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 intratracheal 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 assessed 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).
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-7 activity 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.