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

General Considerations
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 antioxidant 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, PGE2, 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.
50 male Wistar rats of 10-12 weeks
Housed in individual cages
Acclimatized for 9 days

Control group
Normal saline on Day 0
Intratracheally instilled

Bleomycin group
Bleomycin in saline on Day 0
Intratracheally instilled

Sulindac
20mg/Kg BW
Oral gavage

Quercetin
100mg/Kg BW
Oral gavage

SLD+QUE
((10+50)mg/Kg)
Oral gavage

Treatment for 20 days, Analyses
### DIAGRAMMATIC SUMMARY

#### Part Two: Parameters Evaluated

<table>
<thead>
<tr>
<th>Collagen deposition</th>
<th>Histological profile</th>
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<tr>
<td>Hydroxyproline content</td>
<td>Hematoxylin-Eosin staining</td>
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<tr>
<td>Masson’s trichome staining</td>
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</tbody>
</table>

#### 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

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<th>SLD</th>
<th>QUE+SLD</th>
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<td>▼</td>
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<td>↑, ▼</td>
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<td>↑, ▼</td>
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</tr>
</tbody>
</table>

↑ Significantly higher compared to Control, ↓ Significantly lower compared to Control,
▲Significantly higher compared to Bleomycin, ▼ Significantly lower compared to Bleomycin,