal.

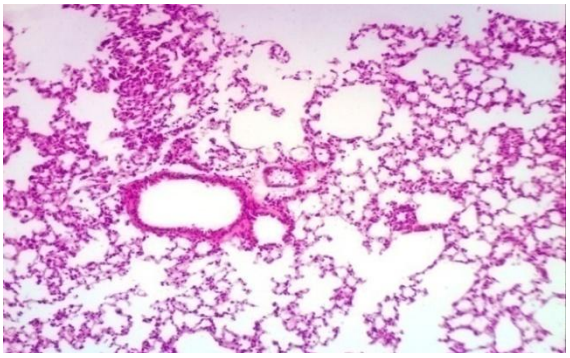

Figure (4): Lung of mice from control (-ve) group showed no histopathological changes. (H & E x 100)

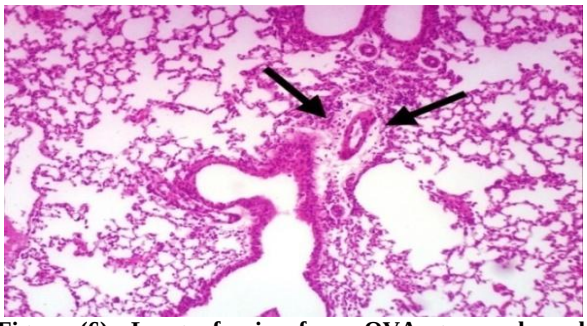

Figure (6): Lung of mice from OVA group showed perivascular oedema associated with inflammatory cells infiltration. (H & E x 100)

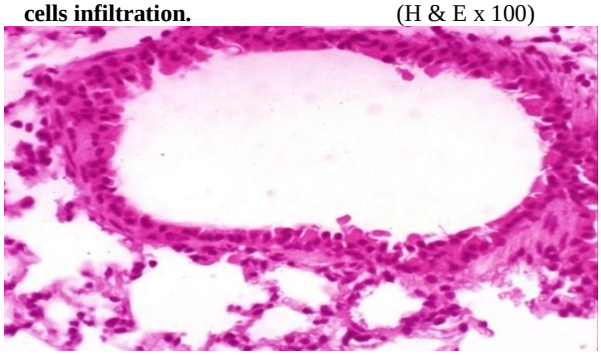

Figure (5): Lung of mice from control (-ve) group showed no histopathological changes. (H & E x 400)

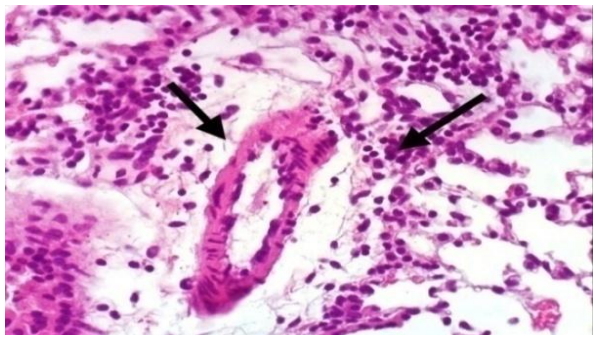

Figure (7): Lung of mice from OVA group showed perivascular oedema associated with inflammatory cells infiltration. (H & E x 400)

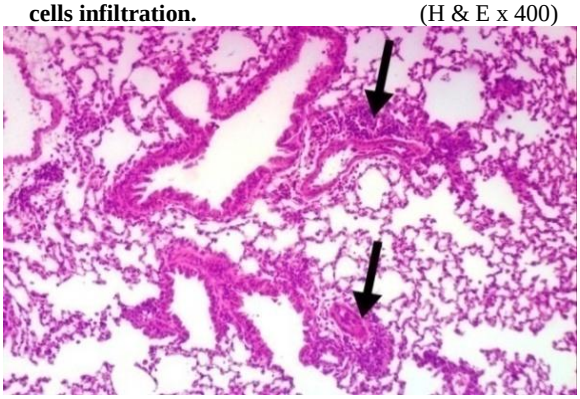

Figure (8): Lung of mice from OVA group showed perivascular inflammatory cells infiltration. (H & E x 100)

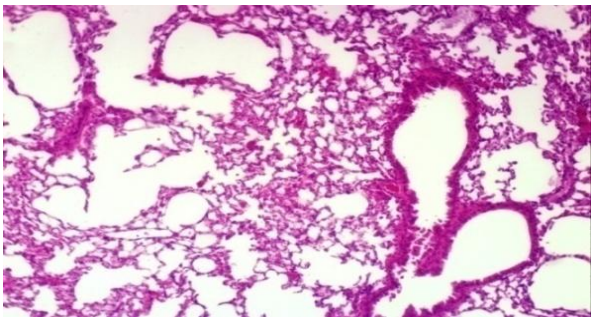

Figure (9): Lung of mice from group montelukast showed no histopathological changes. (H & E X 100)

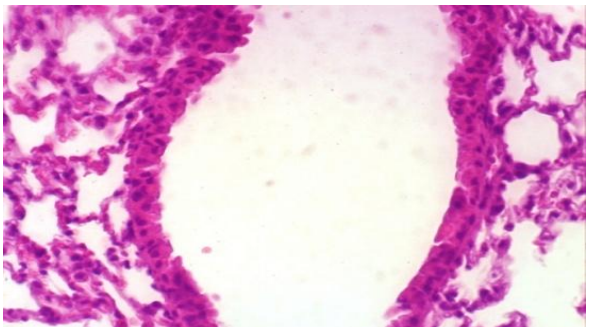

Figure (10): Lung of mice from group montelukast showed no histopathological changes. (H & E X 400)

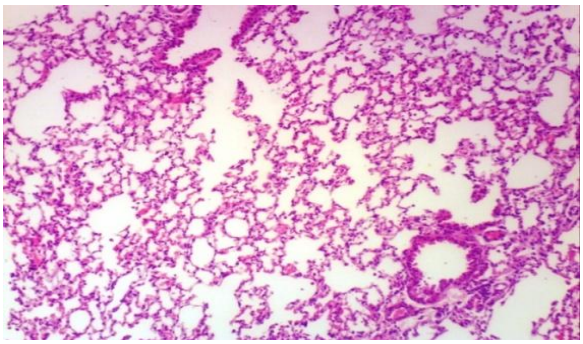

Figure (11): Lung of mice from group GSE showed no histopathological changes. (H & E X 100)

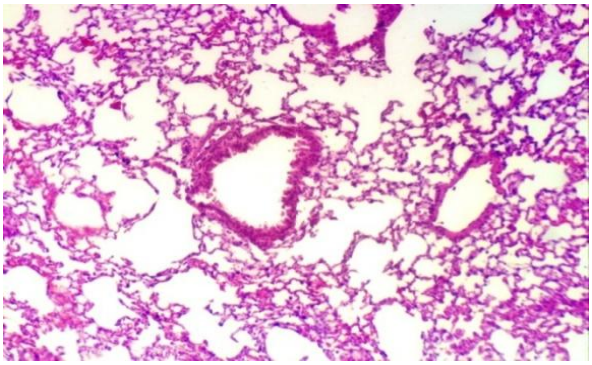

Figure (12): Lung of mice from group GSE+ montelukast showed no histopathological changes. (H & E X 100)

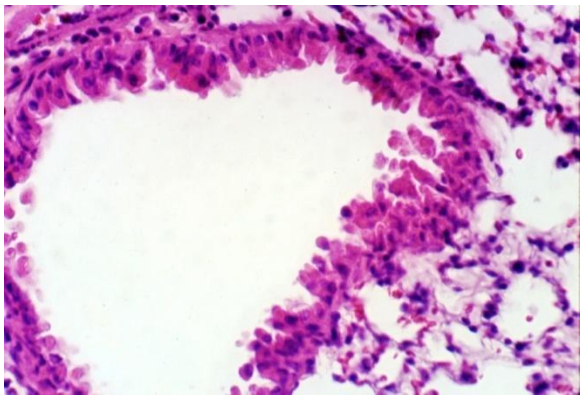

Figure (13): Lung of mice from group GSE+ montelukast showed no histopathological changes. (H & E X 400).

4. Discussion:

Asthma is a chronic inflammation of the bronchial airways and it is characterized by reversible airway obstruction, increased mucus production and infiltration of the airway with eosinophils, neutrophils, mastcells and T-lymphocytes, airway hyper-responsiveness and allergen-specific CD4" **(Houssen *et al*., 2010)**. Asthma is a growing major public health problem worldwide. Studies have shown that the burden of asthma might be significantly higher than previously estimated **(Rabe *et al*., 2004 and Terra *et al*., 2009)**.Medications for asthma are not completely effective and there are concerns regarding the known side effects of corticosteroids **(Bolleddula *et al*., 2007)**. Grape seed extract exhibits chemoprotective properties against ROS **(Rabe *et al*., 2004)**, anti-inflammatory **(Terra *et al*., 2009)**, anti-cancer **(Kaur*et al*., 2006)**, anti-ulcer **(Abbas and Sakr, 2013)** and anti-diabetic potentials **(Pinent *et al*., 2004)**. Grape seed extract has been found to contain phenolic compounds which have many protective properties due to their powerful antioxidant activity **(Clouatre and Kandaswami, 2005)**. The focus of our interest was to investigate the potent therapeutic role of GSE in ameliorating inflammatory against ovalbumin-induced bronchial asthma in male mice.

In an immune-mediated disease such as asthma, T helper (Th) cells played a very crucial role in induction and maintenance of disease state. Amongst two different Th cells, Th1 responsible for secretion of interleukin (IL)-2 and interferon (IFN)-c whereas Th2 is for IL-4, IL-13, and IL-5. Th2 also responsible for switching from IgG to IgE, an important mediator of asthma and which is present abundantly on the cell surface of basophils. Thus, in the present investigation, OVA-sensitized mice caused a significant release and increase BALF levels of cytokines as IL-5 and IL-13 along with plasma level of IgE in OVA untreated mice as compared with control group. In addition, IL-5 is essential for the maturation of eosinophils, and it accumulates in the lung during the inflammatory process of asthma **(Uhm *et al*., 2012)**. Mucus hypersecretion is an important characteristic of asthma, which contributes to the exacerbation of symptoms. While IL-13 is considered a major stimulus for this phenomenon **(DiGiovanni *et al*., 2009).**

However, administration of GSE significantly inhibited these elevated levels which demonstrated it's Th inhibitory potential. These findings are in consistency with **AL-Hanbali *et al*. (2009)** who demonstrated that epicatechin (EC) from GSE suppressed pro-inflammatory cytokines and enhanced the production of the anti-inflammatory cytokine in whole blood cultures which were stimulated with phytohemeagglutinin (PHA) plus lipopolysaccharide (LPS). This indicates that EC possesses anti-inflammatory action and coincides with what is known traditionally or experimentally about grape as an anti-inflammatory plant **(Terra *et al*., 2009)**. Moreover, **Kim *et al*. (2006**) stated that catechins suppressed the production of some proinflammatory cytokines in microvascular endothelial cells in a concentration dependent manner.

The release of inflammatory mediators after allergen challenge is associated with the elevated level of ROS, which in turn caused depletion of intracellular antioxidant **(Adil *et al*., 2014, Kandhare *et al*., 2014, Aswar *et al*., 2015** and **Kandhare *et al*., 2015a)**. Superoxide dismutase is an important enzymatic antioxidant defense. It is responsible for detoxification of ROS *via* reduction of superoxide anion to form hydrogen peroxide **(Kandhare *et al*., 2012 a** and **Visnagri *et al*., 2013)**. In corroboration of the previous study, administration of allergen like OVA caused a significant depletion of SOD level **(Kandhare *et al*., 2015b)**. Furthermore, elevated level of MDA is an indicator of lipoperoxidation is which causes rearrangement of the double bond in the unsaturated fatty acids which then leads to lipid cell membrane destruction **(Kandhare *et al*., 2012 c, Visnagri *et al*., 2012, Kandhare *et al*., 2013** and**Kandhare *et al*., 2015a)**.

Furthermore, elevated ROS also associated with the release of pro-inflammatory mediators such as nitric oxide (NO) which is an unconventional intracellular messenger **(Kandhare *et al*., 2012 b** and **Kandhare *et al*., 2015a)**. Nitric oxide reacts with ROS to gives rise a vicious cycle that leads to nonspecific tissue damage (**Kandhare *et al*., 2012 d** and **Kandhare *et al*., 2015a)**. Clinically it has been proven that elevated production of NO has been associated with increased mucus production and

infiltration of inflammatory **(Hart, 1999** and **Ricciardolo, 2003)**.

However, administration of GSE caused a significant increase in the lung level of SOD along with a decrease in both NO and MDA levels in the lung tissues suggesting its potential antioxidant properties. In accordance with these findings, experimental studies have shown that oral administration of GSE lowered ROS generation and enhanced the activity of the endogenous antioxidant system **(Busserolles *et al*., 2006)**. In particular, proanthocyanidins (PC) the active constituent of GSE have been reported to be able to scavenge free radicals and NO and reduction of their levels **(Bagchi *et al*., 2003)**. **Kim *et al*. (2004)** demonstrated that the pre-treatment of rats with epicatechin (EC), the second active component of GSE, inhibited both IL-1β induced nitrite production and iNOS gene expression *via* the inhibition of nuclear factor κB inhibitor protein. **Mantena and Katiyar (2006)** reported that the antioxidant property of GSE contributed to the inhibition of phosphorylation of mitogen-activated protein kinase (MAPK) through inhibition of H2O2 production and inhibition of the depletion of antioxidant defense enzymes. Consequently, the inhibition of the H2O2-mediated phosphorylation of MAPK in NHEK by GSE indicates that GSEs have the ability to neutralize the effect of H2O2.

Several studies have shown that ROS and RNS, produced by the inflammatory and immune cells **(Chapple, 1996)**, have a specific role in tissue destruction associated with inflammatory diseases **(Waddington *et al*., 2000)**. In the present study, lung of OVA untreated mice showed widespread per-bronchiolar, perivascular infiltrates, and oedema. This result is in accordance with that of **Kumar and Shah (2015)** who found the presence of intense inflammatory cell infiltration in the peribronchiolar region compared with normal mice, while montelukast treatment attenuated the inflammatory cell infiltration in lung tissue after OVA challenge. Treatment with GSE caused a significant inhibition in the OVA-induced pathological alteration in lung tissues. These findings are in agreement with **Hemmati *et al*. (2006)** who attributed the protective effect of grape seed extract to its ability to inhibit the formation of the inflammatory cytokines. **Ahmed *et al.* (2015)** who suggested that GSE could inhibit apoptotic cells death induced by formaldehyde inhalation through increasing the anti-apoptotic capacity in concomitant with decreasing the pro-apoptotic activity of the lung cells.

In conclusion, results of the present study reveal that the administration of GSE in an animal model of asthma could blunt the cytokine response to OVA in sensitized animals *via* down-regulation of nitroso-oxidative stress, cytokine and IgE release which decreases the airway resistance thus supporting its anti-inflammatory as well as bronchodilator role during the allergic response in the lung. These effects suggest a potential for a new asthma treatment since GSE controls the exaggerated inflammatory response observed in this model.

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