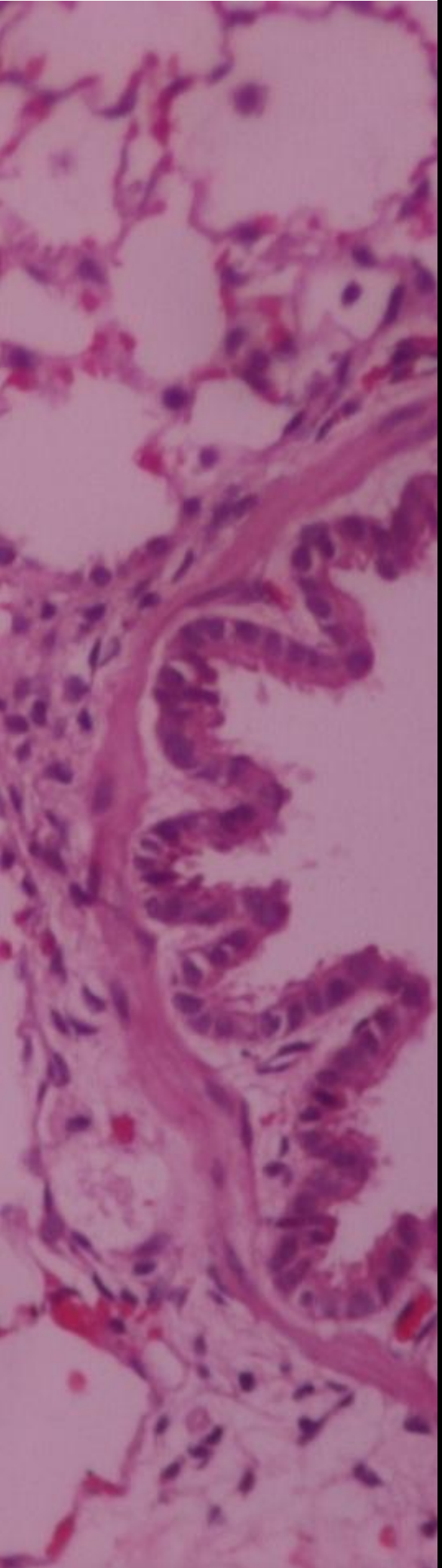

Bleomycin induced pulmonary fibrosis in rats:
Pathophysiology and therapeutic approaches

Ramesh Verma

A Ph.D. thesis submitted to
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BLEOMYCIN INDUCED PULMONARY FIBROSIS IN RATS: PATHOPHYSIOLOGY AND THERAPEUTIC APPROACHES

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CERTIFICATE

This is to certify that the thesis "BLEOMYCIN INDUCED PULMONARY FIBROSIS IN RATS: PATHOPHYSIOLOGY AND THERAPEUTIC APPROACHES" incorporates the results of investigation carried out by the candidate himself and analyzed in the Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara.

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List of Abbreviations

ANOVA:	Analysis of variance
BAL:	Bronchoalveolar lavage
BLM:	Bleomycin
CON:	Control
COX:	Cyclooxygenase
DTNB:	5,5'-Dithiobis-(2-nitrobenzoic acid)
EDTA:	Ethylenediaminetetra acetic acid
ELISA:	Enzyme-linked immunosorbent assay
ERK:	Extracellular signal-regulated kinases
GPx:	Glutathione peroxidase
GSSG:	Oxidised glutathione reductase
H-E staining:	Hematoxylin and eosin staining
IL:	Interleukin
IPF:	Idiopathic pulmonary fibrosis
JNK:	c-Jun N-terminal kinases
KCl:	Potassium chloride
MAPK:	Mitogen-activated protein kinases
MDA:	Malondialdehyde
MMP:	Matrix metalloproteinase
NADH:	Nicotinamide adenine dinucleotide
NBT:	Nitroblue tetrazolium chloride

Nrf2:	Nuclear factor 2-erythroid related factor-2
NSAID:	Non-steroidal anti-inflammatory drug
PBS:	Phosphate buffered saline
PDGF:	Platelet-derived growth factor
PGE:	Prostaglandin E
QUE:	Quercetin
SDS:	Sodium dodecylsulphate
SLD:	Sulindac
SOD:	Superoxide dismutase
TBA-RS:	Thiobarbituric acid reactive substance
TCA:	Trichloroacetic acid
TGF-β:	Transforming growth factor beta
TNFRs:	Tumor necrosis factor receptors
TNF-α:	Tumor necrosis factor-α

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive and ultimately fatal lung disease of unknown aetiology and is reported to be refractory to the existing medical therapy (ATS, 2000). The poor prognosis and hence, unexpected fatality makes IPF among the dreaded pulmonary diseases in the present time. Moreover, IPF is cited as one of the most frequent interstitial lung diseases, which is characterized by the histological pattern of usual interstitial pneumonia (ATS, 2000).

In the extensive literature on idiopathic pulmonary fibrosis, controversy prevails as who first documented the disease. Hamman and Rich are generally recognized as the first to describe idiopathic pulmonary fibrosis as a new clinical and pathological entity. However, several earlier papers published in German have reported necropsy findings consistent with idiopathic pulmonary fibrosis from a contemporary point of view (Homolka, 1987).

Von Buhl (1872) argued that desquamative pneumonia is a distinct form of lung disease, with degeneration and desquamation of alveolar and bronchiolar epithelium. His histological findings included infiltration of the lung stroma by spindle and star cells (i.e., fibroblasts) and an excess of connective tissue. For more chronic changes he used the term ‘chronic interstitial pneumonia’. He considered tuberculosis or syphilis as the probable aetiological agent.

Rindfleisch (1898) published a conclusive necropsy finding of idiopathic pulmonary fibrosis in a 40-year-old clergyman who had progressively worsening cough and dyspnea. He noticed a hypertrophied right ventricle and small, stiff lungs without pleural adhesions. The lung interstitium contained an enormous amount of fibrous tissue with round cells, as well as multiple cystic spaces lined by a single layer of epithelium and connected to small bronchioli. The term he used to describe the fibrous and cystic changes was ‘cirrhosis cystic pulmonum’.

However, even though there are certain important findings reported by a myriad of scientists the most important contribution came from Hamman and Rich when they first

published their observations on idiopathic pulmonary fibrosis in the year 1933 (Hamman and Rich, 1933). A testimony to their growing experience with the disease was their additional publications for the next one decade or so (Hamman and Rich, 1935; Hamman and Rich, 1944). The main contribution of Hamman and Rich was the recognition and detailed description of the clinical and pathological features of a form of lung disease that they called 'acute diffuse interstitial fibrosis of the lungs'. In their cases the disease was clinically characterized by extreme dyspnea, cyanosis and cough. Death occurred between 31 days and 24 weeks after the first admission to hospital. Histologic findings included alveolar edema, erythrocytes, hyaline membrane formation and cuboidal proliferation of alveolar cells. Interstitial infiltrates included occasional leukocytes and in three of four cases there were an excessive number of eosinophils. Excessive proliferation of fibrous tissue in the interstitium was described as a striking histologic feature in all cases. In some cases they also observed necrosis of alveolar and bronchiolar walls.

With further pathologic analysis, several distinct types of pulmonary fibrosis were described, and the terms diffuse fibrosing alveolitis, diffuse interstitial fibrosis and idiopathic pulmonary fibrosis were introduced to describe a more insidious, yet still debilitating form of chronic pulmonary fibrosis (Scadding and Hinson, 1967; Crystal *et al*., 1976). Currently, idiopathic pulmonary fibrosis is considered the most common and severe form of pulmonary fibrosis, with a startling median survival of nearly three years, with no proven effective therapy and with lung transplantation remaining the only viable intervention in end-stage disease (Raghu *et al*., 2011).

Moreover, it is now well recognized that the natural history of idiopathic pulmonary fibrosis is unknown and the onset of symptoms is gradual, starting usually with non-productive cough and exertional dyspnea. With involvement of larger areas of the lung, severe dyspnea at rest and signs of right heart failure develop (ATS, 2002). It has been reported that only in few cases the clinical state is preserved for a period of several years, but in majority of patients health deteriorate more rapidly and during acute exacerbation chances of mortality remain high (ATS, 2002). The prevalence of idiopathic pulmonary fibrosis is estimated at 20/100,000 for males and 13/100,000 for females, and survival time from diagnosis ranges from 2 to 4 years (Kim *et al*., 2006).

Because of stealthy rapid progression, most patients present at an advanced stage of disease. Histological characteristics include remodelling of lung architecture with fibroblastic foci and "honeycombing". The lung involvement is patchy with a predominantly basal and subpleural pattern of matrix deposition and tissue distortion within the pulmonary interstitium, leading to impaired gas transfer and respiratory failure (ATS, 2002; Zhao *et al*., 2010). Treatment options for pulmonary fibrosis are limited. The clinical management focuses on treatment of complications (e.g. right heart failure, infections, etc.), supportive care and in few cases involves lung transplantation. Anti-inflammatory drugs such as prednisone may carry symptomatic relief, but they do not appear to halt progression of fibrosis and their beneficial effects in idiopathic pulmonary fibrosis remain in question. Cytotoxic drugs (cyclophosphamide, azathioprin, etc.) have not been shown to improve lung function or life expectancy and may be associated with harmful side effects (Moeller *et al*., 2008).

Pathogenesis of pulmonary fibrosis

The review of literature so far made it apparent that the underlying mechanisms for the onset and progression of fibrosis or even the natural history of idiopathic pulmonary fibrosis are poorly understood. Epithelial damage, tissue injury and inflammation are clearly involved in the initiation of repair, but how much these initiating events contribute to the chronicity and progression of disease is unknown (Moeller *et al*., 2006).

One hypothesis suggests a role for resident intrapulmonary fibroblasts, responding to a variety of stimuli such as transforming growth factor beta (TGF-β) and differentiating into myofibroblasts, with matrix deposition and scar formation as a result. Repeated microinjuries associated with inflammatory processes are thought to be triggers of progression of the fibrotic response (Ask *et al*., 2006). However, the importance of an ongoing inflammatory component raises questions since anti-inflammatory therapy evoked no significant reversal in the clinical setting associated with idiopathic pulmonary fibrosis (Cook *et al*., 2002). On the basis of the clinical and histopathologic findings in human disease, it is likely that inflammation is important but dispensable (Selman *et al*., 2002). However, the involvement of excessive extra-cellular matrix deposition and abnormal wound healing is widely accepted (Selman *et al*., 2001).

Several factors, including age, genetic susceptibility and environmental agents are known to contribute to lung fibrosis. There are other conditions that might also cause

lung scarring. The lung insult as a result of other conditions is often referred to as "pulmonary fibrosis" and these conditions include the following:

- Diseases, like rheumatoid arthritis and sarcoidosis
- Medicines, such as those used for certain heart conditions
- Breathing in mineral dusts, such as asbestos or silica
- Allergies or overexposure to dusts, animals or moulds.

There are many names for this condition, such as "bird breeder's lung," "farmer's lung," or "humidifier lung." These conditions are called hypersensitivity pneumonitis (Mannino *et al*., 1996; Baumgartner *et al*., 2000; Xaubet *et al*., 2003; Yang *et al*., 2007).
The suggested mechanisms involved in progressive fibrosis are summarized in figure 1.

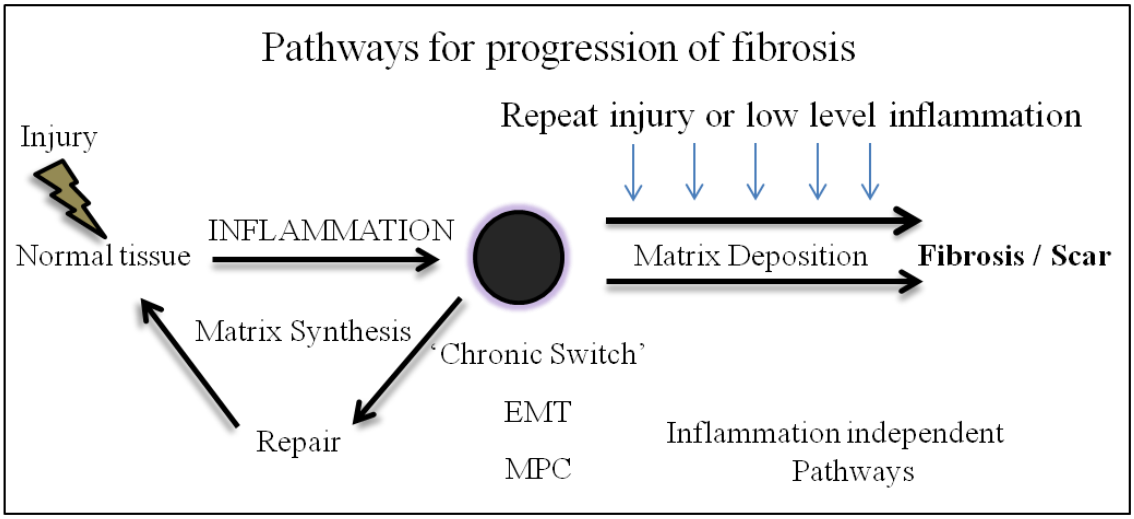


Figure 1. Schematic representation of pathways leading to the progression of fibrosis. Tissue damage initiates inflammation and repair, resulting usually in normal tissue. Repeat injury and inflammation can progress to excessive matrix deposition and scar. Other inflammation independent pathways may involve aberrant epithelial-fibroblast interactions, epithelial mesenchymal transition (EMT), mesenchymal precursor cells (MPC) or autocrine pathways of stimulation resulting in progressive scar formation [Adopted from Moeller *et al*., 2006].

An increased interest exists in mesenchymal cells and in particular, in the fibroblastic foci that are associated with disease progression (Kasai *et al*., 2005). These fibroblastic foci are also associated with increased levels of active transforming growth factor beta (TGF-β) in the fibrotic lung. This cytokine is an important mediator of fibroblast differentiation into the myofibroblast phenotype (Hashimoto *et al*., 2001). Over expression of active TGF-β produces lung fibrosis in animals (Sime *et al*., 1997). TGF-β

is a major regulator of wound repair and a stimulant of reactive oxygen species production in fibroblasts (Waghray *et al*., 2005). Oxidative stress is often defined as an imbalance between reactive oxygen species production and antioxidant defences. Oxidative stress can deregulate cell signalling (Rahman *et al*., 2006) and is a potential target for the development of therapeutics to treat lung fibrosis (Kinnula *et al*., 2005).

Oxidative Stress and Lung Fibrosis

Part of the altered alveolar environment in lung fibrosis involves oxidative stress that is driven by an imbalance between oxidant production and antioxidant defences. Reactive oxygen species are normal byproducts of cellular metabolism and are continually produced at low levels under basal conditions. Biologically, the reactive oxygen species superoxide (O_2^-) is commonly generated from the uncoupling of the cellular electron-transport systems (McCord and Fridovich, 1978). Superoxide can also rapidly react with nitric oxide (NO) to form the strong oxidizing and nitrating agent, peroxynitrite ($ONOO^-$). The reactive oxygen species hydrogen peroxide (H_2O_2) is generated directly from O_2^- through a rapid dismutation reaction that can occur either enzymatically with superoxide dismutases or spontaneously. This means that wherever O_2^- is generated, formation of H_2O_2 also occurs. In addition, H_2O_2 is formed enzymatically as a byproduct of lipid metabolism in peroxisomes (Langan *et al*., 2006). H_2O_2 is stable at biologic pH and easily crosses lipid membranes. Hydrogen peroxide can participate in hydroxyl radical ($HO^{\cdot}$) formation in the presence of metals (Gutteridge, 1994). H_2O_2 readily reacts with thiol functional groups and this type of reaction is proposed to be a key mechanism by which reactive oxygen species modulate cell-signalling events (Dickinson and Forman, 2002).

The impact of reactive oxygen species may be especially important in the lung because of its large surface area and its exposure to higher oxygen levels than other tissues. The lung counters this with a formable array of antioxidant defense systems, starting with high levels of antioxidants in the epithelial lining fluid (Cantin *et al*., 1987). Glutathione is a major water-soluble antioxidant thiol in the lung epithelial lining fluid (Cantin *et al*., 1987), and its levels are lower in subjects with idiopathic pulmonary fibrosis (Cantin *et al*., 1989). The lung also has a number of antioxidant enzyme systems including superoxide dismutase, catalase and glutathione peroxidase (Rahman *et al*., 2006). Overexpression of many of these antioxidant enzyme systems is protective against lung

fibrosis (Gao *et al*., 2008). Many of these antioxidant systems are upregulated during lung fibrosis *via* the nuclear factor erythroid 2–related factor 2 (Nrf2) a redox-sensitive transcription factor (Itoh *et al*., 1999) that when deficient, enhances lung fibrotic responses (Cho *et al*., 2004). It is likely that inadequate antioxidant adaptive responses play a key role in lung fibrosis.

When reactive oxygen species production and antioxidant defences are mismatched, an increase in reactive oxygen species steady state levels leads to an increase in the oxidation of cellular macromolecules. Reactive oxygen species are difficult to measure directly and often are assessed by measuring oxidative footprints in fluids and tissues, such as markers of protein, lipid and DNA oxidation. Subjects with idiopathic pulmonary fibrosis have increased levels of oxidized proteins in their epithelial lining fluid that correlate with the percentage of neutrophils in the epithelial lining fluid (Behr *et al*., 1991; Lenz *et al*., 1996; Rottoli *et al*., 2005). Idiopathic pulmonary fibrosis subjects are also reported to have lower antioxidant capacity in their epithelial lining fluid than do healthy subjects (Rahman *et al*., 1999). Moreover, IPF subjects have higher levels of exhaled ethane (a marker of lipid peroxidation) than do normal subjects and these levels are inversely correlated with partial pressure of oxygen (PaO_2) in arterial blood (Kanoh *et al*., 2005).

Animal models of pulmonary fibrosis

Animal models play an important role in the investigation of diseases and many models are established to examine pulmonary pathobiology. Nevertheless, chronic diseases are more difficult to model. The situation with idiopathic pulmonary fibrosis is even more complicated, since the etiology and natural history of the disease is unclear and no single trigger is known that is able to induce “idiopathic pulmonary fibrosis” in animals. Different models of pulmonary fibrosis have been developed over the years. Most of them mimic some, but never all features of human idiopathic pulmonary fibrosis, especially the progressive and irreversible nature of the condition. A large majority of lung fibrosis animal models involve the overproduction of oxidants and the fibrotic effects are potentiated in antioxidant-deficient animals. However, a number of drugs are known to produce lung fibrosis in humans and animals (Zimmerman *et al*., 1984; Kehrer *et al*., 1986). Many of these drugs are chemotherapeutic agents that stimulate oxidative stress. Ionizing radiation is also a well-characterized method of producing lung fibrosis

in animals, as well as a known adverse effect of cancer radiation treatment (Coggle *et al*., 1986). A number of environmental exposures produce lung oxidative stress and fibrosis, including exposure to asbestos and silica (Yamano *et al*., 1995; Gulumian, 1999). In addition, known cytokines, such as TGF-β, when overproduced, result in lung fibrosis. Most of these models have been shown to stimulate lung-injury responses and oxidative stress. An ideal animal model would mimic human disease as closely as possible, be highly reproducible and consistent, easy to perform, not too costly and widely accessible.

Advantages of animal models in general, as opposed to *in vitro* studies, are the ability to replicate the complex genetic, biochemical and environmental interactions in pulmonary fibrosis in lung tissue, whereas *in vitro* systems are limited to investigate specific cellular or molecular responses. However, no ideal experimental model of idiopathic pulmonary fibrosis, representing all aspects of human disease, exists. Major histologic and biochemical changes in fibrotic tissue can be replicated satisfactorily, whereas the slow progressive character of human disease, an aspect that is poorly understood, is more difficult to mimic. A major limitation with currently available animal models of lung fibrosis is that they do not closely mimic human interstitial pneumonias and many spontaneously resolve over time (Chua *et al*., 2005).

Common methods include radiation damage, instillation of bleomycin, silica or asbestos and transgenic mice or gene transfer employing fibrogenic cytokines.

Irradiation model

In humans, irradiation-induced lung fibrosis occurs as complication of treating thoracic malignancies such as esophageal and bronchial carcinomas, lymphomas or total body irradiation for bone marrow transplantation (Gross and Hunninghake, 2001). TGF-β has been shown to be involved in acute and chronic radiation-induced fibrosis (Epperly *et al*., 2006).

Ionizing radiation produces fibrotic responses and generates hydrogen atom radical $H^{\cdot}$, hydroxyl radical $OH^{\cdot}$, and hydrated electrons from the ionization of water in tissues. All three of these species are highly reactive and can generate and propagate a cascade of different reactive oxygen species mediated DNA damage and induction of TGF-β. Whole-body radiation decreases the levels of endogenous antioxidants and increases markers of lipid oxidation in animals and humans (Clemens *et al*., 1989; Arterbery *et al*.,

1994). Increased oxidative stress has been reported in radiation pneumonitis in humans (Jack *et al*., 1996) and in radiation-induced lung injury in rats (Vujaskovic *et al*., 2001; Fleckenstein *et al*., 2007). Several animal hemithoracic irradiation models of lung fibrosis have been developed and used to screen compounds for antifibrotic effects.

Both catalytic and scavenger antioxidants have been shown to attenuate radiation-induced lung injury and fibrosis in animals. Radiation-induced lung fibrosis is worsened in antioxidant-deficient animals (Thanislass *et al*., 1995; Epperly *et al*., 2000) and attenuated in superoxide dismutases-overexpression models (Malaker and Das, 1988; Epperly *et al*., 2000; Kang *et al*., 2003).

Irradiation models present a reliable tool for induction of fibrosis, as the radiation effect is dependent on dose and volume and therefore, predictable. The long period of time needed to see fibrotic changes comes close to human disease; however, this represents a practical limitation of the model (Moeller *et al*., 2006).

Gene overexpression models - Fibrogenic cytokines

A second approach to models of fibrosis involves transgenic modulation to produce animals with genetic defects, such as tissue specific overexpression of cytokines and growth factors or other extracellular matrix components, leading to downstream activation of specific cytokine pathways. Conventional constitutive transgenic models do not fit well with the adult nature of the human disease, so most useful data makes use of tissue specific inducible transgenic systems to provide temporal and spatial control over transgene expression, initiating or terminating it rapidly and reversibly. The most widely used inducible transgenic system for the lung is based on the tetracycline-controlled transcriptional regulator controlling pneumocyte specific gene promoter sequences (Zhu *et al*. 2002; Lee *et al*. 2003). Transient transgenic models, using adenoviral vector mediated cytokine gene transfer to bronchial, bronchiolar and alveolar epithelium, have been successfully developed (Sime, *et al*., 1997; Kolb, *et al*., 2001; Kolb *et al*., 2002) and can be applied to all ages of rodents (Gauldie *et al*., 2003). Both of these transgenic systems have provided data on several key molecular regulators of the fibrotic process, along with data showing other molecules which apparently do not directly contribute to the fibrotic process.

A number of cytokines have been shown to stimulate fibrotic events and include TGF-β, tumor necrosis factor alpha, platelet-derived growth factor, connective tissue growth factor, endothelin, granulocyte–macrophage colony-stimulating factor, interleukin (IL-1β), IL-6, IL-10, and IL-13 (Ask *et al*., 2006). The best studied of these various cytokines in lung fibrosis is TGF-β produced by a variety of different cell types namely thrombocytes, macrophages, lymphocytes, epithelial and endothelial cells and fibroblasts. TGF-β is secreted to the extracellular space in an inactive form, bound to latency associated peptide (LAP), which is also bound to latent TGF-β -binding protein (LTBP) and is directly connected to the extracellular matrix (Leitlein *et al*., 2001). Multiple enzymes, extracellular proteases or physical/chemical factors such as ionizing radiation, are able to activate TGF-β (Sheppard, 2001). TGF-β isoforms have a number of effects on cellular responses including modulating cell growth, migration, differentiation and apoptosis (Safayhi *et al*., 1985). TGF-β induces myofibroblast differentiation, extracellular matrix synthesis and inhibits extracellular matrix breakdown (Zhang and Phan, 1996). TGF-β1 is abundant in bronchoalveolar lavage fluid and present in fibroblastic foci biopsies from idiopathic pulmonary fibrosis subjects (Broekelmann *et al*., 1991). Overexpression of TGF-β1 in animals induces a progressive lung fibrosis that is largely independent of inflammation (Smith, 1971). TGF-β1 produces oxidative stress by the induction of reactive oxygen species production and a decrease in expression of cellular antioxidants (Koli *et al*., 2008). TGF-β1 induces reactive oxygen species production by activation of NADPH oxidases and through mitochondrial dysfunction (Thannickal and Fanburg, 1995; Sturrock *et al*., 2006). Very few studies have been reported on the effects of antioxidants in this relatively new animal model of lung fibrosis.

Fibrogenic environmental agents

A number of environmental dust and fibre exposures have been associated with the development of lung fibrosis (Mossman *et al*., 1991). Both silica and asbestos exposures produce lung fibrosis in animals (Driscoll *et al*., 1995; Kawanami *et al*., 1995) and pneumoconiosis in humans (Ross and Murray, 2004). Both silica and asbestos produce injury and oxidative stress in the lungs of animals leading to lung fibrosis (Abidi *et al*., 1999).

Several asbestos and silica induced animal models of lung fibrosis have been developed. Silica and asbestos-induced lung fibrosis are worsened in antioxidant-deficient animals (Lombard-Gillooly and Hubbard, 1993; Fattman *et al*., 2006) and attenuated in catalase overexpression models (Mossman *et al*., 1990). Catalytic and scavenger antioxidants have been shown to attenuate asbestos- and silica-induced lung injury and fibrosis in animals. Catalytic antioxidants have been shown to have protective effects against silica induced injury (Day, 2008).

Fibrogenic drugs

Paraquat is a redox-active herbicide that also is known to produce fatal pulmonary fibrosis in humans (Toner *et al*., 1970; Copland *et al*., 1974) and animals (Smith, 1971; Smith *et al*., 1974). Paraquat is thought to redox cycle with cellular enzymes to produce the paraquat cation radical that rapidly reacts with oxygen to form O_2^- (Bus *et al*., 1976; Adam *et al*., 1990; Gray *et al*., 2007). Paraquat produces lung oxidative stress in animals (Kornbrust and Mavis, 1980; Brigelius *et al*., 1986; Dusinska *et al*., 1998; Wilhelm *et al*., 1999; Adachi *et al*., 2003) and humans (Minakata *et al*., 1993; Ishii *et al*., 2002). Both catalytic and scavenger antioxidants have been shown to attenuate paraquat induced lung injury and fibrosis in animals. Administration of superoxide dismutases has been shown to attenuate paraquat-induced lung injury *in vitro* (Ishii *et al*., 2002) and *in vivo* (Wasserman and Block, 1978; Ogata *et al*., 1994).

The antiarrhythmic drug amiodarone produces lung fibrosis in humans (Sobol and Rakita, 1982) and animals (Cantor *et al*., 1984). Some data suggest a role for oxidative stress in amiodarone-induced lung fibrosis. Amiodarone inhibits mitochondrial complex I and II respiration and produces mitochondrial dysfunction in lung epithelial cells and macrophages (Bolt *et al*., 2001). In the ventilated perfused rabbit lung system, amiodarone increases the levels of reactive oxygen species and oxidized glutathione (Kennedy *et al*., 1988). Further studies have revealed that amiodarone is metabolized to an aryl radical that may give rise to other reactive oxygen species (Vereckei *et al*., 1993; Nicolescu *et al*., 2007). Both catalytic and scavenger antioxidants have been shown to attenuate amiodarone-induced lung injury and fibrosis in animals.

Bleomycin model

Bleomycin, first discovered in 1962 is a glycosylated linear nonribosomal peptidechemotherapeutic antibiotic, produced by the bacterium "*Streptomyces*

verticillus" (Umezawa, 1967; Adamson, 1976). Its use in animal models of pulmonary fibrosis is based on the fact that fibrosis is one of the major adverse effects of bleomycin in human cancer therapy. Bleomycin plays an important role in the treatment of lymphoma, squamous cell carcinomas, germ cell tumors and malignant pleural effusion, where it is injected intrapleurally. It is believed that bleomycin acts by causing single and double-strand DNA breaks in tumor cells and thereby interrupting the cell cycle. This happens by chelation of metal ions and reaction of the formed pseudoenzyme with oxygen, which leads to production of DNA-cleaving superoxide and hydroxide free radicals (Claussen and Long, 1999). An overproduction of reactive oxygen species can lead to an inflammatory response causing pulmonary toxicity, activation of fibroblasts and subsequent fibrosis (Grande *et al*., 1998; Chaudhary *et al*., 2006). Bleomycin hydrolase, a bleomycin inactivating enzyme, critically influences the effects of this drug on different tissues. The lungs maintain low levels of the enzyme and therefore, are more susceptible to bleomycin-induced tissue injury (Sebti *et al*., 1989).

Pulmonary side effects in patients are dose-dependent, age-related and occur more often in the presence of pre-existing pulmonary diseases or smoking. Lung toxicity develops in approximately 10% of patients receiving bleomycin and is clinically associated with cough, dyspnea, fever, cyanosis and deterioration of lung function parameters. Within weeks to months this response might progress to pulmonary fibrosis in nearly 1% of patients (Compendium of Pharmaceuticals and Specialties, 2006).

Bleomycin as an agent to induce experimental lung fibrosis was first described in dogs (Fleischman *et al*., 1971), later in mice (Adamson and Bowden, 1974), hamsters (Snider *et al*., 1978) and rats (Thrall *et al*., 1979).

It causes inflammatory and fibrotic reactions within a short period of time, even more so after intratracheal instillation. The initial elevation of pro-inflammatory cytokines (interleukin-1, tumor necrosis factor-α, interleukin-6, interferon-γ) is followed by increased expression of pro-fibrotic markers (transforming growth factor-β1, fibronectin, procollagen-1) with a peak around day 14. The "switch" between inflammation and fibrosis appears to occur around day 9 after bleomycin challenge (Chaudhary *et al*., 2006).

It has been reported that histological hallmarks, such as intra-alveolar buds, mural incorporation of collagen and obliteration of the alveolar space are present in bleomycin-treated animals similar to idiopathic pulmonary fibrosis patients (Usuki, 1995). This observation has led to the assumption, that bleomycin reproduces typical features of the human disease and hence, the use of this model has become very popular. Further, the bleomycin model has the advantage that it is quite easy to perform, widely accessible and reproducible, and therefore, fulfils important criteria expected from a good animal model. Fairly consistent dosages have been established for each species to achieve a fibrotic response and dependent on the route of administration, different fibrotic patterns develop. Intratracheal instillation of bleomycin, the standard route of administration, results in bronchiocentric accentuated fibrosis, whereas intravenous or intraperitoneal administration induces subpleural scarring similar to human disease (Chua *et al*., 2005). The bleomycin model has contributed tremendously to elucidate the roles of cytokines, growth factors and signalling pathways involved in pulmonary fibrosis. For instance, it has helped to determine TGF-β as one of the key factors in the development of pulmonary fibrosis (Zhao *et al*., 2002).

However, despite undisputed qualities and some similarities in histological alterations, the bleomycin model has significant limitations with regard to understanding the progressive nature of human idiopathic pulmonary fibrosis. As mentioned, bleomycin causes an inflammatory response, triggered by overproduction of free radicals, with induction of proinflammatory cytokines and activation of macrophages and neutrophils, thus resembling acute lung injury in some way. The subsequent development of fibrosis, however, is at least partially reversible, independent from any intervention (Izbicki *et al*., 2002).

The above review of literature suggests that the standard pharmacological agent for induction of experimental pulmonary fibrosis in animals is bleomycin hence the same was used as an inducing agent to develop the animal model in the present study. A schematic representation of the major cellular events leading to lung fibrosis is depicted in figure 2.

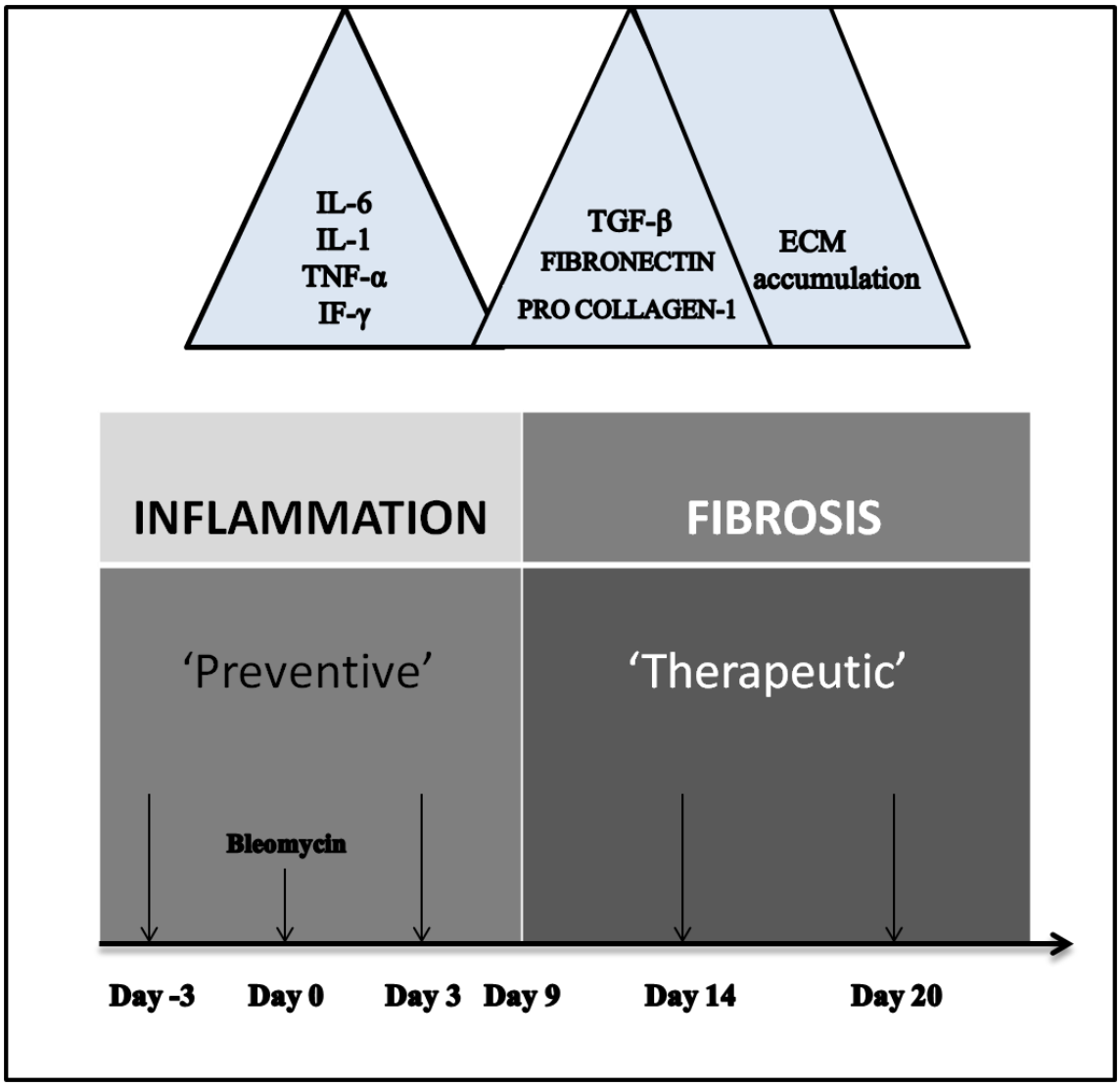


Figure 2. Pictorial representation of sequence of events in bleomycin-induced pulmonary fibrosis. After administration of bleomycin, there is the onset of an acute inflammatory response lasting up to 8 days, followed by fibrogenic changes resulting in deposition of matrix and distortion of lung structure out to 28 or 35 days. Treatments during the first seven days would be considered "preventive" while treatments during the later stages after days 7–10 would be considered "therapeutic" [adopted from Moeller *et al*., 2008]

The entire current animal models of lung fibrosis have clear involvement of inflammation and reactive oxygen species in their pathogenesis. The evidence of inflammation and redox imbalance in lung fibrosis is substantial and thus the rationale for testing anti-inflammatory and antioxidants as potential new therapeutics for lung fibrosis is appealing. In the literature there are number of compounds which have antioxidant effect and have effectively attenuated the bleomycin induced fibrosis. Soumyakrishnan, and Sudhandiran, (2011) reported that the isoflavone daidzein possesses anti-fibrotic effect against bleomycin induced fibrosis in rats. The garlic

derived antioxidant diallyl sulfide effectively thwarted bleomycin induces pulmonary fibrosis (Kalayarasan *et al*., 2008). Also there are ample evidences of anti-inflammatory drugs attenuating the bleomycin induced fibrosis. Arafa and co-workers (2007) reported the anti-fibrotic action of meloxicam, a non steroidal anti inflammatory drug, in Swiss albino mice. Another anti inflammatory drug namely montelukast has been known to arrest the development of bleomycin-induced pulmonary fibrosis in mice (Shimbori *et al*., 2011). The dexamethasone, a corticosteroid and a drug used to reduce inflammation in many conditions was found to delay bleomycin-induced lung fibrosis in rats (Chen *et al*., 2006). An anti-inflammatory Chinese herbal formulation feining was reported to be effective against bleomycin-induced pulmonary fibrosis Sprague–Dawley rats (Liang *et al*., 2011).

The detrimental role of reactive oxygen species in many disease states has led to the development of new antioxidants. One such group of compounds with potential antioxidant property is the flavonoids present in fruits and vegetables, of which quercetin (3,3',4',5,7- pentahydroxyflavone) (Figure 3) has attracted much attention for its beneficial health effects (Hollman and Katan, 1999; Skibola and Smith, 2000; Boots *et al*., 2008; Jagtap *et al*., 2009). Quercetin, a typical flavonoid ubiquitously present in fruits and vegetables, such as onion, tea, apples and berries. It exhibits antioxidative, anti-inflammatory and vasodilating effects, and has been proposed to be a potential anti-cancer agent (Erlund, 2004).

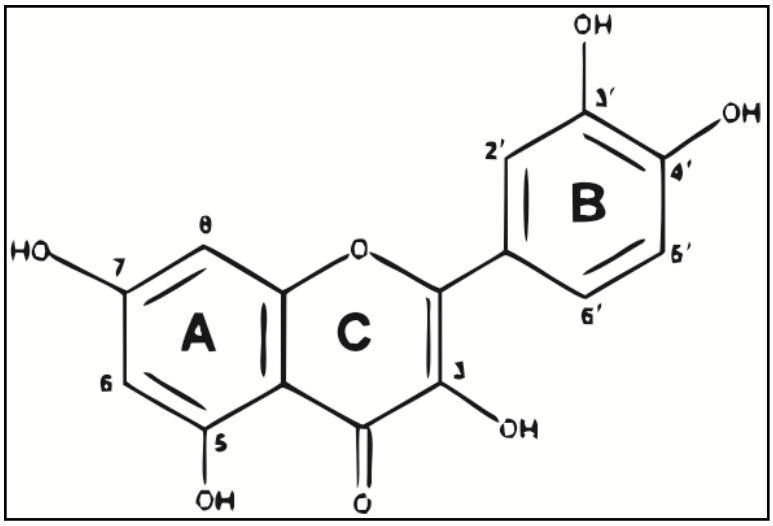


Figure 3. Molecular structure of quercetin- 3,3',4,5,7-Pentahydroxyflavone (from Kroon *et al.*, 2004).

Chemistry of quercetin

Flavonoids are characterized by 2 benzene rings (A and B) which are connected by an oxygen-containing pyrene ring (C). The three rings are planar and the molecule is relatively polarized. Three intermolecular hydrogen bonds are observed: two with the carbonyl group and the other between the hydroxyl groups in ring B (Mendoza-Wilson and Glossman-Mitnik, 2004). Analysis of the structure–activity relationships showed that the physical properties of quercetin are determined by its chemical structure. Quercetin can participate in the complex reactions with metals, affecting the transportation, reactivity, bioavailability and toxicity of metal ions. It possesses three possible chelating sites in competition which could be classified in the following way: catechol >α-hydroxycarbonyl>β-hydroxycarbonyl (Cornard *et al*., 2005).

Quercetin possesses all the structural elements characteristic of an anti-oxidant: (1) an ortho-dihydroxy or catechol group in ring B, (2) a 2, 3-double bond, and (3) the 3- and 5-OH groups with the 4-oxo group (Bors *et al*., 1990; Silva *et al*., 2002).

Pharmacokinetics of quercetin

At present, the pharmacokinetics of quercetin has not been fully characterized, although a number of studies have been carried out both in animals and humans. Flavonoid glycosides from diet are believed to pass through the small intestine be hydrolyzed to aglycone by enterobacteria in the caecum and colon and absorbed into epithelial cells via lipophilicity-dependent simple diffusion (Bokkenheuser *et al*., 1987). Quercetin glucosides can also be directly absorbed via the sodium-dependent glucose transporter-1 or excreted into the lumen via multidrug resistance protein 2 (Murota and Terao, 2003). After their facilitated uptake by means of carrier-mediated transport, quercetin glycosides are often hydrolyzed by intracellular β-glucosidases (Nemeth *et al*., 2003). The intestinal lactase phlorizin hydrolase displays a specific activity towards flavonoid glycosides (Day *et al*., 2000). Hydrolysis to aglycone by enterocytes or enterobacteria is crucial for the efficient absorption of quercetin glucosides in the intestinal tract (Nemeth *et al*., 2003).

Quercetin absorbed from the intestinal lumen is mostly converted to conjugated metabolites before entering circulation, and the major metabolites present in human plasma are quercetin 3′-O-β-Dglucuronide (Q3′GA) and quercetin 4′-O-β-D-glucuronide

(Q4′GA). Some metabolites still possess considerable activity, including Q3GA, Q3′GA and Q4′GA (Williamson *et al*. 2005).

Regarding the tissue distribution, a recent study observed that quercetin is found concentrated in lungs, testes, kidneys, thymus, heart and liver, with the highest concentrations of quercetin and its methylated derivatives detected in the pulmonary tissue (de Boer *et al*. 2005).

Urinary elimination of quercetin is not the main excretion routes in human subjects or in rats. A substantial portion of the metabolites may be excreted in the bile (Murota and Terao, 2003). Quercetin can undergo microbial degradation in the colon to phenolic acids and CO_2, which is exhaled in the breath (Abrahamse *et al*. 2005).

Beneficial health effects of quercetin against various oxidative stress related diseases have been documented (Flora, 2009). However, studies examining its potential pneumoprotective effects are limited. Therefore, it was of interest to determine the ameliorative role, if any, of quercetin against bleomycin-induced lung injury because of the former's potent antioxidant activity.

Further, it is suggested that along with reactive oxygen species, inflammation also plays an important role in the development of lung fibrosis. From the earliest descriptions of patients with pulmonary fibrosis, cellular inflammation in the lung parenchyma has been a consistent pathologic finding (Hamman and Rich, 1935 and 1944; Scadding and Hinson, 1967; Crystal *et al*., 1976). Histological analysis has shown varied accumulations of lymphocytes, macrophages, plasma cells, eosinophils and neutrophils, and the presence of lymphoid follicles with germinal centres has been observed in many patients in the lung interstitium (Scadding and Hinson, 1967; Crystal *et al*., 1976). Also, the forerunner for bleomycin-induced pulmonary fibrosis has been considered the inflammatory response induced by the anti-neoplastic agent (Arafa *et al*., 2007).

The non-steroidal anti-inflammatory drug (NSAID), sulindac ([Z]-5-fluoro-2-methyl-1-[*p*-(methylsulfinyl)-benzylidene]indene-3-acetic acid), is well known for its anti-inflammatory activity, which is due to its ability to inhibit the cyclooxygenases enzymes thereby inhibiting prostaglandin synthesis (Vane *et al*., 1998).

The mechanism of action of NSAIDs involves reduction of prostaglandin synthesis by inhibition of cyclooxygenase enzyme (COX) enzyme through competitive antagonism for arachidonic acid binding to the COX. For a drug to be an effective competitive inhibitor for arachidonic acid binding to COX, the drug must possess both high lipophilic and acidic properties to mimic the natural substrate chemistry. Sulindac possess a polar group in the lipophilic tail (Vane *et al*., 1998). Moreover, sulindac is a sulfoxide prodrug, upon consumption it is converted into the metabolites (figure 4) sulindac sulfide and sulindac sulfone (Duggan *et al*., 1977).

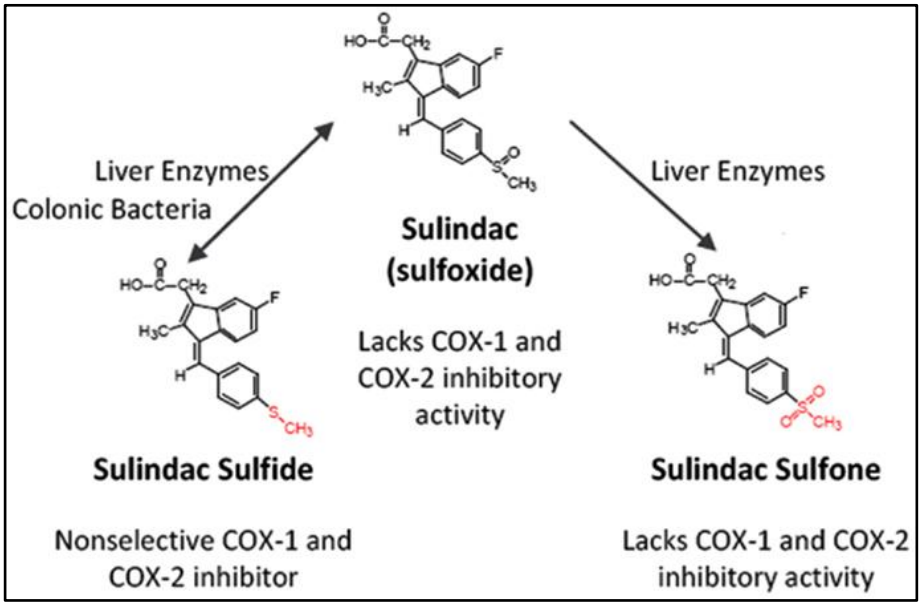


Figure 4. Metabolism of Sulindac to the sulphide and sulphone derivatives (Gurpinar *et al*., 2013)

Pharmacokinetics of Sulindac

Since all NSAIDs are highly lipophilic substances, members of the class share similar, if not identical, absorption properties. Drug absorption after oral administration is generally rapid and complete. Sulindac, and its sulfone and sulfide metabolites, are bound to plasma proteins, predominantly to albumin. In humans, after oral intake of sulindac, peak plasma concentration of this drug is reached after 1 h and after 2 to 4 h for its sulfide and sulfone metabolites. All three forms of sulindac undergo varying degrees of entero-hepatic recirculation. There are little data regarding the distribution of sulindac into human tissues and fluids (Davies and Watson, 1997).

The original findings of cellular inflammation in the lung have been supplemented with an extensive accumulation of scientific studies which have implicated numerous inflammation-related cytokines and cell surface molecules in profibrotic mechanisms (Barnes and Adcock, 2009). Stemming from the observations on inflammatory cells, cytokines, chemokines, and cell surface molecules, and the inflammation hypothesis has dominated the field of pulmonary fibrosis for nearly four decades (Homer *et al*., 2011; Wynn, 2011; Crystal *et al*., 2002). Therefore, it was thought pertinent to study the modulatory role, if any, of sulindac, a known cyclooxygenases inhibitor which has also reported to have antiradical effects too (Fernandes *et al*., 2003) on belomycin-induced pulmonary fibrosis in rats.

Moreover, as it has been observed in many complex disorders, it is likely that combination of molecules, rather than a single drug, will be more effective as therapeutic agent. Therefore, the present study was extended to evaluate the inhibitory effects of quercetin in combination with sulindac on bleomycin induced rat pulmonary fibrosis as quercetin possess an antioxidant while sulindac is well known for its anti-inflammatory activity. To the best of our knowledge, so far no scientific data is available regarding the combined effects of quercetin and sulindac on experimentally induced pulmonary fibrosis.

MATERIAL AND METHODS

2. Material and methods

2.1 Materials

Test Substance

Inducing Agent : Bleomycin

Test Drug : Quercetin, Sulindac and a Combination of Quercetin plus Sulindac

Vehicle

Normal saline solution by intratracheal instillation on day 0 and 0.5% Carboxymethylcellulose solution in distilled water for oral gavage from day 1 to day 20.

Preparation of Inducing Agent

Bleomycin at the dose of 6.5 U/kg body weight was dissolved in normal saline in the concentration of 3 U/mL (Wang *et al.,* 2002).

Preparation of Test Drug

The drug was freshly prepared in 0.5% Carboxymethylcellulose solution in distilled water and the concentration was adjusted so that each animal received 10 ml/kg body weight. The dose volume changed as per the change in the body weight.

- Quercetin at the dose of 100 mg/kg body weight/day (Tang *et al.,* 2012)
- Sulindac within its therapeutic anti-inflammatory dose (ED_{50} for rats, 20 mg/kg body weight) (Vaish and Sanyal, 2012)
- Quercetin and Sulindac at the combined group dosed with 50 and 10 mg/kg body weight/day, respectively.

Materials used in the study were of Analytical Grade (AR). Materials and instruments that were used in during the study are listed in Table 2.1.

Table: 2.1 Materials used in study

Chemicals	**Company**
Bleomycin	Biochem Pharmaceutical Industries Ltd, Mumbai
Quercetin	Sigma Aldrich Chemie GmbH, Germany
Sulindac	Sigma Aldrich Chemie GmbH, Germany
Normal Saline	Merck Limited, Mumbai
Thiobarbituric Acid	Hi-Media, Mumbai
Sodium Nitroprusside	Hi-Media, Mumbai
Phenazine Methosulphate	Hi-Media, Mumbai
Nitroblue Tetrazolium Chloride	Hi-Media, Mumbai
Hematoxylin stain powder	Qualigens fine chemicals, Mumbai
Eosin	Merck limited, Mumbai
Formaldehyde	Qualigens fine chemicals, Mumbai
Disodium hydrogen phosphate, anhydrous purified	Merck specialist, Mumbai
Sodium dihydrogen phosphate monohydrate	Merck specialist, Mumbai
Paraffin wax	Qualigens fine chemicals, Mumbai
Ethylene diamine tetra acetic acid	RANKEM, RFCL Ltd, New Delhi
Trichloroacetic acid.	RANKEM, RFCL Ltd, New Delhi
Pyridine	S. D. Fine chemicals Ltd , Mumbai
Potassium hydroxide	Qualigens Fine chemicals , Mumbai
Potassium dihydrogen phosphate	Qualigens Fine chemicals , Mumbai
Sodium hydroxide pallets	Qualigens Fine chemicals , Mumbai
Cupric Sulphate	Qualigens Fine chemicals , Mumbai
Hydrogen peroxide	Qualigens Fine chemicals , Mumbai
Folin CioCalateau Reagent	SISCO Research lab Pvt. Ltd, Mumbai
n – butanol	SISCO Research Lab Pvt. Ltd, Mumbai
Nicotinamide adenine dinucleotide phosphate	Hi-Media, Mumbai
Reduced Glutathione	SISCO Research Lab Pvt. Ltd, Mumbai
5,5’-dithiobis(2-nitrobenzoicacid nitrobenzoic acid	SISCO Research Lab Pvt. Ltd, Mumbai

Chemicals	Company
Sodiun potassium tartrate	SamirTech- Chem Pvt Ltd, Vadodara
Thiobarbituric acid	Titan Biotech Ltd, Bhiwadi
Sodium Lauryl Sulphate	Qualigens Fine Chemicals, Mumbai

Instrument	Company
Tattoo machine	AIMS^{TM} Tattoo Machine, USA
Weighing Balance	Sartorius AG, Germany
Spectrophotometer	Jasco V-650 Series, USA
ELISA Reader	Biotek EL808, USA
Microplate Washer	ELX 50, USA
Tissue Homogeniser	York Scientific Industry, Delhi
Centrifugation machine	ThermoScientific, Germany
Embedder	Medite, India
Microtome	Leica, Germany
Tissue processor	Leica, Germany
Incubator	Tempo, Mumbai

2.2 Animals

Test Species	:	Rat *(Rattus norvegicus)*
Strain	:	Wistar (RCCHan: WIST)
Animal Source	:	Animal Breeding Facility, Jai Research Foundation, Vapi
Number of Animals	:	50 animals
Age of the Animals	:	10-12 weeks old at the time of dosing
No. of animals per group	:	10 rats (10 males)

Acclimatization

Rats were allowed to acclimatize to the experimental room conditions for a period of nine days prior to randomization. During the acclimatization period, the rats were observed for clinical signs of any disease.

Husbandry Practices

Caging **:**	Polypropylene rat cages covered with stainless steel grid top were used. Autoclaved clean rice husk was used as the bedding material.
Water Bottle**:**	Each cage was supplied with a polypropylene water bottle (capacity 300 ml) with a stainless steel nozzle.
Room Sanitation:	Each day, floor of the Single Corridor Room (SCR) experimental room was swept and all work tops and floor were mopped with a disinfectant solution.

Animal Identification

In each cage, animals were identified with tattooing and coloured cage label showing Research Number, Group Number, Dose, Cage Number and Animal Number.

Feed and Water

Animals were provided with feed and water, *ad libitum.* The certificate of nutrient content of the Teklad Certified Global rat feed was supplied by feed manufacturer – Harlan, U.S.A. The quality of feed and water is regularly monitored at Jai Research Foundation. There were no known contaminants in the feed and water at the levels that would have interfered with the experimental results obtained.

Feed	:	Teklad Certified Global rat feed manufactured by Harlan, U.S.A.
Water	:	U.V. sterilised water filtered through Kent Reverse Osmosis water filtration system

Environmental Conditions

Temperature Range : 20 - 26°C

Relative Humidity Range : 30 - 70%

Photoperiod : The photoperiod was artificially maintained for 12 hours light and 12 hours dark, light hours being 0600 – 1800 hours

2.3 Method

Animals

Specific pathogen-free, healthy young adult male Wistar rats (RCCHan:WIST), of 10 to 12 weeks were used in this study. They were obtained from the Barrier Maintained Rodent Animal Breeding Facility, Jai Research Foundation, Vapi, India. The animals used in this study were handled and treated in accordance with the strict guiding principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The test facility at Jai Research Foundation is AAALAC accredited and is also complies with the National Good Laboratory Practice, India. The experimental protocols were approved by the Institutional Animal Ethics Committee (JRF CPCSEA Approval no. 35/1999/CPCSEA: JRF Research Number - R-570/2010).

Induction of pulmonary fibrosis

Briefly, after the weight was recorded, the rats were anesthetized using a combination of Ketamin (80 mg/kg body weight, i.p.) and Xylazine (20 mg/kg body weight, i.p.) as per standard protocol (Teixeira *et al.,* 2008). A midline incision was made in the neck and the exposed trachea was intubated with tracheal cannula under direct visualization. For induction of pulmonary fibrosis, the rats received a single dose of 6.5 U/kg body weights, Bleomycin sulfate dissolved in 0.5 mL of 0.9% NaCl solution by intratracheal instillation on day 0 of the experiment (Wang *et al.,* 2002). Control rats were given a single intratracheal dose of sterile saline alone.

Experimental groups

The animals were randomized into 5 groups each consisting of 10 animals (Gad and Weil, 1994):

- Group I - Vehicle control rats were given 0.5% Carboxymethylcellulose solution orally from day 1 to day 20 of the experiment.
- Group II - Bleomycin treated rats were administered 0.5% Carboxymethylcellulose solution orally from day 1 to day 20 of the experiment.
- Group III - Animals were orally administered with Quercetin (100 mg/kg body weight/day) in 0.5% Carboxymethylcellulose solution from day 1 to day 20 of the experiment after Bleomycin instillation (Tang *et al.*, 2012).
- Group IV - Animals were treated with Sulindac within its therapeutic anti-inflammatory dose (ED_{50} for rats, 20 mg/kg body weight) in 0.5% Carboxymethylcellulose solution from day 1 to day 20 of the experiment after Bleomycin instillation (Vaish and Sanyal, 2012).
- Group V - Animals were treated with Quercetin (50 mg/kg body weight/day) and Sulindac (10 mg/kg body weight/day) in 0.5% Carboxymethylcellulose solution from day 1 to day 20 of the experiment after Bleomycin instillation.

Preparation of lung tissue homogenate

On day 21 of the experiment, six animals from each group were sacrificed with thiopentone sodium and the lung lobes were excised and weighed. Left lung was used for biochemical estimation while the right lung was used for histological evaluation. Broncholaveolar lavage was performed in four animals from each group under anaesthesia with thiopentone sodium.

2.4 Observations

Mortality

All animals were observed twice a day for mortality and signs of morbidity.

Clinical Signs

All the animals were observed for any abnormal signs at least twice a day.

Body Weight

The animals were weighed at the beginning, through and at the end of experiments. The changes in body weight were recorded.

2.5 Bronchoalveolar lavage fluid

Broncholaveolar lavage fluid was obtained by the injection of 3 mL saline (three times, total 9 mL) followed by gentle aspiration of the fluid from the lung after securing an intratracheal catheter within a trachea. With this catheter, the ratio of the recovery of lavage fluid was approximately 80% and did not significantly differ among the groups. The total numbers of cells in the broncholaveolar lavage fluid were counted with a hemocytometer. For differential counts of leukocytes in the broncholaveolar lavage fluid, smear slides were prepared and stained with Giemsa solution. Differential cell counts were performed on 300 cells per smear.

2.6 Biochemical Assays

Determination of Lung Hydroxyproline

The hydroxyproline assay was performed as described by Edwards and O'Brien (1980). Briefly, the lung was dried and hydrolysed at 120°C in a pressure vessel for 2-4 h. The acid hydrolysates and standards were added to 1.5-ml tubes, along with the same volume of citric/acetate buffer (citric acid, sodium acetate, sodium hydroxide, glacial acetic acid and n-propanol, pH 6.0) and chloramine T solution (chloramine T dissolved in Milli-Q water). The tubes were incubated for 20 min at room temperature and Ehrlich's solution [aldehyde-perchloric acid reagent (p-dimethyl-amino-benzaldehyde) perchloric acid and n-propanol] was added to the tubes, which were then incubated at 60°C for 15 min. The absorbance of the reaction product was read at 550 nm. Sample concentrations were determined from the standard addition curve and the concentration was expressed as mg/g dried tissue.

Determination of Lipid Peroxidation

The lipid peroxidation was determined as described by Ohkawa *et al.* (1979). The method is based on the formation of a red chromophore that absorbs light at 532 nm following the reaction of thiobarbituric acid (TBA) with malonedialdehyde (MDA) and other breakdown

products of peroxidised lipid collectively called as thiobarbituric acid reactive substance (TBA-RS).

A known amount of tissue was washed with 0.1 M phosphate buffered saline (PBS) (pH 7.4) several times. The tissue was then blotted dry, weighed and analyzed immediately. The tissue was then homogenized in PBS (pH 7.4) to prepare 10% homogenate. 0.2 mL of 10% tissue homogenate was then added to the tube containing 0.2 mL of 8.1% sodium dodecylsulphate (SDS), 1.5 mL of 20% acetic acid solution (adjusted to pH 3.5 with 1N NaoH) and 1.5 mL of 1% thiobarbituric acid (TBA) solution. The blank was prepared for each sample by substituting the TBA solution with distilled water. The final volume was made up to 4.0 mL with distilled water. The solution was mixed and heated in a water bath at 95°C for 60 minutes. The tubes were then immediately cooled and 2.0 mL of the aliquot was transferred to a centrifuge tube to which an equal volume of 10% TCA was added. The solution was mixed and centrifuged at 1000 g for 15 minutes. The aliquot of the resulting supernatant fraction was read against blank on a Jasco V-650 Series Spectrophotometer at 532 nm. The lipid peroxidation was calculated using the formula:

$$X = \frac{\text{O. D. of sample} \times \text{Dilution factor}}{E \times \text{Tissue weight (mg)}}$$

Where,

Dilution factor = 0.5

Extinction coefficient of MDA (E) = 1.56×10^5

The results were expressed as n moles of MDA/mg tissue/60 minutes.

Glutathione Content

The concentration of glutathione in lungs of control and treated groups of rats was assayed by the method of Grunert and Philips (1951). Glutathione present in the tissue reacts with sodium nitroprusside to give a red coloured complex in saturated alkaline medium.

A known amount of tissue was homogenized in 3 mL of 3% metaphosphoric acid and 1mL of distilled water and saturated with salt solution (1.5g NaCl crystals) and centrifuged. 2 mL of aliquot was added to the sample tube containing 6 mL of saturated NaCl solution and allowed to stand for 10 minutes at 20°C for equilibrium. The blank tube was run with 2 mL of 2% metaphosphoric acid instead of the aliquot. Then 1 mL of each

0.067 M sodium nitroprusside and 1.5 M sodium cyanide mixture were added to blank and sample tubes respectively. The colored complex developed was measured at 520 nm. The concentration of glutathione was calculated using the regression formula:

X = 272.01 (Y) – 2.32

Where,

X = concentration of glutathione obtained from the regression formula

Y = optical density of the sample

The value of X was substituted in the formula

$$\text{Glutathione concentration} = \frac{\text{Concentration of sample} \times \text{Dilution} \times 1000}{\text{Tissue weight (mg)} \times \text{Aliquot volume}}$$

Where,

Dilution = 3 mL

Aliquot volume = 2 mL

The glutathione content was expressed as µg/g tissue weight.

Glutathione Peroxidase (GPx) (E.C.1.11.1.9) activity

The glutathione peroxidase activity in lungs of control and all the treatment group rats was based on the method of Paglia and Valentine (1967).

A known amount of tissue was homogenised in a known volume of 0.01% digitonin and centrifuged at 12000 g for 30 minutes at 0 to 1.5°C. The supernatant was used as an aliquot. The reaction mixture consisted of 0.3 mL 50 mM potassium phosphate buffer (pH 7.0), 0.1 mL 1 mM ethylenediaminetetra acetic acid (EDTA), 0.1 mL 1 mM sodium azide (NaN_3), 0.1 mL 0.2 mM nicotinamide adenine dinucleotide hydrogen phosphate (NADPH), 0.1 mL E.U./mL oxidised glutathione reductase (GSSG), 0.1 mL 1 mM reduced glutathione, 0.1 mL 25 mM H_2O_2 and 0.1 mL aliquot. The tubes were then incubated for 5 minutes at room temperature. Decrease in absorbance at 340 nm was recorded for 3 minutes. The blank tube contained complete reaction mixture without the

enzyme source. The blank readings were subtracted from the test readings for obtaining the correction factor for the spontaneous reaction. The enzyme activity was calculated using the formula:

$$X = \frac{\text{Sample O. D.} \times \text{Standard concentration} \times \text{Dilution factor}}{\text{Standard O. D.}}$$

Where,

Standard concentration =1.5

Dilution factor = 20

The enzyme activity was expressed as U/g tissue.

Catalase (E.C.1.11.1.6) Activity

The catalase activity was assessed in the tissue of control and all treated groups of rats by the modified method of Luck (1963).

A known volume of tissue was homogenised in a known volume of 0.01% chilled digitonin and centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant was used as an aliquot. The assay mixture consisted of 0.5 mL 50 mM phosphate buffer (pH 7.0) and 1.0 mL aliquot. The decrease in absorbance was noted every 5 seconds. The blank tube contained complete reaction mixture without the enzyme source. The non-enzymatic readings were subtracted from the test readings for obtaining the correction factor for the spontaneous reaction. The enzyme activity was calculated using the formula:

$$X = \frac{\text{O. D. of samples} \times \text{Total volume of assay}}{\text{Aliquot volume} \times E}$$

Where, E = Extinction coefficient = 0.0041 m mol^{-1} x mm^{-1}

The enzyme activity was expressed as µ moles H_2O_2 consumed/mg tissue/min.

Superoxide Dismutase (SOD) (E.C.1.15.11) Activity

The superoxide dismutase activity was assessed in the tissue of control and all treated groups of rats by the modified spectrophotometric method of Kakkar *et al.* (1984). In this method, the formazan formed at the end of the reaction indicates the presence of the enzyme. The activity was calculated wherein one unit of the enzyme concentration

required to inhibit 50% of the optical density of chromogen formed in one minute at 560 nm under the assay condition.

A known amount of the tissue was homogenized in cold normal saline. The tubes were then centrifuged. The supernatant was discarded and to the pellet 2 mL of hypotonic solution (0.56% KCl) was added to yield the enzyme extract. After 15 minutes incubation this was centrifuged at 1000 g and the supernatant was used as the sample. In the assay mixture, the control consisted of 2.4 mL pyrophosphate buffer (0.052 M sodium pyrophosphate buffer at a pH 8.4 adjusted with 0.052 M NaH_2PO_4) 0.1 mL of freshly prepared phenazine methosulphate (186 µ moles phenazine methosulphate in double distilled water), 0.3 mL of nitroblue tetrazolium chloride (30 µ moles freshly prepared NBT) and 0.2 mL of fresh NADH (780 µ moles in double distilled water). To the sample tube 0.2 mL of enzyme was added prior to the addition of NADH in a total of 3 mL of assay system. Then the reaction was stopped by the addition of 1 mL of acetic acid exactly 90 seconds after the addition of NADH. Four mL of n-butanol was added to the tubes and shaken vigorously to extract the formazan. Then the tubes were centrifuged for 10 minutes at 1000 g and supernatant was used for the measurement of optical density at 560 nm against butanol via spectrophotometer. The enzyme activity was calculated by the formula:

$$X = \left\{\frac{\text{O. D. of Control}}{\text{O. D. of Sample}} - \text{Accuracy factor}\right\} \times \frac{\text{Dilution}}{\text{Standard enzyme units}}$$

Where,

Accuracy factor = 1.0

Dilution = 10.0

Standard enzyme unit = 3.0

The activity was calculated and expressed as units/mg tissue.

Reduced Glutathione (GSH)

Reduced glutathione level was measured by the method of Ellman (1959). To the homogenate 5% trichloro acetic acid was added to precipitate the protein content of the homogenate. After centrifugation (at 3000 rpm for 10 min) the supernatant was taken and DTNB solution (Ellman's reagent) was added to it. The absorbance was measured at 412 nm. The level was calculated using the formula.

$$C = \frac{A}{E} D$$

Where,

A = absorbance

E = extinction coefficient

D = dilution factor

The results were expressed in µg/g tissue.

2.7 Measurement of Tumor Necrosis Factor-α

Tumor necrosis factor-α in plasma was assayed by specific enzyme-linked immunosorbent assay using commercially available ELISA test kits (XpressBio Life Science Products, USA). The kit contains a tumor necrosis factor- α coat monoclonal antibody to tumor necrosis factor-α. The representative standard curve was generated using the tumor necrosis factor-α standard with the kit and was expressed as pg/mL.

2.8 Pathology

After sacrifice, each lung tissue weighed to determine the relative organ weights and then was perfused and fixed in 10% neutral buffer formalin and routinely processed and embedded in paraffin. Serial sections (4 µm) were cut and stained with hematoxylin & eosin and Masson's trichrome for light microscopic evaluation to examine the degree of fibrosis. The severity of fibrosis was individually assessed using the semi-quantitative grading system described by Szapiel *et al.* (1979). According to this system the fibrosis in lung specimens were graded as none (0) when no alveolitis was observed; mild (+) - when focal lesions occupying less than 25% of the lung was noticed in alveolar septum; moderate (++) - when widespread alveolitis involving 25-50% of the lung was observed; severe (+++) - when a diffused alveolitis spanning more than 50% of the lung, with occasional consolidation of air spaces and patches of hemorrhagic areas within the interstitium was observed. The entire lung section was reviewed at a magnification of 10X. Each of the 25 random microscopic fields per section were detected, a score ranging from 0 to 3 was assigned. All assessments were performed in double-blind fashion.

2.9 Statistical Analysis

Statistical analyses were carried out by analysis of variance (ANOVA) followed by appropriate post hoc test [multiple comparison tests (LSD)]. All analyses of data were performed using SPSS for windows version 12.0 and probability values of 0.05 or less were considered statistically significant.

RESULTS

The present study was conducted to elucidate the possible inhibitory effects of a dietary flavonoid (quercetin) and a NSAID (sulindac) individually at high yet pharmacologically acceptable doses on idiopathic pulmonary fibrosis in rat. The drugs were administered orally for 20 days post intra-tracheal instillation of a single dose of bleomycin on day 0. In addition to the above mentioned individual dosages of the potential ameliorative agents, the present study was also extended to evaluate the inhibitory effects of quercetin in combination with sulindac at low doses (half the dose when administered individually) on bleomycin induced rat pulmonary fibrosis. The rationale for selecting the above combination therapy was the assumption derived from literature that the pulmonary fibrotic response upon bleomycin challenge is a function of both prolonged inflammation as well as heightened expression of free radicals.

3.1 Clinical Observations

Animals from all the treatment groups appeared to be normal throughout the experimental period (Annexure I-V).

3.2 Changes in Body Weight and percent body weight

Table 1 shows the mean body weight (Figure 1A) and percent body weight changes (Figure 1B) in each group. The body weights of all the treatment groups were comparable to the control group on days 3 and 7. Single intra-tracheal administration of bleomycin (6.5 U/kg) resulted in a marked decrease in their body weight on days 14 and 21 as compared to the control group possibly because of severe lung tissue injury caused by generation of free radicals due to bleomycin instillation. These reductions in body weights associated with bleomycin administration were significantly restored ($p \leq 0.05$) by the treatment with either quercetin or sulindac. There was no significant difference observed between the mean body weights of two treated groups. Co-administration of quercetin and sulindac in combination at the lower doses was associated with significant restoration ($p \leq 0.05$) of body weight compared to bleomycin treated rats and that remained comparable to that of the control group rats on day 21.

A significant increase ($p \leq 0.05$) in the percent body weight change was observed in all the three treatment groups on days 14 and 21 of the experiment which was found to be comparable to the bleomycin treated group on days 3 and 7 of the experiment respectively. A significant decrease ($p \leq 0.05$) in percent body weight change was observed in all the treatment groups on days 3, 7 and 14, post instillation of bleomycin on day 0 when compared to control group rats. On day 21 a significant decrease ($P \leq 0.05$) was observed in the bleomycin treated group as well as in quercetin treated and sulindac treated groups when compared to that of control group. However, the percent body weight change in quercetin and sulindac in combination at the lower doses was found to be comparable to that of the control group rats which showed that the combination treatment was more effective than the individual treatment of both the drugs.

3.3. Change in percent Relative Lung Weight

Bleomycin instillation was accompanied by significant increase ($p \leq 0.05$) in the percent relative weight of lungs when compared with the control group as shown in Figure 1C and Table 2. This deleterious effect due to treatment of bleomycin instillation was ameliorated by treatment with either quercetin or sulindac compared to bleomycin treated group with no significant difference ($p \leq 0.05$) between the two treated groups. Co-administration of quercetin and sulindac in combination at the lower doses was also found to be equally effective ($p \leq 0.05$).

3.4 Biochemical Estimations

3.4.1 Hydroxyproline Content

The effect of various treatments on the hydroxyproline content of lung homogenate is shown in Figure 1D and Table 2. Measurement of hydroxyproline is an efficient index of collagen deposition, since collagen contains significant amount of this amino acid. After 20 days, mean lung hydroxyproline content in bleomycin treated group and sulindac treated group were found to be significantly higher ($p \leq 0.05$) than that in the control group. Both quercetin and sulindac treated groups nevertheless, showed apparent reduction in hydroxyproline content in comparison with bleomycin treated group with no significant difference between the two treated groups. However, co-administration of quercetin and sulindac reduced the hydroxyproline content to a near normal level highlighting the positive influence of combination therapy over individual treatment.

3.4.2 Lipid Peroxidation

Malondialdehyde (MDA) was assayed spectrophotometrically as a marker to lipid peroxidation. Bleomycin treatment produced a significant increase ($p \leq 0.05$) in the lung tissue MDA content, an index for lipid peroxidation when compared with control group. As shown in Figure 1E and Table 3, bleomycin induced increments in MDA content of the lung were significantly reversed by quercetin and sulindac treatments, when compared to that of bleomycin group. Results of the sulindac treatment did show a significant decrease to that of the bleomycin treated group but were significantly higher ($p \leq 0.05$) than that of control group at the end of the experiment. Co-administration of quercetin and sulindac appreciably reduced the level of MDA content in the lung tissue and the level remained comparable to the control group.

3.4.3 Glutathione Content

As shown in Figure 1F and Table 3, a significant decrease ($p \leq 0.05$) in the level of glutathione was observed in bleomycin group when compared to that of the control group rats at the end of the experiment on day 21. The level of glutathione in the remaining treatment groups showed signs of improvement. However, the level of glutathione in the sulindac treated group remained low at the end of the experiment while in the quercetin treated group, a significant increase in the level of glutathione was observed when compared to that of bleomycin treated group. Co-administration of quercetin and sulindac in combination at the lower doses also showed a marked increase in the level of glutathione when compared to that of bleomycin treated group ($p \leq 0.05$) on day 21 of the experiment.

3.4.4 Glutathione Peroxidase (GPx) Activity

Single intra-tracheal instillation of bleomycin produced a significant reduction in the glutathione peroxidase activity in lung tissue after 21 days when compared with control groups (Figure 1G and Table 3). The depletion in glutathione peroxidase activity in the tissue reflects indirectly the generation of free radicals. However, treatment with quercetin and sulindac improved the activity of glutathione peroxidase as is evident from the significantly higher ($p \leq 0.05$) values of glutathione peroxidase activity in the lungs of quercetin and sulindac group when compared to bleomycin group. Co-administration of quercetin and sulindac in combination at the lower doses evoked remarkable recovery in

the level of glutathione peroxidase activity and the value for the same remained comparable to that of control rats on day 21 of the experiment.

Although there was a significant increase ($p \leq 0.05$) in the level of glutathione peroxidase activity in lung tissue after 21 days of quercetin and sulindac treated groups when compared to bleomycin group but it was still found to be significantly low when compared to that of the control group rats. However, the level of glutathione peroxidase activity in quercetin and sulindacin combination at the lower doses was found to be comparable to that of the control group at the end of the experiment which shows that the quercetin and sulindac in combination at the lower doses was found to be more effective than their individual high doses.

3.4.5 Catalase Activity

Depletion in the levels of catalase activity in the tissue reflects indirectly the generation of free radicals. Single intratracheal instillation of bleomycin produced a significant decrease in the catalase activity in lung tissue after 21 days when compared with the control group rats (Figure 1H and Table 3). Even though there was a certain sign of recovery from the bleomycin challenge, the activity of catalase remained significantly low ($P \leq 0.05$) in the quercetin and sulindac treated groups too when compared to that of the controls. However, co-administration of quercetin and sulindac at the given dose showed a significant increase ($P \leq 0.05$) in the activity of catalase at the end of the experiment when compared to that of bleomycin treated rats and the values of catalase activity were comparable to that of the control group.

3.4.6. Superoxide Dismutase (SOD) Activity

As in the case of former enzyme, depletion in the levels of superoxide dismutase activity in the pulmonary tissue reflects the compromised lung antioxidant defence capacity due to the generation of free radicals in the wake of bleomycin administration. A single intratracheal instillation of bleomycin lead to a significant decrease in the superoxide dismutase activity in lung tissue on day 21 of treatment compared to that of control group rats (Figure 1I and Table 3). Individual treatment of quercetin and sulindac though significantly ($p \leq 0.05$) prevented the depletion of superoxide dismutase activity when compared to the bleomycin treated animals, the values were still remarkably higher than that of the control rats. The combination therapy of quercetin plus sulindac nevertheless, effectively revived the levels of superoxide dismutase activity even at the lower dose and

the intensity of SOD activity was found equivalent to that of control group at the end of the experiment.

3.4.7 Reduced Glutathione (GSH)

Figure 1J and Table 3 shows the changes in the levels of reduced glutathione in the lung tissues of control, bleomycin administered, quercetin treated, sulindac treated and quercetin plus sulindac administered rats on day 21 post-treatment. The results imply that bleomycin has significantly decreased the levels of reduced glutathione when compared to that of control group. Both quercetin and sulindac treatment significantly ($p \leq 0.05$) improved the levels of reduced glutathione, compared to the control values on day 21 of treatment, the amount of GSH in these two treatment groups still remained low at a statistically significant level. However, when both the drugs were administered in combination at the lower doses, the treatment was found to be effective as the level of reduced glutathione was found to be comparable to that of the control group.

3.5 Total and Differential Cell Count in Bronchoalveolar Lavage Fluid

Table 4 shows the effect of various treatments on total and differential cell count in bronchoalveolar lavage fluid of rats from control as well as from various treatment groups. Bleomycin treatment caused a significant increase in the total cell count in the bronchoalveolar lavage fluid as compared to control rats. In rats treated with quercetin, total cell count remained more or less similar to the levels of control rats. However, in rats treated with sulindac, the total cell count remained significantly high compared to that of controls even after 20 days of treatment. Nonetheless, in both the treatment groups a significant decrease in the total cell count was observed when compared to that of bleomycin treated group.

When quercetin and sulindac were administered in combination at the lower doses a significant decrease ($p \leq 0.05$) in the total cell count was observed at the end of the experiment, when compared to that of the bleomycin challenged rats. Moreover, the total cell count in the BAL of this treatment group was found to be comparable to that of the control group.

Table 4 also shows the effect of various treatments on bronchoalveolar lavage fluid on differential cell count in control and various experimental groups of rats. Under normal conditions, the count of macrophages is higher in bronchoalveolar lavage as it serves as a

first line of defence against respiratory pathogens. However, in bleomycin treated group, a significant decrease was observed in the percentage of alveolar macrophages. The treatment with quercetin, sulindac and co-administration of both these drugs in combination at the lower doses on day 21 showed a significant increase ($p \leq 0.05$) in percentage of alveolar macrophages when compared to that of the bleomycin treated group all though it was still significantly lower ($p \leq 0.05$) than the control group rats at the scheduled end of the experiment.

The differential cell count showed a significant increase in the percentage of neutrophils in the lungs of rats exposed to bleomycin which is an indication of acute lung injury. However, the treatment with quercetin, sulindac and co-administration of both these drugs on day 21 showed a significant reduction ($p \leq 0.05$) in bleomycin-induced surge of blood cells in the bronchoalveolar lavage but the level of neutrophils in the lungs of rats still remained significantly higher ($p \leq 0.05$) in all the treatment groups when compared to that of the control group rats indicating subtle signs of lung injury even after 21 days of bleomycin challenge (Table 4).

Percentage of eosinophils showed a significant hike ($p \leq 0.05$) in the BAL from the lungs of rats exposed to bleomycin when compared to that of the controls. The data for the eosinophil cell count in the treatment groups namely quercetin, sulindac and co-administration of both these drugs at the lower doses also showed a significant increase ($p \leq 0.05$) in percentage of eosinophil count in comparison to that of the control group rats. However a remarkable decrease in the percentage of eosinophil count was observed in the combination treatment group of quercetin plus sulindac when compared to that of the bleomycin treated group (Table 4).

Percentage of lymphocytes showed a significant drop ($p \leq 0.05$) in bronchoalveolar lavage from the bleomycin treated rats when compared to that of the control group rats. Nevertheless, a significant increase ($p \leq 0.05$), in the level of lymphocytes was evident in rats subjected to quercetin, sulindac and co-administration of both these drugs when compared to that of the bleomycin treated group. Moreover, the level of lymphocytes in the three drug treatment groups mentioned above was found to be comparable to that of the control group at the end of the experiment (Table 4).

3.6 Tumor Necrosis Factor-α (TNF-α) Concentration

The plasma levels of tumor necrosis factor-α is presented in Figure 1K and Table 5. The tumor necrosis factor-α level in the plasma of bleomycin-administered group remained elevated on day 21 when compared with the control group ($P \leq 0.05$). Treatment with quercetin as well as sulindac decreased the bleomycin induced increase in TNF-α level in plasma at the end of the experiment when compared to bleomycin treated group. However, the levels of tumor necrosis factor-α remained elevated in sulindac treated group on day 21 when compared to that of control group ($P \leq 0.05$). Though quercetin appreciably reduced the bleomycin induced hike in plasma TNF-α level, co-administration of quercetin plus sulindac was found to be a more effective ameliorative agent since the combination therapy brought the plasma concentration of TNF-α to that of control rats.

3.7 Histopathological Examination of Lung Tissue

Histopathological abnormalities in lungs were assessed on day 21 using hematoxylin and eosin staining (Figures 2 and 3).

Sections from vehicle group displayed normal histoarchitecture with no apparent pathological changes (Figure 2A). However, in bleomycin treated group, marked histopathological changes such as interstitial thickening with inflammatory cell infiltration, extensive collagen deposition and collapsed alveolar spaces were observed (Figures 2B and 2C). Inflammatory infiltrates were localized to the alveolar spaces and peribronchial wall which consisted of mainly fibroblasts, lymphocytes, macrophages and few neutrophils. Furthermore, there were few foci of alveolar epithelialization and mild hyperplastic bronchial epithelium.

Although fibrotic lesions were observed in quercetin and sulindac treated groups, the extent of alveolitis and fibrosis was markedly less severe compared to that of bleomycin treated group, which indicated ameliorative effect of these treatment regimens to reduce pulmonary fibrosis (Figure 2D to 2G). Efficacy of quercetin and sulindac to abrogate fibrotic changes when accessed histologically revealed better effect of quercetin than sulindac to reduce inflammatory and fibrotic changes. Lung sections from rats treated with combination therapy of quercetin and sulindac displayed nearly normal alveolar structure except few inflammatory infiltrates in the interstitium (Figure 2H and 2I).

Furthermore, the semi-quantitative assessment of fibrosis in lung sections was performed by scoring pathological lesions as per the Szapiel method of examination (Table 5). The

Szapiel score of bleomycin induced group was found significantly higher on day 21 when compared with control group. However, Szapiel scores on day 21 of quercetin and sulindac treated groups showed marked decrease compared to the bleomycin treated group reaffirming ameliorative role of quercetin and sulindac against bleomycin induced lung fibrosis. Scores for the combination therapy of quercetin and sulindac displayed significant improvement and was found to be near normal (Table 5).

Figure 3 shows the Masson's trichrome staining. Tissue injury due to administration of bleomycin resulted in the excess deposition of collagen, as evident in Figure 3B when compared to that of control group in Figure 3A. However, both quercetin and sulindac treated groups exhibited noticeable reduction in the collagen deposition in the lung tissues (Figures 3C and 3D). Co-administration of quercetin and sulindac also displayed a noteworthy reduction in the deposition of collagen which can be correlated with the decrease in the hydroxyproline content as shown in Figure 1D and Table 2 and as observed in Figure 3E.

Table 1: Mean Body Weight and Percent Body Weight Changes

Treatment Groups	Mean Body Weight (g)					Percent Body Weight Change			
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 0 - 3**	**Day 0 - 7**	**Day 0 - 14**	**Day 0 - 21**
Control Group	310.40 ± 17.68	301.70 ± 17.91	326.70 ± 22.16	359.80 ± 27.11	366.00 ± 25.07	-2.80 ± 1.98	5.22 ± 2.91	15.88 ± 4.75	17.88 ± 3.25
Bleomycin Control	320.40 ± 25.36	303.80 ± 26.72	314.80 ± 21.21	320.50* ± 19.31	331.40* ± 15.50	-5.22* ± 1.99	-1.63* ± 2.89	0.27* ± 5.05	3.72* ± 4.75
Quercetin Treatment	318.60 ± 26.88	300.90 ± 27.07	308.30 ± 28.52	338.50 ± 23.58	348.80 ± 25.48	-5.59* ± 1.69	-3.25* ± 2.78	6.38$^{*, \#}$ ± 2.32	9.59$^{*, \#}$ ± 2.40
Sulindac Treatment	320.40 ± 24.63	301.40 ± 27.44	311.20 ± 25.07	346.10$^{\#}$ ± 27.02	359.90 ± 27.78	-6.00* ± 2.67	-2.87* ± 2.11	8.05$^{*, \#}$ ± 3.16	12.37$^{*, \#}$ ± 3.16
Quercetin + Sulindac Treatment	318.30 ± 23.54	302.30 ± 21.09	316.40 ± 23.59	349.50$^{\#}$ ± 24.80	364.20 ± 26.70	-4.97* ± 2.38	-0.29* ± 8.41	10.30$^{*, \#}$ ± 10.80	14.93$^{\#}$ ± 11.42

Values are given as Mean ± SE for groups of ten rats each, $^{*}p < 0.05$ vs. Control Group, # $p < 0.05$ vs. bleomycin Group.

Table 2: Relative Organ weight and Hydroxyproline Content

Treatment Groups	Relative Organ Weight (%)	Hydroxyproline Content (mg/g dried tissue)
Control Group	0.63 ± 0.01	1.71 ± 0.05
Bleomycin Control	0.86 ± 0.05*	2.59 ± 0.23*
Quercetin Treatment	0.66 ± 0.06#	1.93 ± 0.12#
Sulindac Treatment	0.70 ± 0.12#	1.94 ± 0.12*, #
Quercetin + Sulindac Treatment	0.65 ± 0.03#	1.82 ± 0.07#

Values are given as Mean ± SE for groups of six rats each, * $p < 0.05$ vs. Control Group, # $p < 0.05$ vs. bleomycin Group.

Table 3: Oxidative stress status of lung tissue of rats subjected to various treatments.

Biochemical Estimations	Control Group	Bleomycin Control	Quercetin Treatment	Sulindac Treatment	Quercetin + Sulindac Treatment
Malondialdehyde level (nmoles MDA/mg tissue/60 min)	45.41 ± 2.00	60.63 ± 1.87*	48.77 ± 2.76$^{\#}$	49.63 ± 1.85$^{*,\#}$	47.49 ± 2.53$^{\#}$
Glutathione Content (µg tissue)	37.96 ± 7.48	23.00 ± 6.12*	33.85 ± 6.33$^{\#}$	31.84 ± 5.44	36.60 ± 4.08$^{\#}$
Glutathione peroxidase (U/g tissue)	5.45 ± 0.14	2.49 ± 0.19*	4.33 ± 0.13$^{*,\#}$	3.30 ± 0.14$^{*,\#}$	5.24 ± 0.12$^{\#}$
Catalase (µM H_2O_2 consumed/mg tissue/min)	19.84 ± 2.22	12.68 ± 0.82*	17.32 ± 1.46$^{*,\#}$	16.83 ± 1.38$^{*,\#}$	18.94 ± 1.08$^{\#}$
Superoxide Dismutase (U/mg tissue)	0.34 ± 0.03	0.21 ± 0.01*	0.31 ± 0.02$^{*,\#}$	0.31 ± 0.02$^{*,\#}$	0.33 ± 0.02$^{\#}$
Reduced glutathione (µg/g tissue)	1.80 ± 0.05	1.06 ± 0.07*	1.66 ± 0.07$^{*,\#}$	1.64 ± 0.06$^{*,\#}$	1.72 ± 0.06$^{\#}$

Values are given as Mean ± SE for groups of six rats each, $^{*}p < 0.05$ vs. Control Group, # $p < 0.05$ vs. bleomycin Group.

Table 4: The total and differential blood cell count of rats from various study groups

Experimental Group	Total cells ($\times 10^6$ ml^{-1})	Macrophage (%)	Neutrophils (%)	Eosinophil (%)	Lymphocytes (%)	Basophil (%)
Control Group	0.65 ± 0.12	85.17 ± 2.00	1.83 ± 0.50	0.83 ± 0.17	11.67 ± 1.50	0.50 ± 0.25
Bleomycin Control	1.64 ± 0.15*	49.33 ± 3.83*	38.33 ± 1.83*	4.08 ± 0.58*	5.42 ± 0.92*	2.83 ± 1.17*
Quercetin Treatment	0.87 ± 0.14#	71.00 ± 3.00*,#	13.00 ± 2.00*,#	4.08 ± 0.96*	10.50 ± 1.25*,#	1.42 ± 0.75#
Sulindac Treatment	0.89 ± 0.08*,#	70.08 ± 1.96*,#	12.17 ± 1.50*,#	3.92 ± 0.46*	13.17 ± 0.42*,#	0.67 ± 0.17#
Quercetin + Sulindac Treatment	0.73 ± 0.08#	76.25 ± 3.25*,#	8.08 ± 1.63*,#	2.58 ± 0.63*,#	12.50 ± 1.50*,#	0.58 ± 0.13#

Values are given as Mean ± SE for groups of four rats each, $^{*}p < 0.05$ vs. Control Group, #$p < 0.05$ vs. bleomycin Group.

Table 5: The tumor necrosis factor-α and grade of fibrosis in rats subjected to various treatments.

Experimental Group	Tumor Necrosis Factor-α (pg/mL)	Grade of fibrosis
Control Group	22.89 ± 2.56	0.00 ± 0.00
Bleomycin Control	53.23 ± 3.83*	2.48 ± 0.07*
Quercetin Treatment	26.05 ± 1.41$^{\#}$	1.44 ± 0.13$^{*,\#}$
Sulindac Treatment	28.33 ± 1.91$^{*,\#}$	1.58 ± 0.15$^{*,\#}$
Quercetin + Sulindac Treatment	22.86 ± 2.45$^{\#}$	0.51 ± 0.12$^{*,\#}$

Values are given as Mean ± SE for groups of six rats for TNF-α and four rats for grading of fibrosis, $^{*}p < 0.05$ vs. Control Group, $\#p < 0.05$ vs. bleomycin Group.

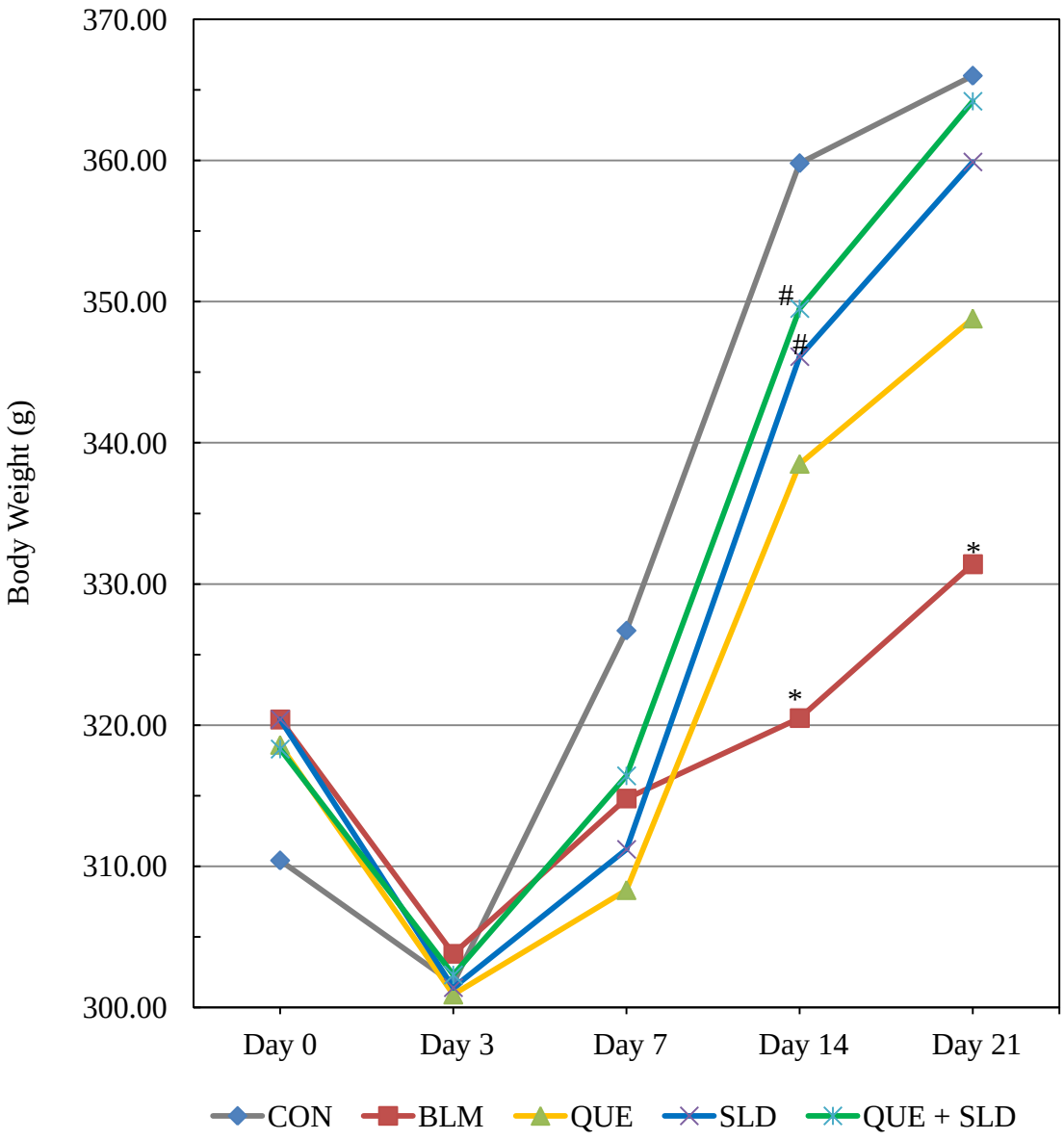


Figure: 1A. Effect of various treatments on bodyweight of experimental groups on days 0, 3, 7, 14 and 21. CON: Control; BLM: Bleomycin; QUE: Quercetin; SLD: Sulindac. n = 10.

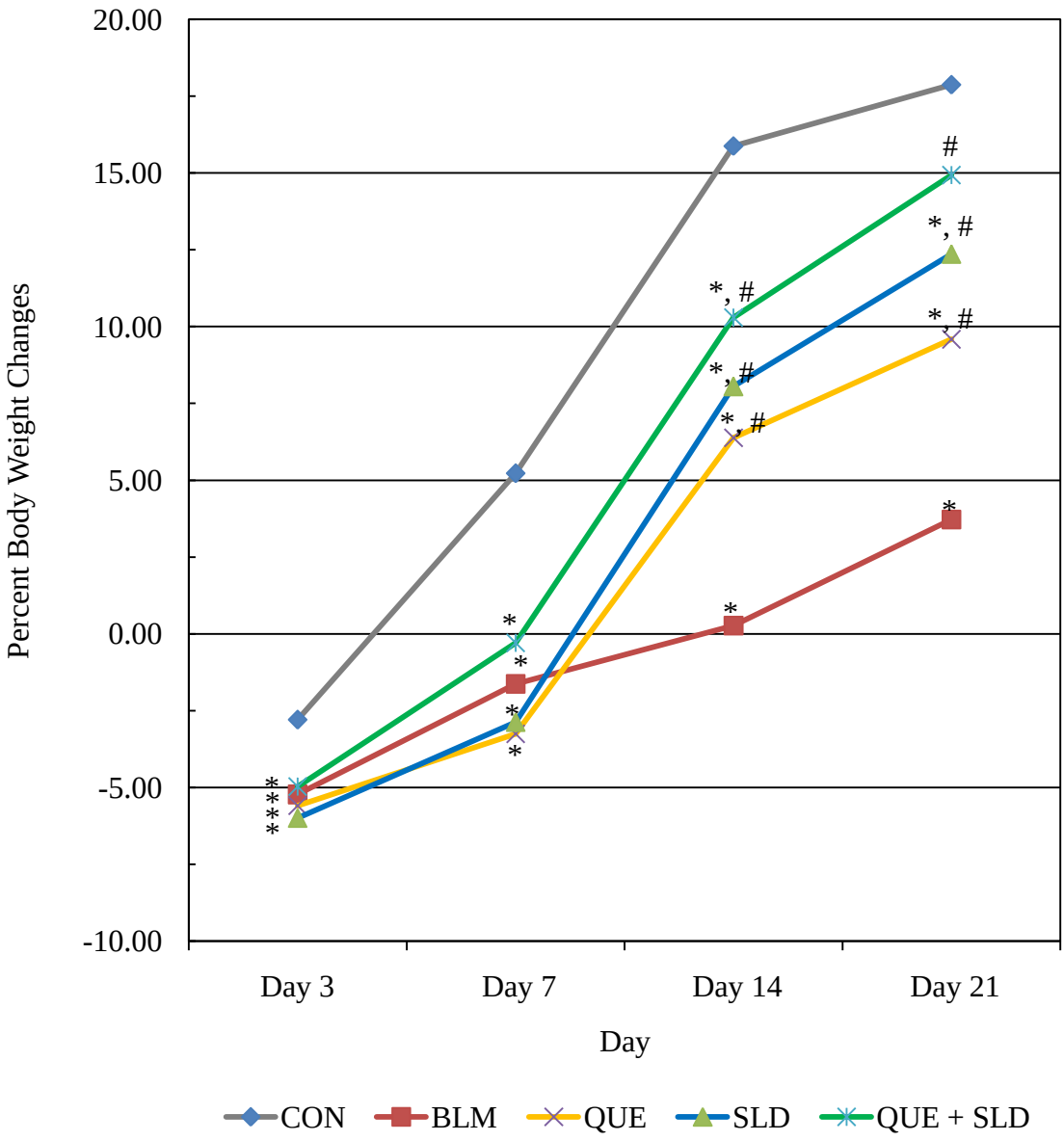


Figure: 1B Effect of various treatments on percent bodyweight changes of experimental groups on days 3, 7, 14 and 21. CON: Control; BLM: Bleomycin; QUE: Quercetin; SLD: Sulindac. n = 10.

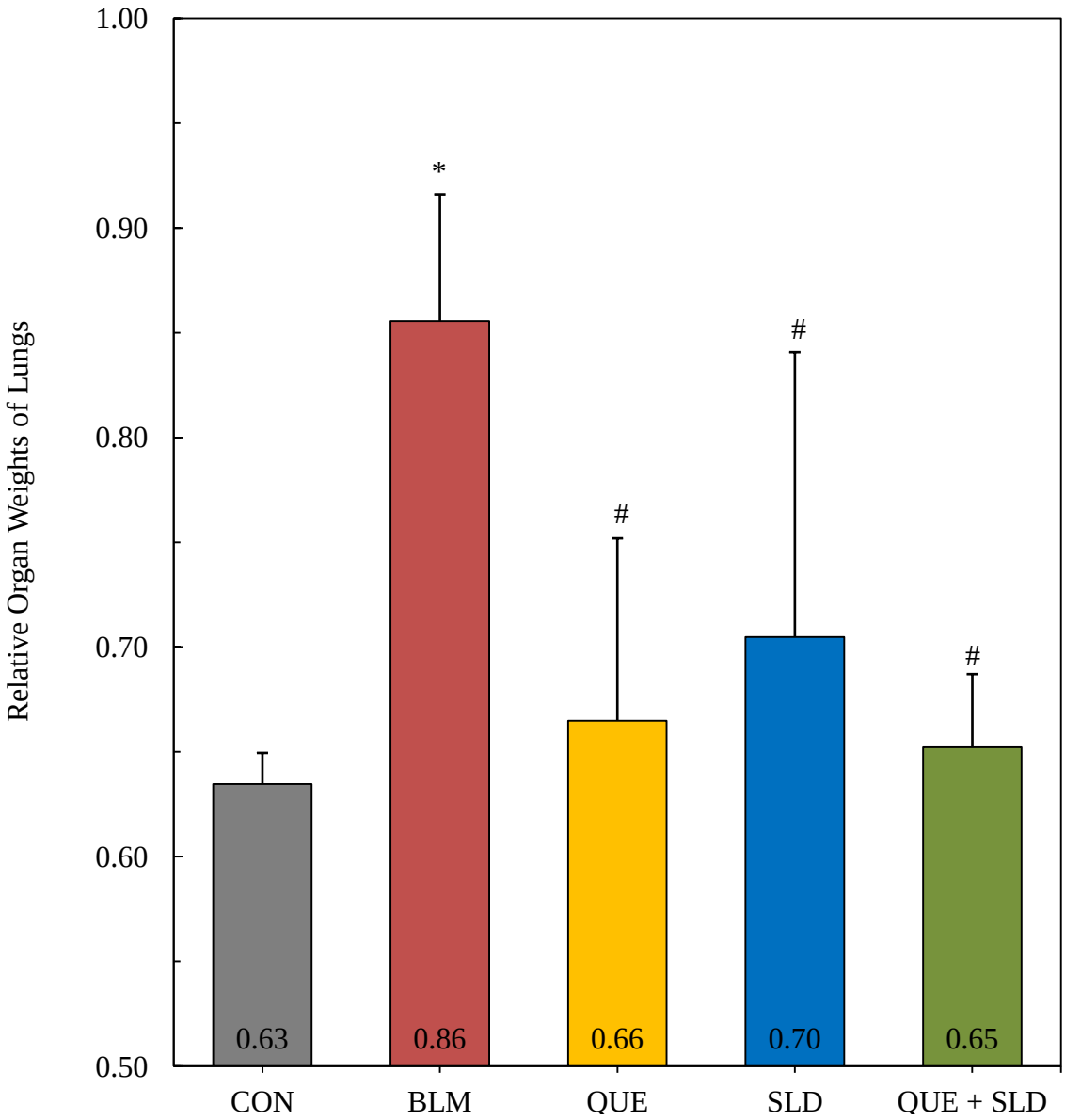


Figure: 1C. Effect of various treatments on the relative organ weight of lungs on day 21 of Experiment. CON: Control; BLM: Bleomycin; QUE: Quercetin; SLD: Sulindac. n = 6

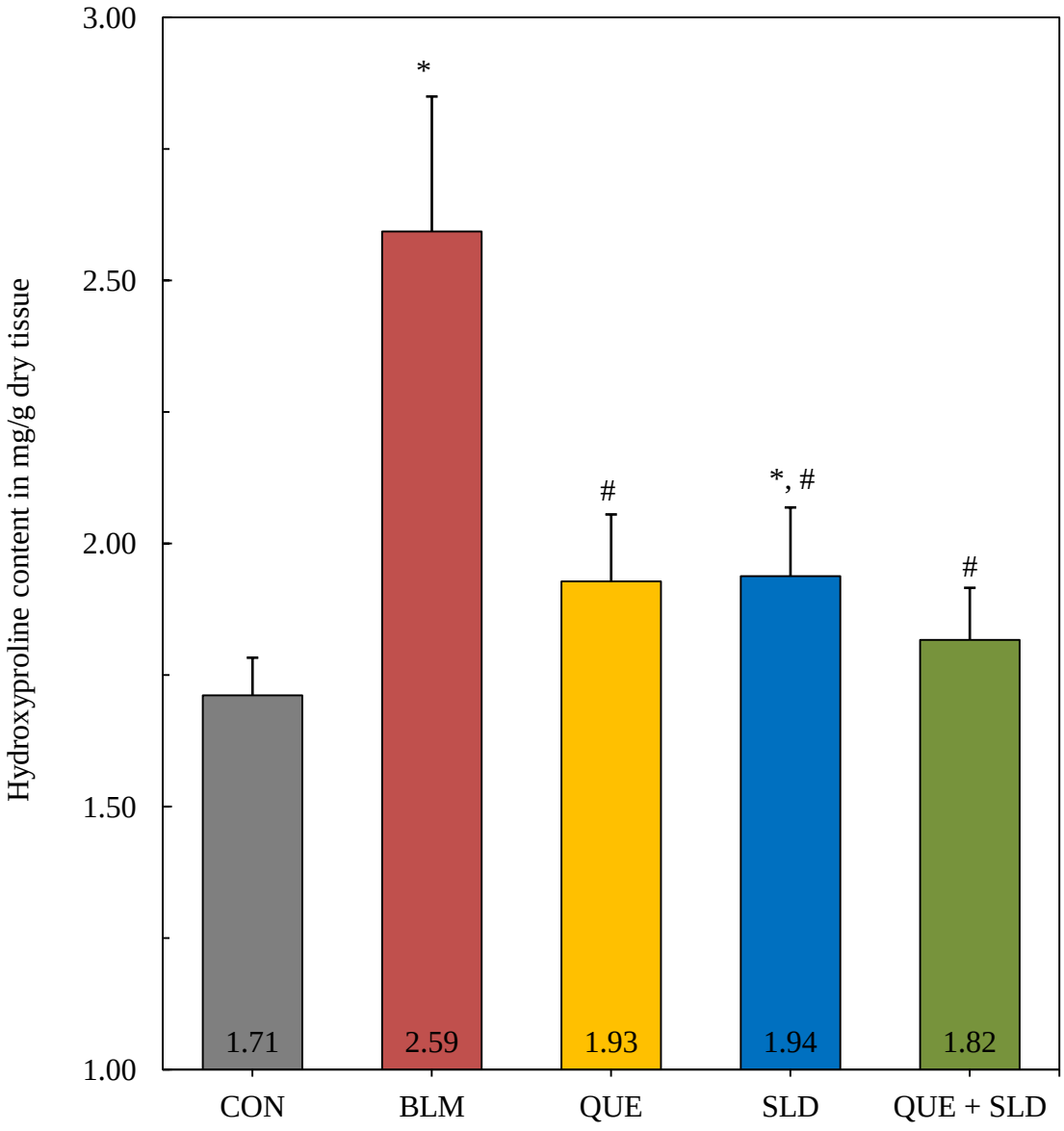


Figure: 1D. Effect of various treatments on the hydroxyproline content in lungs on Day 21 of Experiment. CON: Control; BLM: Bleomycin; QUE: Quercetin; SLD: Sulindac. n = 6.

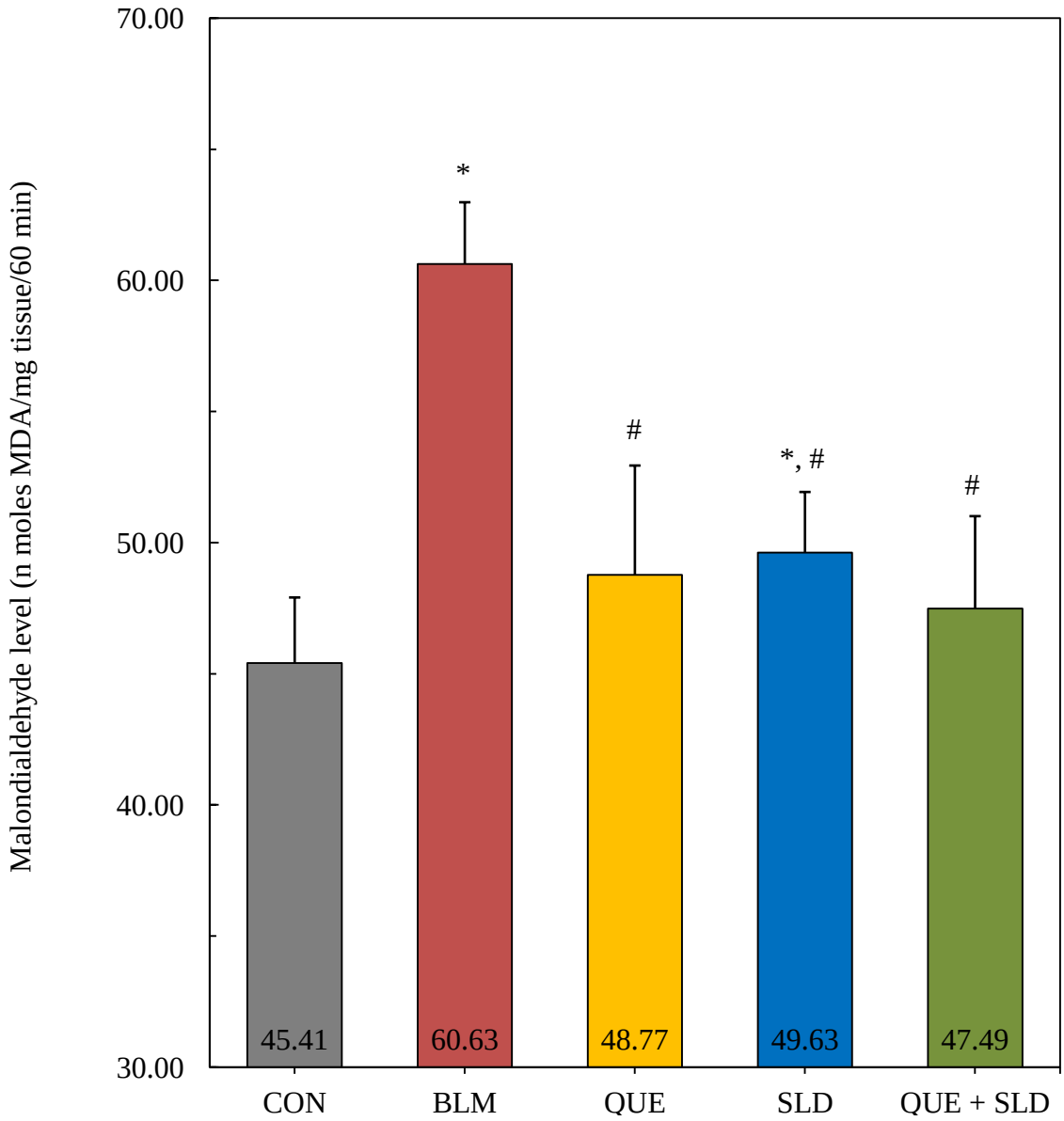


Figure: 1E. Effect of various treatments on the lipid peroxidation in lungs on day 21 of experiment. CON: Control; BLM: Bleomycin; QUE: Quercetin; SLD: Sulindac. n = 6.

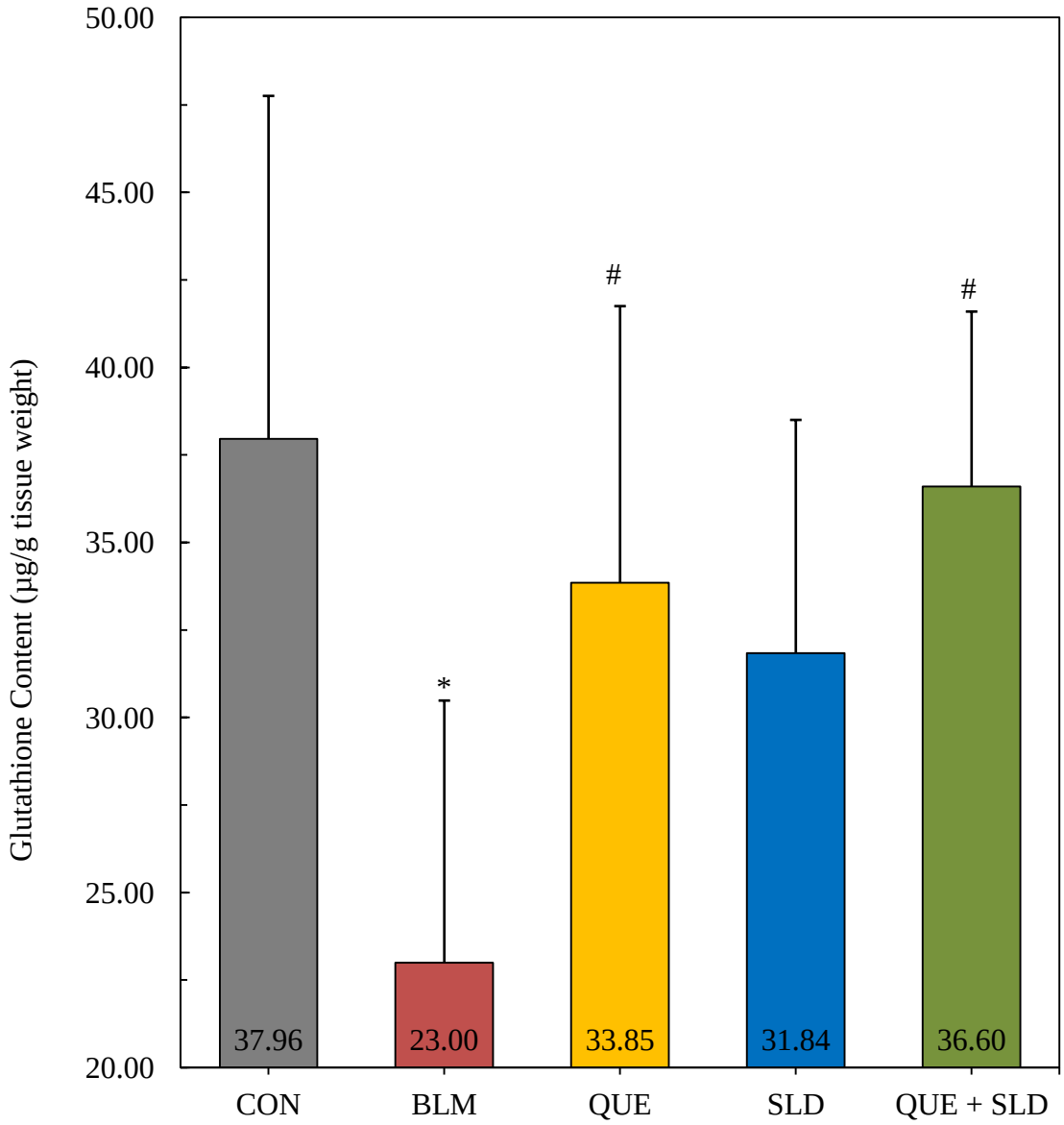


Figure: 1F. Effect of various treatments on the glutathione content in lungs on day 21 of experiment. CON: Control; BLM: Bleomycin; QUE: Quercetin; SLD: Sulindac. n = 6.

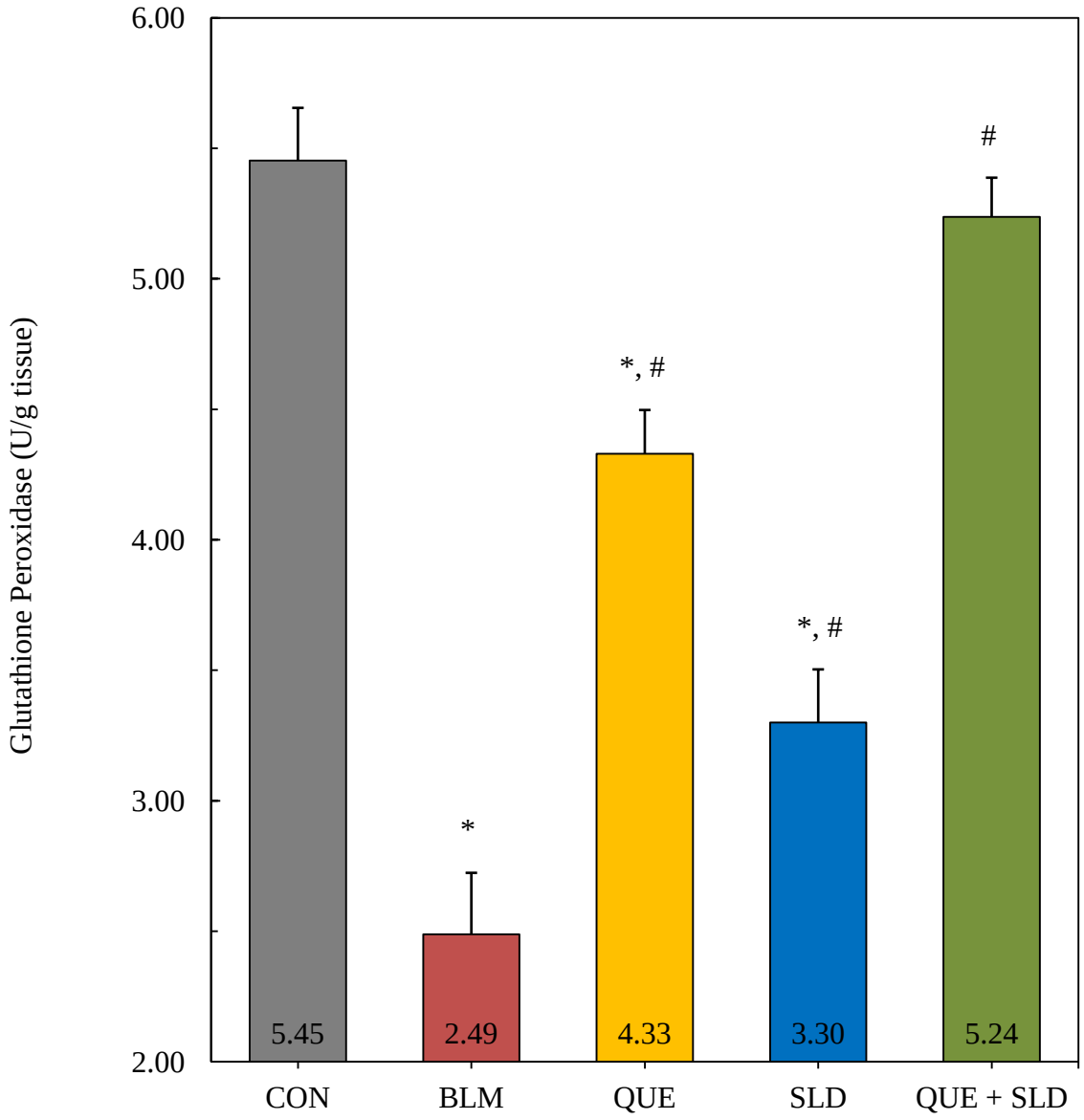


Figure: 1G. Effect of various treatments on the glutathione peroxidase in lungs on Day 21 of experiment. CON: Control; BLM: Bleomycin; QUE: Quercetin; SLD: Sulindac. n = 6.

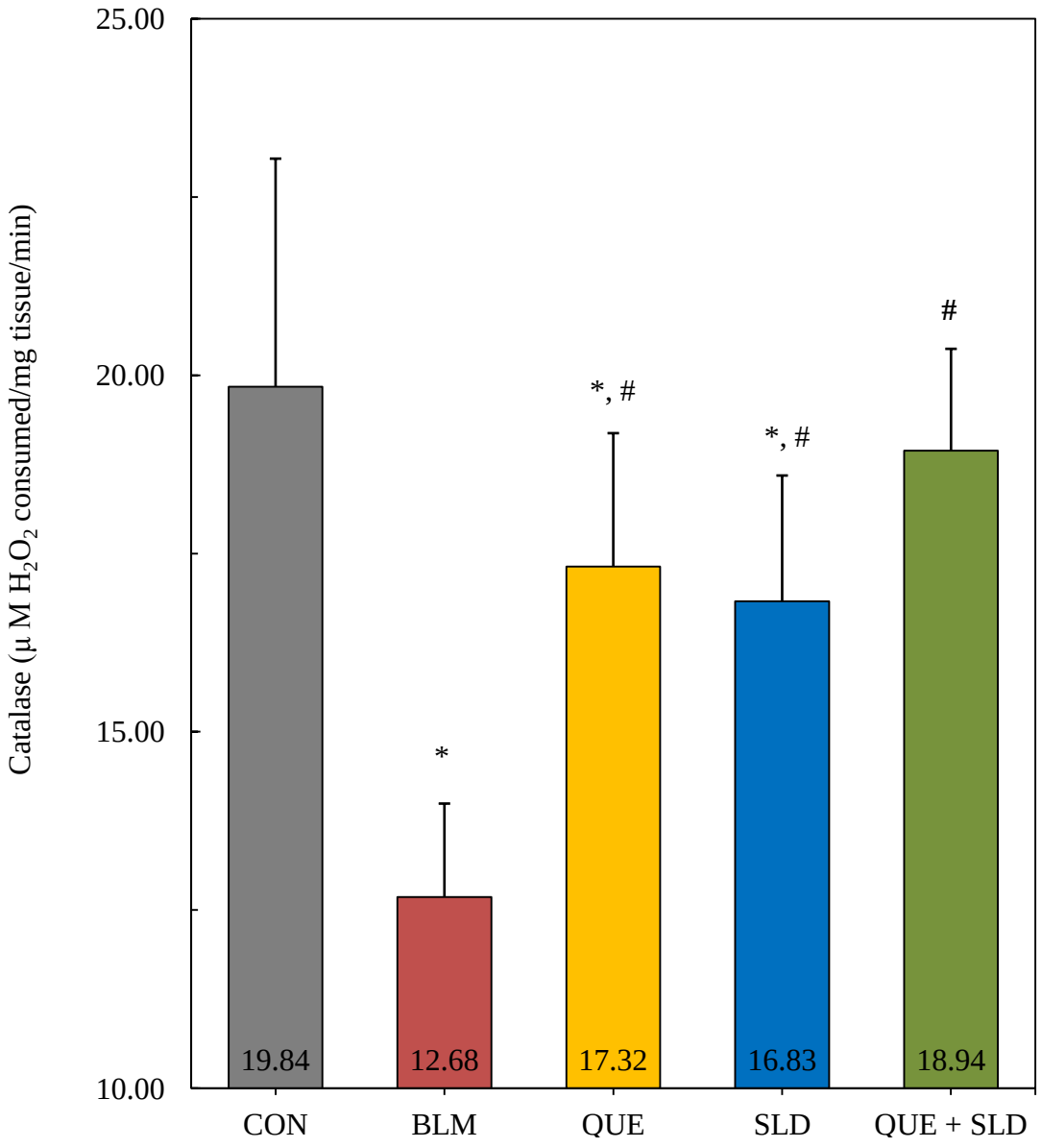


Figure: 1H. Effect of various treatments on catalase at Day 21 of experiment. CON: Control; BLM: Bleomycin; QUE: Quercetin; SLD: Sulindac. n = 6.

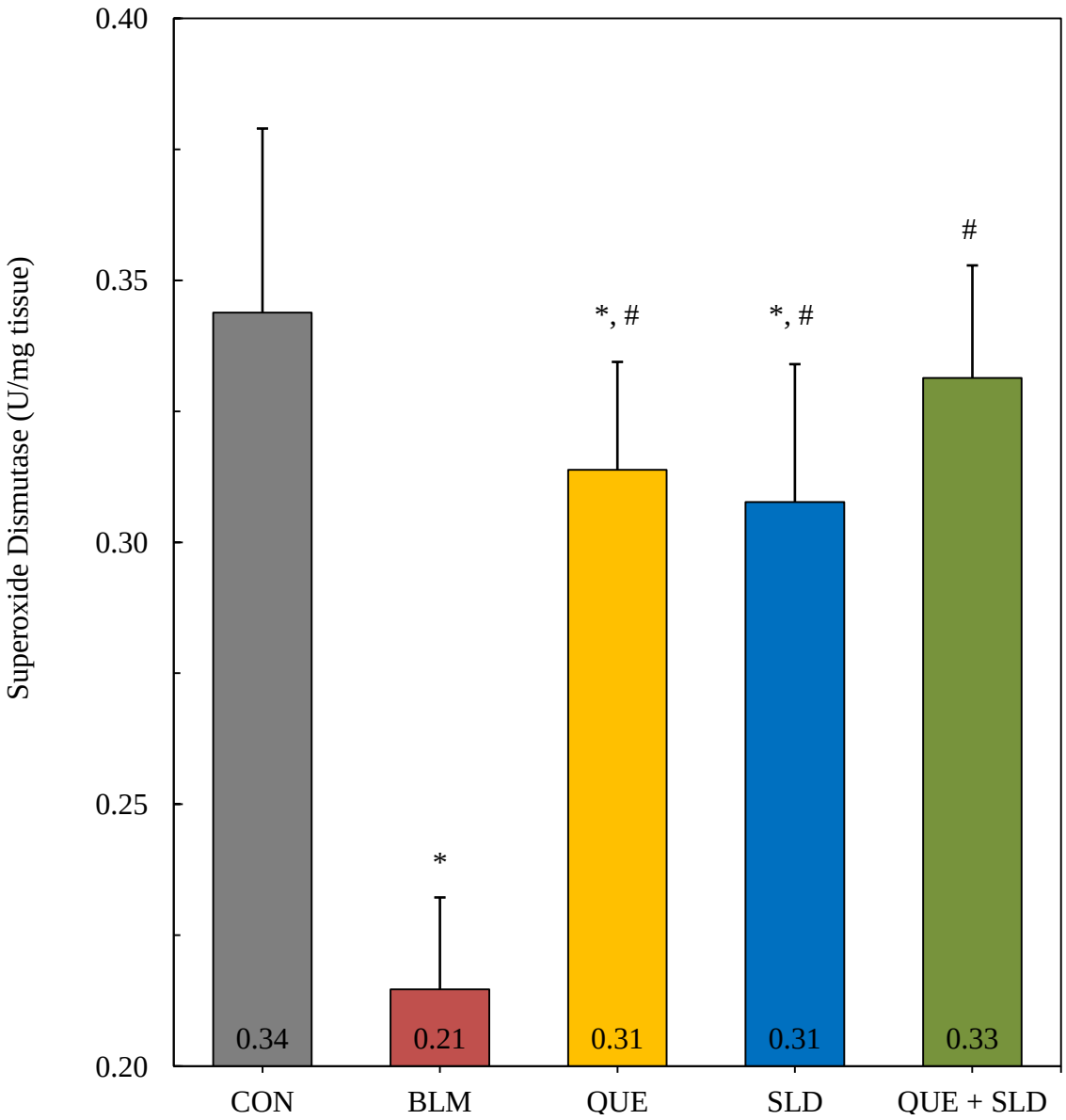


Figure: 1I. Effect of various treatments on superoxide dismutase at day 21 of experiment. CON: Control; BLM: Bleomycin; QUE: Quercetin; SLD: Sulindac. n = 6.

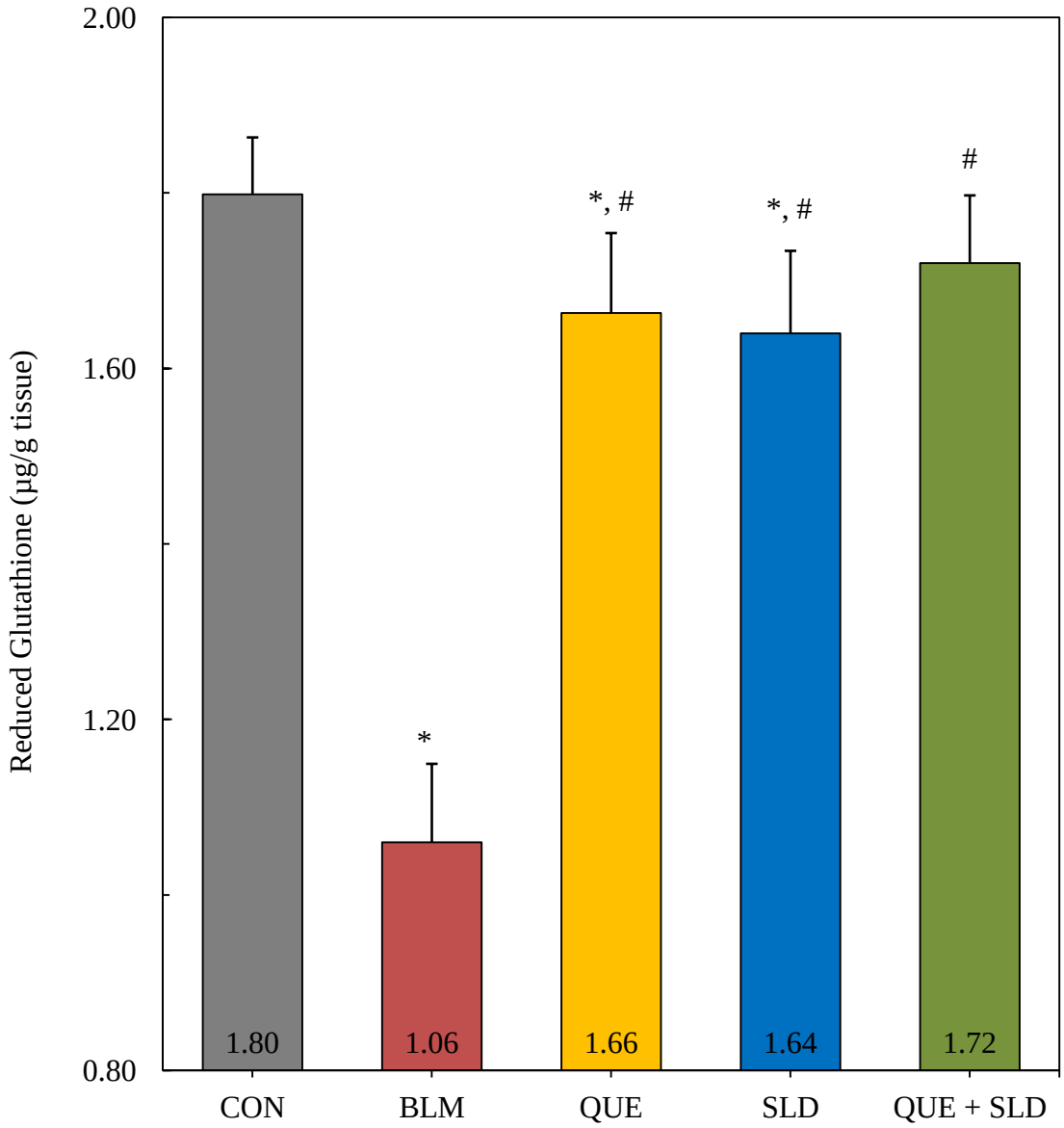


Figure: 1J. Effect of various treatments on reduced glutathione on day 21 of experiment. CON: Control; BLM: Bleomycin; QUE: Quercetin; SLD: Sulindac. n = 6.

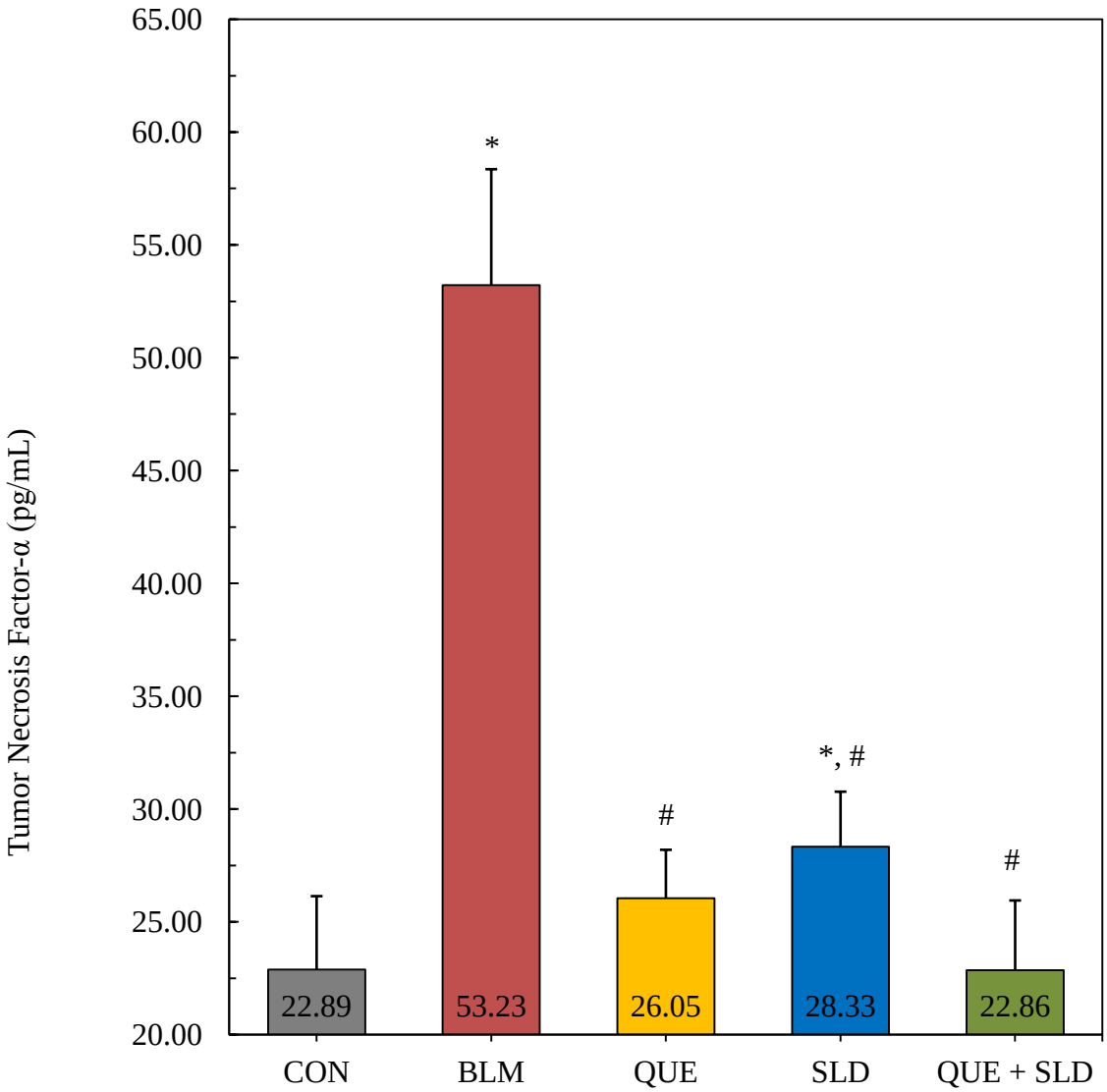


Figure: 1K. Effect of various treatments on tumor necrosis factor-α on day 21 of experiment. CON: Control; BLM: Bleomycin; QUE: Quercetin; SLD: Sulindac. n = 6.

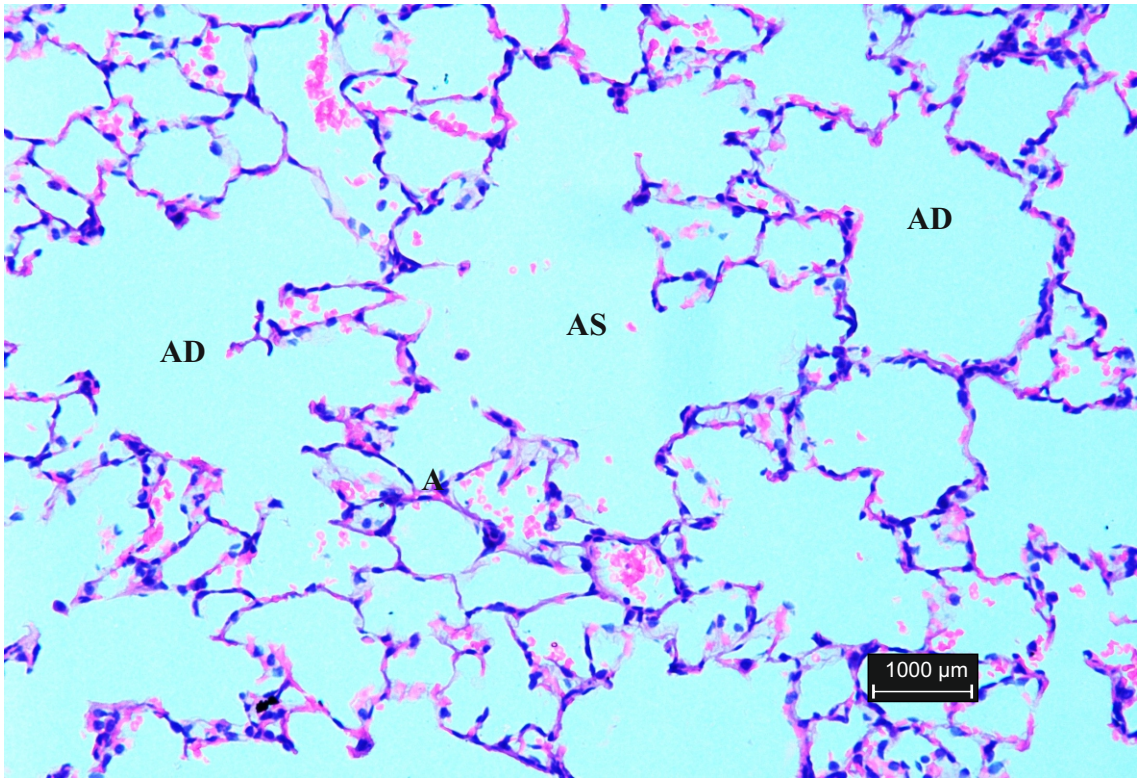


Figure 2A. Photomicrograph of the representative lung section showing normal histological profile (original magnification 200x; Hematoxylin-Eosin staining). A: Alveoli; AD: Alveolar Duct; AS: Alveolar Sac.

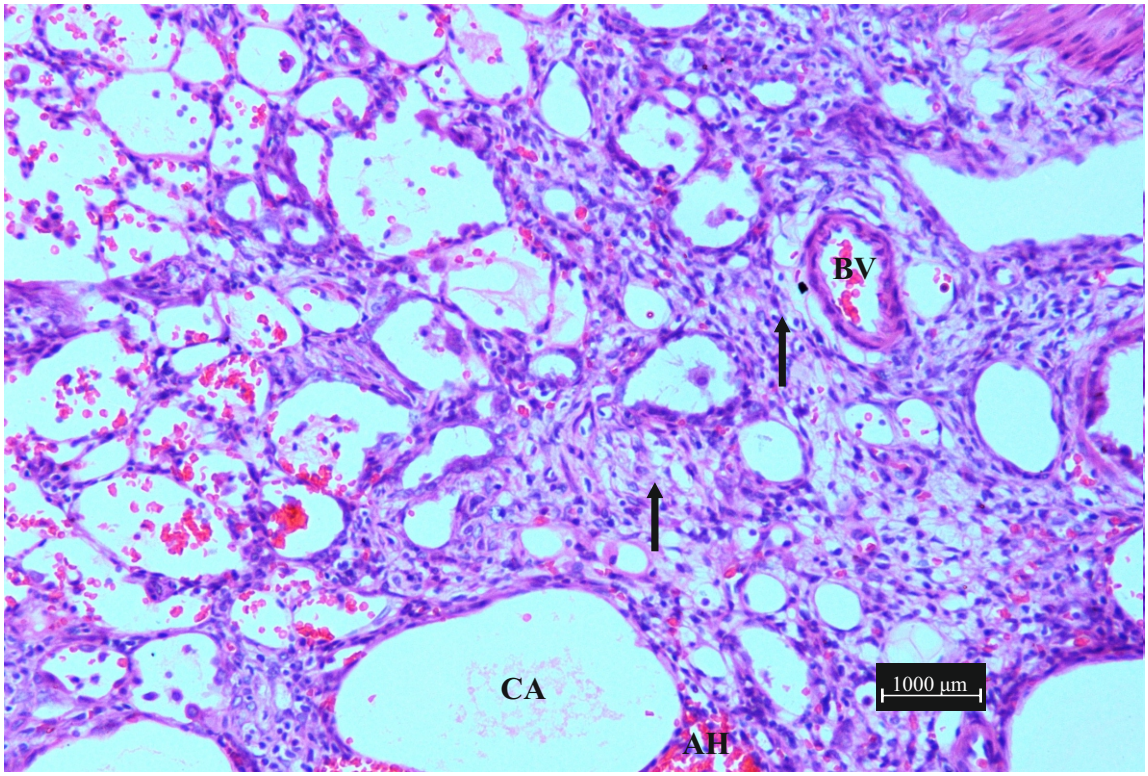


Figure 2B. Photomicrograph of the representative lung section showing distorted architecture induced by Bleomycin. Arrows indicate severe thickening of alveolar septa with extensive infiltration of lymphocytes, macrophages and fibroplasia (original magnification 200x; H-E staining). AH: Alveolar Haemorrhages; BV: Blood Vessel; CA: Collapsed (emphysematous) Alveoli.

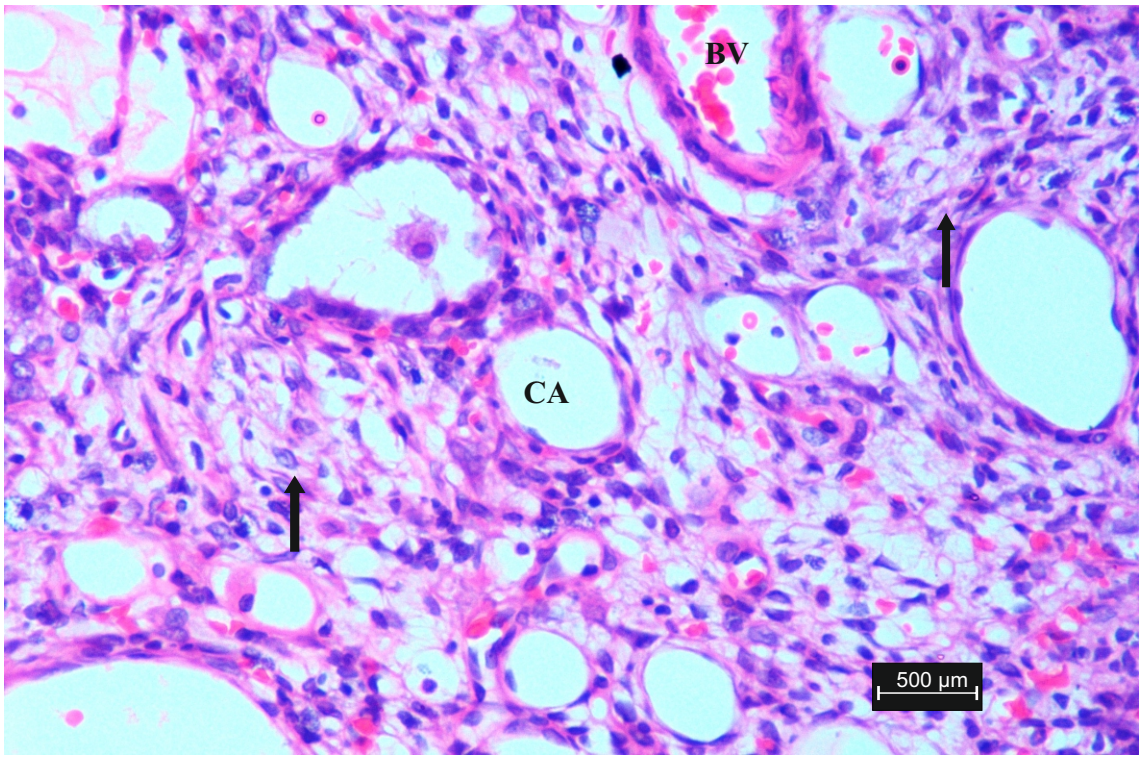


Figure 2C. Photomicrograph of the representative lung section showing distorted architecture induced by Bleomycin. Arrows indicate severe thickening of alveolar septa with extensive infiltration of lymphocytes, macrophages and fibroplasia (original magnification 400x; H-E staining). BV: Blood Vessel; CA: Collapsed (emphysematous) Alveoli.

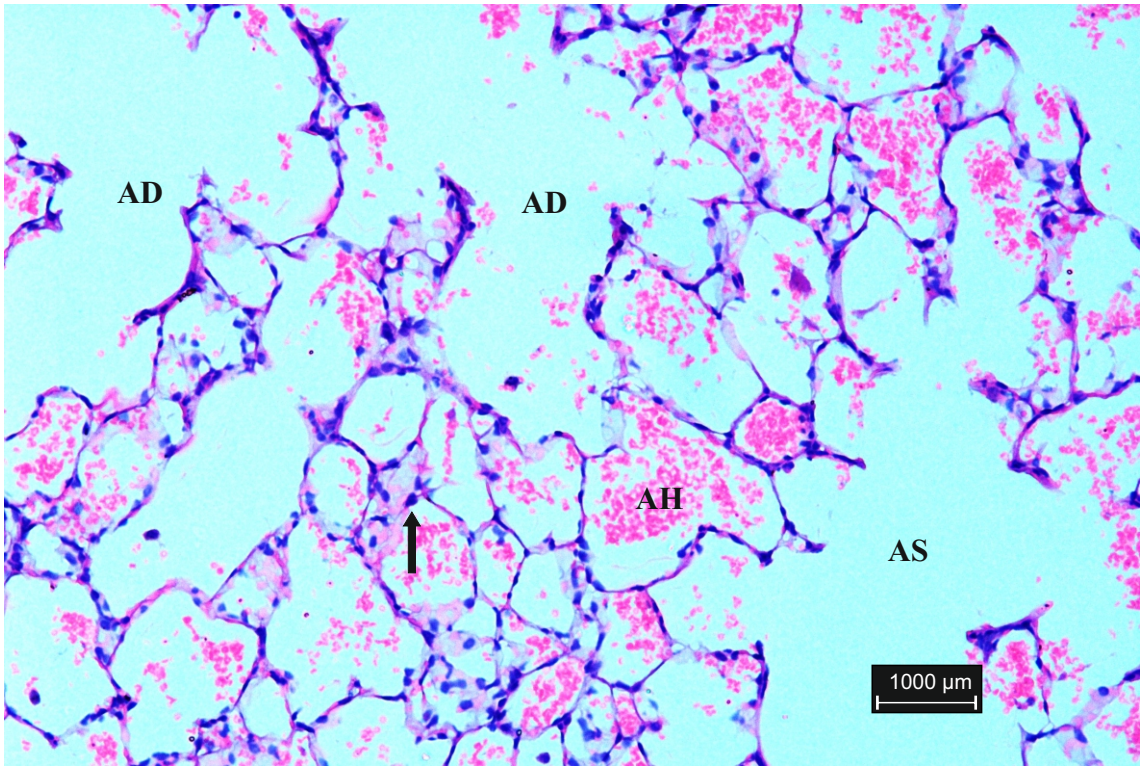


Figure 2D. Photomicrograph of the representative lung section showing ameliorative effect of quercetin. Arrow indicate minimal septal thickening with mild inflammatory infiltrates and fibrosis (original magnification 200x; H-E staining). AD: Alveolar Duct; AH: Alveolar Haemorrhages; AS: Alveolar Sac.

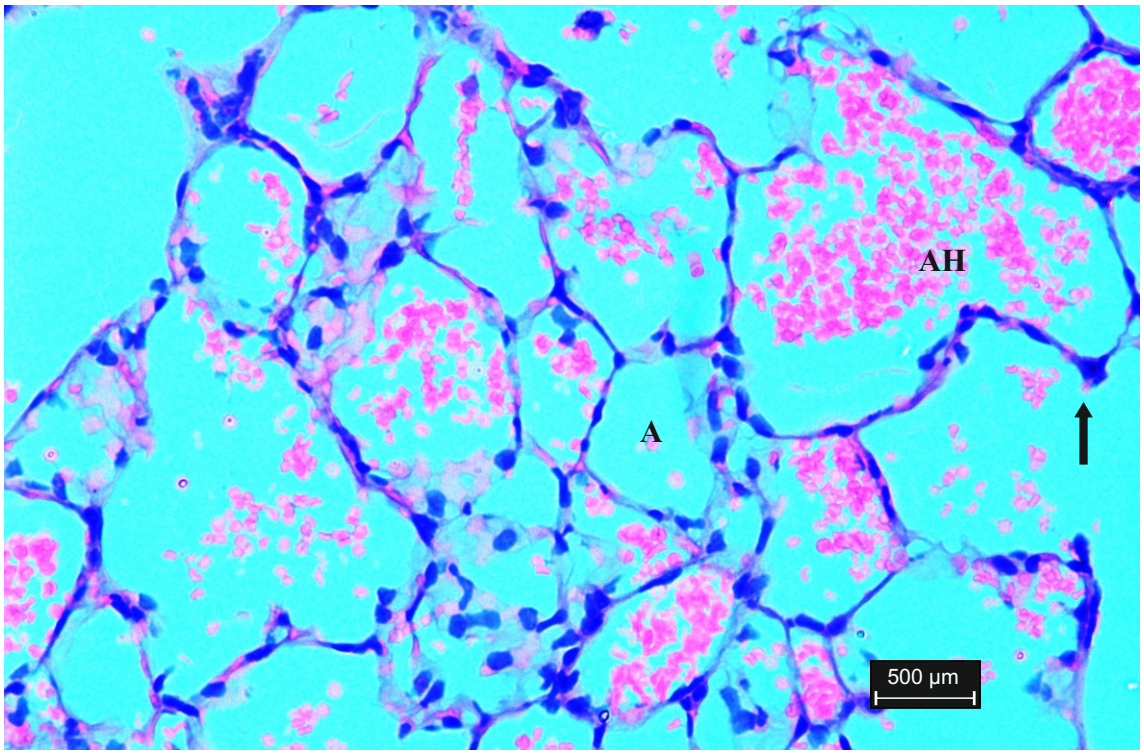


Figure 2E. Photomicrograph of the representative lung section showing ameliorative effect of quercetin. Arrow indicate minimal septal thickening with few inflammatory infiltrates (original magnification 400x; H-E staining). A: Alveoli; AH: Alveolar Haemorrhages.

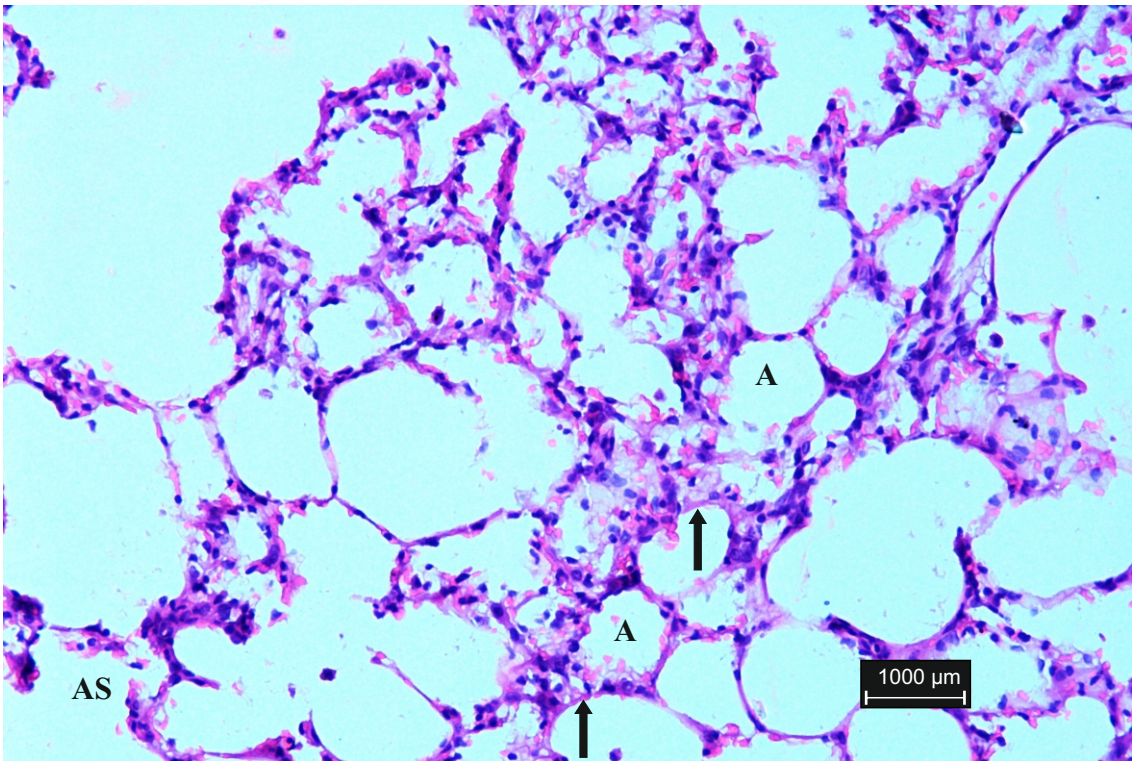


Figure 2F. Photomicrograph of the representative lung section showing ameliorative effect of sulindac. Arrows indicate mild thickening of alveolar septa with mononuclear cell infiltration and fibroplasia (original magnification 200x; H-E staining). A: Alveoli; AS: Alveolar Sac.

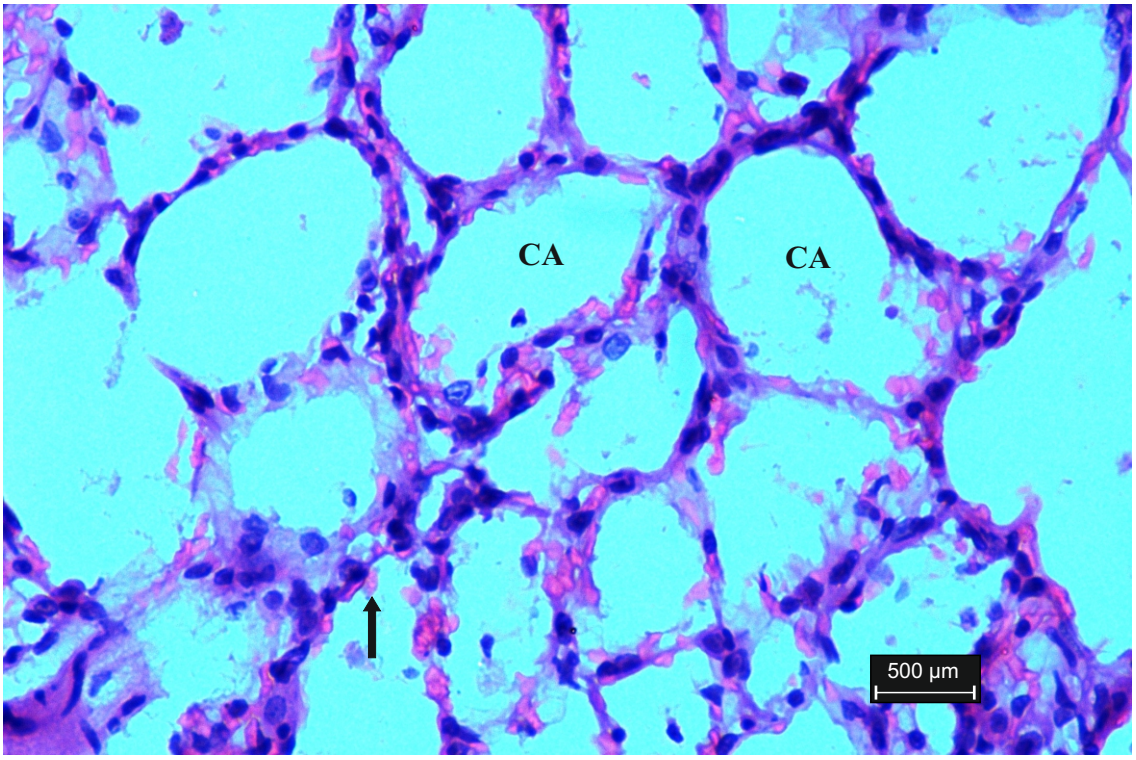


Figure 2G. Photomicrograph of the representative lung section showing ameliorative effect of sulindac. Arrows indicate mild thickening of alveolar septa with mononuclear cell infiltration and fibroplasia (original magnification 400x; H-E staining). CA: Collapsed (emphysematous) Alveoli.

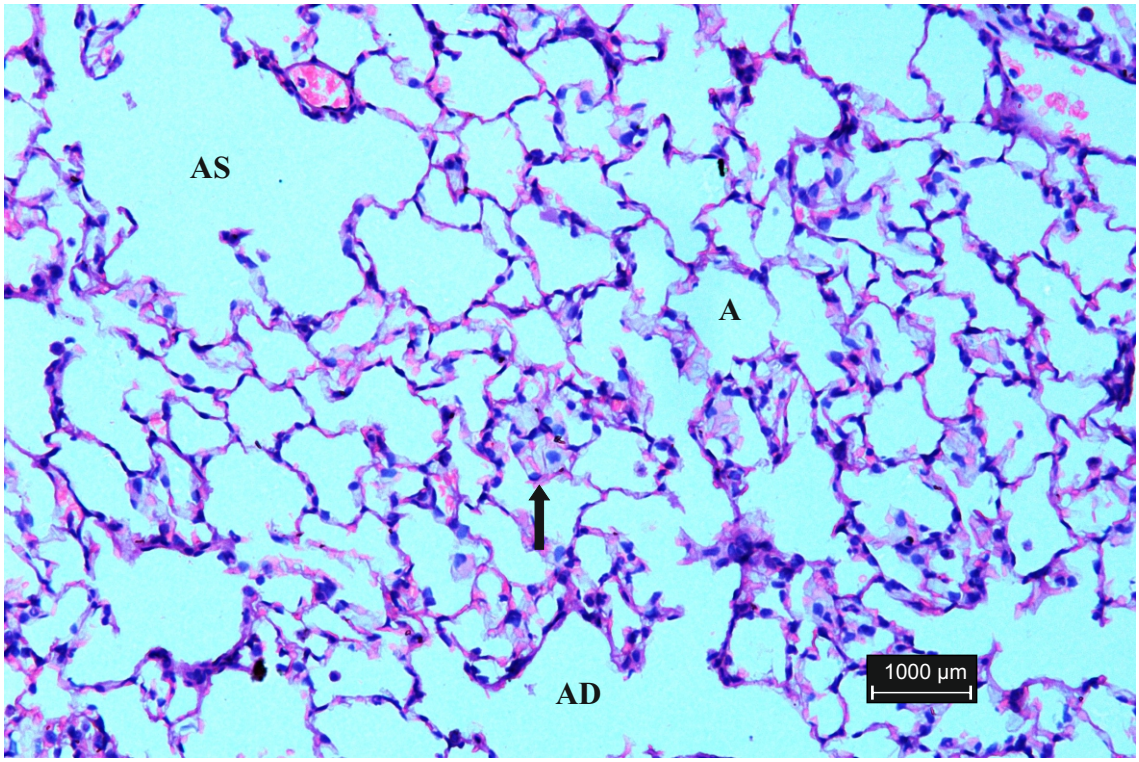


Figure 2H. Photomicrograph of the representative lung section showing ameliorative effect of quercetin in combination with Sulindac. Arrows indicate near normal histoarchitecture of lung with few mononuclear cell infiltration in alveolar septa (original magnification 200x; H-E staining). A: Alveoli; AD: Alveolar Duct; AS: Alveolar Sac.

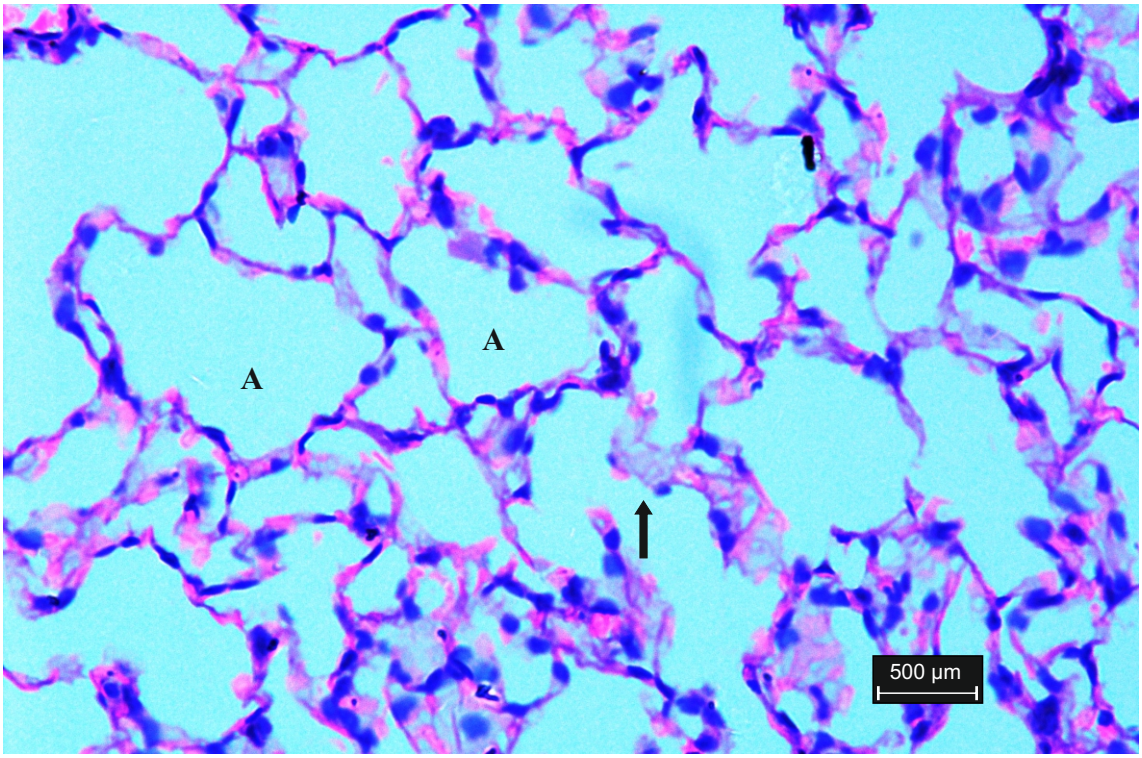


Figure 2I. Photomicrograph of the representative lung section showing ameliorative effect of quercetin in combination with Sulindac. Arrows indicate near normal histoarchitecture of lung with few mononuclear cell infiltration in alveolar septa (original magnification 400x; H-E staining). A: Alveoli.

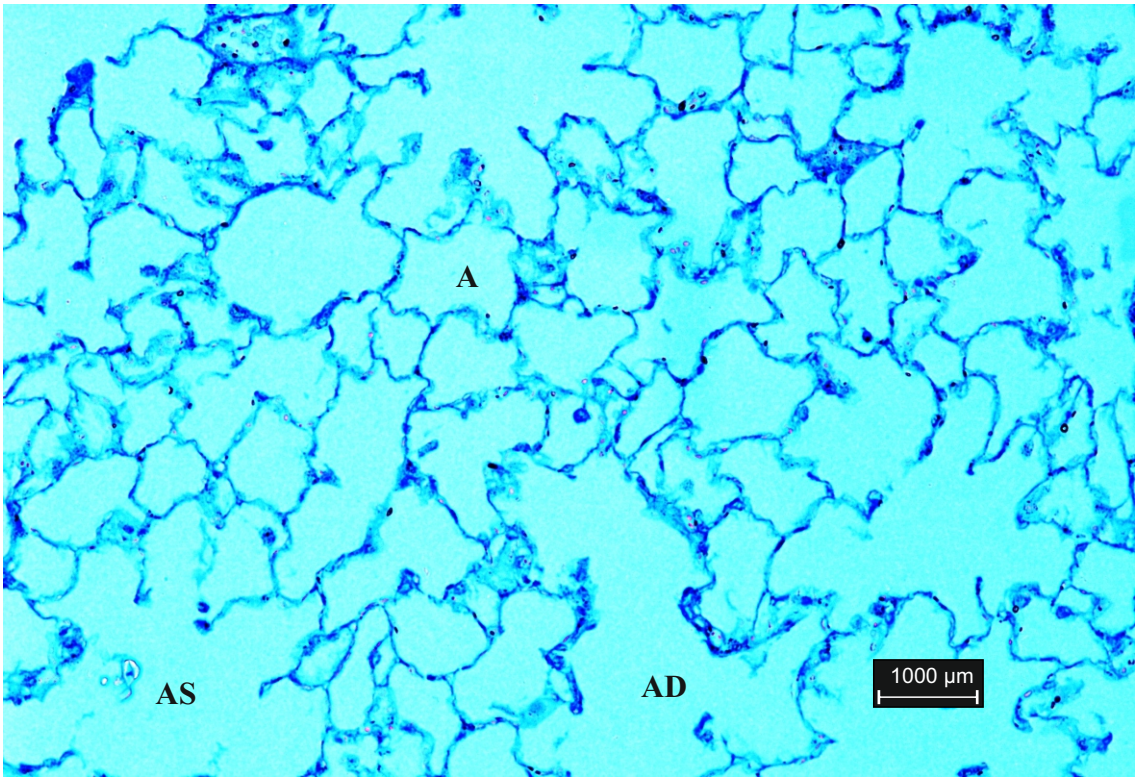


Figure 3A. Photomicrograph of the representative lung section showing normal histological profile (original magnification 200x; Masson's Trichrome staining). A: Alveoli; AD: Alveolar Duct; AS: Alveolar Sac.

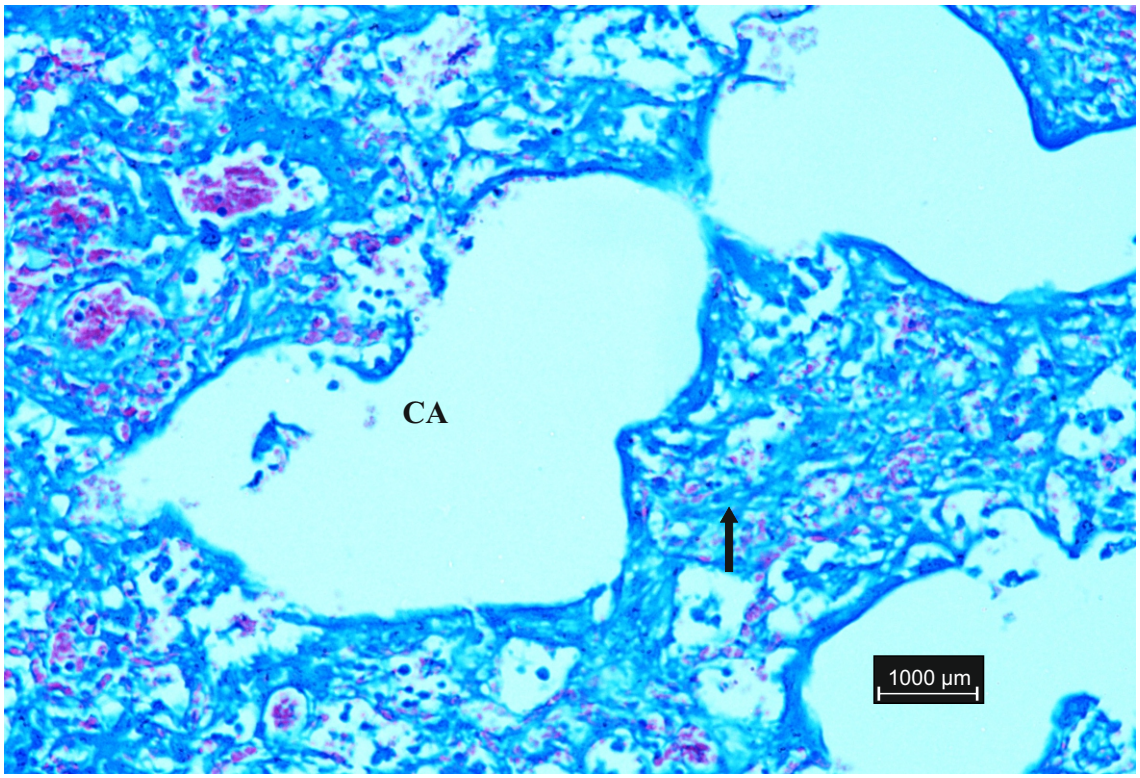


Figure 3B. Photomicrograph of the representative lung section showing distorted architecture induced by Bleomycin. Arrows indicate severe thickening of alveolar septa with extensive collagen deposition (original magnification 200x; Masson's Trichrome staining). CA: Collapsed (emphysematous) Alveoli.

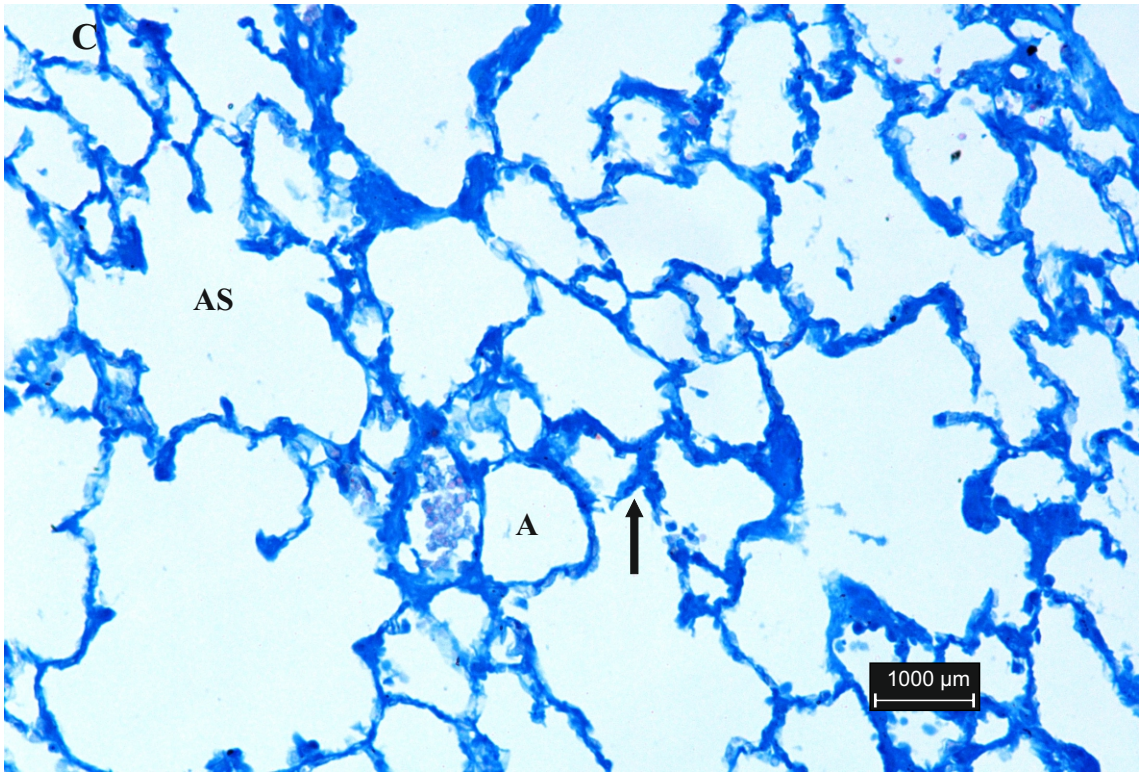


Figure 3C. Photomicrograph of the representative lung section showing ameliorative effect of quercetin. Arrow showing minimal thickening of alveolar septa with collagen deposition (original magnification 200x; Masson's Trichrome staining). A: Alveoli; AS: Alveolar Sac.

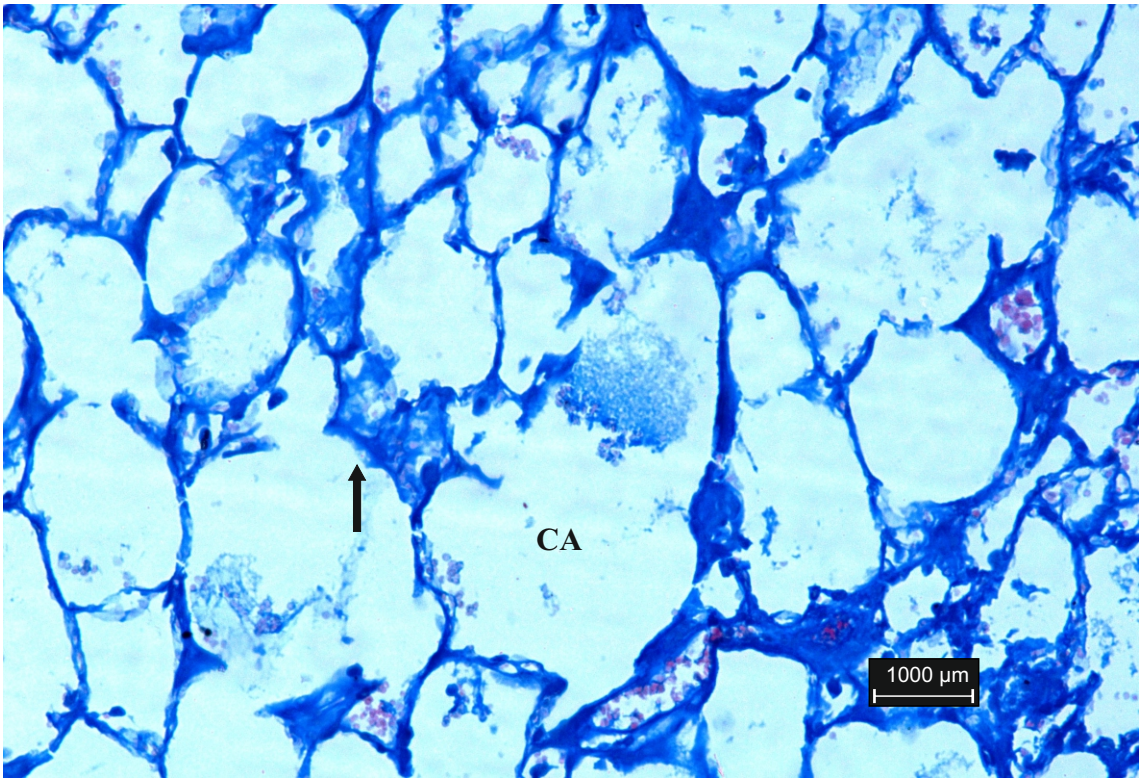


Figure 3D. Photomicrograph of the representative lung section showing ameliorative effect of sulindac. Arrow showing mild thickening of alveolar septa with collagen deposition (original magnification 200x; Masson's Trichrome staining). CA: Collapsed (emphysematous) Alveoli.

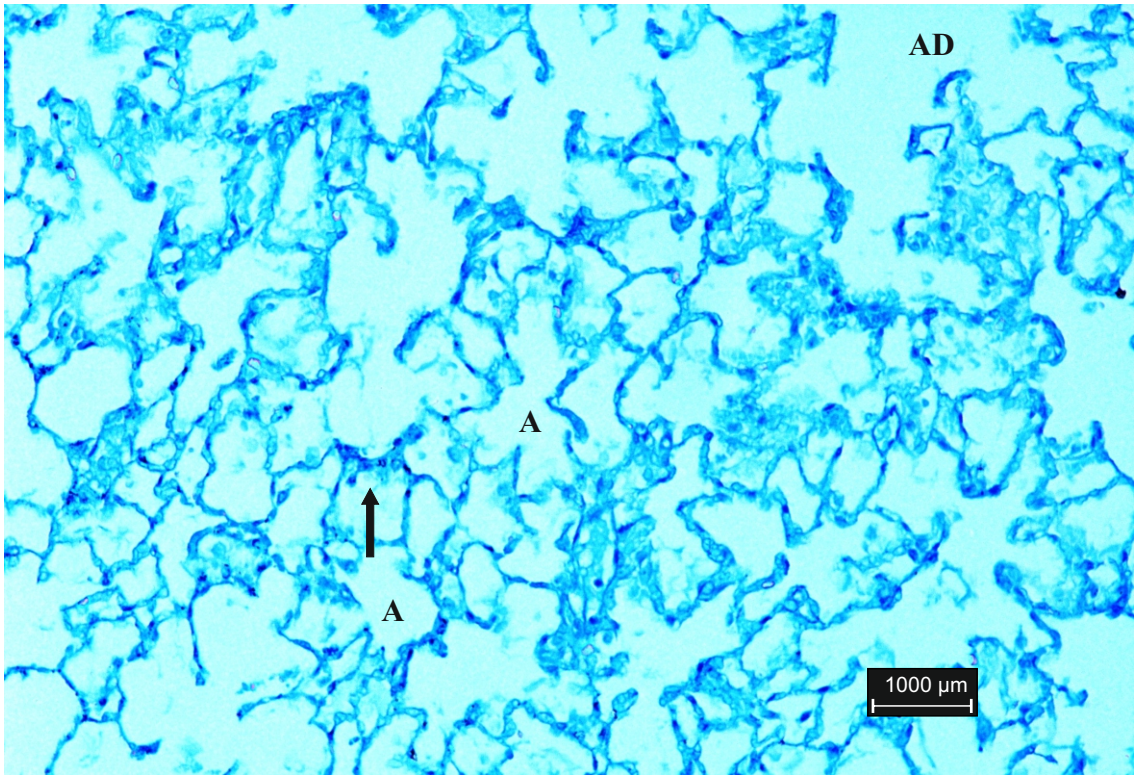


Figure 3E. Photomicrograph of the representative lung section showing ameliorative effect of quercetin in combination with Sulindac. Arrow indicate near normal pulmonary structure with no evidence of collagen deposition (original magnification 200x; Masson's Trichrome staining). A: Alveoli; AD: Alveolar Duct.

DISCUSSION

Idiopathic pulmonary fibrosis (IPF) is an end-stage pulmonary disease for which efficient treatment is not available. The disease is characterized by failure of alveolar re-epithelialization, deposition of excessive collagen and distortion of the lung architecture, which leads to respiratory failure (Oku *et al*., 2008). In order to understand the finer mechanisms of development of pulmonary fibrosis as well as to screen the efficacy of various compounds as potential therapeutic agent against this pathological manifestation, animal experimentations are inevitable. The use of these animal models has helped in partly establishing the pathways of lung damage leading to fibrosis, and by comparison studies of fibrosis models with lung pneumopathy patients, has validated many of these animal studies (Cooper, 2000).

The bleomycin model of lung fibrosis is a widely accepted test system and is a standardized experimental model for human lung fibrosis (Keane *et al*., 2001). The clinical usefulness of bleomycin - an anti-cancer drug for human malignancies including germ-cell tumors, lymphomas, Kaposi's sarcoma, cervical cancer and squamous cell carcinomas of the head and neck - has been hampered due to its detrimental effects (Sleijfer, 2001).

In the current study we have used Wistar rat model of lung fibrosis created by challenging the rats with a single dose of bleomycin sulfate by intratracheal instillation. Further, a scan through the available literature suggests that bleomycin induces pulmonary fibrosis through a combined effect of sustained inflammation and excessive production of free radicals in lung tissues. Therefore, it was thought pertinent to evaluate the ameliorative effect, if any, of a potential antioxidant like quercetin and an anti-inflammatory compound namely sulindac individually at a pharmacologically accepted high dose on idiopathic pulmonary fibrosis in rat. The drugs were administered orally for 20 days post intra-tracheal instillation of a single dose of bleomycin. In addition to the above mentioned individual dosages of the potential pneumo-protective agents, the present study was also extended to evaluate the possible beneficial effect of both these compounds in

combination (quercetin + sulindac) but at half the dose when administered individually on bleomycin induced pulmonary fibrosis in rats.

Analysis of the results revealed a marked reduction in the body weight on days 14 and 21 in the bleomycin treated group when compared to that of the control group rats, which could be attributed to the progression of fibrosis as opined by Zhou *et al*. (2007). However, the body weights of the various treatment groups remained more or less comparable to that of the control group rats throughout the experiment. Moreover the percent body weight changes showed that though quercetin treated and sulindac treated groups exhibited a marked improvement compared to the bleomycin challenged rats, a statistically significant difference was still evident when compared to controls. However, the percent body weight change in quercetin and sulindac in combination at the lower doses not only showed a significant increase when compared to that of the bleomycin treated group but also was found to be comparable to that of the control group rats at the end of the experiment. In this respect, the combination treatment was found to be more effective then the individual treatment of both the drugs.

Twenty one days after single intratracheal instillation of bleomycin, an obvious increase in the relative weight of lungs was observed in the experimental animals compared to that of control rats. Soumyakrishnan and Sudhandiran (2011) have reported a similar increase in lung weight in bleomycin treated animals. This is a clear indication of lung fibrosis that is characterised by excessive deposition of collagen. All the three treatment regimes were found to be effective as the relative organ weights of the three treatment regimes showed a significant decrease in the relative organ weight when compared to that of the bleomycin treated group and were found to be comparable with the control group rats with no significant difference within all the three treatment groups.

After analysing the physical end point of injury like lung weight in all the treatment groups, lung injury was quantitatively assessed biochemically (hydroxyproline - an index of collagen deposition, malondialdehyde - a measure of lipid peroxidation, lung contents of reduced glutathione, glutathione peroxidase activity, glutathione content, superoxide dismutase and catalase) and cytologically (total and differential cell count in bronchoalveolar lavage fluid). Histochemical localization of collagen in lung tissue has been further done to confirm the extent of collagen deposition. Lung histopathology was

also done to confirm the model and to unravel the possible inhibitory activities of the various treatment regimes mentioned earlier.

Deposition of excess or abnormal collagen is a characteristic of lung fibrosis as reported by many previous studies (Daba *et al*., 2002; Pardo *et al*., 2003; Serrano-Molar *et al*., 2003). In bleomycin induced lung fibrosis, the major deposits of connective tissue especially collagen is considered as the responsible element in the fibrotic tissue. In circumscribed areas of the fibrotic lung, it has been reported that collagen fibrils are positioned in between the basement membrane and the surface of endothelial cells. This invasion of the air-blood space will increase the distance between the air and blood compartments and thus may hamper gas exchange in the alveoli (Grande *et al*., 1998). Since, the amino acid hydroxyproline is the precursor for collagen, the estimation of the amino acid following acid digestion of collagen is a good biochemical index of collagen content. The present observation is in accordance with the previous findings, which too demonstrated remarkable increases in lung hydroxyproline content as an index of collagen accumulation and deposition (El-Medany *et al*., 2005; Gazdhar *et al*., 2007; Zhao *et al*., 2010). This finding was further confirmed by using the collagen-specific Masson's trichrome method of staining on lung sections for collagen deposition. Bleomycin, in the present work, induced collagen accumulation and deposition in peribronchial and perialveolar tissues that obliterated alveolar spaces as tiny fibrils.

However, it was noticed that individual administration of quercetin and sulindac effectively thwarted the progression of bleomycin induced lung fibrosis by significant decrease in the hydroxyproline level as shown in Table 2 and Figure 1D. The same was also confirmed visually by Masson's trichrome staining of lung sections as shown in Figure 3. Co-administration of quercetin and sulindac also prevented, to a great extent, the increase in collagen accumulation and the mean values of hydroxyproline content were in concurrence with those of vehicle control, remaining within 95% confidence limits.

Lipid peroxidation, a marker of oxidative stress, is an autocatalytic, free radical mediated, destructive process wherein polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (Kalayarasan *et al*., 2008). It is well documented that free radicals target biomacromolecules such as DNA, proteins and lipids, thereby causing increased lipid peroxidation and ultimately damage to the lung (Liang *et al*., 2011). In the current study, single intra tracheal instillation of bleomycin lead to

heightened malondialdehyde (MDA) [an indicator of lipid peroxidation] activity in the lungs, which can be attributed to free radical-mediated membrane damage. Treatment with sulindac significantly decreased the observed levels of malondialdehyde in bleomycin-treated rats, although these were still significantly higher than the control group rats. However, treatment with quercetin and co-administration of quercetin with sulindac showed significant decrease in the levels of MDA and the values of MDA in these groups were comparable to that of the control rats at the end of the experiment.

Extracellular superoxide dismutase is the only antioxidant enzyme in the extracellular matrix and extracellular space that is known to enzymatically scavenge superoxide radicals and thereby prevent the formation of many other reactive oxygen metabolites (Gao *et al*., 2008). This enzyme is expressed at a high level in the lung, compared to most other tissues. Its ability to directly bind several components in the extracellular matrix allows it to exist in high concentrations with specific matrix components (Gao *et al*., 2008). Moreover, it has been reported that superoxide dismutase plays an important role in preventing oxidant-induced extracellular matrix degradation and abolishing fibrotic cytokine TGF-β activation, which plays an important role in the development of fibrosis. Importantly, superoxide dismutase has been shown to protect against experimental pulmonary fibrosis, and its levels are low in the fibrotic areas of human IPF. This low level of interstitial superoxide dismutase in lungs may therefore contribute to antioxidant imbalances that result in further progression of this relentless disease (Bowler *et al*., 2002; Fattman *et al*., 2003).

In the current study a decline in the activity of superoxide dismutase was evident in bleomycin-treated rats, which is in concordance with previous studies (Ozyurt *et al*., 2004). However, all the three ameliorative treatments showed significant increase in the level of superoxide dismutase when compared to that of the bleomycin treated group. Moreover, the level of superoxide dismutase in the group which was treated with both quercetin and sulindac at lower dose was found to be comparable to that of the control group rats.

Catalase, a 240-kD tetrameric heme protein, is one of the major intracellular antioxidant enzymes responsible for detoxifying the hydrogen peroxide produced under physiological conditions to oxygen and water (Deisseroth and Dounce, 1970). Excessive hydrogen peroxide is harmful to almost all cell components, and thus its rapid and efficient removal

is vitally important for aerobic organisms. In the lungs, catalase is expressed during the later stages of development. It is constitutively expressed in airway and alveolar epithelial cells and in macrophages, and plays an important role in the endogenous antioxidant defence system (Zamocky *et al*., 2008). Moreover, it has been reported that the activity of this enzyme is found diminished in human pulmonary fibrosis and in bleomycin-induced lung injury (Odajima *et al*., 2010). A decrease in catalase particularly occurs in bronchiolar epithelial cells and/or in various types of abnormal re-epithelialization in fibrotic lungs (Odajima *et al*., 2010). The current study showed that a single intratracheal instillation of bleomycin lead to a significant decrease in the activity of this enzyme as compared to that of the vehicle control group, which is in accordance with findings of Sogut *et al.,* (2004). Treatment with quercetin and sulindac in this study improved the catalase activity compared to bleomycin treated rats however it remained still significantly lower to that of the control group rats. Moreover, the positive effect of co-administration of quercetin and sulindac at low dose proved to be statistically more significant than that of the individually administered drugs, as the levels of this enzyme in the case of the former were found to be comparable to that of the control group at the end of the experimental period.

Glutathione, a low molecular weight antioxidant synthesized in cells, is secreted by epithelial cells and plays a major role in the removal of many reactive oxygen species (Sutherland *et al*., 1985; Cantin *et al*., 1987). Imbalances in the expression of glutathione and associated enzymes have been implicated in a variety of pathological conditions. Besides enzymatic antioxidants, the level of glutathione, a non-enzymatic reducing agent that traps free radicals and prevents oxidative stress, was also found decreased in bleomycin-treated group. It has been well documented that a decrease in glutathione reductase activity often leads to decrease in reduced glutathione levels (Dairam *et al*., 2007). In the current study, a notable descent in the levels of reduced glutathione was observed in bleomycin-challenged rats, which could be due to overproduction of reactive oxygen species that exert an inhibitory effect on the enzyme Glutathione reductase, as opined by many (Blum and Fridovich, 1985; Sogut *et al*., 2004). Individual administration of quercetin and sulindac by and large restored the activities of these enzymatic antioxidants close to normal values. This might be due to the inhibitory action of both the drugs on reactive-oxygen species, which therefore reduces the oxidative stress often associated with pulmonary fibrosis. Co-administration of quercetin with sulindac showed

a significant increase in the levels of glutathione and reduced glutathione as compared to those in the bleomycin group. Moreover, the values were found comparable to those of the control group rats at the end of the experiment.

Similarly, glutathione peroxidase is also a powerful endogenous antioxidant enzyme, which contains the non-metallic element selenium. This enzyme protects the system from the harmful effect of free radicals by reducing these into alcohol and water (Soumyakrishnan and Sudhandiran, 2011). Treatment of either quercetin or sulindac post bleomycin challenge significantly increased the activity of this enzyme when compared to the bleomycin treated group. This may be due to the antioxidant property of quercetin which can be attributed to its phenolic hydroxyl groups. In case of sulindac the effect might be due to its anti-inflammatory property. However, the activity level of the glutathione peroxidase at the end of the experimental period was still found to be significantly lower than the control group rats. Co-administration of quercetin with sulindac also showed a significant increase in the activity of glutathione peroxidase when compared to that of the bleomycin control and was found to be comparable to that of the control group rats at the end of the experiment.

In addition to the oxidative stress mentioned earlier, intratracheal administration of bleomycin leads to interstitial inflammation, with a marked increase in the recruitment of leukocytes. The leukocytes such as macrophages, neutrophils and lymphocytes play a key role in inflammation and tissue remodelling (Xin *et al*., 2010). Alveolar macrophages are derived from monocytes and serve multiple immunological functions. They defend the lungs by phagocytic activity, taking part in specific mechanisms of defence as well as specific immune responses via secretory activity (Geiser, 2002; Geissmann *et al.,* 2010). Under normal conditions, alveolar macrophages are the most abundant cell population in bronchoalveolar lavage while during acute lung injury, of the kind induced by bleomycin here, there is a significant increase in the neutrophils. The same has also been observed in the idiopathic pulmonary fibrosis (Gadek *et al.,* 1980; Rudd *et al.,* 1981; Haellgren *et al.,* 1989; Mantovani *et al.,* 2002). During the present study a significant increase in the total number of cells, neutrophils and lymphocytes, while significant decrease in the number of macrophages in bronchoalveolar lavage fluid was seen in the case of the bleomycin treated rats. This is in accordance with previous studies of Gong *et al*., (2005) and Sriram *et al*., (2009). However, the total cell count, neutrophils, lymphocyte and macrophages count in quercetin, sulindac and combination group at the low doses showed definite improvement

from the bleomycin treated group. An inhibited recruitment of leukocytes, which directly impacted inflammation and tissue repair, might partly account for the preventive effect of quercetin and sulindac on bleomycin-induced pulmonary fibrosis, which may be due to their ability to interfere with free radical-mediated reactions.

Tumor necrosis factor-α (TNF-α), a potent pro-inflammatory cytokine acts as one major molecule among the multifaceted networks of cellular and molecular interactions that regulate the fibrotic process (Razzaque and Taguchi, 2003). It is widely accepted that tumor necrosis factor-α plays a pivotal role in bleomycin-induced lung injury and fibrosis. Neutralization of TNF-α with Anti-tumor necrosis factor-α antibody or administration of soluble tumor necrosis factor receptors (TNFRs) can prevent the development of lung fibrosis resulting from bleomycin exposure in mice (Phan and Kunkel, 1992; Piguet and Vesin, 1994; Swope and Lolis, 1999). Moreover, bleomycin exposure induces minimal lung inflammation or lung collagen deposition in double-TNFR (p75 and p55) knockout mice (Ortiz *et al*., 1999). Thus it could be hypothesized that suppression of TNF-α in the acute phase of lung inflammation would lead to attenuation of subsequent lung fibrosis.

In this study, a significant elevation in the TNF-α expression was observed in the bleomycin-treated group, which is concomitant with the findings of El-Medany *et al*. (2005). The tissue injury caused by bleomycin is found to be inflammation-mediated, which might be due to the production of free radicals, possibly leading to activation of nuclear factor kappa-B and increase in synthesis of TNF-α (Ortiz *et al*., 2002; Kalayarasan *et al*., 2008).

Moreover, during the course of this study it has been observed that oral administration of quercetin for twenty days post local instillation of bleomycin reduced the expression of pro-inflammatory cytokine TNF-α to the basal level indicating marked recovery from the induced lung injury caused by the drug in question. The decrease in the level of tumor necrosis factor-α in the quercetin treated group is due to its strong antioxidant effect, due to which there is a decrease in the production of reactive oxygen species. This probably works by inhibiting Nuclear Factor κ-B, which is an inducer of TNF-α expression.

In case of sulindac, a significant decrease in the level of TNF-α was observed when compared to that of the bleomycin treated group. The decrease in the level of tumor necrosis factor-α in sulindac treated group is also speculated to be due to its inhibitory effect on nuclear factor kappa-B activity as suggested by Berman *et al*., 2002. Although

TNF-α levels were substantially reduced in the sulindac group, they were not comparable to that of the control group rats. Therefore, on a relative scale, quercetin was more effective than sulindac in rescuing the symptoms of lung fibrosis.

The present study also demonstrated that co-administration of quercetin and sulindac at low doses are more beneficial in a fibrosis condition than are the individual treatments. This observation could well be a result of the anti-inflammatory effect of sulindac being potentiated by the anti-oxidant effect of quercetin.

Histopathological profiling of the lungs was carried out on day 21 using hematoxylin and eosin staining and Masson's trichrome staining.

Normal lung tissues showed typical open alveoli, interalveolar spaces with customary terminal bronchi, normal appearance of bronchiolar epithelium, thin interalveolar septa, lack of inflammatory cells and fibrosis (Liang *et al*., 2011). On the other hand, the bleomycin treated group displayed marked histopathological changes such as moderate to severe hemorrhages, congestion, emphysema, sloughing of bronchial epithelium from basement membrane, interstitial thickening with inflammatory cell infiltration, extensive collagen deposition and collapsed alveolar spaces (Figure 2B and 2C). Inflammatory infiltrates were localized to the alveolar spaces and peribronchial wall which consisted of mainly fibroblasts, lymphocytes, macrophages and few neutrophils. Furthermore, there were few foci of alveolar epithelialization and mild hyperplastic bronchial epithelium.

Although fibrotic lesions were observed in quercetin and sulindac treated groups, the extent of alveolitis and fibrosis was far less severe compared to that of bleomycin treated group (Figure 2D to 2G). Efficacy of quercetin and sulindac to abrogate fibrotic and associated inflammatory changes when accessed histologically revealed better effect of quercetin than sulindac. Lung sections from rats treated with combination therapy of quercetin and sulindac displayed nearly normal alveolar structure except a few inflammatory infiltrates in interstitium (Figure 2H and 2I).

Furthermore, a semi-quantitative assessment of fibrosis in lung sections was performed by scoring pathological lesions as per the Szapiel method of examination (Szapiel *et al*., 1979) (Table 5). The Szapiel score of bleomycin induced group was found significantly higher on day 21 when compared with control group. However, Szapiel scores on day 21 of quercetin and sulindac treated groups showed marked decrease from the bleomycin

treated group, reaffirming their ameliorative role against bleomycin induced lung fibrosis. Scores for the combination therapy of quercetin and sulindac displayed significant improvement and was found to be near normal (Table 5).

Masson's staining is considered a reliable method for localising collagen as specific areas in a histological preparation (Shimbori *et al*., 2011). Figure 3 shows the Masson's trichrome stained lung tissue section. Tissue injury due to administration of bleomycin resulted in excessive deposition of collagen, when compared to that of control group, as evident in Figures 3B and 3A. However, both quercetin and sulindac treated groups exhibited noticeable reduction in the collagen deposition in lung tissue (Figure 3C and 3D). Co-administration of quercetin and sulindac also displayed a noteworthy reduction in the deposition of collagen, which can be correlated with the decrease in the hydroxyproline content as shown in Figure 1D and Table 2 and as observed in Figure 3E.

The ameliorative potential of quercetin is due to its strong free radical scavenging activity. It is also a good metal chelator as reported by Jovanovic *et al*., (1998). It has also been previously shown that quercetin acts through various mechanisms including the antioxidative activity, the inhibition of enzymes that activate carcinogens, the modification of signal transduction pathways and interactions with receptors and other proteins (Chen *et al*., 2010). *In vitro* studies have demonstrated that quercetin inhibited the production of reactive oxygen species in lipopolysaccharide-stimulated Kupffer cells (Kawada *et al*., 1998). Moreover, reports have also shown that quercetin treatment effectively reduced superoxide anions (Huk *et al*., 1998) and could inhibit lipid peroxidation and hence, alterations in lung morphology during pulmonary injury or infection (Huk *et al*., 1998; Kumar *et al*., 2003).

On the other side, the effect of sulindac seems to be due to its anti-inflammatory activity, which is due to its ability to inhibit the cyclooxygenases enzymes, thereby blocking prostaglandin synthesis (Vane *et al*., 1998). Moreover, it has also been reported that nitric oxide plays an important role in pathogenesis of lung fibrosis and idiopathic pulmonary fibrosis (Yildirim *et al*., 2004). Although we have not undertaken the estimation of the nitric oxide in the present study, a report does state that sulindac has an inhibitory effect on the production of nitric oxide (Fernandes *et al*., 2003), which may be one of the possible mechanisms acting here in the current case. It has also been reported that sulindac is effective in scavenging reactive oxygen species free radicals (Dairam *et al*., 2007). In

the present findings, elevated level of reactive oxygen species was observed in bleomycin treated group but this was considerably reduced in sulindac-treated rats, signifying its antioxidant potential.

Moreover, MAPK pathways such as ERK1/2 and JNK 6 are activated in response to a high oxidative status, leading to increase in the levels of MMP-7 (Ho *et al*., 2011). The MAPK pathway is a known target for potential fibrosis therapy, as several fibrogenic cytokines signal through MEK/ERK, including non-canonical TGF-β, PDGF, IL-13 and TNF-α (Madala *et al*., 2012). We therefore suggest that quercetin and sulindac, due to its antioxidative and anti-inflammatory property and inhibitory effect on TNF-α, targets the above-mentioned pathway whereby downregulating the MMP-7activity and hence prevent extensive tissue remodelling a process associated with the initiation of lung fibrosis (Verma *et al*., 2013).

The results of the present study also suggest that each of quercetin and sulindac exerted a significant attenuation of the extent and severity of bleomycin induced lung fibrosis almost similarly although the antioxidant was found to score slightly better over the NSAID. Additionally, it could also be presumed that the attenuating effect of either quercetin or sulindac is partly due to their inhibitory effect on hydroxyproline contents and tumor necrosis factor-α. These ameliorative effects associated with quercetin and sulindac treatments could be due to the cumulative action of both the drugs over the inflammatory phase of bleomycin challenge.

What also comes to the fore here in this study is that co-administration of quercetin and sulindac at low doses proved to be more beneficial than each of them individually. In wake of the above observation, it is but logical to presume that as hypothesised and later vividly exemplified by the current results the pulmonary fibrosis is a function of augmented production of reactive oxygen species as well as prolonged inflammation at lung tissue upon bleomycin challenge. Hence, it is not surprising that a combination of anti-inflammatory and anti-oxidant drug as in case of the current one worked well together even at a low dose than each of them in isolation.

In conclusion, the present work provided an indication of the possible use of anti-inflammatory and anti-oxidant compounds in attenuating bleomycin induced lung fibrosis. These are believed to be acting by decreasing hydroxyproline levels, down regulating tumor necrosis factor-α mediated collagen deposition and more importantly by improving

the anti-oxidant defence as well as reducing the inflammatory phase in a bleomycin challenged rat. Also, the co-administration of quercetin and sulindac enhanced the beneficial effects afforded by either quercetin or sulindac. The results presented here may help us open up a whole new perspective for the possible roles of such drugs in preventing bleomycin-induced lung damage.

GENERAL CONSIDERATIONS

Idiopathic pulmonary fibrosis (IPF) is a prototype of idiopathic interstitial pneumonias, a chronic disease of the lung parenchyma that leads to diffuse scarring and end-stage tissue fibrosis (ATS, 2002). Typical features in this disease include dyspnea, diffused interstitial infiltrates, progressive lung fibrosis, and poor prognosis. The pathological changes in IPF include patchy fibrotic lesions that vary both in age and activity, and only weak inflammation. The focal zones of fibroblast proliferation are called "fibroblastic foci" and appear to occur at sites of recent alveolar injury (Gross and Hunninghake, 2001; ATS, 2002). The biochemical mechanisms in the pathogenesis of IPF are still poorly understood and medical therapies have thus far offered little, if any, benefit against the progression of this disease (ATS, 2002; Raghu *et al.*, 2004; Selman *et al.*, 2004).

The underlying mechanisms for the onset and progression of fibrosis or even the natural history of IPF are poorly understood. Inflammation and immune processes are, however, considered among the major mechanisms that injure lung tissue and induce fibrosis (Hagiwara *et al.*, 2000). There is also considerable evidence that oxidative stress due to oxygen-generated free radicals plays a major role in inflammatory and immune-mediated bleomycin-induced lung damage (Oury *et al.*, 2001).

Over the years a variety of animal models of pulmonary fibrosis have been developed to examine potential therapies for idiopathic pulmonary fibrosis. Bleomycin - a chemotherapeutic antibiotic drug, which induces pulmonary parenchymal fibrosis, is the most established experimental model of IPF in rodents (Moeller *et al.*, 2008).

Bleomycin induces the genesis of reactive oxygen species upon binding to DNA and iron, which in turn causes DNA damage (Atzori *et al.*, 2004). The interaction of bleomycin with DNA is postulated to initiate the inflammatory changes through a concerted action of various cytokines leading to excessive accumulation of collagen in the lung tissue (Wang *et al.*, 1991). Further, it is reported that bleomycin promotes the depletion of endogenous antioxidant defences thus exacerbating oxidant mediated tissue injury (Atzori *et al.*, 2004). The lung is selectively affected by bleomycin because it lacks an enzyme that hydrolyzes

the β-aminoalanine moiety of bleomycin, which prevents its metabolite from binding to metals such as iron (Filderman *et al.*, 1988).

Strategies aimed at reducing oxidative stress have been found to be successful in declining bleomycin induced lung fibrosis (Galvan *et al.*, 1981; Sriram *et al.*, 2009; Soumyakrishnan and Sudhandiran, 2011). Moreover, many anti-inflammatory compounds have also reported to have exhibited ameliorative potential against lung scarring (Chen *et al*., 2006, Shimbori *et al*., 2011). Thus indicating that oxidative stress coupled with prolonged inflammation could be that reason behind bleomycin induced pulmonary fibrosis in rats. Hence, it was hypothesized that mitigating either one or both of these established pathological manifestation of bleomycin intoxication might save the lung from getting scarred. The current study therefore, was directed at understanding the ameliorative property of quercetin an established antioxidant and sulindac a non steroidal anti-inflammatory drug (NSAID) individually as well as in combination in bleomycin instilled rats.

Of the two drugs selected for the study, quercetin (3,3',4',5,7-pentahydroxyflavone), a flavanoid present in fruits and vegetables, has attracted much attention for its beneficial health effects due to its multiple mechanisms including antioxidant activity, anti-inflammation, modification of signal transduction pathways and interactions with receptors and other proteins. The antioxidant activity of quercetin is primarily credited to its phenolic hydroxyl groups (Materska and Perucka, 2005). Sulindac ([Z]-5-fluoro-2-methyl-1-[*p*-(methylsulfinyl)-benzylidene] indene-3-acetic acid), a non-steroidal anti-inflammatory drug (NSAID), is well known for its anti-inflammatory activity, which is due to its ability to inhibit the cyclooxygenases enzymes and thereby inhibits prostaglandin synthesis (Vane *et al.*, 1998).

So, the present study was conducted to elucidate the possible inhibitory effects of quercetin on bleomycin induced rat pulmonary fibrosis compared to that afforded by sulindac. As observed in many complex ailments, it is likely that combination of agents, rather than single treatments will be more effective in curbing the health issue. Specific pathogen-free, healthy young adult male Wistar rats (RCCHan:WIST) of 10 to 12 weeks were used in this study which were kept in standard laboratory conditions as per NIH Guide for the Care and Use of Laboratory Animals and AAALAC principle. The test facility where experiments were conducted is AAALAC accredited and also complies with

the GLP, India. The experimental protocols were approved by the Institutional Animal Ethics Committee (JRF CPCSEA Approval no. 35/1999/CPCSEA: JRF Research Number - R-570/2010).

Briefly, after the weight was recorded, the rats were anesthetized using a combination of ketamin (80 mg/kg body weight, i.p.) and xylazine (20 mg/kg body weight, i.p.) as per standard protocol (Teixeira *et al.*, 2008). A midline incision was made in the neck and the exposed trachea was intubated with tracheal cannula under direct visualization. For induction of pulmonary fibrosis, the rats received a single dose of 6.5 U/kg body weights, bleomycin sulfate dissolved in 0.5 mL of 0.9% NaCl solution by intratracheal instillation on day 0 of the experiment (Wang *et al.*, 2002). Control rats were given a single intratracheal dose of sterile saline alone.

The animals were randomized into 5 groups each consisting of 10 animals (Gad and Weil, 1994):

- Group I - Vehicle control rats were given 0.5% carboxymethylcellulose solution orally from day 1 to day 20 of the experiment.
- Group II - Bleomycin treated rats were treated with 0.5% carboxymethylcellulose solution orally from day 1 to day 20 of the experiment.
- Group III - Animals were orally administered with quercetin (100 mg/kg body weight/day) in 0.5% carboxymethylcellulose solution from day 1 to day 20 of the experiment after bleomycin instillation (Tang *et al.*, 2012).
- Group IV - Animals were treated with sulindac within its therapeutic anti-inflammatory dose (ED_{50} for rats, 20 mg/kg body weight) in 0.5% carboxymethylcellulose solution from day 1 to day 20 of the experiment after bleomycin instillation (Vaish and Sanyal, 2012).
- Group V - Animals were treated with quercetin (50 mg/kg body weight/day) and sulindac (10 mg/kg body weight/day) in 0.5% carboxymethylcellulose solution from day 1 to day 20 of the experiment after bleomycin instillation.

The drug was freshly prepared and the concentration was adjusted so that each animal received 10 ml/kg body weight.

The animals were weighed at the beginning, through and at the end of experiments. The changes in body weight were recorded. Single intratracheal administration of bleomycin (6.5 U/kg) resulted in a marked decrease in their body weight on days 14 and 21 as compared to the saline treated control group because of severe tissue damage caused by generation of free radicals which could be attributed to the progression of the fibrosis due to bleomycin instillation and are in accordance with the findings of Zhou *et al.* (2007). These reductions in body weights associated with bleomycin administration were significantly restored by treatment with quercetin or sulindac when compared to the bleomycin treated group. Co-administration of quercetin and sulindac in combination at the lower doses was associated with significant restoration of body weights when compared with the bleomycin treated group and remained comparable to that of the control group rats.

A significant increase in the percent body weight change was observed in all the three drug treatment groups on days 14 and 21 of the experiment which was found to be comparable to the bleomycin treated group on days 3 and 7 of the experiment. Moreover, percent body weight change revealed that there was a significant decrease in the percent body weight in the entire treatment group all through the experimental schedule but for the group that received quercetin plus sulindac wherein the change in body weight was akin to that of controls. This amply testifies that the combination treatment was more effective than the individual treatment of both the drugs.

Twenty one days after single intratracheal instillation of bleomycin, an obvious increase in the relative weight of lungs was observed in the experimental animals compared to that of control rats. Soumyakrishnan and Sudhandiran (2011) have also reported a similar increase in lung weight in bleomycin treated animals. This increase in lung weight is a clear indication of lung fibrosis that is characterised by excessive deposition of collagen, which was evident from the increased hydroxyproline levels observed in the lungs of bleomycin treated rats. This deleterious effect due to treatment of bleomycin instillation was ameliorated by treatment with either quercetin or sulindac. Co-administration of quercetin and sulindac in combination at the lower doses was also found to be effective.

On day 21 of the experiment, six animals from each group were sacrificed with Thiopentone sodium and the lung lobes were excised and weighed and were used for

biochemical estimations. Lungs of four animals from each group were used for histopathological evaluation.

Lung injury was assessed biochemically by quantifying hydroxyproline content, an index of collagen deposition, present locally. Lung tissue homogenate was prepared as described by Edwards and O'Brien (1980). Lipid peroxidation was assessed by measuring the level of malondialdehyde following the method of Ohkawa *et al.* (1979). The activity of superoxide dismutase was assayed by the method described by Kakkar and co-workers (1984). The activity of catalase was assessed by Luck (1963). The concentration of glutathione in the lung was assayed by the method of Grunert and Philips (1951). Reduced glutathione level was evaluated by the method of Ellman (1959). The glutathione peroxidase activity was based on the method of Paglia and Valentine (1967). The extent of inflammation was evaluated cytologically by counting the total and differential cell count in bronchoalveolar lavage fluid.

Deposition of excess or abnormal collagen is a characteristic of lung fibrosis as reported by many previous studies (Daba *et al.*, 2002; Pardo *et al.*, 2003; Serrano-Molar *et al.*, 2003). Since the amino acid hydroxyproline is the precursor for collagen, estimation of this amino acid following acid digestion of collagen is considered a good biochemical marker of collagen content. The result of this study is in accordance with previous findings, which too demonstrate remarkable increase in lung hydroxyproline content as an index of collagen accumulation and deposition (El-Medany *et al.*, 2005; Gazdhar *et al.*, 2007; Zhao *et al.*, 2010).

Biochemical estimation of hydroxyproline revealed that the level of hydroxyproline in bleomycin and sulindac treated groups were significantly higher than those in control group rats. Both quercetin and sulindac treated groups exhibited significant reduction in hydroxyproline content in comparison with bleomycin control group. Co-administration of quercetin and sulindac treated group also exhibited significant reduction in hydroxyproline content in comparison with bleomycin control group.

Further, it is known that reactive oxygen species play an important role in the development of fibrotic responses in the lung, especially in those induced due to bleomycin challenge. Bleomycin binds to iron (Fe^{2+}), undergoes redox cycling and catalyzes the formation of reactive-oxygen species resulting in lung damage (Liang *et al.*, 2011) Therefore, in the

current study several established markers of oxidative stress were evaluated to gain a mechanistic understanding on the ameliorative potential of the drugs in question.

Lipid peroxidation, a marker of oxidative stress is an autocatalytic, free radical mediated, destructive process, wherein polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (Kalayarasan *et al.*, 2008). Bleomycin treatment produced a significant increase in the lung tissue MDA content, an index for lipid peroxidation when compared with control groups. Bleomycin-induced increments in MDA content of the lung were significantly prevented by quercetin and sulindac treatments. Results of the sulindac treatment did show significantly reduced levels than the bleomycin treated group, but these were still not comparable to those of the vehicle control group at the end of the experiment. Co-administration of quercetin and sulindac, on the other hand, reduced MDA content in the lung tissue to levels comparable to the control group.

Imbalances in the expression of glutathione and associated enzymes have been implicated in a variety of pathological conditions. Beside enzymatic antioxidants, the level of glutathione, a nonenzymatic reducing agent that traps free radicals and prevents oxidative stress, was found reduced in the bleomycin-treated group. It has been well documented that decrease in glutathione reductase activity often leads to decrease in glutathione levels (Dairam *et al.*, 2007). A notable drop in the activity of glutathione was observed in bleomycin-challenged rats, which might be due to overproduction of reactive oxygen species that exerts inhibitory effect on this enzyme (Blum and Fridovich, 1985; Sogut *et al.*, 2004). However, the level of glutathione in the sulindac treated group was found to be comparable to that of the bleomycin treated group at the end of the experiment, while in the quercetin treated group, a significant increase in the level of glutathione was observed at the end of the experiment when compared to that of bleomycin treated group. Co-administration of quercetin and sulindac in combination at the lower doses also showed a marked increase in the level of glutathione when compared to that of bleomycin treated group on day 21 of the experiment.

Similarly, depletion of superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione activities in the tissue reflects indirectly the generation of free radical produced by bleomycin administration (Ozyurt *et al.*, 2004; Sogut *et al.*, 2004). Intratracheal instillation of bleomycin produced a significant decrease in the superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione activities in lung

tissue on day 21 when compared with the control group rats. Quercetin and sulindac treatments significantly prevented the depletion of superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione when compared to that of the bleomycin control group, but these values were still not comparable to those of the vehicle control group. Quercetin and sulindac in combination also prevented the decrease in the levels of superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione at the lower doses when compared to that of the bleomycin treated group and remained comparable to that of the control group rats which shows that the combination of quercetin and sulindac at the low doses have a greater effect than the individually administered doses.

In addition to the oxidative stress mentioned earlier, interstitial inflammation is a direct result of bleomycin administration, accompanied with a marked increase in the recruitment of leukocytes. The leukocytes such as macrophages, neutrophils and lymphocytes play a key role in inflammation and tissue remodelling (Xin *et al.*, 2010). A significant increase in the total number of cells, neutrophils and lymphocytes while significant decrease in macrophages in broncholaveolar lavage fluid was seen in bleomycin treated group. This is in accordance with previous studies of Gong *et al.* (2005) and Sriram *et al.* (2009). In rats treated with quercetin and sulindac, the total cell count remained similar to the levels of control rats. Moreover, in combined treatment group at the lower dose levels, a significant decrease in the total cell count was observed when compared to that of bleomycin treated group, which was also comparable to that of the control group rats.

Single dose of intratracheal administration of bleomycin caused significant alterations in the differential cell count in the bleomycin group with respect to the control group rats. These alterations were inhibited or brought to near normal in all the three treatment groups. These results also suggested that combination of quercetin and sulindac at the low doses appeared to be more effective than the full separate doses of each.

Moreover, tumor necrosis factor-α (TNF-α), a potent pro-inflammatory cytokine which is one of the major molecules involved a multifaceted network of cellular and molecular interactions that regulates the fibrotic process (Razzaque and Taguchi, 2003), was also estimated using specific enzyme-linked immunosorbent assay by commercially available test kits. In this study, a significant elevation in the TNF-α expression was observed in the

bleomycin-treated group, which is in accordance with the findings of El-Medany *et al.* (2005). Treatment with quercetin and sulindac countered the bleomycin-induced increase in TNF-α level in plasma at the end of the experiment. However, it remained elevated in sulindac treated group on day 21 when compared to that of control group, while in the quercetin treated group, it remained comparable to that of the control group on day 21. Co-administration of quercetin and sulindac was associated with a significant reduction in TNF-α concentration in comparison to other treatment groups.

In another experiment, the lung tissue was screened for any histopathological changes in response to the various treatments. Immediately after sacrificing the rats, each lung was perfused and fixed in 10% neutral buffered formalin and routinely processed and embedded in paraffin. Serial sections (of 4μm thickness) were cut and stained with hematoxylin and eosin for light microscopic observation of pathological changes associated with bleomycin induced lung fibrosis and efficacy of quercetin and sulindac independently and in combination against it. The severity of fibrosis was individually assessed using the semi-quantitative grading system described by Szapiel *et al.* (1979). The scores of fibrosis in lung specimens were graded from − to +++ and correspondingly numbered from 0 to 3. The entire lung section was reviewed at a magnification of 100X. Histochemical localization of collagen in lung was further carried out to confirm the assessment of collagen deposition.

Histopathological examination of the lung tissue revealed that bleomycin treated group showed marked histopathological changes such as interstitial thickening with inflammatory cell infiltration, extensive collagen deposition and collapsed alveolar spaces. Inflammatory infiltrates were localized to the alveolar spaces and peribronchial wall which consisted of mainly fibroblasts, lymphocytes, macrophages and few neutrophils. Furthermore, there were few foci of alveolar epithelialization and mild hyperplastic bronchial epithelium. Similar histopathological changes reported by others give credence to the present observation (Teixeira *et al.*, 2008; Liang *et al.*, 2011). Although fibrotic lesions were also observed in quercetin and sulindac treated groups, the extent of alveolitis and fibrosis were markedly less severe as compared to the kind found in the bleomycin treated group, indicating ameliorative effect of these regimens to reduce pulmonary fibrosis. Efficacy of quercetin and sulindac to abrogate fibrotic changes, when assessed histologically, revealed better effect of quercetin than sulindac to reduce inflammatory and

fibrotic changes. Lung sections from rats treated with combination therapy of quercetin and sulindac displayed nearly normal alveolar structure except for a few inflammatory infiltrates in interstitium.

Furthermore, the semi-quantitative assessment of fibrosis in lung sections was performed by scoring pathological lesions as per the Szapiel method of examination. The Szapiel score of bleomycin induced group was found significantly higher on day 21 when compared with control group. However, Szapiel scores on day 21 of quercetin and sulindac treated groups showed marked decrease compared to the bleomycin treated group reaffirming ameliorative role of quercetin and sulindac against bleomycin induced lung fibrosis. Scores for the combination therapy of quercetin and sulindac displayed significant improvement and was found to be near normal.

Also, tissue injury due to administration of bleomycin resulted in the excess deposition of collagen when compared to that of control group (El-Medany *et al.*, 2005; Zhao *et al.*, 2010). However, both quercetin and sulindac treated groups exhibited noticeable reduction in collagen deposition in the lung tissues. Co-administration of quercetin and sulindac also displayed a noteworthy reduction in the deposition of collagen which can be correlated with the decrease in the hydroxyproline content.

The ameliorative potential of quercetin is due to its strong free radical scavenging activity. It is also a good metal chelator as reported by Jovanovic *et al.* (1998). It has also been reported that quercetin acts through various mechanisms including the antioxidative activity, the inhibition of enzymes that activate carcinogens, the modification of signal transduction pathways and interactions with receptors and other proteins (Chen *et al.*, 2010). *In vitro* studies have demonstrated that quercetin in fact inhibited the production of reactive oxygen species in lipopolysaccharide-stimulated Kupffer cells (Kawada *et al.*, 1998). Moreover, reports show that quercetin treatment effectively reduces superoxide anions (Huk *et al.*, 1998) and inhibits lipid peroxidation and hence, alterations in lung morphology during pulmonary injury or infection (Huk *et al.*, 1998; Kumar *et al.*, 2003).

On the other side, the effect of sulindac seems to be due to its anti-inflammatory activity, which is due to its ability to inhibit the cyclooxygenases enzymes, thereby blocking prostaglandin synthesis (Vane *et al.*, 1998). Moreover, it has also been reported that nitric oxide plays an important role in pathogenesis of lung fibrosis and idiopathic pulmonary

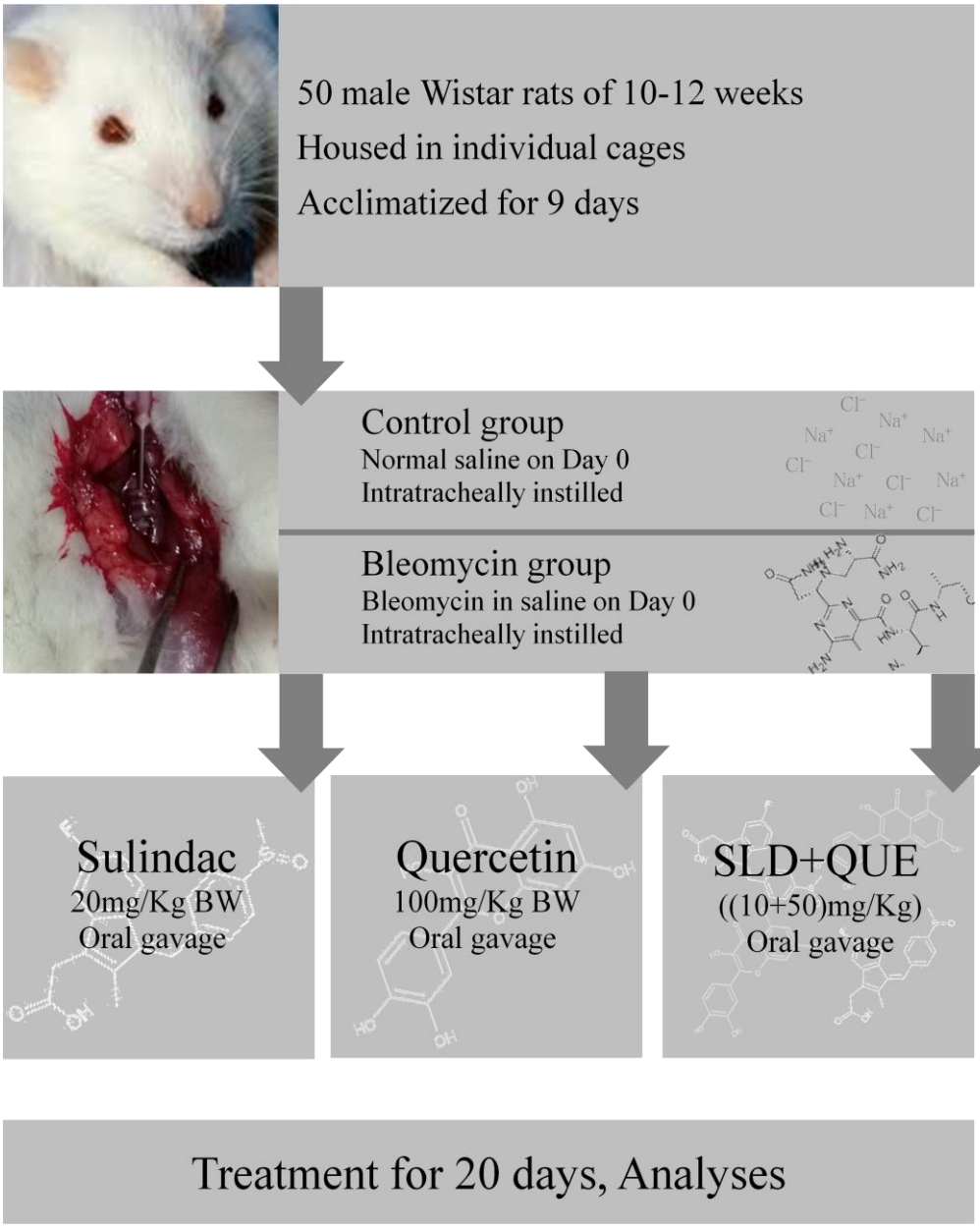
fibrosis (Yildirim *et al*., 2004). Although we have not undertaken the estimation of the nitric oxide in the present study, a report does state that sulindac has an inhibitory effect on the production of nitric oxide (Fernandes *et al*., 2003), which may be one of the possible mechanisms acting here in our case. It has also been reported that sulindac is effective in scavenging reactive oxygen species free radicals (Dairam *et al*., 2007). In our findings, elevated level of reactive oxygen species was observed in bleomycin treated group but this was considerably reduced in sulindac-treated rats, signifying its antioxidant potential.

What this study highlights is that treatment with quercetin and sulindac in combination at low doses is statistically more beneficial than each of the individual treatments. It seems logical to speculate that this is due to the anti-inflammatory effect of sulindac which is being potentiated by the anti-oxidant effect of quercetin.

In summary, the histophysiological evidences from the current study suggest without doubt that single intratracheal instillation of bleomycin induces lung fibrosis in rats, which can be used as an ideal animal model to test the efficacy of new therapeutic agents as potent drugs of choice to treat idiopathic pulmonary fibrosis. Presently two compounds were tested for their possible amelioratory property against bleomycin induced lung scarring, of which quercetin with its excellent antioxident property coupled with subtle anti-inflammatory property was found to be more effective than the NSAID sulindac. However, a combination of both these compounds even at lower doses effectively curbed the fibrotic response of the bleomycin challenged pulmonary tissue by reviving the otherwise compromised antioxidant defence and also by downplaying the local inflammatory response. Analysis of the histological profile of lung from various treatment groups reaffirms the above notion. Nevertheless, mechanistic studies including the timed expression pattern of collagenase enzymes (MMP7, MMP2), nitric oxide synthase-2, NO, $PGE_{2,}$ IL1, IL6,IL10, etc. need to be further undertaken to unearth the precise pathways that the drugs in question exploit to halt the progression of bleomycin induced pulmonary fibrosis.

DIAGRAMMATIC SUMMARY

Part One: Experimental Design



DIAGRAMMATIC SUMMARY

Part Two: Parameters Evaluated

Collagen deposition
Hydroxyproline content Masson's trichome staining

Histological profile
Hematoxylin-Eosin staining

Oxidative Status
Lipid Peroxidation Glutathione content Glutathione peroxidase Glutathione peroxidase Catalase Superoxide dismutase Reduced Glutathione content

Inflammatory marker
Tumor necrosis factor- α

Cytological profile
Total cell count Differential cell count

DIAGRAMMATIC SUMMARY

Part Three: Summary of Results

	BLE	QUE	SLD	QUE+SLD
Hydroxyproline Content	↑	▼	↑, ▼	▼
Lipid Peroxidation	↑	▼	↑, ▼	▼
Glutathione Content	↓	▲	-	▲
Glutathione peroxidise	↓	↓, ▲	↓, ▲	▲
Catalase	↓	↓, ▲	↓, ▲	▲
Superoxide Dismutase	↓	↓, ▲	↓, ▲	▲
Reduced glutathione	↓	↓, ▲	↓, ▲	▲
Grading of fibrosis	↑	↑, ▼	↑, ▼	↑, ▼

↑ Significantly higher compared to Control, ↓ Significantly lower compared to Control,

▲Significantly higher compared to Bleomycin, ▼ Significantly lower compared to Bleomycin,

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Annexure I: Daily Clinical Observations of Control Group Rats

Animal Number	Day																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Note: 1 = Normal, 2 = Dead, 23 = Dyspnea, 24 = Abdominal Breathing, 25 = Gasping, 27 = Tachypnea, 28 = Nasal Discharge, 146 = Coughing

Annexure II: Daily Clinical Observations of Bleomycin Treated Rats

Animal Number	Day																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
14	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
18	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Note: 1 = Normal, 2 = Dead, 23 = Dyspnea, 24 = Abdominal Breathing, 25 = Gasping, 27 = Tachypnea, 28 = Nasal Discharge, 146 = Coughing

Annexure III: Daily Clinical Observations of Quercetin Treated Rats

Animal Number	Day																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
21	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
25	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
26	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
27	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
28	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
29	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Note: 1 = Normal, 2 = Dead, 23 = Dyspnea, 24 = Abdominal Breathing, 25 = Gasping, 27 = Tachypnea, 28 = Nasal Discharge, 146 = Coughing

Annexure IV: Daily Clinical Observations of Sulindac Treated Rats

Animal Number	Day																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
31	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
32	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
33	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
34	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
35	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
37	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
38	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
39	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
40	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Note: 1 = Normal, 2 = Dead, 23 = Dyspnea, 24 = Abdominal Breathing, 25 = Gasping, 27 = Tachypnea, 28 = Nasal Discharge, 146 = Coughing

Annexure V: Daily Clinical Observations of Rats treated with Quercetin and Sulindac in combination

Animal Number	Day																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
41	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
42	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
43	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
44	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
45	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
46	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
47	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
48	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
49	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Note: 1 = Normal, 2 = Dead, 23 = Dyspnea, 24 = Abdominal Breathing, 25 = Gasping, 27 = Tachypnea, 28 = Nasal Discharge, 146 = Coughing

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Evaluating the inhibitory potential of sulindac against the bleomycin-induced pulmonary fibrosis in wistar rats

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ABSTRACT

The present study examined the protective effect of sulindac on bleomycin-induced lung fibrosis in rats. Animals were divided into saline group, bleomycin group (single intratracheal instillation of bleomycin) and bleomycin + sulindac (orally from day 1 to day 20). Bleomycin administration reduced the body weight, altered antioxidant status (such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione) while it increased the lung weight, hydroxyproline content, collagen deposition and lipid peroxidation. However, simultaneous administration of sulindac improved the body weight, antioxidant status and decreased the collagen deposition in lungs. Moreover, the levels of inflammatory cytokine tumour necrosis factor-α increased in bleomycin-induced group, whereas, on treatment with sulindac the levels of tumour necrosis factor-α were found reduced. Finally, histological evidence also supported the ability of sulindac to inhibit bleomycin-induced lung fibrosis. The results of the present study indicate that sulindac can be used as an agent against bleomycin-induced pulmonary fibrosis.

1. Introduction

Pulmonary fibrosis is a severe chronic disease with various causes and poor prognosis. Its main histological features include lesions of the alveolar septa, fibroblast and myofibroblast proliferation in lung parenchyma, abnormal re-epithelialization, and excessive extracellular matrix deposition. Lung fibrosis is associated with chronic inflammation and is characterized by the recruitment of macrophages, neutrophils, and lymphocytes in the airways. During lung inflammation, activated phagocytes release large amounts of reactive oxygen species, which may be involved in tissue injury and in impeding tissue repair, both of which lead to pulmonary fibrosis. Recent studies show that antioxidant compounds protect rats against tissue damage and pulmonary fibrosis because these compounds can attenuate the oxidant burden on tissue (Manoury et al., 2005).

Bleomycin is a commonly used chemotherapeutic agent that can cause dose-dependent pulmonary fibrosis (Jules-Elysee and White, 1990). Bleomycin-induced pulmonary injury and lung fibrosis has been documented in several animal species (Wang et al., 1991; Tzurel et al., 2002). This model has been widely used for studying the mechanisms involved in

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the progression of human pulmonary fibrosis and the impact of various drugs on this progression (Yara et al., 2001; El-Khatib, 2002). Bleomycin is known to generate reactive oxygen species upon binding to DNA and iron, which cause DNA damage. The interaction of bleomycin with DNA appears to initiate the inflammatory and fibroproliferative changes via a concerted action of various cytokines leading to collagen accumulation in the lungs. Bleomycin also promotes the depletion of endogenous antioxidant defences thus exacerbating oxidant mediated tissue injury (Atzori et al., 2004). The lung is selectively affected by bleomycin because this tissue lacks an enzyme that hydrolyzes the β-aminoalanine moiety of bleomycin, which prevents its metabolite from binding metals such as iron (Filderman et al., 1988).

Sulindac ([Z]-5-fluoro-2-methyl-1-[*p*-(methylsulfinyl)-benzylidene]indene-3-acetic acid), a non-steroidal anti-inflammatory drug, is well known for its anti-inflammatory activity, which is due to its ability to inhibit the cyclooxygenases enzymes thereby inhibiting prostaglandin synthesis (Vane et al., 1998). Sulindac is a sulfoxide prodrug, which is converted *in vivo* to the metabolites sulindac sulfide and sulindac sulfone. In addition to their anti-inflammatory properties, sulindac and its metabolites have been shown to play an important role in the prevention of colonic carcinogenesis (Fernandes et al., 2003).

The forerunner for bleomycin-induced pulmonary fibrosis has been considered the inflammatory response induced by the anti-neoplastic agent (Arafa et al., 2007). Therefore, sulindac has been utilized in the current study primarily for its anti-inflammatory effects. Besides, the notion that the cyclooxygenases inhibitor has shown some antiradical effects prompted us to investigate its modulatory effects on bleomycin-induced lung fibrosis possibly by regulating the oxidant/anti-oxidant imbalance (Fernandes et al., 2003).

Taking all that into consideration, we have addressed in the present work whether or not sulindac can inhibit bleomycin-induced lung injury using a rat model of lung fibrosis.

2. Materials and methods

2.1. Animals

Specific pathogen-free, healthy young adult male Wistar rats (RCCHan:WIST), weighing 274–362 g, were used in this study. They were obtained from the Barrier Maintained Rodent Animal Breeding Facility, Jai Research Foundation, Vapi, India. All the animals were fed with standard Teklad Certified Global High Fibre rat feed manufactured by Harlan, USA and provided U.V. sterilized water filtered through Kent Reverse Osmosis water filtration system *ad libitum*. The rats were kept in a controlled environment (temperature: 22 ± 2 °C and relative humidity: 30–70%) with an alternating cycle of 12-h light and dark. The animals used in this study were handled and treated strictly in accordance with the guiding principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The test facility at Jai Research Foundation is AAALAC accredited and also complies with the National Good Laboratory Practice, India. The experimental protocols were approved by the Institutional Animal Ethics Committee (Approval no. 35/1999/CPCSEA).

2.2. Experimental design

Rats were randomized into 3 groups, each consisting of 8 animals (Gad and Weil, 1994). Briefly, after the weights were recorded, the rats were anesthetized using a combination of ketamin (80 mg/kg body weight, i.p.) and xylazine (20 mg/kg body weight, i.p.) as per standard protocol (Teixeira et al., 2008). A midline incision was made in the neck and the trachea was exposed. A tracheal cannula was inserted into the trachea under direct visualization. For induction of pulmonary fibrosis, the rats received a single dose of 6.5 U/kg body weights bleomycin sulfate dissolved in 0.5 mL of 0.9% NaCl solution by intratracheal instillation on day 0 of the experiment (Wang et al., 2002). Control group rats were given a single intratracheal dose of sterile saline. Group 1 (vehicle control) and Group II (bleomycin treated) rats were treated with 0.5% carboxymethylcellulose solution (10 ml/kg body weight) from day 1 to day 20 of the experiment. Animals from Group III were treated with sulindac within its therapeutic anti-inflammatory dose (ED_{50} for rats, 20 mg/kg body weight) in carboxymethylcellulose solution for day 1 to day 20 of the experiment after bleomycin instillation (Vaish and Sanyal, 2012). The drug was freshly prepared and the concentration was adjusted so that each animal received 10 ml/kg body weight. The animals were weighed at the beginning, through and at the end of experiments. The changes in body weight were recorded.

2.3. Biochemical assays

2.3.1. Preparation of lung tissue for biochemical studies

On day 21 of the experiment, five animals from each group were sacrificed using thiopentone sodium and the lung lobes were excised. Broncholaveolar lavage was performed in three animals from each group under anaesthesia with thiopentone sodium.

2.3.2. Determination of lung hydroxyproline

The hydroxyproline assay was performed as described by Edwards and O'brien (1980). Briefly, the lung was dried and hydrolysed at 120 °C in a pressure vessel for 2–4 h. The acid hydrolysates and standards were added to 1.5-ml tubes, along with the same volume of citric/acetate buffer (citric acid, sodium acetate, sodium hydroxide, glacial acetic acid and *n*-propanol, pH 6.0) and chloramine T solution (chloramine T dissolved in Milli-Q water). The tubes were incubated for 20 min at room temperature and Ehrlich's solution [aldehyde-perchloric acid reagent (p-dimethyl-amino-benzaldehyde) perchloric acid and *n*-propanol] was added to the tubes, which were then incubated at 60 °C for 15 min. The absorbance (OD at 550 nm) of the reaction product was read.

2.3.3. Determination of lipid peroxidation

Malondialdehyde is the most abundant individual aldehyde resulting from lipid peroxidation breakdown in biological systems and is commonly used as an indirect method for

estimation of lipid peroxidation. Malondialdehyde content was assayed using the thiobarbituric acid test as described by Ohkawa et al. (1979). Malondialdehyde reacts with thiobarbituric acid to form a coloured complex. Absorbance was measured at 532 nm.

2.3.4. *Determination of reduced glutathione*

Reduced glutathione level was measured by the method of Ellman (1959). To the homogenate 5% trichloro acetic acid was added to precipitate the protein content of the homogenate. After centrifugation (at 3000 rpm for 10 min) the supernatant was taken and DTNB solution (Ellman's reagent) was added to it. The absorbance was measured at 412 nm.

2.3.5. *Determination of glutathione peroxidase activity*

The glutathione peroxidase activity was based on the method of Paglia and Valentine (1967). Tert-butylhydroperoxide was used as substrate. The assay measures the enzymatic reduction of H_2O_2 by glutathione peroxidase through consumption of reduced glutathione that is restored from oxidized glutathione GSSG in a coupled enzymatic reaction by glutathione reductase (GR). GR reduces GSSG to GSH using NADPH as a reducing agent. The decrease in absorbance was recorded at 340 nm was recorded.

2.3.6. *Estimation of glutathione content*

The concentration of glutathione in the lung was assayed by the method of Grunert and Philips (1951). Glutathione present in the tissue reacts with sodium nitroprusside to give a red coloured complex in saturated alkaline medium. The absorbance was measured at 520 nm.

2.3.7. *Measurement of superoxide dismutase*

The activity of superoxide dismutase was measured following the method of Kakkar et al. (1984). A known amount tissue homogenates was mixed with sodium pyrophosphate buffer, phenazine methosulphate and nitroblue tetrazolium chloride. The reaction was started by the addition of NADH. The reaction mixture was incubated at 30 °C for 90 s and stopped by the addition of 1 ml of glacial acetic acid. The absorbance of the chromogen formed was measured at 560 nm.

2.3.8. *Determination of catalase activity*

Catalase activity was assessed by the method of Luck (1963), wherein the breakdown of hydrogen peroxide is measured. In brief, the assay mixture consisted of 3 mL of H_2O_2-phosphate buffer and 0.05 mL of the supernatant of the tissue homogenate. The change in absorbance was recorded for a minutes at 30-s interval at 240 nm.

2.3.9. *Broncholaveolar lavage*

Broncholaveolar lavage fluid was obtained by the injection of 3 ml saline (three times, total 9 ml) followed by gentle aspiration of the fluid from the lung after securing an intratracheal catheter within a trachea. With this catheter, the ratio of the recovery of lavage fluid was approximately 80% and did not significantly differ among the groups. The total numbers of cells in the broncholaveolar lavage fluid were counted with a hemocytometer. For differential counts of leukocytes in the broncholaveolar lavage fluid, smear slides were prepared and stained with Giemsa solution. Differential cell counts were performed on 300 cells per smear.

2.3.10. *Measurement of tumour necrosis factor-α*

Tumour necrosis factor-α concentration was measured using an enzyme-linked immunosorbent assay kit (Xpressbio Life Scientific Products). The determinations were done according to the Test Kit instructions.

2.4. *Histological studies*

After sacrifice, each lung tissue was perfused and fixed in 10% neutral buffer formalin and routinely processed and embedded in paraffin. Serial sections (4 μm) were cut and stained with haematoxylin & eosin and Masson trichrome for light microscopic evaluation to examine the degree of fibrosis. The severity of fibrosis was individually assessed using the semi-quantitative grading system described by Szapiel et al. (1979). The scores of fibrosis in lung specimens were graded from − to +++ and correspondingly numbered as from 0 to 3. The entire lung section was reviewed at a magnification of 10×. Each of the 25 random microscopic fields per section were detected, a score ranging from 0 to 3 was assigned. All assessments were performed in blind fashion.

2.5. *Materials*

Sulindac, chloramine-T and hydroxyproline were procured from Sigma–Aldrich Chemie GmbH. Bleomycin hydrochloride was procured from the market and was in the form of bleomycin ampoules (15 units) manufactured by Biochem Pharmaceutical Industries Ltd., Mumbai, India. All other chemicals were of analytical grade and procured from reputed manufactures of India, *viz.*, Sisco Research Laboratories Pvt. Ltd., Qualigens Fine Chemicals Pvt. Ltd. and HiMedia Laboratory Pvt. Ltd.

2.6. *Statistical analysis*

Statistical analyses were carried out by analysis of variance (ANOVA) followed by post hoc test (Dunn's test). All analyses of data were performed using SPSS for windows version 12.0 and probability values of 0.05 or less were considered statistically significant.

3. Results

3.1. *Changes in body weight*

Fig. 1 shows the effect of sulindac on the body weight of bleomycin administered groups of rats. Single intratracheal administration of bleomycin (6.5 U/kg) resulted in a marked decrease in their body weight on day 14 as compared to the saline treated control group because of severe tissue damage caused by free radicals. However, body weight of sulindac-treated rats remained comparable to the control group rats throughout the experiment.

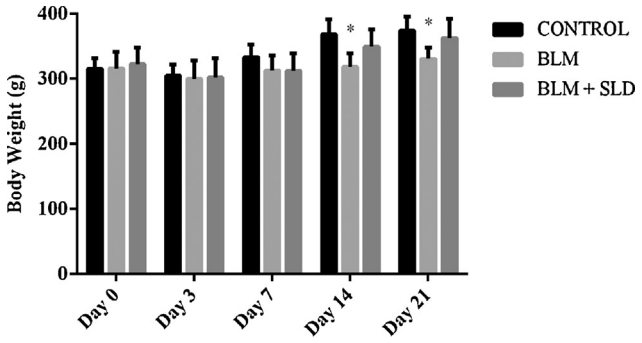

Fig. 1 – The mean body weight of rats during the course of experiment. $n = 8$; $^{*}p < 0.05$ vs. control group.

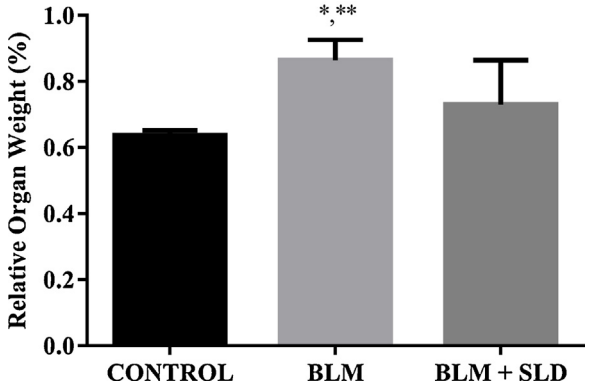

Fig. 2 – The relative weights of lungs in rats subjected to various treatments. BLM – bleomycin; SLD – sulindac; $^{**}p < 0.01$ vs. control group. $^{\#}p < 0.05$ vs. bleomycin Group. $^{\#\#}p < 0.01$ vs. bleomycin group.

3.2. *Change in percent relative organ weight*

As shown in Fig. 2 the percent relative organ weight of the sulindac group showed a marked decrease at the end of the experiment as compared to the bleomycin treatment group. Nevertheless, there was no significant variation in relative lung weight between sulindac treated group and control group.

3.3. *Hydroxyproline content*

The effect of sulindac on the hydroxyproline content of lung homogenates is presented in Table 1. It is well known that the hallmark of fibrosis is collagen deposition. Measurement of hydroxyproline is an efficient index of collagen deposition, since collagen contains significant amounts of the amino acid. After 21 days, the hydroxyproline content of the lungs in the bleomycin group increased significantly when compared to the control group. Lung hydroxyproline content in the bleomycin + sulindac group was found significantly lower than that of bleomycin group.

3.4. *Lipid peroxidation*

The result of this study showed an increase in the level of lipid peroxidation in bleomycin administered group when compared to the control group, which might be due to tissue injury and damage. Sulindac treatment however, significantly lowered the bleomycin induced lipid peroxidation in the lung of rats as evident from the MDA levels (Table 1).

3.5. *Reduced glutathione*

Table 1 shows the changes in the level of reduced glutathione in control, bleomycin-administered and sulindac treated lung tissues. The results amply testify that bleomycin has significantly decreased the levels of reduced glutathione compared to that of control group. Sulindac treated group of rats showed significantly higher levels of reduced glutathione when compared with corresponding bleomycin treated group.

3.6. *Glutathione peroxidase activity*

Bleomycin produced a significant reduction in the glutathione peroxidase activities in lung tissue after 21 days when compared with control groups (Table 1). The depletion in glutathione peroxidase activity in the tissue reflects indirectly the generation of free radicals. However, treatment with sulindac improved the activity of glutathione peroxidase as is evident from the significantly higher values of glutathione peroxidase activity in the lungs of sulindac group compared to bleomycin group.

Table 1 – The hydroxyproline content and oxidative stress status of lung tissue of rats subjected to various treatments.

Biochemical estimations	Control	Bleomycin	Bleomycin + sulindac
Hydroxyproline content (mg/g dried tissue)	1.77 ± 0.07	2.54 ± 0.19**	1.93 ± 0.14##
Malondialdehyde level (nmoles MDA/mg tissue/60 min)	44.62 ± 1.23	60.06 ± 1.64**	46.60 ± 2.16##
Reduced glutathione (μg/g tissue)	1.78 ± 0.04	1.07 ± 0.07**	1.67 ± 0.05##
Glutathione peroxidise (U/g tissue)	5.51 ± 0.13	2.44 ± 0.17**	3.33 ± 0.15**,##
Glutathione content (μ/g tissue)	38.64 ± 3.26	24.77 ± 1.96**	35.38 ± 2.61##
Superoxide dismutase (U/mg tissue)	0.34 ± 0.03	0.22 ± 0.01**	0.31 ± 0.02##
Catalase (μM H_2O_2 consumed/mg tissue/min)	19.22 ± 1.13	12.58 ± 0.94**	17.37 ± 1.01##

Values are given as Mean ± SE for groups of six rats each.
** $p < 0.01$ vs. control group.
$p < 0.01$ vs. bleomycin group.

Table 2 – The total and differential blood cell count of rats from various study groups.

Experimental group	Total cells ($\times 10^6$ ml^{-1})	Macrophage (%)	Neutrophils (%)	Eosinophil (%)	Lymphocytes (%)
Control	0.68 ± 0.13	84.22 ± 1.41	2.11 ± 0.37	0.78 ± 0.15	12.33 ± 1.11
Bleomycin	1.63 ± 0.18**	48.00 ± 3.33**	39.00 ± 1.56**	4.00 ± 0.67**	5.67 ± 0.89**
Bleomycin + sulindac	0.93 ± 0.05##	71.11 ± 1.93**,##	11.44 ± 1.41**,##	3.89 ± 0.59**	12.89 ± 0.15##

Values are given as Mean ± SE for groups of four rats each.
** $p < 0.01$ vs. control group.
$p < 0.01$ vs. bleomycin group.

Table 3 – The tumour necrosis factor-α and grade of fibrosis in rats subjected to various treatments.

Experimental Group	Tumour necrosis factor-α (pg/mL)	Grade of fibrosis
Control	22.82 ± 2.10	0.00 ± 0.00
Bleomycin	53.89 ± 3.04**	2.50 ± 0.07**
Bleomycin + sulindac	26.69 ± 2.42##	1.58 ± 0.15**,##

Values are given as Mean ± SE for groups of six rats each.
** $p < 0.01$ vs. control group.
$p < 0.01$ vs. bleomycin group.

3.7. *Glutathione content*

Significantly ($p < 0.01$) lower level of glutathione was observed in the lung tissues of bleomycin administered rats as compared to that of the controls. However, treatment with sulindac enhanced the glutathione content significantly by day 21 (Table 1).

3.8. *Superoxide dismutase activity*

Effect of bleomycin and bleomycin plus sulindac on lung tissue superoxide dismutase activity is presented in Table 1. The superoxide dismutase activity of bleomycin-treated rats significantly decreased as compared to the control group. Administration of sulindac was found to significantly restore the activity of antioxidant enzyme superoxide dismutase.

3.9. *Catalase activity*

The catalase activity in the lung homogenate of bleomycin-treated rats was considerably lower than that of vehicle control rats. In the sulindac-treated group, the catalase activity was significantly higher as compared to the bleomycin-treated group (Table 1).

3.10. *Total and differential cell count in broncholaveolar lavage fluid*

Table 2 shows the effect of sulindac on broncholaveolar lavage fluid differential and total cell count in control and experimental groups of rats. Bleomycin treatment caused a significant increase in the total cell count in the broncholaveolar lavage fluid as compared to control rats. In rats treated with sulindac, total cell count remained similar to the levels of control rats. The differential cell count showed a significant increase in neutrophils and eosinophils in the lungs of rats exposed to bleomycin. The sulindac treatment for 21 days significantly reduced the bleomycin-induced hike in the blood cells in the bronchoalveolar lavage. While the percentage of lymphocytes and alveolar macrophages were decreased in bleomycin-induced group, treatment with sulindac upturned these changes significantly.

3.11. *Tumour necrosis factor-α concentration*

Plasma levels of tumour necrosis factor-α are presented in Table 3. The tumour necrosis factor-α protein levels in plasma from rats in bleomycin administered group remained elevated on day 21 when compared with the sham group. Treatment with sulindac was found to decrease the bleomycin-induced increase in tumour necrosis factor-α level at the end of the experiment.

3.12. *Hisopathological examination of lung tissue*

Histopathological abnormalities in lungs were detected on day 21 using haematoxylin and eosin staining (Figs. 3–5) and Masson's trichrome staining (Figs. 6–8).

Normal lung tissues showed typical open alveoli, interalveolar spaces with customary terminal bronchi, normal appearance of bronchiolar epithelium, thin interalveolar septa, lack of inflammatory cells and fibrosis.

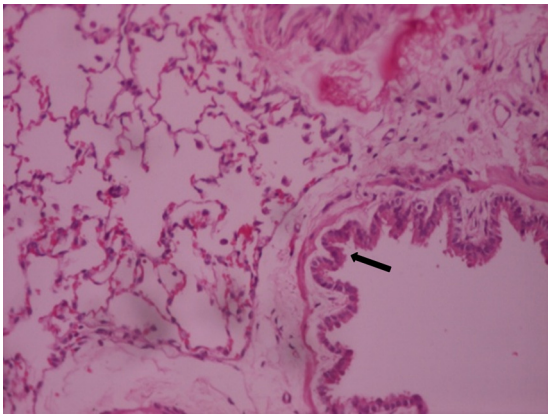

Fig. 3 – Control lung tissue showing normal open alveoli and interalveolar space (Haematoxylin and Eosin staining, 10×).

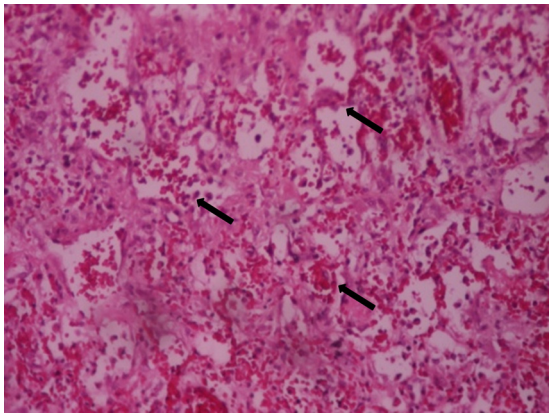

Fig. 4 – Bleomycin-treated lungs showing macrophage infiltration, haemorrhages, congestion and thickening of alveolar lining (Haematoxylin and Eosin staining – 10×).

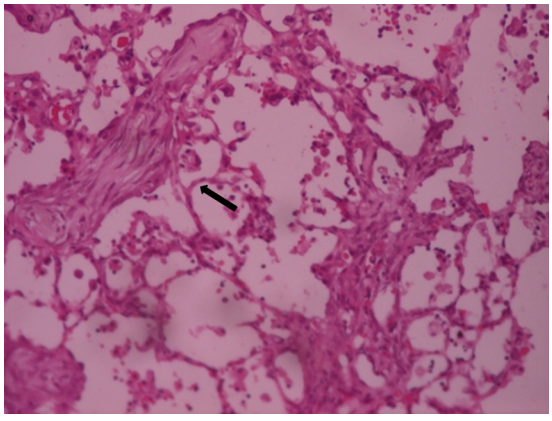

Fig. 5 – Sulindac treated lungs showing decrease in the thickening of alveolar lining and decrease in the macrophage infiltration as compared to bleomycin tissue (Haematoxylin and Eosin staining, 10×).

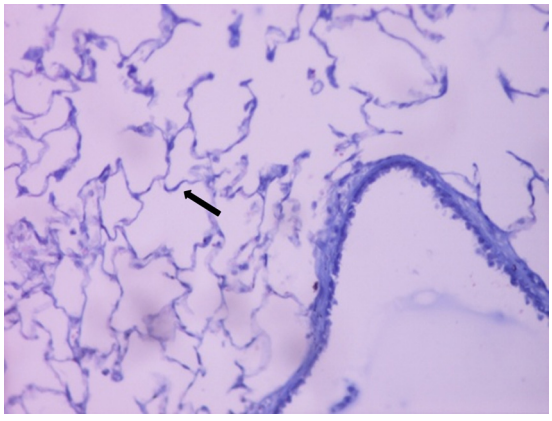

Fig. 6 – Normal lung tissue showing normal open pattern of alveoli and interalveolar space with minimal collagen deposition (Masson's trichrome staining, 10×).

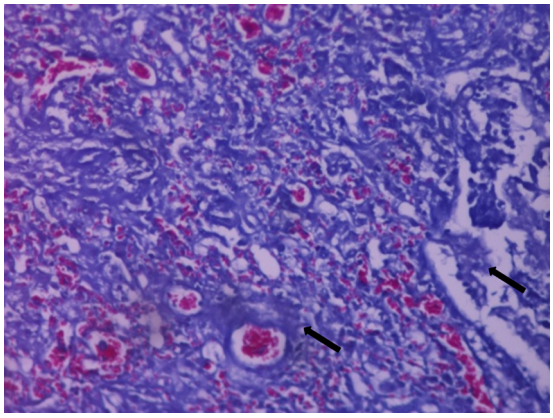

Fig. 7 – Bleomycin-treated lungs showing extensive collagen deposition as compared to the control group lungs (Masson's trichrome staining, 10×).

Bleomycin-administered group rats showed distorted architecture of the tissue i.e. moderate to severe haemorrhages, congestion, emphysema, sloughing of bronchial epithelium from basement membrane, areas of increased alveolar thickening, leukocytes accumulation in alveolar walls and increased fibrosis. However, lungs from sulindac-treated rats showed decreased amount of leukocytes and less thickening of the alveoli compared to the bleomycin-treated group.

Masson staining is regarded as a reliable method for localizing collagen as defined area in a histological preparation. Bleomycin-treated group displayed an increased grade of collagen deposition and large fibrotic areas, compared to the control group. However, collagen accumulation was remarkably decreased in sulindac-treated groups when compared to the bleomycin group. Furthermore, the semi-quantitative assessment of lung sections was performed to number pathology score as per the Szapiel examination (Table 3). The Szapiel score of bleomycin-induced group was found significantly higher on day 21 when compared with control group. However, Szapiel scores on day 21 of sulindac-treated group showed remarkable decrease when compared to the bleomycin-treated group.

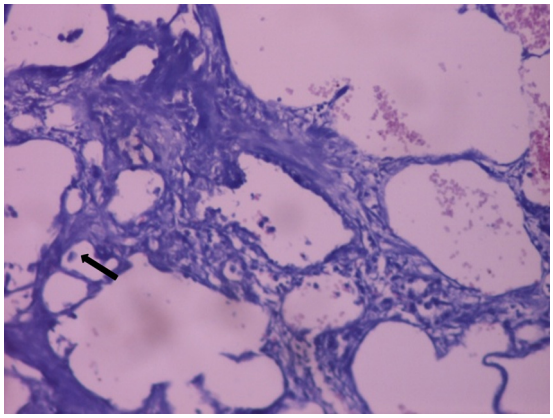

Fig. 8 – Sulindac-treated lungs showing mild collagen deposition as compared bleomycin treated lungs (Masson's trichrome staining, 10×).

4. Discussion

The clinical usefulness of bleomycin, an anti-cancer drug for human malignancies including germ-cell tumours, lymphomas, Kaposi's sarcoma, cervical cancer and squamous cell carcinomas of the head and neck has been hampered due to its detrimental effects (Sleijfer, 2001). The major side-effect of this drug is the induction of lung fibrosis in patients treated with bleomycin. Pulmonary fibrosis is commonly progressive and essentially an untreatable disease with an increasingly fatal outcome (Coker and Laurent, 1998). The bleomycin animal model of lung fibrosis is an established and widely used surrogate model of human lung fibrosis. There have been a number of studies employing bleomycin in different animal models including mice, rats, hamsters and dogs (Keane et al., 2001). The use of these animal models has helped in partly establishing the pathways of lung damage leading to fibrosis and by comparison studies of patients with lung pneumopathy, have validated many of these animal studies (Cooper, 2000).

In the current study, we have used wistar rat model of lung fibrosis by challenging the rats with a single dose of bleomycin sulfate by intratracheal instillation. A marked reduction in the body weight was observed in the bleomycin treated group, which might be due to the progression of the fibrosis (Zhou et al., 2007). Moreover there was increase in the relative organ weight of the lungs of the bleomycin-challenged rats when compared to the control rats, which may be due to the excessive deposition of collagen. This is in accordance with the finding of Soumyakrishnan and Sudhandiran (2011). However, the body weight and relative organ weight of the lungs of the sulindac treated group remained comparable to the control group rats.

Lung injury was quantitatively assessed biochemically (hydroxyproline, an index of collagen deposition; malondialdehyde, as a measure of lipid peroxidation; lung contents of reduced glutathione, glutathione peroxidase activity, glutathione content; superoxide dismutase and catalase) and cytologically (total and differential cell counts in broncholaveolar lavage fluid). Further, histochemical localization of collagen in lung tissue was done to confirm the assessment of collagen deposition. Lung histopathology was also done to confirm the model and to unravel the possible inhibitory activity of sulindac.

Deposition of excess or abnormal collagen is a characteristic of lung fibrosis as reported by many previous studies (Daba et al., 2002; Pardo et al., 2003; Serrano-Molar et al., 2003). Since, the amino acid hydroxyproline is the precursor for collagen, the estimation of the amino acid following acid digestion of collagen is a good biochemical index of collagen content. Our result is in accordance with previous findings, which too demonstrated remarkable increase in lung hydroxyproline content as an index of collagen accumulation and deposition (Gazdhar et al., 2007; El-Medany et al., 2005; Zhao et al., 2010). This finding was further confirmed by collagen-specific staining using Masson's trichrome staining of lung sections for collagen deposition. Bleomycin, in the present work, induced collagen accumulation and deposition in peribronchial and perialveolar tissues that obliterated alveolar spaces as tiny fibrils. However, intensity of collagen deposition was considerably reduced in sulindac-treated group, which might be due to the inhibitory effect of sulindac.

Further, it is known that reactive oxygen species play an important role in the development of fibrotic responses in the lung, especially in those induced due to bleomycin challenge. Bleomycin binds to iron (Fe^{2+}), undergoes redox cycling and catalyzes the formation of reactive-oxygen species, ultimately increasing lipid peroxidation and resulting in lung damage (Liang et al., 2011). Sulindac, a non-steroidal anti-inflammatory drug, is effective in scavenging reactive oxygen and nitrogen species free radicals (Dairam et al., 2007). In our findings, elevated level of reactive oxygen species was observed in bleomycin treated group but this was considerably reduced in sulindac-treated rats thus signifying its antioxidant potential.

Lipid peroxidation, a marker of oxidative stress is an autocatalytic, free radical mediated, destructive process, wherein polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (Kalayarasan et al., 2008). In our study, the observed high levels of lipid peroxidation in bleomycin-injured rats can be attributed to free radical-mediated membrane damage. However, treatment with sulindac significantly decreased the observed levels of malondialdehyde in bleomycin-treated rats.

Imbalances in the expression of glutathione and associated enzymes have been implicated in a variety of pathological conditions. Beside enzymatic antioxidants, the level of glutathione, a nonenzymatic reducing agent that traps free radicals and prevents oxidative stress, was also decreased in bleomycin-treated group. It has been well documented that decrease in glutathione reductase activity often leads to decrease in reduced glutathione levels (Dairam et al., 2007). A notable descent in the activity of glutathione peroxidise was observed in bleomycin-challenged rats, which might be due to overproduction of reactive oxygen species that exerts inhibitory effect on this enzyme (Sogut et al., 2004; Blum and Fridovich, 1985). Administration of sulindac restored the activities of these enzymatic antioxidants close to normal values. This might be due to the inhibitory action of sulindac on reactive oxygen species, consequently decreasing the oxidative stress produced during pulmonary fibrosis.

Superoxide dismutase catalyzes the dismutation of superoxide into oxygen and hydroperoxides, thereby acting as a potent antioxidant. A decline in the activity of superoxide dismutase was evident in bleomycin-treated rats, which is in concordance with previous studies (Ozyurt et al., 2004). Catalase is another antioxidant enzyme found in peroxisomes. This enzyme functions as the catalyst for the conversion of hydrogen peroxide, formed previously by the dismutation of superoxide dismutase, into water and molecular oxygen (Sogut et al., 2004). Decrease in the activity of this enzyme was also observed in bleomycin-treated group. Treatment with sulindac brought the levels of these enzymes too, close to that of control group.

Similarly, glutathione peroxidase is also a powerful endogenous antioxidant enzyme, which contains the non-metallic element selenium. This enzyme protects the system from the harmful effect of free radicals by reducing these into alcohol and water (Soumyakrishnan and Sudhandiran, 2011).

Treatment with sulindac significantly increased the level of this enzyme when compared to the bleomycin treated group. This may be due the antioxidant property of sulindac which may inhibit reactive oxygen radical production in the lungs.

In addition to the oxidative stress mentioned earlier, intratracheal administration of bleomycin leads to interstitial inflammation, with the marked increase in the recruitment of leukocytes. The leukocytes such as macrophages, neutrophils and lymphocytes play a key role in inflammation and tissue remodelling (Xin et al., 2010). A significant increase in the total number of cells, neutrophils and lymphocytes while significant decrease in macrophages in broncholaveolar lavage fluid was seen in bleomycin treated group. This is in accordance with previous studies of Gong et al. (2005) and Sriram et al. (2009). However, the total cell count, neutrophils, lymphocyte and macrophages count in sulindac treated rats remained comparable to the control group rats. Inhibited leukocytes recruitment, which directly impacted inflammation and tissue repair, might partly account for the preventive effect of sulindac on bleomycin-induced pulmonary fibrosis, which may be due to its ability to interfere with free radical-mediated reactions.

Moreover, tumour necrosis factor-α, a potent pro-inflammatory cytokine acts as one major molecule among the multifaceted networks of cellular and molecular interactions that regulate the fibrotic process (Razzaque and Taguchi, 2003). In this study, a significant elevation in the tumour necrosis factor-α expression was observed in the bleomycin-treated group, which is in accordance with the findings of El-Medany et al. (2005). The tissue injury caused by bleomycin is found to be inflammation-mediated, which might be due to the production of free radicals, possibly leading to activation of nuclear factor kappa-B and increase in synthesis of tumour necrosis factor-α (Ortiz et al., 2002; Kalayarasan et al., 2008). Sulindac has an inhibitory effect on nuclear factor kappa-B activity (Berman et al., 2002). Sulindac substantially reduced the expression of tumour necrosis factor-α, perhaps by inhibitory effect on nuclear factor kappa-B activity.

The subsequent corroboratory histopathological observation showed marked structural distortion of the alveolar space with collapsed alveolae, interalveolar inflammation, thickened alveolar wall and abnormal collagen deposition in bleomycin-induced rats. Similar histopathological changes reported by others give credence to the present observation (Liang et al., 2011; Teixeira et al., 2008). Moreover, in the present study it was observed that sulindac could hinder the structural distortion caused by bleomycin as indicated by the improvement in lung fibrosis scores that might be due to its antioxidant potency of the former.

Increase in the number of fibroblasts leads to excessive deposition of collagen content in the lung interstitium. One of the strategies to attenuate fibrosis is to inhibit the overproduction of collagen and proliferation in fibroblasts (Gong et al., 2005). Decrease in the deposition of collagen was observed in the sulindac-treated group as observed in Masson's trichrome stained section of the lungs.

Nitric oxide plays an important role in pathogenesis of lung fibrosis and idiopathic pulmonary fibrosis (Yildirim et al., 2004). Although we have not undertaken the estimation of the nitric oxide in the present study, a report does state that sulindac has an inhibitory effect on the production of nitric oxide (Fernandes et al., 2003).

In the present study, the pulmonary response to bleomycin challenge includes a rapid development of oxidative stress in combination with reduction of antioxidant capacity in lung. Inhibitory effect of sulindac reduced oxidative stress by anti-inflammatory and reactive oxygen species scavenging capacity. Additional studies are required to specify the protective mechanism of sulindac on this model, as well as to investigate the effect of sulindac on other animal model of lung fibrosis.

5. Conclusion

Pulmonary fibrosis is generally non-responsive to conventional corticosteroid therapy (Green, 2002). While instillation of bleomycin resulted in reduction of antioxidant capacity, elevation of inflammatory cytokines, fibrotic changes and collagen accumulation in lung tissue, Sulindac displayed pneumoprotective property through enhancement of antioxidant defence, decrease in the level of inflammatory cytokines and collagen accumulation. Thus, the present results suggest that sulindac effectively prevents the pulmonary injury induced by bleomycin challenge.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Research Article

Evaluating the Ameliorative Potential of Quercetin against the Bleomycin-Induced Pulmonary Fibrosis in Wistar Rats

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The current study deals with the effect of a dietary flavanoid quercetin on fibrotic lung tissue in rats. Bleomycin was administered by single intratracheal instillation to Wistar rats to induce lung fibrosis. The pathologies associated with this included significantly reduced antioxidant capacity, ultimately leading to protracted inflammation of the lung tissue. The hallmark of this induced fibrosis condition was an excessive collagen deposition in peribronchial and perialveolar regions of the lung. Oral quercetin treatment over a period of twenty days resulted in significant reversal of the pathologies. The antioxidant defense in lung tissue was revived. Moreover, activity of the collagenase MMP-7, which was high in fibrotic tissue, was seen restored after quercetin administration. Trichome staining of lung tissue sections showed high collagen deposition in fibrotic rats, which may be a direct result of increased mobilization of collagen by MMP-7. This was appreciably reduced in quercetin treated animals. These results point towards an important protective role of quercetin against idiopathic lung fibrosis, which remains a widely prevalent yet incurable condition in the present times.

1. Introduction

Bleomycin is a commonly used chemotherapeutic agent which, however, induces dose-dependent pulmonary fibrosis upon long-term administration [1]. Interstitial pulmonary fibrosis is characterized by an altered cellular composition of the alveolar region with excessive deposition of collagen. However, lung inflammation is considered to be a major underlying factor for the induction of pulmonary fibrosis [2]. Reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radical are reported as major mediators of lung inflammatory processes [3]. Nevertheless, the direct linkage between reactive oxygen species formation and pulmonary fibrosis has not been established conclusively. Bleomycin-induced pulmonary injury and lung fibrosis have been documented in studies using several animal models [4, 5]. These models have been widely used for studying the mechanisms involved in the progression of human pulmonary fibrosis and the impact of various drugs on this progression [6, 7]. The bleomycin induces the genesis of reactive oxygen species upon binding to DNA and iron, which in turn causes DNA damage [8]. The interaction of bleomycin with DNA is postulated to initiate the inflammatory and fibroproliferative changes through a concerted action of various cytokines leading to collagen accumulation in the lung [5]. Further, it is reported that bleomycin promotes the depletion of endogenous antioxidant defences, thus exacerbating oxidant mediated tissue injury [8]. The lung is selectively affected by bleomycin as it lacks an enzyme that hydrolyzes the β-aminoalanine moiety of bleomycin, which prevents its metabolite from binding to metals such as iron [9]. Strategies aimed at reducing oxidative stress have been found to be successful in decreasing bleomycin-induced lung injury and fibrosis [10–12].

The detrimental role of reactive oxygen species in many disease states has led to the development of new antioxidants. One such group of compounds with potential antioxidant property is the flavonoids present in fruits and vegetables, of which quercetin (3,3′,4′,5,7-pentahydroxyflavone) has attracted much attention for its beneficial health effects.

It has been suggested that daily intake of these substances may reduce the risk of various chronic health disorders such as cardiovascular disease, diabetes, tumor development, stroke, and neurodegenerative disease [13–16]. The beneficial effects of quercetin have been attributed to multiple mechanisms including antioxidant activity, anti-inflammation, modification of signal transduction pathways and interactions with receptors and other proteins [17]. The antioxidant activity of quercetin is primarily credited to its phenolic hydroxyl groups [18].

Beneficial health effects of quercetin against various oxidative stress related diseases have been documented [19]. However, studies examining its potential pneumoprotective effects are limited. Although experimental evidence for the role of a redox imbalance in lung fibrogenesis is substantial, this chronic disease is generally nonresponsive to conventional anti-inflammatory and immunomodulatory therapy [20]. Therefore, it was of interest to determine the ameliorative role, if any, of quercetin against bleomycin-induced lung injury because of the former's potent antioxidant activity.

2. Materials and Methods

2.1. Animals. Specific pathogen-free, healthy young adult male Wistar rats (RCCHan:WIST) of 10 to 12 weeks were used in this study. They were obtained from the Barrier Maintained Rodent Animal Breeding Facility, Jai Research Foundation, Vapi, India. All the animals were fed with Teklad certified global rat feed manufactured by Harlan, USA and UV sterilised water filtered through Kent Reverse Osmosis water filtration system was provided *ad libitum*. The rats were kept in a controlled environment (temperature: 22 ± 2°C and relative humidity: 30 to 70%) with an alternating cycle of 12 h light and dark. The animals used in this study were handled and treated in accordance with the strict guiding principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The test facility at Jai Research Foundation is AAALAC accredited and also complies with the National Good Laboratory Practice, India. The experimental protocols were approved by the Institutional Animal Ethics Committee (approval no. 35/1999/CPCSEA).

2.2. Experimental Design. Rats were randomized into three groups each consisting of 10 animals [21]. Briefly, the rats were anesthetized using a combination of ketamine (80 mg/kg body weight, i.p.) and xylazine (20 mg/kg body weight, i.p.) as per standard protocol [22]. A midline incision was made in the neck and the exposed trachea was intubated with tracheal cannula under direct visualization. For induction of pulmonary fibrosis, the rats received a single dose of 6.5 U/kg body weight bleomycin sulfate dissolved in 0.5 mL of 0.9% NaCl solution on day 0 of the experiment [23]. Control rats received a single intratracheal dose of sterile saline alone. Group 1 (vehicle control) and group II (bleomycin treated) rats were treated with 0.5% carboxymethylcellulose solution orally from day 1 to day 20 of the experiment. Animals from group III were orally administered with quercetin (100 mg/kg body weight/day) in 0.5% carboxymethylcellulose solution from day 1 to day 20 of the experiment after bleomycin instillation [24]. The drug was freshly prepared in 0.5% carboxymethylcellulose solution and the concentration was adjusted so that each animal received 10 mL/kg body weight. The animals were weighed at the beginning, through and at the end of experiment. The changes in body weight were recorded.

2.3. Biochemical Assays

2.3.1. Preparation of Lung Tissue for Biochemical Studies. On day 21 of the experiment, six animals from each group were sacrificed with thiopentone sodium and the lung lobes were excised and the tissue was used for the biochemical estimations. BAL was performed in four animals from each group under anaesthesia with thiopentone sodium and the tissue was used for the preparation of the slides to be used for histological examinations.

2.3.2. Determination of Lung Hydroxyproline. The hydroxyproline assay was performed as described by Edwards and O'Brien [25]. The lung was dried and then hydrolysed at 120°C in a pressure vessel for 2–4 h. The acid hydrolysates and standards were added to 1.5 mL tubes, along with the same volume of citric/acetate buffer (citric acid, sodium acetate, sodium hydroxide, glacial acetic acid, and n-propanol pH 6.0) and chloramine T solution (chloramine T dissolved in Milli Q water). The tubes were incubated for 20 min at room temperature and Ehrlich's solution (p-dimethyl-aminobenzaldehyde, perchloric acid and n-propanol) was added to the tubes which were then incubated at 60°C for 15 min. The absorbance of the reaction product was read at 550 nm.

2.3.3. Determination of Lipid Peroxidation. Malondialdehyde (MDA) is the most abundant individual aldehyde resulting from lipid peroxidation breakdown in biological systems and is commonly used as an indirect method for the estimation of lipid peroxidation. MDA content was assayed using the thiobarbituric acid test as described by Ohkawa et al. [26]. MDA reacts with thiobarbituric acid to form a coloured complex. Known amount of tissue homogenate was added to the tubes containing dodecyl sulphate, acetic acid, and thiobarbituric acid solution and heated in a water bath at 95°C for 60 minutes. Trichloroacetic acid was added to the tubes and absorbance was measured at 532 nm to determine the malondialdehyde content.

2.3.4. Measurement of Superoxide Dismutase Activity. The activity of superoxide dismutase was measured following the method of Kakkar et al. [27]. A known amount of tissue homogenates was mixed with sodium pyrophosphate buffer, phenazine methosulphate, and nitroblue tetrazolium chloride. The reaction was started by the addition of NADH. The reaction mixture was incubated at 30°C for 90 seconds and stopped by the addition of glacial acetic acid. The absorbance of the chromogen formed was measured at 560 nm.

2.3.5. Determination of Catalase Activity. Catalase activity was assessed by the method of Luck [28], wherein the breakdown of hydrogen peroxide is measured. Briefly, a known amount of tissue homogenate is added to 0.01% chilled digitonin and was centrifuged at 4°C and 0.05 mL of the supernatant of the tissue homogenate is mixed with 3 mL of H_2O_2 phosphate buffer. The change in absorbance was recorded at 30-second interval at 240 nm.

2.3.6. Bronchoalveolar Lavage (BAL). BAL fluid was obtained by the injection of 3 mL saline (three times, total 9 mL) followed by gentle aspiration of the fluid from the lungs after securing an intratracheal catheter within a trachea. With this catheter, the ratio of the recovery of lavage fluid was approximately 80% and did not significantly differ among the groups. The total numbers of cells in the bronchoalveolar lavage fluid were counted with a hemocytometer. For differential counts of leukocytes in the bronchoalveolar lavage fluid, smear slides were prepared and stained with Giemsa solution. Differential cell counts were obtained from a count of 300 cells per smear.

2.3.7. Measurement of Tumor Necrosis Factor- (TNF-) α. TNF-α in plasma was assayed by specific enzyme-linked immunosorbent assay using commercially available ELISA test kits (XpressBio Life Science Products, USA). The kit contains a TNF-α monoclonal antibody coat for a 96-well microtitre plate and polyclonal antibody to TNF-α. The representative standard curve was generated using the TNF-α standard supplied with the kit.

2.3.8. Estimation of MMP-7. MMP-7 levels in plasma and BAL were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA). The samples were added in duplicate to 96-well plates coated with the MMP-7 antibody and incubated for 2 h. After washing three times with washing buffer, the conjugated secondary antibody was added and the plate was further incubated for 2 h. Plates were washed again prior to incubation with the substrate solution for 1 h. The amplifier solution was then added, and the plate was incubated for additional 30 min. All incubation cycles were performed at room temperature. Following termination of the reaction with the stop solution; the optical density was measured at 490 nm using a microplate reader. The concentration of MMP-7 in each sample was calculated from a standard curve.

2.4. Histological Studies. After sacrifice, each lung tissue was perfused and fixed in 10% neutral buffer formalin and routinely processed and embedded in paraffin. Serial sections (4 μm) were cut and stained with hematoxylin and eosin and Masson's trichrome for light microscopic evaluation to examine the degree of fibrosis. The severity of fibrosis was individually assessed using the semiquantitative grading system described by Szapiel [29]. According to this system, the fibrosis in lung specimens was graded as none (0) when no alveolitis was observed; mild (+)—when focal lesions occupying less than 25% of the lung was noticed in alveolar septum; moderate (++)—when widespread alveolitis involving 25–50% of the lung was observed; and severe (+++)—when a diffused alveolitis spanning more than 50% of the lung, with occasional consolidation of air spaces and patches of hemorrhagic areas within the interstitium, was observed. The entire lung section was reviewed at a magnification of s10X. Each of the 20 random microscopic fields per section was detected; a score ranging from 0 to 3 was assigned. All assessments were performed in double-blind fashion.

2.5. Material. Quercetin, Chloramine-T and Hydroxyproline were procured from Sigma-Aldrich Chemie GmbH. Bleomycin hydrochloride was procured from the local market and was in the form of bleomycin ampoules (15 units) manufactured by Biochem Pharmaceutical Industries Ltd., Mumbai, India. All other chemicals were of analytical grade and procured from reputed manufactures of India, namely, Sisco Research Laboratories Pvt. Ltd., Qualigens Fine Chemicals Pvt. Ltd., and HiMedia Laboratories Pvt. Ltd.

2.6. Statistical Analysis. Statistical analyses were carried out by analysis of variance (ANOVA) followed by Dunn's post hoc test. All analyses of data were performed using SPSS for windows version 12.0 and a probability value of 0.05 or less was considered as statistically significant.

3. Results

3.1. Changes in Body Weight. Figure 1 shows the effect of quercetin on the body weight of bleomycin administered groups of rats. Single intratracheal administration of bleomycin (6.5 U/kg) resulted in a marked decrease in their body weight on days 14 and 21 as compared to the saline-treated control group possibly because of severe tissue damage caused by free radicals. However, body weight of quercetin-treated rats remained comparable to the control group rats throughout the period of experiment.

3.2. Change in Percent Relative Lung Weight. As evident from Figure 2, the percent relative lung weight of bleomycin-treated animals remained significantly high compared to the other two groups of animals. However, twenty-day quercetin treatment protected the lung tissue from bleomycin-induced fibrotic response as evident from the statistically comparable mean percent relative lung weight in both quercetin-treated as well as control animals.

3.3. Hydroxyproline Content. The effect of quercetin on the hydroxyproline content of lung homogenate is presented in Figure 3. Measurement of hydroxyproline is an efficient index of collagen deposition since collagen contains significant amount of this amino acid. After 20 days, the hydroxyproline content in the lungs of bleomycin group increased significantly compared to that in the control group. However, an apparent reduction ($P \leq 0.05$) in lung hydroxyproline content was observed in the bleomycin plus quercetin group compared to the bleomycin group, indicating the possible protective role of quercetin against lung collagen deposition.

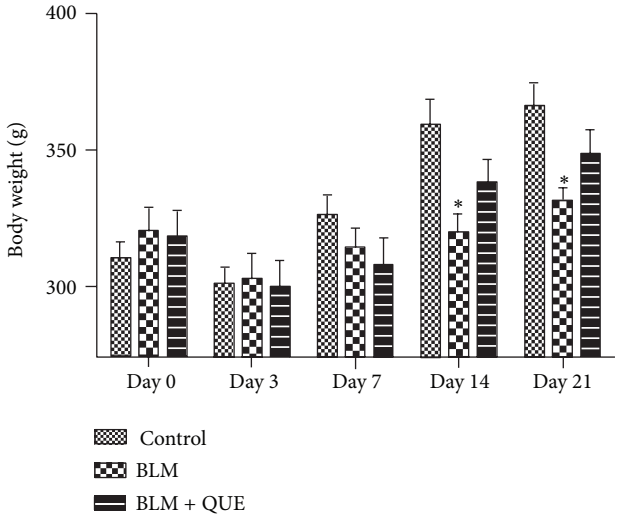

Figure 1: Effect of quercetin (QUE) on body weight of bleomycin- (BLM-) treated rats. $n = 10$. $^{*}P < 0.05$ versus control group.

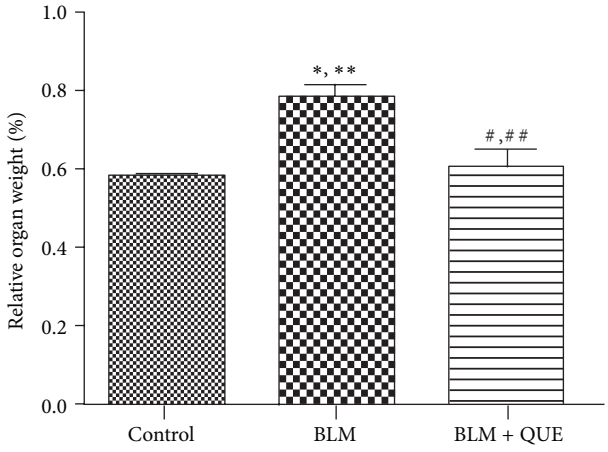

Figure 2: Relative weight of rat lung on day 21 of treatment. $n = 6$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ versus control group. $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ versus bleomycin group.

3.4. Lipid Peroxidation. The result of this study showed an increase in the level of lipid peroxidation in bleomycin-administered group over that of control group, which could be a manifestation of bleomycin induced tissue injury and damage. Nevertheless, quercetin treatment significantly lowered the bleomycin-induced lipid peroxidation in the lung of rats as evident from the near normal MDA levels (Table 1).

3.5. Superoxide Dismutase Activity. Effect of bleomycin and bleomycin plus quercetin on lung tissue superoxide dismutase activity is presented in Table 1. The superoxide dismutase activity in the lung of bleomycin-treated rat was found significantly reduced as compared to the control group. Administration of quercetin proved to be effective in restoring the altered activity of this antioxidant enzyme.

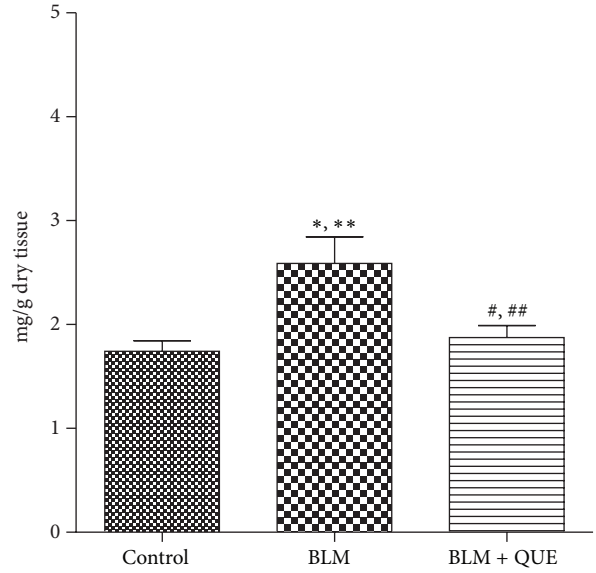

Figure 3: Hydroxyproline content in lung tissue on day 21 of treatment. $n = 6$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ versus control group. $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ versus bleomycin group.

3.6. Catalase Activity. The catalase activity in the lung homogenate of bleomycin-treated rats was found considerably lower than that of vehicle control rats. The quercetin administration which improved the catalase activity on day 21 of treatment of the animals in this group showed comparable catalase activity with that of controls (Table 1).

3.7. Total and Differential Leukocyte Count in BAL. The total leukocyte count and the subset proportion in the bronchoalveolar lavage of control and experimental group of rats are given in Table 2. Bleomycin induction caused a statistically significant ($P \leq 0.05$) increase in the total leukocyte count in the BAL as compared to that in control animals. However, in rats treated with quercetin, total leukocyte count remained similar to that observed in control rats at the end of experimental regime. The differential cell count showed a significant increase in proportion of neutrophils and eosinophils in the lungs of rats exposed to bleomycin. It was observed that the quercetin treatment for 20 days significantly reduced the bleomycin induced hike in neutrophils in the BAL. While the percentage of lymphocytes and alveolar macrophages was decreased in bleomycin-induced group, treatment with quercetin reversed these changes significantly.

3.8. Tumor Necrosis Factor- (TNF-) α Concentration. Figure 4 shows the effect of quercetin on plasma levels of TNF-α. The plasma TNF-α level of bleomycin-administered rats remained elevated on day 21 of experiment when compared with the sham-treated group. Treatment with quercetin, however, decreased bleomycin-induced increase in plasma TNF-α level by the end of the experiment.

Table 1: Levels or activities of various markers of oxidative stress in the lung tissue of the rats from control and treated groups.

Biochemical estimations	Control	Bleomycin	Bleomycin + quercetin
Malondialdehyde level (nmoles MDA/mg tissue)	44.34 ± 1.28	60.63 ± 1.87*	46.64 ± 1.13#
Superoxide dismutase (U/mg tissue)	0.34 ± 0.03	0.21 ± 0.01*	0.31 ± 0.02#
Catalase (μM H_2O_2 consumed/mg tissue/min)	18.94 ± 1.22	12.68 ± 0.82*	17.32 ± 1.46#

Values are given as mean ± SE for groups of six rats each, $^{*}P \leq 0.05$ versus control group, $^{\#}P \leq 0.05$ versus bleomycin group.

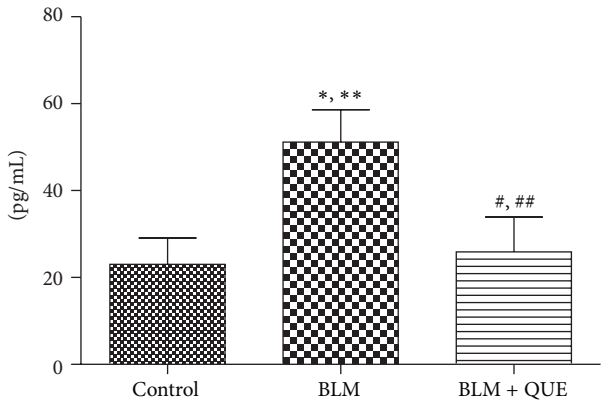

Figure 4: Plasma levels of tumor necrosis factor-α on day 21 of treatment. $n = 10$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ versus control group. $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ versus bleomycin group.

3.9. Estimation of MMP-7. The levels of MMP-7 were estimated in blood plasma and bronchoalveolar lavage fluid in the various stated experimental groups with the help of an ELISA-based method. Our results (Table 3) showed a significant increase in the levels of MMP-7 in both plasma and bronchoalveolar lavage fluid in response to bleomycin challenge. The level of this extracellular matrix digesting enzyme in animals that received quercetin treatment after bleomycin administration was found to be markedly lower than that in the bleomycin-treated group, and, at the same time, comparable to that in the control group.

3.10. Histopathological Examination of Lung Tissue. Histopathological abnormalities in lungs were studied at the end of the experiment using hematoxylin and eosin staining as well as Masson's trichrome staining.

Bleomycin administered rats showed distorted architecture of the lung tissue which included moderate to severe hemorrhages, emphysema, areas of increased thickening of alveolar septa, leukocytic infiltration in alveolar walls, and fibroplasia (Figures 5(b) and 5(c)) when compared with control group (Figure 5(a)). Nevertheless, querctin showed to have ameliorative effect on the inflammatory lesions developed by bleomycin treatment. Pulmonary histoarchitectural changes in animals treated with quercetin showed mild to moderate degree of septal thickening with few inflammatory cells. Emphysematous changes and alveolar hemorrhages were remarkably reduced in querctin-treated group of animals (Figures 5(d) and 5(e)).

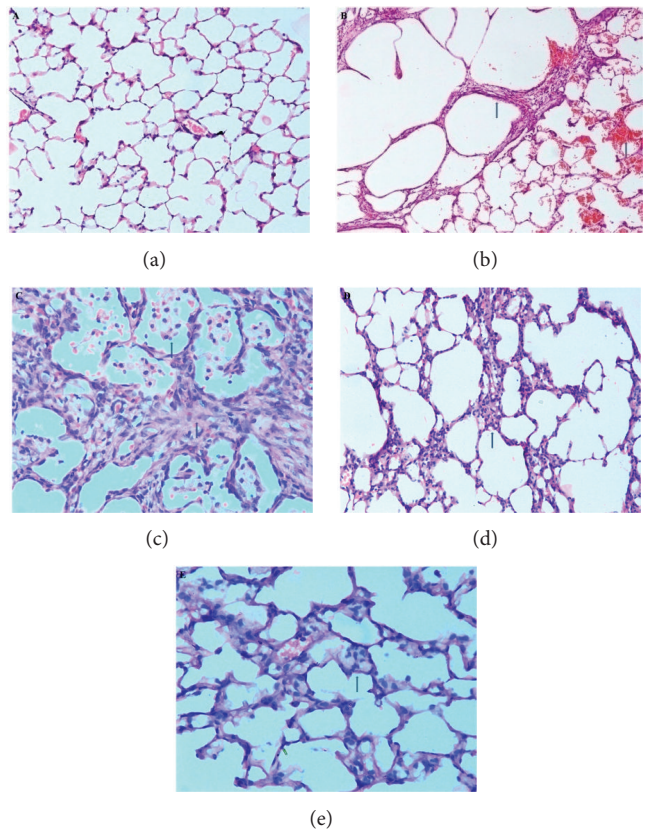

Figure 5: (Hematoxylin and eosin staining.) (a) Normal histoarchitecture of lung 200x. (b) Bleomycin-treated lung showing thickening of alveolar septa with hemorrhages and marked exphysematous change 200x. (c) Bleomycin-treated lung showing severe thickening of alveolar septa with fibroblast proliferation and infiltration of foamy macrophages and mononuclear cells 400x. (d) Quercetin-treated lungs showing mild to moderate thickening of alveolar septa 200x. (e) Quercetin-treated lungs showing mild thickening of alveolar septa with few inflammatory cells 400x.

In order to understand the degree of lung fibrosis in various treatment groups, the extent of collagen deposition was studied as a marker of fibrosis through Masson's trichrome staining. As expected, bleomycin-treated group displayed an increased grade of collagen deposition, compressed alveoli and large fibrotic areas, compared to that in the control group

Table 2: Total leukocyte count and percentage leukocyte subset in the bronchoalveolar lavage fluid of rats from control and treatment groups.

Experimental group	Total cells ($\times 10^6$ mL^{-1})	Macrophage (%)	Neutrophils (%)	Eosinophil (%)	Lymphocytes (%)
Control	0.65 ± 0.12	85.17 ± 2.00	1.83 ± 0.50	0.83 ± 0.17	11.67 ± 1.50
Bleomycin	1.64 ± 0.15*	49.33 ± 3.83*	38.33 ± 1.83*	4.08 ± 0.58*	5.42 ± 0.92*
Bleomycin + quercetin	0.87 ± 0.14$^{\#}$	71.00 ± 3.00$^{*,\#}$	13.00 ± 2.00$^{*,\#}$	4.08 ± 0.96*	10.50 ± 1.25

Values are given as mean ± SE for groups of four rats each, $^*P \leq 0.05$ versus control group, $^{\#}P \leq 0.05$ versus bleomycin group.

Table 3: Level of MMP-7 in blood plasma and bronchoalveolar lavage fluid.

Treatment groups	MMP-7 (ng/mL)	
	BAL	Plasma
Control	7.65 ± 0.15	5.59 ± 0.19
Bleomycin	10.58 ± 0.56*	7.28 ± 0.49*
Bleomycin + quercetin	8.16 ± 0.25$^{\#}$	6.19 ± 0.17$^{\#}$

Values are given as mean ± SE for groups of four rats each, $^*P \leq 0.05$ versus control group, $^{\#}P \leq 0.05$ versus bleomycin group.

Table 4: Szapiel examination scores of lung tissue from rats subjected to various treatments.

Experimental group	Grade of fibrosis
Control	0.00 ± 0.00
Bleomycin	2.51 ± 0.06*
Bleomycin + quercetin	1.48 ± 0.15$^{*,\#}$

Values are given as mean ± SE for groups of six rats each, $^*P \leq 0.05$ versus control group, $^{\#}P \leq 0.05$ versus bleomycin group.

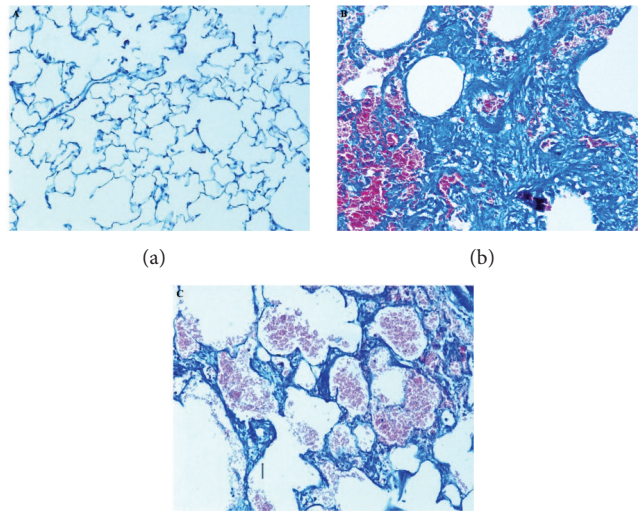

Figure 6: (Masson's trichrome staining.) (a) Normal histoarchitecture of lung 200x. (b) Bleomycin-treated lung showing excessive collagen deposition leading to septal thickening and compressed alveoli 200x. (c) Quercetin-treated lungs showing mild to moderate collagen deposition 200x.

(Figures 6(a) and 6(b)). However, collagen accumulation was remarkably decreased in rats from quercetin-treated group (Figure 6(c)) when compared to the bleomycin group.

Furthermore, the semiquantitative assessment of fibrosis in lung sections was performed by scoring pathological lesions as per the Szapiel method of examination (Table 4). The Szapiel score of bleomycin-induced group was found significantly higher on day 21 when compared with control group. However, Szapiel scores on day 21 of quercetin treated group showed marked decrease compared to the bleomycin-treated group reaffirming protective role of quercetin against bleomycin-induced lung fibrosis.

4. Discussion

The clinical use of bleomycin, as an anticancer drug for a myriad of human malignancies, has been hampered due to its detrimental effects [30]. The major side effect is the induction of lung fibrosis in patients treated with bleomycin. Pulmonary fibrosis is commonly progressive and essentially an untreatable disease with an increasingly fatal outcome [31]. In order to understand the finer mechanisms of development of pulmonary fibrosis as well as to screen the efficacy of various compounds as potential therapeutic agent against this pathological manifestation, animal experimentations are inevitable. The bleomycin-rodent animal model of lung fibrosis is an established and widely used surrogate model of human lung fibrosis [32]. Comparison studies of patients with lung pneumopathy and experimentally induced lung fibrosis animal model have validated effectiveness of this experimental system as an acceptable model [33]. Subsequently, the use of this animal model has helped in partly establishing the pathways of lung damage leading to fibrosis [33].

In the current study, we have used Wistar rat model of lung fibrosis created by challenging the rats with a single dose of bleomycin sulfate by intratracheal instillation. A marked reduction in the body weight was observed in the bleomycin-treated group, which could be attributed to the progression of the fibrosis [34]. Twenty-one days after single intratracheal installation of bleomycin, an obvious increase in the relative weight of lungs was observed in the experimental animals compared to that of control rats. Soumyakrishnan and Sudhandiran [12] have also reported a similar increase in lung weight in bleomycin-treated animals. This increase in lung weight is a clear indication of lung fibrosis that is characterised by excessive deposition of collagen as evident from the increased hydroxyproline levels observed in the lungs of bleomycin-treated rats. However, it was noticed that administration of quercetin effectively thwarted the progression of bleomycin-induced lung fibrosis, and, hence, body weight and relative weight of the lungs in the animals

of quercetin-treated group was in concurrence with that of vehicle control and remained within 95% confidence limits.

Further, the local tissue response to bleomycin-induced lung injury was evaluated by assessing various biochemical markers, namely, hydroxyproline—an index of collagen deposition, malondialdehyde—as a measure of lipid peroxidation and superoxide dismutase, as well as catalase—two key antioxidant enzymes in the lungs of rats from various study groups. In order to complement the biochemical response of bleomycin-challenged lung tissue, cytological analyses such as total and differential leukocyte counts in bronchoalveolar lavage fluid were also conducted. In addition, histochemical localization of collagen in lung tissue was done to confirm the extent of lung fibrosis in various groups of animals. Lung histopathology also was done to confirm the progression of lung fibrosis in bleomycin-instilled rats and also to unravel the possible inhibitory potential of quercetin against the bleomycin-induced lung fibrosis.

Since the amino acid hydroxyproline is the precursor for collagen, the estimation of this amino acid following acid digestion of collagen is considered a good biochemical index of collagen content. Bleomycin-treated group exhibited statistically significant increase in lung hydroxyproline content as compared to control group. This result is in accordance with previous reports, which also demonstrated remarkable increase in lung hydroxyproline content in bleomycin-instilled pulmonary fibrosis models [2, 35, 36]. Deposition of excess or abnormal collagen is a characteristic of lung fibrosis as stated by many while studying the mechanisms behind xenobiotic-induced lung fibrosis [37–39]. The present biochemical indication of bleomycin-induced lung fibrosis was further confirmed visually by collagen-specific Masson's trichrome staining of lung sections. The microscopical observation of the trichrome stained histological sections revealed that bleomycin treatment induced collagen accumulation and deposition in peribronchial and perialveolar tissues that obliterated alveolar spaces as tiny fibrils. However, the lung tissues from quercetin-treated rats showed much improved histological profile with near normal collagen deposition and a basal level of hydroxyproline content indicating quercetin's ameliorative role against bleomycin-induced pulmonary fibrosis.

Further, it is known that reactive oxygen species play an important role in the development of fibrotic responses in the lung of the subject upon bleomycin challenge. Bleomycin binds to iron (FeII), undergoes redox cycling, and catalyzes the formation of reactive oxygen species (11). It is well documented that these free radicals, once produced, target biomacromolecules such as DNA, protein, and lipid, with the ultimate progression of lipid peroxidation, resulting in damage to the lung [40]. In the current study too, we observed signs of oxidative stress as exemplified by heightened MDA activity in the lungs of animals subjected to bleomycin. Moreover, the drug-treated rats also showed compromised antioxidant response as evident from subdued activity of antioxidant enzymes such as catalase and superoxide dismutase. Similar observations made by others give credence to the present notion [41, 42]. However, it was observed that the extent of oxidative damage was well within the normal level in the lungs of quercetin-treated rats signifying this flavonoid's antioxidant potential. Flavonoids are reported to be powerful antioxidants providing remarkable protection against oxidative stress and free radical damage [13]. Moreover, quercetin, a member of the flavonoid family, is cited as one of the most prominent dietary antioxidants [13]. A study regarding the tissue distribution of quercetin in rats has shown that, upon quercetin treatment, the highest accumulation of this flavonoid and its metabolites was observed in rat lungs [43]. Hence, it is prudent to presume that quercetin could emerge as a pneumoprotective agent against local oxidative stress inducers like the one that is addressed in the current study.

In addition to the oxidative stress mentioned earlier, intratracheal administration of bleomycin might also results in interstitial inflammation characterised by an increase in the recruitment of leukocytes [44]. It is known that the recruitment of inflammatory cells to the site of inflammation plays a pivotal role in the pathogenesis of several inflammatory conditions. Further, it is also reported that the leukocytes such as macrophages, neutrophils, and lymphocytes play a key role in inflammation and tissue remodelling [44]. In the current study, a significant increase in the total leukocytes count with a marked increase in the proportion of neutrophils and eosinophils was apparent in the bronchoalveolar lavage of bleomycin treated animals. At the same time, the proportion of macrophages and lymphocytes was found low in the bronchoalveolar lavage fluid of bleomycin-challenged rats. The observed trend in the count of inflammatory cells in the bronchoalveolar lavage fluid during bleomycin induced pulmonary injury is in accordance with the previous reports of experimental pulmonary fibrosis [11, 45]. However, the anti-inflammatory property of quercetin gained further consolidation from our observation that twenty-day repeated treatment of this flavonoid after intratracheal instillation of bleomycin significantly reduced the recruitment of leukocytes into the alveolar air space. Analysis of extravasated cells from lung revealed that the recruitment of neutrophils was significantly reduced in the quercetin-treated rats compared to bleomycin-administered ones. The obvious reduction in the extravasation of leukocytes upon quercetin treatment indicates substantial recovery from bleomycin-induced lung inflammation, and hence, underlining the ameliorative property of this flavonoid against the drug-induced lung injury.

Moreover, it is reported that TNF-α, a potent proinflammatory cytokine, acts as a major component of a multifaceted network of cellular and molecular interactions that regulate the progression of fibrotic process [46]. In this study, a significant elevation in the expression of TNF-α was observed in the bleomycin-treated group, which is in accordance with previous findings [2, 47]. Furthermore, it has been reported that the reactive oxygen species generated as a result of bleomycin treatment might induce nuclear factor κ-B and increase the synthesis of TNF-α [48, 49]. However, we noticed that oral administration of quercetin for twenty-days after local instillation of bleomycin reduced the expression of proinflammatory cytokine TNF-α to the basal level indicating marked recovery from the induced lung injury caused by the drug in question.

Further, it is well documented that fibrosis is a consequence of repair of damaged tissue and is known to be closely associated with the remodelling of extracellular matrix tissue [50]. Also, as has been reported by others, pulmonary fibrosis patients show elevated levels of MMP-7 [51–53]. Moreover, it has been reported that mice lacking MMP-7 are protected from bleomycin-induced lung injury suggesting a key role of MMP-7 in the induction of pulmonary fibrosis by bleomycin [53]. On the line of these reports, we took up the estimation of MMP-7 to throw light on a possible role of this enzyme as a direct or indirect target of quercetin. Our results indeed do suggest an association of MMP-7 levels with induction of fibrosis by bleomycin. This increase in expression was found to be prevented by quercetin treatment. Whether MMP-7 is a direct target of quercetin, however, needs to be assessed.

The subsequent corroboratory histopathological observation showed abnormal histological profile of the lung tissue in bleomycin-administered rats. Notable deviant histoarchitecture of the lung from the drug-treated animals includes collapsed alveolae, thickened alveolar wall, and abnormal collagen deposition. Similar structural changes reported by others from bleomycin-induced lung fibrosis rat models consolidate the credence of the present experimental model [22, 40, 54]. Moreover, in the present study, it was observed that quercetin treatment decelerated the progression of bleomycin-induced structural deformation as exemplified by the low Szapiel scores in this group of animals compared to bleomycin-treated ones. It is well documented that quercetin is a strong free radical scavenger and also a good metal chelator [55]. It has also been reported that quercetin acts through various mechanisms including the antioxidative activity, the inhibition of enzymes that activate carcinogens, the modification of signal transduction pathways, and interactions with receptors and other proteins [17]. *In vitro* studies have demonstrated that quercetin, in fact, inhibited the production of reactive oxygen species in lipopolysaccharide-stimulated Kupffer cells [56]. Moreover, reports have also shown that quercetin treatment effectively reduced superoxide anions [57] and could inhibit lipid peroxidation, and hence, alterations in lung morphology during pulmonary injury or infection [57, 58].

MAPK pathways such as ERK1/2 and JNK 6 are activated in response to a high oxidative status, leading to increase in the levels of MMP-7 [59]. The MAPK pathway is a known target for potential fibrosis therapy as several fibrogenic cytokines signal through MEK/ERK, including noncanonical TGF-β, PDGF, IL-13, and TNF-α [60]. We, therefore, suggest that quercetin, due to its antioxidative property and inhibitory effect on TNF-α, targets the above mentioned pathway to ultimately decrease the MMP-7 levels in quercetin-treated rats.

In the light of the current observations as well as the above cited reports, it is pertinent to presume that quercetin, being an antioxidant, effectively reduces the formation of bleomycin-induced free radicals which are known to trigger a cascade of inflammatory responses culminating in moderate to severe lung injury in treated subjects, and hence, could be used as an effective inhibitor of the bleomycin-induced pulmonary fibrosis.

5. Conclusion

Pulmonary fibrosis is generally nonresponsive to conventional therapy. The bleomycin-induced fibrosis model displayed reduced antioxidant capacity, elevation of inflammatory cytokines, increased MMP-7 expression, fibrotic changes, and collagen accumulation in lung tissue. Quercetin appeared to have a pneumoprotective effect through enhancement of antioxidant status, decrease in the level of inflammatory cytokines, reduction of MMP-7 expression, and minimisation of collagen accumulation. We, therefore, suggest that quercetin effectively attenuates the pulmonary injury induced by bleomycin challenge. Additional studies, however, will be required to understand the comprehensive role of quercetin in the pathogenesis of pulmonary fibrosis.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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