Discussion
6. DISCUSSION

Asthma is an inflammatory disease characterized by airflow obstruction, acute or chronic inflammation, airway hyperresponsiveness (AHR) and structural remodeling (Kumar, 2001). With regards to pathophysiology, asthma is recognized by T-helper type 2 (Th2)-cell-driven chronic inflammation, and a variety of inflammatory mediators (cytokines, chemokines, signaling molecules, adhesion molecules and growth factors) from immune cells and structural cells in the airways are involved in various stages of asthma (Elias et al., 2003). In numerous studies using animal models and clinical research, the critical observations in asthma could fall into several characteristic parameters, such as bronchoconstriction, IgE-mediated anaphylaxis, immune responses, eosinophilia, neutrophilia, AHR and structural changes (Moffatt, 2005; Spina and Page, 2002). Further, IgE mediated immune responses and eosinophilia are prominent symptoms in the airways of allergic asthma (Bochner et al., 1994; Bousquet et al., 1990), and the produced cytokines (such as TNF-α, IL-4, IL-5 and IL-6) in the allergic process also play an important role in the AHR development and airway remodeling (Riffo-Vasquez and Spina, 2002).

Indeed asthma is a result of orchestrated inflammatory events, many of which involve specific inhibitors that act on the pathway of asthma—for example, histamine H1 antagonists, thromboxane antagonists, platelet-activating-factor antagonists, cyclooxygenase inhibitors and prostaglandin inhibitors—have been tried but have failed in clinical trials (Moffatt, 2005). In contrast, glucocorticoids, which suppress the progenitor levels of inflammatory cells to baseline by widespread inhibition of cytokine synthesis and cytokine-mediated immune-cell survival, have so far been used to manage the symptoms of asthmatic patients over a period of 30 years (Baatjes et al., 2002). These reports suggest that the therapeutic approach for asthma management should focus on restoring the balance of asthmatic parameters rather than searching for potent inhibitors of specific pathways of the asthmatic process. Moreover, natural sources have been neglected for searching antiasthmatic compounds, in spite of information available for several plants used in traditional medicine for the treatment of asthma (Dorsch and Wagner, 1991). Although some promising results were obtained, such studies have not
reached preclinical stages and in some cases not even the active principle has been determined (Addy and Burka, 1988; Dorsch et al., 1992).

Indian systems of traditional medicine are well systematized but are largely unrecognized in the West. Ayurveda is gaining greater visibility; related systems, such as Unani-Tibb, Siddha and Tibetan are likely to remain obscure (Ziment, 1997a; Ziment, 1997b). Ayurveda has recommended a number of drugs in the form of complementary and alternative medicine (CAM) from indigenous plants sources to treat bronchial asthma and other allergic disorders for hundreds of years and has been successful in controlling the disease as well (Chung, 1996). Large numbers of medicinal plant preparations have been reported to possess anti-asthmatic effects; these include *Datura* plants (Ziment and Tashkin, 2000), *Albizia lebbeck* (Tripathi and Das, 1977), *Tinospora asthmatica* (Nayampalli et al., 1986), *Solanum xanthocarpum* (Gupta et al., 1967), *Boswellia serrata* (Ammon et al., 1993; Ammon et al., 1991), *Vitex negundo* (Nair and Saraf, 1995), *Tephrosia purpurea* (Gokhale et al., 2000), *Cissampelos sympodiahs* (Bezerra-Santos et al., 2006) and *Ganoderma tsugae* (Lin et al., 2006) The majority of herbal products found to be useful in the management of asthma in the experimental studies have yet to undergo clinical trials In this connection, in the light of above facts, as a part of our continuing search for biologically active anti-asthmatic agents from the medicinal resources, we have selected the plant; *Moringa oleifera* and evaluated its efficacy to reveal its potential usefulness in the prevention/amelioration of symptoms of asthma and related allergic disorders.

In the present investigation we have authenticated the quality of *M. oleifera* (drumstick). The authentication tests of the dried seeds used in the present study were done by comparing dried seeds of *M. oleifera* morphologically and microscopically as mentioned in different standard texts (Kirtikar and Basu, 1935; Gupta, 2003) and the sample used in our study We found our sample was authentic. Quantitative limit tests like ash and extractive values are another parameters used to standardize the herbal drugs (Bhutani, 2000) Seeds used in the study contained 1.95% of foreign matter, 3.51% of total ash, 0.52% of acid-insoluble ash, 0.9% of water soluble ash, 18.4% of ethanol soluble extractives, 31.2% of water soluble extractives, and 4.5% moisture All these tests
justified that the seeds used under study pass the quantitative limits tests as prescribed by various monographs.

After having the authenticated sample of *M. oleifera* used in the present study, extract and various fractions were prepared for carrying out the pharmacological activities. The extraction of the powder of the dried seeds of *M. oleifera* was carried out using ethanol solvent. The yield of ethanolic extract was found to be 20%. Our preliminary clinical studies on dried seed powder of *M. oleifera* showed decrease in severity of asthma symptoms and simultaneous improvement in peak expiratory flow rate (Agrawal and Mehta, 2006; Agrawal and Mehta, 2008). Further, earlier reports from our laboratory showed that ethanolic extract possesses significant bronchodilatory activity (Mehta and Agrawal, 2008). We have also reported the protective effect of ethanolic extract of seeds of *M. oleifera* against inflammation associated with development of arthritis in rats (Mahajan et al., 2007b). Since, dried seed powder and ethanolic extract showed greater bronchodilatory effect in both clinical and preclinical studies, we decided to go for its fractionation to identify a more active fraction. The obtained ethanolic extract was therefore subjected for successive fractionation using solvents of different polarity like chloroform, ethyl acetate and n-butanol. The extract remained after n-butanol fractionation was called as marc (residual fraction). Successive fractionation of 100g of ethanolic extract with the above mentioned solvents gave a yield of 7.20g with chloroform, 6.36g with ethyl acetate, 12.24g with n-butanol and remaining residual fraction was found to be 74.20g. The ethanolic extract and its fractions were then studied for their pharmacological activities.

In the present study, ethanolic extract and its all fractions were screened for pharmacological studies. Bronchodilatory activity was studied using two spasmogens; histamine and acetylcholine respectively in guinea pigs, mast cell stabilizing study was carried out with two stimuli; compound 48/80 and egg albumin on isolated rat peritoneal mast cells, delayed type hypersensitivity reaction and humoral antibody responses were studied in mice using SRBC as antigen for immunomodulatory activities. Further, antianaphylactic activity was studied by systemic anaphylactic reaction in mice and passive cutaneous anaphylaxis reaction (PCA) in Wistar rats.
Discussion

During an acute asthmatic episode, exposure to allergens and irritants like pollens, moulds, house dust mites, animals’ dander, occupational chemicals cause activation of inflammatory cells. These cells can synthesize and secrete a vast numbers of mediators like histamine, tryptase, leukotrienes and prostaglandins that directly cause bronchoconstriction, submucosal gland secretion and vasodilation resulting in the *early phase asthmatic response* (Hamid et al., 2003). Further, sensitized guinea pigs respond to allergen with acute allergic bronchospasm that may be sufficiently intense to cause obstruction of airflow and death. It might be presumed that hyperreactivity to allergic mediators contributes to allergic bronchospasm (Hoshiko and Morley, 1993). In the present study histamine hydrochloride and acetylcholine were used as spasmogens in the form of aerosols (0.25%) to cause immediate bronchospasm in guinea pigs. Bronchodilatory activity was evaluated by observing effects of ethanolic extract and its fractions on pre-convulsion dyspnoea (PCD) time. The standard antihistamine; ketotifen fumarate and anticholinergic; atropine sulphate offered a significant protection by increasing the mean PCD time in animals exposed to histamine and acetylcholine respectively. Pretreatment with ethanolic extract and its fractions were found to significantly increase the preconvulsion time against histamine and acetylcholine aerosol as compared to control. Among various fractions tested n-butanol fraction produced greater increase in percentage of PCD time as compared to ethanolic extract, chloroform fraction and ethyl acetate fraction. The effects produced by ethanolic extract and its n-butanol fraction were comparable to ketotifen fumarate and atropine sulphate suggesting their protective effect might be due to their bronchodilatory activity. *Albizia lebbeck* (Tripathi and Das, 1977), *Benincasa hispida* (Kumar and Ramu, 2002), *Ephedra sinica* (Akiba et al., 1979), *Picrorrhiza kurroa* (Dorsch et al., 1991), *Rosmarinus officinalis* (Aqel, 1991), *Ocimum sanctum* (Singh and Agarwal, 1991), which are well known anti-asthmatic herbal drugs also showed bronchodilatory activity against histamine and acetylcholine induced bronchospasm in guinea pigs.

In addition to bronchodilating activity, a significant number of therapeutic approaches for bronchial asthma have been designed based on the antagonism of specific mediators released from mast cells. Mast cell degranulation in response to IgE dependent stimuli participate in inflammatory changes of asthma through the elaboration of
cytokines (Hamid et al., 2003). Mast cells are found throughout the walls of the respiratory tract, and increased numbers of these cells have been described in the airways of asthmatics (Foresi et al., 1990; Dunhill, 1960). These cells are activated via an antigen or chemical stimulation and cause subsequent mobilization of calcium and degranulation of the cell (Conard et al., 1975). Degranulated mast cells liberate pro-inflammatory and nociceptive mediators that include histamine, cytokines, leukotrienes, platelet activating factors, proteolytic enzymes and chemotactic factors for eosinophils and neutrophils (Kobayashi et al., 2000; Galli et al., 2002). Subsequently, these mediators play a pivotal role in airway inflammatory responses such as airway eosinophilia, late asthmatic response and airway hyperresponsiveness as well as in immediate hypersensitivity reaction like bronchoconstriction (Theoharides and Cochrane, 2004; Reuter et al., 2008).

Degranulation of mast cells has been taken as the criteria of positive anaphylaxis. Anaphylaxis and Compound 48/80 induced secretion from mast cell share a common requirement as far as the presence of calcium is concerned. However, Compound 48/80 can utilize intracellular calcium stores to initiate the release process, even in the absence of calcium from the extracellular medium (Burka, 1984). On the other hand, anaphylaxis requires the presence of calcium in the extracellular medium which moves into the cell via calcium gates in the membrane (West, 1983). Disodium cromoglycate, a well-known mast cell stabilizer, inhibits the release of pre-formed inflammatory mediators like histamine and serotonin from mast cells (Ward et al., 1969; Shin et al., 2004). In the present study, a significant protection of rat peritoneal mast cells from disruption by antigen; egg albumin and Compound 48/80 by ethanolic extract and its fractions indicating their mast cell stabilizing activity. Ethanolic extract and n-butanol fraction showed very significant protection at all concentrations, while chloroform and ethyl acetate fraction showed significant protection at higher concentration only. *Adhatoda vasica* (Tripathi et al., 1979), *Albizia lebbeck* (Johri et al., 1985), *Coleus forskohlii* (Marone et al., 1987), *Tylophora asthmatica* (Geetha et al., 1981), *Allium cepa* (Johri et al., 1985), *Bacopa monnieri* (Samiulla et al., 2001), *Citrus unshiu* (Kim et al., 1999) etc are several well known indigenous plants used in asthma and have been reported to have mast cell stabilizing activity.
Discussion

Treating asthma through immunomodulation has been attempted only in the last few years. The mechanisms include inhibition of specific immune molecules, signaling pathways and immune reactions that regulate airway disease. Delayed type immune reactions are based on the activation of antigen specific CD\(^{4+}\) and CD\(^{8+}\) T cells and need 24 hr to 48 hr to develop. Upon recurrent contact with identical antigens, recruitment of CD\(^{4+}\) and CD\(^{8+}\) T cells cause inflammation and cytotoxic induced apoptosis in target cells as well as cytokine mediated leukocyte infiltration (Averbeck et al., 2007). In the delayed type hypersensitivity reaction (DTH, cell mediated) i.e. type-IV T lymphocytes and activated macrophage mediated reaction, sensitized animals, when challenged with same allergen, resulted in a significant increase in paw edema when compared with contralateral hind paw receiving normal saline only as control (Askenase et al., 1975). Further, DTH requires the specific recognition of a given antigen by activated T lymphocytes which subsequently proliferate and release different inflammatory mediators. The antigen is known to be associated with the release of mediators such as histamine, cytokines, products of arachidonic acid metabolism and eventually interferon-\(\gamma\) leading to DTH (Griswold et al., 1982). These in turn, increase vascular permeability, induce vasodilatation, macrophage accumulation (Descotes, 1999) and increased concentration of lytic enzymes for more effective killing (Kuby, 1997). In the present study, delayed type hypersensitivity reaction was measured as change in the percent edema of footpad of mice. Treatment with ethanolic extract and n-butanol fraction showed significant reduction in percent edema at 48 hr compared to control. However, treatment with chloroform and ethyl acetate fraction failed to produce any significant decrease in edema volume. This indicates the immunosuppressant nature of the drug and its inhibitory action could be due to an influence of constituents of extract/fraction on biological mediators.

Humoral immune reactions are mediated by IgG and IgM antibodies which are directed against membrane associated antigens. The humoral immunity involves interaction of B-lymphocytes with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells (Gokhale et al., 2003). Antibody functions as the effector of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily
ingested by phagocytic cells (Benacerraf, 1978). We have also studied humoral response on sheep erythrocyte specific haemagglutination antibody titre in mice. Treatment with ethanolic extract and n-butanol fraction showed significant dose dependent inhibition in antibody titre as compared to control animals those received normal saline only; this indicates the decreased responsiveness of macrophages and T and B lymphocyte subsets involved in antibody synthesis. Treatment with ethyl acetate fraction also produced significant inhibition but at higher dose only. In contrast, chloroform fraction failed to produce any significant inhibition of antibody titre.

During both systemic and local immunologic reactions, mediators including histamine, leukotrienes, and prostaglandin D, a substance known to be produced by mast cells are released systemically as well as locally into respiratory secretions (Zweiman, 1988). Immediate-type allergic reactions (anaphylactic allergic reactions) are a life-threatening syndrome induced by the sudden systemic release of inflammatory mediators from mast cells. Further, mast cells play a major role in the development of many physiological changes during allergic responses (Shin et al., 2005). These cells are located throughout the human body, and upon allergen exposure, they are stimulated via the IgE-receptors (Kemp and Lockey, 2002).

Numerous reports established that stimulation of mast cells with Compound 48/80 initiates the activation of a signal transduction pathway that leads to histamine release (Moon et al., 2005). Several studies also have shown that Compound 48/80 and other polybasic compounds are able, to activate G proteins (Mousli et al., 1990). The evidence indicates that the protein is Gi-like and that the activation is inhibited by benzalkonium chloride (Bueb et al., 1990). Compound 48/80 increases the permeability of the lipid bilayer membrane of cell by causing a perturbation in the membrane. This result indicates that the increase in membrane permeability may be an essential trigger for the release of the mediator from mast cells. In this regard, anti-allergic agents having a membrane stabilizing action may be desirable. Our data demonstrated that, pre-treatment with ethanolic extract and n-butanol fraction of M. oleifera significantly inhibited Compound 48/80-induced systemic anaphylactic reaction. Further, treatment with chloroform and ethyl acetate fraction failed to produce any significant effect on systemic anaphylaxis reaction. However, in in vitro mast cell stabilizing studies we found significant inhibition
Discussion

of mast cell degranulation at higher concentration of these fractions. Thus, it is possible to hypothesize that both ethanolic extract and n-butanol fraction might have an ability to stabilize the lipid bilayer membrane, thereby preventing the perturbation being induced by Compound 48/80, and regulating the degranulation of mast cells in rat skin by stabilizing membrane fluidity.

In spite of increasing evidence of the role of several other mediators (Rafferty and Holgate, 1989, Rimmers and Church, 1990), histamine is still regarded to be the principal mediator of antigen-induced skin reactions. In addition, intradermal and intranasal application of chemical mediators and chemical mediator releasers increase vascular permeability in a manner similar to that of allergic models (Inagaki et al., 1989). Passive cutaneous anaphylaxis (PCA) is very effective way to test skin allergic reactions and has been successfully applied in murine models to assess the effects of oriental medicines (Lee et al., 1997; Kim and Lee, 1999; Kim et al., 2000). The present study also utilized PCA for testing effect of extract/fraction on IgE-mediated local allergic reaction. Ethanolic extract and n-butanol fraction administered rats were protected from IgE mediated local anaphylaxis. However, treatment with chloroform and ethyl acetate fraction failed to produce any significant inhibition of passive cutaneous anaphylaxis reaction. Ethanolic extract and n-butanol fraction showed significant protection against systemic as well as local anaphylactic reaction suggesting their anti-allergic properties.

Various plants and plant-based preparations have been used in traditional medicine for centuries to treat allergic diseases in the Indian subcontinent. Crude extracts of *Syzygium aromaticum* (Kim et al., 1998b), *Vitex rotundifolia* (Shin et al., 2000), *Crinum glaucum* (Okpo and Adeyemi, 2002), *Striga orobanchioides* (Harish et al., 2001) have been studied for effects upon mast cell-mediated anaphylactic reactions. The protective effects of the extracts of *Lycopus lucidus* against mast cell-mediated allergic reactions has been reported by Yun et al. (2003). Among the various components from *Lycopus lucidus*, betulinic acid, a pentacyclic triterpene modulated the production of TNF-α and IL-6 by monocytes and macrophages during immune responses. Anti-allergic activities of oligopeptide (Hossen et al., 2006; Singh et al., 1998) and eugenol (Kim et al., 1997b) have also been demonstrated in murine models of IgE-mediated reactions. These compounds have mast cell stabilizing activity and thus are able to inhibit the release of
Discussion

histamine. In the present study we also found similar results suggestive of antianaphylactic nature of extract/fraction and also indicate that *M. oleifera* may be applicable to the treatment of allergic skin reactions.

Standardization of the extracts and fractions of the herbal drug can be performed by estimation of marker compounds that may be chemical markers or biomarkers using modern analytical techniques like HPTLC and HPLC. These techniques are considered as emerged tools for the quantitative and qualitative assessment of the herbal drugs (Borris, 1996). In the present study, the ethanolic extract of seeds of *M. oleifera* and its fractions were subjected to HPTLC finger printing to trace out the possible number of components in each of them. Further, the quantitative estimation of ascorbic acid, quercetin, benzylisothiocyanate and glycerol-1-(9-octadecanoate), a marker compounds present in *M. oleifera* was carried out by HPTLC analysis. The HPLC was performed for estimation of β-sitosterol in ethanolic extract and its each fraction. On performing HPTLC analysis of the ethanolic extract of seeds and its fractions following percentage weight by weight quantity of ascorbic acid, quercetin, benzylisothiocyanate and glycerol-1-(9-octadecanoate) was found 0.52%, 2.57%, 2.77%, 0.47% in ethanolic extract; 0.02%, 2.23%, 0.00%, 0.21% in chloroform fraction, 0.05%, 0.00%, 2.53%, 0.16% in ethyl acetate fraction, 0.45%, 2.38%, 2.72%, 0.29% in n-butanol fraction; 0.00%, 0.00%, 1.79% and 0.00% in residual fraction respectively. The concentration of β-sitosterol was found to be 2.59% in ethanolic extract, 0.84% in chloroform fraction, 0.33% in ethyl acetate fraction, 3.42% in n-butanol fraction and 0.15% in residual fraction. **Thus, the ethanolic extract was found to contain higher concentration of above mentioned marker compounds except β-sitosterol.** Among various fractions of ethanolic extract, n-butanol fraction contained higher concentration of these constituents as compared to chloroform and ethyl acetate fractions. The concentration of β-sitosterol was found to be higher in n-butanol fraction.

In nutshell, study with ethanolic extract and n-butanol fraction showed significant bronchodilatory, mast cell stabilizing, immunosuppressive and antianaphylactic activities as compared to chloroform and ethyl acetate fractions. The differences in the effects among the extract and fractions of *M. oleifera* can be attributed to the corresponding differences in concentration of marker compounds in
these extract and fractions. Further, a good co-relation of all these activities and concentration of marker compounds in ethanolic extract and n-butanol fraction was observed.

On the basis of the phytochemical and pharmacological results, we have made an attempt to investigate the mechanism of action for antiasthmatic activity of effective extracts i.e. ethanolic extract and n-butanol fraction using chemical- toluene diisocyanate (TDI) induced-immune mediated inflammatory responses in rats and ovalbumin induced airway inflammation in guinea pigs.

Chronic exposure to toluene diisocyanate (TDI), a low molecular weight compound widely used as an industrial chemical, has been associated with asthma (Bernstein, 1982). Although the pathogenesis of diisocyanate-induced asthma is still largely unknown (Baur et al., 1994; Bolognesi et al., 2001), increasing evidences from human and laboratory animal models has indicated that the occupational asthma (OA) induced by TDI shares several features with human allergic asthma (Wisnewski and Redlich, 2001). Indeed intranasal instillation of TDI resulted in systemic Th2-dominated immune responses characterized by lower Th1 cytokines (IFN-γ) associated with a rise in Th2 cytokines (TNF-α, IL-4, IL-5, IL-6 and IL-13). It also causes an allergic inflammation throughout the conducting airways characterized by a proliferation of goblet cells and an influx of eosinophils (Ban et al., 2006).

TDI-induced asthma is characterized by hyperresponsiveness and inflammation of the airways (Mapp et al., 1987). This inflammation is associated with infiltration of lymphocytes, eosinophils and neutrophils into the bronchial lumen (Boschetto et al., 1987). In the present study, the airway inflammation observed in the TDI-control rats showed respiratory hyperreactivity symptoms (sneezing, rhinorrhea and nasal obstruction) that were similar to asthma. A variety of inflammatory mediators, released through the activation of many inflammatory and structural cells, result in the typical pathophyslogic changes of asthma (Barnes, 1996, Barnes et al., 1998). Activated eosinophils can release different cationic proteins including major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN). There is evidence from a number of studies that these proteins can damage the respiratory epithelium (Motojima et al., 1989, Frigas et al., 1980). Some
evidences have indicated a possible correlation between activation status of eosinophils and immune mediated responses (Filipovic and Cekic, 2001). Further, several studies have provided evidence of involvement of neutrophils and its association with airway neutrophilia in the pathogenesis of TDI-induced asthma (Seltzer et al., 1986; Lee et al., 2002). Other clinical studies reported that measures of chronic asthma severity such as FEV1 correlate with the degree of neutrophilia in sputum or bronchial biopsy specimens (Jatakanon et al., 1999; Louis et al., 2000). Moreover, neutrophilic inflammation in the airway is also increasingly recognized in acute exacerbations of asthma and in status asthmaticus (Sur et al., 1993; Ordonez et al., 2000; Ying et al., 1997).

In our study we found the significant increased in number of eosinophils and neutrophils in blood of TDI-control rats suggesting the presence of eosinophilia and neutrophilia. However, it does not show any increase in total cell, lymphocyte and monocytes count. Further, TDI-control rats showed significant increase in the number of total cell count as well as each differential count in BAL fluid, suggestive of leukocytosis; has been used as indicator of lung inflammation particularly of the late phase response in human asthma (Wenzel, 1994; Bousquet et al., 1990). Treatment of rats with ethanolic extract and n-butanol fraction suppresses hyperreactivity in the form of decreasing the score of symptoms; sneezing, rhinorrhea and nasal obstruction. It also alleviates bronchoalveolar inflammation via decreasing the infiltration of total and differential inflammatory cells in blood (particularly eosinophils and neutrophils) as well as in BAL fluid.

Cytokines play a critical role in orchestrating and perpetuating inflammation in asthmatic airways and several specific cytokine and chemokine inhibitors are now in development in treatment of asthma (Chung and Barnes, 1999). In particular, T cell derived TNF-α, IL-6 and Th2-type cytokine IL-4 and IL-5 (Martin et al., 2004) TNF-α may have an important amplifying effect in immune mediated inflammation (Kips et al., 1992). There is evidence for increased TNF-α expression in asthmatic airways (Bradding et al., 1994). IgE triggering in sensitized lungs leads to increased TNF-α expression in epithelial cells in both the rat and human lung (Ohno et al., 1990; Ohkawara et al., 1992) TNF-α is also present in bronchoalveolar lavage fluid obtained from asthmatic patients (Broide et al., 1992). Infusion of TNF-α causes increased airway responsiveness in
Brown-Norway rats (Kips et al., 1992). Further, TNF-α may be an important mediator in the initiation of chronic inflammation, by activating the secretion of cytokines from a variety of cells in the airways (Hughes et al., 1995).

IL-4 was reported to be produced by CD41 and CD81 T-lymphocytes, eosinophils, and mast cells in both atopic and nonatopic asthma (Bradding et al., 1994; Ying et al., 1997). IL-4 is well known to be required for the priming of Th2 cells; it promotes the production of IgE antibodies that play a key role in the elicitation of immediate hypersensitivity reactions associated with respiratory allergy (Ahn et al., 2007). IL-4 receptor blockade prevents the development of antigen-induced airway hyperreactivity, goblet cell metaplasia, and pulmonary eosinophilia in a mouse model (Gavett et al., 1995). Thus, IL-4 appears to be important in the early stages of Th2 cell development.

IL-6 has been reported to have activities on a wide range of cellular processes, including T-lymphocyte activation and immunoglobulin production by B-lymphocytes; as such, it can also enhance IL-4-dependent IgE synthesis (Vercelli et al., 1989). There is evidence for increased release of IL-6 from alveolar macrophages from asthmatic patients after allergen challenge and increased basal release, compared with nonasthmatic subjects (Gosset et al., 1991). IgE-dependent triggering stimulates the secretion of IL-6 from both blood monocytes and alveolar macrophages in vitro (Broide et al., 1991). Increased levels of IL-6 can be measured in nasal washings from children after rhinovirus infection (Zhu et al., 1996). Further, in two animal studies, the ability of TDI was demonstrated to induce Th2 cytokines. The authors have used modern methods of molecular immunology to compare cytokine mRNA response in auricular lymph node of BALB/c mice following treatment with isocyanates (Plitnick et al., 2005). Kouadio et al. (2005) exposed Wistar rats to TDI vapour, 4h/day for five consecutive days and quantified IL-2, IL-4 and IL-6 productions in the BAL fluid.

The results obtained in the present study suggest that, the level of TNF-α, IL-4 and IL-6 were increased significantly in TDI-control rats as compared to non-sensitized control animals suggestive of development of the disease in animals through the release of Th2 cell derived cytokines. Treatment with dexamethasone and n-butanol fraction produced significant decreased in the level of TNF-α, IL-6 and IL-4 in both serum and
BAL fluid. Whereas, ethanolic extract produced significant decrease in the level of IL-4 and IL-6 in both serum and BAL fluid. Further, the protective effect of this extract/fraction by reduction in the levels of these mediators in serum, BAL fluid and their release into the local tissues of lungs (confirmed here by histopathological observation) seemed to overshadow its potential anti-inflammatory effects.

Oxidative stress plays an important role in the pathogenesis of airway diseases, particularly when inflammation is prominent (Paredi et al., 2002). Inflammatory and immune cells in the airways, viz eosinophils, monocytes/macrophages and neutrophils release increased amounts of ROS in asthmatic patients (Sedgwick et al., 1990; Kanazawa et al., 1991). It is reported that the generation of ROS in the lungs is enhanced after exposure to exogenous chemical agents including TDI (Janssen et al., 1993). The increased degree of lipid peroxidation (increase level of MDA) is known to correlate with changes in the degree of airway obstruction (Montuschi et al., 2000). Decreased levels of superoxide dismutase (SOD; De Raeve et al., 1997, Tekin et al., 2000; Shanmugasunaram et al; 2001), catalase (Novak et al., 1991) and GSH (Li et al., 1994) in the lungs of asthmatics have been reported. Studies by Varshavski et al. (2003) had revealed suppressed activity of SOD and catalase, as well as reductions in the levels of reduced glutathione in patients with bronchial asthma. Both SOD and catalase () play important roles in the detoxification of superoxide anion and H2O2, respectively, thereby protecting cells against free radical-induced damage (Wood et al., 2003). Reduced glutathione (GSH), in conjunction with glutathione peroxidase (GPx) and glutathione-S-transferase (GST) play a central role in the defense against free radicals, peroxides, and a wide range of xenobiotics (Rahman et al., 2006).

In the present study, an increased level of lung tissue malondialdehyde and decreased activities of superoxide dismutase, catalase and the level of reduced glutathione were observed in TDI-control rats. Treatment with dexamethasone, ethanolic extract and n-butanol fraction showed significant decrease in level of MDA and significant improvement in the activities of SOD, CAT and level of GSH as compared to those levels seen in TDI-control rats. This suggests that *M. oleifera* reduces oxidative stress, thereby prevents generation of free radical and finally inhibits development of immune mediated inflammation. Furthermore, increases in dietary antioxidant status.
Discussion

particularly that of α-tocopherol, correlated with lower levels of lipid peroxidation and improved lung function (Britton et al., 1995). A beneficial link between polyphenols intake and lower disease risk is likely, with many of the clinical benefits being attributed to both their antioxidant and anti-inflammatory properties (Arts and Hollman, 2005). In one double blind cross-over study some authors evaluated the protective effect of dietary antioxidants (i.e. vitamin E and vitamin C) on ozone-induced bronchial hyperresponsiveness in adult subjects with asthma (Trenga et al., 2001). Moreover, *M. oleifera* have been reported to be a rich source of antioxidants, including flavanoids and polyphenolic compounds (Sanchez et al., 2006b) that might be responsible for the anti-oxidant defense mechanism.

We also used ovalbumin (OVA) induced asthma model in guinea pigs to find out the mechanism of action as it shows similar effects like human allergic asthma (Smith and Broadley, 2007). Ovalbumin challenge evoked anaphylactic reactions in sensitized animals which result from the action of various mediators, histamine, leukotrienes, prostaglandins, thromboxane A$_2$, and platelet activating factor from inflammatory cells (Ogunbiyi and Eyre, 1985).

In the present study, all the OVA-sensitized animals did not show any change in body weight. OVA-control animals showed significant decrease in the tidal volume and increase in respiration rate was indicative of exertional breathing due to bronchoconstriction, a symptom of asthma. Treatment with ethanolic extract and n-butanol fraction showed protective effect by significant improvement in these parameters. Protection against acetylcholine-induced bronchoconstriction before and after lung function parameters (on day 29) in drug treated animals showed bronchodilatory effect of extract/fraction.

Allergen induced asthma is an important form of the inflammatory disease of the airways. Upon exposure to an allergen, inflammatory cells including eosinophils (Azzawi et al., 1990), neutrophils (Sur et al., 1993), lymphocytes (Robinson et al., 1993), macrophages/monocytes (Bentley et al., 1992) and mast cells infiltrate the airways (Fan and Mustafa, 2006). The eosinophilic inflammation of the airways, with an increase in activated and degranulated eosinophils is the key feature of allergic asthma (Bousquet et al., 1990). It has been shown in the guinea pigs (Pretolani et al., 1994), non-human
Discussion

primates (Gundel et al., 1992) and humans (Wardlaw et al., 1988) that there is a
correlation between the activation status of eosinophils in the airways and the
development of bronchial hyperresponsiveness. Neutrophils are prominent inflammatory
cells in the airways of patients with allergic asthma (Conese et al., 2003) and capable of
producing PGs, TXA$_2$, LTB$_4$ and PAF are thought to play an important role in disease
pathogenesis (Anticevic et al., 1996). Increased numbers of lymphocytes that express
mRNA for IL-4 and IL-5 have been observed in the airway submucosa and sputum of
patients with asthma and during allergen-induced late-phase asthmatic reactions, where
they are associated with increased numbers of eosinophils (Till et al., 1995; Ying et al.,
1997). Nevertheless, macrophages may participate in airway inflammation through
multiple mechanisms. Alveolar macrophages express the low-affinity receptor for
IgEFcERII (Melewicz et al., 1982) and expression appears to be increased in asthmatic
subjects relative to healthy persons (Williams et al., 1992). Macrophages produce
inflammatory mediators; such as platelet activating factor, prostaglandinF$_{2\alpha}$ and
thromboxane (MacDermot et al., 1984) Pro-inflammatory cytokines produced by
macrophages include IL-1, TNF-$\alpha$, IL-6 and GM-CSF, which may induce endothelial
cell activation, cellular recruitment, and prolonged eosinophils survival (Gosset et al.,
1992)

In this study we found the increased number of eosinophils, neutrophils,
monocytes/macrophages and lymphocytes in blood as well as in BAL fluid of OVA-
control animals as compared to non-sensitized controls. Among the differentials,
eosinophils counts increased by many folds in the BAL fluid Treatment with
dexamethasone, ethanolic extract and n-butanol fraction significantly decreased these cell
counts as compared to OVA-controls. Further, attenuation of inflammatory cell numbers
in BAL fluid was mirrored by tracheal and lung tissue histology. Therefore, these results
corroborate the evidence that ethanolic extract and n-butanol fraction treatment may be
useful to control activation of the inflammatory processes underlying the exacerbation of
allergic asthma

The antigen challenge of sensitized animals initiates the release of
pharmacological mediators including histamine, leukotrienes, prostaglandins and PAF,
possibly from mast cells and macrophages, which act on smooth muscle to cause
Discussion

Bronchospasm (Brocklehurst, 1971; Anderson et al., 1983a) As we have discussed earlier, allergen can also activate T cells which in turn release cytokines, for example, IL-3, IL-4, IL-5, IL-6, IL-8, GM-CSF and TNF-α causing the migration of eosinophils into the airways and the subsequent activation of these cells (Corrigan and Kay, 1992). In the present study, we have measured the level of elevated inflammatory cell markers IL-4, IL-5, IL-6 and TNF-α in BAL fluid as well as in serum.

The results of our study suggest that, cytokines TNF-α, IL-4, IL-5 and IL-6 were increased predominantly in OVA-control animals. This could be an indication for stronger anaphylactic sensitization creating severe inflammation. Treatment with dexamethasone, ethanolic extract and n-butanol fraction suppressed the level of these mediators significantly as compared to OVA-controls. In contrast, ethanolic extract did not reduce significantly the levels of TNF-α and IL-4 in BAL fluid. However, treatment with n-butanol fraction showed significant protection as compared to ethanolic extract. These results validate the popular use of *M. oleifera* for the treatment of asthma and allergic diseases and indicate that the possible mechanism of its beneficial action is associated with a significant decrease in cytokine production in serum and BAL fluid.

Mast cells can release mediators that have both immediate and chronic effects on airway constriction and inflammation (Reuter and Taube, 2008). The local release of histamine during allergic reactions has long been recognized as an important step in immediate hypersensitivity reactions to antigen. Bartosch et al. (1932) first reported that histamine is released from guinea pig lung during anaphylaxis. Acute release of histamine following an allergic or non-allergic insult may lead to bronchoconstriction, which can be attenuated by selective H1-receptor antagonists (Holgate and Finnerty, 1989). Again sensitization was using ovalbumin and then second exposure to same antigen causes acute anaphylactic shock resembling the acute asthmatic attack causing the release of various inflammatory mediators and cellular infiltration. The degranulation of mast cells and release of pro-inflammatory mediators, cytokines and chemokines (Ryzhov et al., 2004; Church et al., 1988) are also attributed to the influx of leukocytes in BAL fluid which subsequently leads to chronic lung inflammation (Cairns and Walls, 1996). We found that, significant increased in level of histamine in OVA-control animals were found to be significantly decreased in the n-butanol fraction treated animals
Whereas ethanolic extract treated animals did not show any significant inhibition in these levels. Further, histamine has been shown to activate eosinophils (Raible et al., 1994). Taking together, in the present study, significantly increase in level of histamine and numbers of eosinophils due to sensitization with ovalbumin were found to be significantly decreased in the n-butanol fraction treated animals. This depicts that, the n-butanol extract has efficacy to inhibit the release of the inflammatory mediator-like histamine by down regulating the cellular count.

Our data from above mentioned study suggest that, both ethanolic extract and n-butanol fraction showed protection by improvement in airway hypereactivity symptoms, oxidative stress parameters (MDA, SOD, CAT and GSH) and lung function parameters (tidal volume, respiration rate) before and after acetylcholine exposure. The antiasthmatic activity might be due to the inhibition of infiltration of inflammatory cells, release/synthesis of T-cell derived cytokines; TNF-α, IL-4, IL-5, IL-6. Further, these extracts may have an ability to inhibit the release of mediators like histamine into the local tissues of lungs and trachea which was confirmed by histopathogical observations.

In the above two experimental models of asthma, we found that n-butanol fraction has more significant antiasthmatic activity as compared to ethanolic extract. Further, the concentration of β-sitosterol in ethanolic extract and n-butanol fraction of *Moleifera* correlate well with pharmacological activities. As mentioned earlier the concentration of β-sitosterol in n-butanol fraction was found to be higher as compared to ethanolic extract and other fractions. So we have isolated β-sitosterol which was found to be responsible for antiasthmatic activity. The isolation of β-sitosterol from n-butanol fraction of *Moleifera* was performed by preparative Thin Layer Chromatography (TLC) technique. The isolated β-sitosterol was subjected to purification by column chromatography. The yield of the β-sitosterol was found to be 0.34% of the weight of the powder of the dried seeds of *Moleifera*. TLC analysis of the isolated sample along with the reference standard β-sitosterol (Natural Remedies Pvt Ltd, Bangalore, India), carried out in different solvent systems, showed a single spot with the identical Rf values of both the reference standard and isolated compound. Characterization and structure elucidation studies of isolated compound was carried out by melting point, UV, IR, Mass, 1H-NMR.
and $^{13}$C NMR spectral analysis which matched well with data of standard β-sitosterol (Yao et al., 2007). The results confirmed the identity of the isolated compound as β-sitosterol. We undertook to study the pharmacological activities of isolated compound, β-sitosterol.

With the increasing use of small animal models for studying asthma pathophysiology, there is a growing need for new techniques to quantify lung function in these species (Yilmaz et al., 2005a). Most non-invasive parameters that describe the function of respiratory system are well defined but the number of the existing mathematical models is inadequate. Many of the newer instruments such as Spirometry (for humans), Barometric whole body plethysmography and Bronchospasm transducer (for animals) were developed exclusively for lung function parameters have an ability to display waveforms that are the results of pressure, volumes and flows generated via machine in response to patient or animals during both inspiration and expiration, related to time. In the present study we have used Respiromax (model No. 070613-1, Columbus Instrument, OH, USA), an instrument can accommodate 1 to 8 animals for precision measurements of the tidal volume (ml), respiration rate (breaths/min), minute volume (ml/minute), inspiration time (sec) and expiration time (sec) and maximum expiratory flow (L/min) during exposure to allergens (Yilmaz et al., 2005b). One of the important benefits of this instrument is that one can measure all above parameters from breathing pattern (waveform) before and after allergen challenge. Further, these parameters also provide significant information for the assessment of the disease progression in human asthmatic subjects (Colasanti et al., 2004; Menzies et al., 2006). Hence, to understand the mechanism of action of any presume antiasthmatic drug, such lung function parameters showed to be considered in a global as well as in specific contexts (Campos et al., 2001).

Antigen challenge guinea-pig model has proved to be a good asthma model as it displays some similarities with human asthma in response to allergen (Campos and Church, 1992). Allergen challenge causes early phase reaction in the form of acute bronchoconstriction and late phase reactions characterized by an infiltration of inflammatory cell, release of mediators, structural changes of the local tissues (Fernandez et al., 2005). Hence we have studied the effect of β-sitosterol by using acute bronchospasm induced by histamine hydrochloride and acetylcholine and ovalbumin
induced chronic airway inflammation in guinea-pigs. In latter model, we measured the lung function parameters like tidal volume, respiration rate, minute volume, inspiration time, expiration time and maximum expiratory flow by using Respiromax Instrument.

The early airway response in asthma is IgE dependent and caused by the activation and degranulation of pulmonary mast cells by allergen mediated cross-linking of IgE antibodies bound to the high affinity IgE receptor (FceRI). As a result, numerous mediators are released which ultimately cause acute airway constriction after allergen exposure (Turner and Foreman, 1999). In the present study, control animals showed dyspnoea and gasping (within short time); a sign of bronchospasm, when challenge with histamine hydrochloride and acetylcholine (0.25%). β-sitosterol (2.5 and 5mg/kg) produced significant (p<0.01) increase in PCD time at higher doses against both the spasmogens as compared to control animals. However, it failed to produce any significant protection at a lower dose (1.25mg/kg). Further, this protection was comparable with standard drug ketotifen fumarate and atropine sulphate suggestive of bronchodilatory activity of β-sitosterol.

In ovalbumin induced asthma model, all animals present in OVA-control and β-sitosterol treated groups did not show any significant difference in the body weight during the experimental period as compared to those present in nonsensitized controls. Tidal volume and minute volume was found to be decreased significantly, and respiration rate was found to be increased significantly before and after exposure to acetylcholine in OVA-control animals as compared to nonsensitized animals from the day 21 to 29. Whereas inspiration time, expiration time and peak expiratory flow were found to be reduced from day 24 to 29. These changes in the lung function parameters in OVA-control animals suggestive of exertional breathing and airway inflammation; key features of asthma. Treatment with β-sitosterol showed significant improvement in these parameters as compared to OVA-control animals.

It has been documented that the late phase airway response in asthma is associated with the infiltration of the inflammatory cells to the site of inflammation (Williams, 2004). In the present study, OVA-control animals showed the increase in the total and differential cellular count in blood as well as in BAL fluid suggestive of severity of disease in relation to cellular infiltration. Treatment with dexamethasone and β-
sitosterol significantly decreased the number of total cell count in both blood and BAL fluid. However, among the differential cell count, treatment with β-sitosterol decrease each cell count in blood and eosinophil and neutrophils count in BAL fluid as compared to OVA-control animals. Further, amelioration of inflammatory cell numbers in BAL fluid was confirmed by tracheal and lung tissue histology. Therefore, these results suggest the evidence that β-sitosterol treatment may be useful to control activation of the inflammatory processes underlying the exacerbation of allergic asthma.

The initial indication for cytokine involvement in the pathogenesis of asthma came from studies performed in the early 1990s showing that allergic asthma was associated with Th2 cytokine expression. Mast cells are most likely an important immediate source of TNF-α. Further, localization of cytokines to mast cell subsets reveals preferential IL-4 expression by MCt mast cells, with prominently IL-5 and IL-6 expression by the MCr subset (Chung and Barnes, 1999). In the present study, we have confirmed the existence of the prominent Th2 type cytokines; IL-4, TNF-α, IL-5 and IL-6 in OVA-control animals suggestive of chronic airway inflammation. Dexamethasone and β-sitosterol treatment suppressed the level of these mediators significantly. β-sitosterol decrease the level of TNF-α, IL-5 and IL-4 in BAL fluid as well as in serum. However, it failed to produce any significant inhibition on the level of IL-6 in both serum and BAL fluid. These reductions in the level of the cytokines are co-related with mast cell stabilizing activity of the β-sitosterol. Moreover, these results suggest that the possible mechanism of action of β-sitosterol for bronchodilatory action might be due to decrease in cytokine production.

Further, ongoing chronic inflammation is associated with mast cell degranulation as evidenced by increased level of mast cell mediators in lung tissues (Bartosch et al., 1932, Foresi et al., 1990). In the present study, significant increased in histamine levels in OVA-control animals suggestive of inflammation of lung tissues in relation to release of mediators. Treatment with dexamethasone and β-sitosterol significantly decreased these levels as compared to diseased control animals. This depicts that; β-sitosterol might have an ability to inhibit the release of inflammatory mediator-like histamine.

Atopic asthma has been extensively investigated and shown to comprise structural changes in the airways (Amin et al., 2000). In the present study, our histopathological
results suggest that β-sitosterol treatment inhibited angiogenesis, epithelial shedding and leukocyte infiltration into the airway after ovalbumin challenge. Thus it is speculated that β-sitosterol has a prophylactic effect on allergen-induced airway inflammation. β-sitosterol has shown bronchodilatory activity by increasing PCD time against spasmogens (acetylcholine and histamine). It also showed protection against ovalbumin induced chronic airway inflammation by improvement in lung function parameters (tidal volume, respiration rate, minute volume, inspiration time, expiration time and peak expiratory rate). In addition it also produced inhibition of infiltration of inflammatory cells (eosinophils and neutrophils), level of TNF-α, IL-4, IL-6, IL-5 and histamine.

Herbal medicine is readily available in our diverse vegetation, cheap and all carries the potentials of introducing new templates into modern medicine. India is sitting on a gold mine of well-recorded and well practiced knowledge of tradition herbal medicine hence natural product research continues to explore Indian traditional medicines to develop new novel drugs (Kamboj, 2000). Phytochemical investigations of seeds of *M. oleifera* in the present study and by other investigators also have shown the presence of various active components. The active principles estimated in the present study like ascorbic acid, quercetin, β-sitosterol, glycerol 1-9-octadecanoate and benzyl isothiocyanate might be responsible for one or more mechanisms for such pharmacological activities of ethanolic extract and its fractions.

Recently, Christofidou-Solomidou and Muzykantov, (2006) proved that, ascorbic acid is the most extensively investigated antioxidant for effects on asthma and has been shown in numerous case-controls and cross sectional studies to be associated with a reduced risk of asthma. Interventional studies investigating the role of either a single dose or extended antioxidant supplementation in particular ascorbic acid on exercise-induced bronchoconstriction have shown a protective effect (Schachter and Schlesinger, 1882; Cohen et al., 1997; Anderson et al., 1983b). Further, Ting and colleagues (1983) reported the beneficial effect of high dose of ascorbic acid on pulmonary functions in patients with mild asthma. Moreover, the effect of ascorbic acid was also tested on some models of histamine release and experimental anaphylaxis; however they could not find any protective effect after ascorbic acid treatment (Alvarez and Mesa, 1981)
Discussion

Quercetin has been the subject of dozens of scientific reports over the past 30 years. It has shown the greatest activity among the flavanoids studied in experimental models (Kahraman et al., 2003). Quercetin is frequently used therapeutically in allergic conditions, including asthma, hay fever, eczema, and hives. Further, it appears to be key antioxidant in the treatment of asthma (Miller, 2001). In experimental study, quercetin reduced the concentration of PGE\textsubscript{2} and LTB\textsubscript{4} in pleural exudate of rats given carrageenan intrapleurally (Mascolo et al., 1988). It also inhibits mast cell degranulation and subsequent release of histamine (Pearce et al., 1984; Middleton and Drzewiecki, 1984). Clinical studies of quercetin in asthma are lacking; however, this flavonoid is probably useful in the overall treatment due to its antihistaminic activity (Miller, 2001).

Antiasthmatic activity of benzylisothiocyanate in a dose dependent (150mg/kg: 89%; 75mg/kg: 76%; 30mg/kg: 66%; 15mg/kg: 49%) manner has been reported against the bronchial obstruction induced by ovalbumin, measured by whole body plethysmography (Dorsch et al., 1984). The antitumour promoting activity of glycerol-1-(9-octadecanoate) has been reported by testing its inhibitory effects on Epstein-Barr virus-early antigen (EBV-EA) (Guevara et al., 1999).

Phytosteroids possesses many interesting medicinal and pharmacological activities. Chemically these compounds resemble in structure with steroids and plant containing such steroids proved anti-inflammatory agents by modern clinical studies, like *Trigonella foenum graecum, Solanum xanthocarpum, Boswellia serrata, Glycyrrhiza glabra, Commiphora mukul, Withania somnifera, Smilax officinalis* etc. Among the number of steroids, β-sitosterol is found in a variety of plants and is similar to the structure of cholesterol. The scientific literature is replete with reports of the biological activities of sterols or their glycosides in various animal models. The possible use of β-sitosterol as therapeutic drugs in immune-mediated disorders has been reported (Bouic and Lamprecht, 1999). Recently Yuk et al. (2007) reported the beneficial effects of lactose-β-sitosterol and β-sitosterol on ovalbumin-induced lung inflammation in actively sensitized mice. For instance, β-sitosterol and its glycoside have been shown to reduce carcinogen-induced cancer of the colon in rats (Raicht et al., 1980), as well as exhibiting anti-inflammatory activity through cytokine inhibition (Aherne and O’Brien, 2008) anti-pyretic (Gupta et al., 1980) and anti-complement activity (Yamada et al., 1987) *In vivo*
and in vitro studies with β-sitosterol showed to increase in Th1 while dampening Th2 activities (Myers and Bouic, 1998). In the present study, the results obtained from the quantitative estimation of marker compounds suggest that, their concentration is higher in both ethanolic extract and n-butanol fraction. These compounds might be responsible for the one or more mechanism(s) for antiasthmatic activity of ethanolic extract and n-butanol fraction. β-sitosterol appears to be the active principle responsible for the antiasthmatic activity. However, the involvement of other marker compounds; ascorbic acid, quercetin, benzylisothiocyanate and glycerol 1-9-octadecanoate can not be ruled out.