Introduction
2. INTRODUCTION

Asthma has become one of the most common chronic diseases in the industrialized countries and its frequency is predicted to increase throughout the world over the next decade, particularly in developing countries (Barnes, 2003a). Twenty years ago, asthma was viewed as a disease of bronchoconstriction and treated predominantly with bronchodilators. However, at present it is considered as chronic inflammatory disease of the airway, and involves the infiltration of many inflammatory cells (Th2 cells), mediators and inflammatory proteins relative to Th1 cells (Tattersfield et al., 2002). This inflammation ultimately leads to recurrent episodes of asthma symptoms: cough, chest tightness, wheezing and dyspnea. Further, the cells involved in the asthmatic inflammatory process have a major capacity for producing reactive oxygen species (ROS) (Ricciono et al., 2006). In addition, there are characteristic structural changes to the airways (termed remodeling), some of which might even precede the development of the disease. These changes include subepithelial fibrosis (basement membrane thickening), airway smooth muscle hypertrophy and hyperplasia, angiogenesis, goblet-cell hyperplasia and submucosal-gland hyperplasia (Payne et al., 2003). Typically, all asthmatic patients with active disease have hyperresponsive airways, exhibiting an exaggerated bronchoconstrictor response to various stimuli. For many patients, the disease has its roots in infancy, and both genetic factors (Cookson, 1999, Prescott et al., 1998) and environmental factors like viruses, allergens, occupational pollutants, infections, drugs, chemicals, exercise and emotional stress (Stein et al., 1999; Halonen et al., 1999) contribute to its inception and evolution (Venables and Chan-Yeung, 1997).

Asthma affects approximately 8% of the world’s population (Kasserra et al., 2004). Prevalence varies from region to region depending upon the definition used for diagnosis of asthma (Aggarwal et al., 2006). Diagnosed asthma in adults is generally reported as 2.7 to 4.0% in most European countries, 12.0% in England (Chinn et al., 1997) and 9.5 to 17.9% in Australia (Dubois et al., 1998). In United States the prevalence is 7.1%, approximately 4.8 million of these people are children. It is one of the leading causes for both outpatient and hospital care, with approximately 500,000 hospitalizations and more than 5,000 deaths annually. At greatest risk for hospitalization with asthma are African-Americans and children. Death rates from asthma are highest among African-
Americans between the ages of 15 and 24 years (Devereux et al., 1996). The cost for asthma-related treatment is estimated to be $14.5 billion. Approximately half of all cases of asthma develop during childhood and another before 40 years of age (Peat et al., 1992). However, asthma can begin at any age, and it can affect both sexes and all cultures. The overall burden of asthma in India is estimated at more than 15 million patients (WHO Factsheet, 2005).

Asthma is characterized by a complex inflammatory response involving resident cells (e.g. mast cells, macrophages, and nerves), recruited cells (e.g. eosinophils, T-lymphocytes, monocytes, dendritic cells, basophils, neutrophils and platelets) and structural cells (e.g. epithelial cells; smooth muscle cells, endothelial cells, fibroblasts and myofibroblasts) (Hamid et al, 2003). These cells can synthesize and secrete a vast numbers of mediators like histamine, bradykinin, tryptase, leukotrienes and prostaglandins that perpetuate the inflammatory response, causing increased blood flow, vasoconstriction, fluid leak from the vasculature, recruitment of inflammatory cells to the area and bronchoconstriction (Begueret et al., 2007). This is called early phase asthmatic response. The late phase inflammatory reaction occurs 6 to 9 hours after allergen provocation and involves the recruitment and activation of eosinophils, CD4+ T-cells, basophils, neutrophils, and macrophages (Rosenwasser, 2000). This results in epithelial damage, bronchial wall edema, mucus hyper secretion, airway remodeling and bronchial hyper-responsiveness (BHR) of bronchial smooth muscle. This bronchoconstrictive response is associated with inflammation and produces recurrent episodes of wheezing, dyspnea, and shortness of breath that last for a day or so (Hidalgo Castro et al., 2004).

At the cellular level, inflammatory events begin when macrophages present antigen to T lymphocytes that elaborate T-helper type-2 (Th2) cytokines such as interleukins 4, 5, 6 and 13. In turn, IL-4 and IL-13 regulate IgE synthesis. These both interleukins allows B cells to switch to IgE. IgE binding to allergen can cross link the high affinity IgE receptors on the surface of the mast cells, resulting in release of inflammatory mediators including cytokines (Okumura et al., 2003). IL-5 mediates eosinophils recruitment and infiltration, acting in tandem with members of β-chemokine family, especially eotaxin, macrophage inflammatory protein-1-α and monocyte chemoattractant protein-1. Eosinophils are considered pivotal cellular mediators of
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asthma (Berman and Weller, 1992). Recruitment of eosinophils requires the binding of adhesion molecules on the cells to their counter receptors on vascular endothelial cells. Tumor necrosis factor alpha (TNF-α) of mast cells or macrophage origin upregulates endothelial expression of intercellular adhesion molecules-1 and 2, while IL-4 and IL-13 upregulates endothelial expression of vascular cell adhesion molecule-1 (Lee et al., 2003). These interact with their ligands, lymphocyte function associated antigen-1 and very late acting antigen-4, on T cells and eosinophils, allowing cell migration from the vasculature. Subsequent infiltration of these cells into the airways is associated with many pathological changes seen in asthma (Zeng et al., 2001). Eosinophils produce immunoregulatory cytokines (TNF-α, IL-3, 4, 5, 6 and 8 and granulocyte macrophage colony simulating factor), growth factors (transforming growth factor-α), and proteins toxic to cells (major basic protein, eosinophils paraoxidase and eosinophils derived neurotoxin), thereby contributing to airway inflammation and over the long term remodeling (Holgate, 2008).

Currently available drugs for the treatment of asthma include β2 agonists, anticholinergics, corticosteroids, mast cell stabilizers, phosphodiesterase inhibitors, PAF inhibitors, leukotriene modifiers and TXA2 inhibitors. Some newer molecules are being investigated on the basis of the new mechanisms involved such as cell-adhesion blockers, transcription factor inhibitors and enzyme (tyrosine, MAP kinase, metalloprotease, tryptase, phosphoinositide 3-kinase) inhibitors. For many patients, these drugs have been helpful in symptomatic relief. It has been reported that prolonged treatment with conventional drugs produced various adverse effects. Muscle tremor and hypokalemia are major adverse effects of β2 agonists (Nelson, 1986; Haalboom et al., 1985). Theophylline has narrow therapeutic index and requires monitoring of drug levels (Stoloff, 1994, Nasser and Rees, 1993) Inhaled ipratropium bromide had bitter taste and causes cough, nausea, dryness of mouth and throat. Adverse effects of corticosteroids include fluid retention, increased cell mass, increased appetite, weight gain, osteoporosis, capillary fragility, hypertension, peptic ulceration, diabetes, cataract and psychosis (Nanulescu et al., 2004) Also, the efficacy of currently used anti-asthmatic drugs is compromised in several ways. Individual oral agents act only on a part of the pathogenic process of bronchial asthma and hence they may not produce any cure and may not prevent all
complications of bronchial asthma. Thus, the existing classes of anti-asthmatic agents offer a limited variety of actions that can be combined in a complementary and additive manner. Few patients maintain the recommended target for the good asthma control, and a normal physiologic pattern of breathing is rarely reinstated. This emphasizes the need for newer and better therapeutic approaches.

There has been great pressure from the pharmaceutical industry to develop new drugs for asthma as this is an enormous and expanding global market. New treatments in development for asthma include inhibitors of the pro-inflammatory enzymes, such as PDE$_4$, p38 mitogen-activated kinase and nuclear-factor-kB activating kinase (Barnes, 2004). More specific approaches include inhibiting chemokine receptors on eosinophils and T lymphocytes, inhibiting adhesion molecules that recruit key inflammatory cells and inhibiting mast cells with syk kinase inhibitors (Chihara et al, 1999). Antibodies that block IgE have now been introduced in some countries and have clinical efficacy, especially in patients with severe allergic asthma (Barnes, 2006). Combination inhalers with a corticosteroid and a long-acting β$_2$-agonist are the most effective treatment so far available and it is likely that several combination inhalers will become available, including once daily drugs (Beeh et al., 2007). However, none of these agents have long-term effects on airway inflammation or remodeling and therefore are not disease-modifying or curable. So, there is a need to find more effective therapies for patients with more severe asthma, who are not well controlled by current therapies. Although this is a small minority of patients (<5%) they account for more than half of the health care spending on asthma. As a result, there is high prevalence of usage of complementary and alternative medicine (CAM) to treat asthma for hundreds of years.

The use of complementary and alternative medicines for asthma treatment is of great interest and has become an increasing appealing component of standard medical care (Lane and Lane, 1991; Graham and Blass, 2000, Huntley and Ernst, 2000). A major traditional medical system includes Ayurveda, Siddha, Unani-Tibb and Yoga, these systems are still being practiced in all parts of the country (Ziment and Tashkin, 2000). The common forms of CAM used for asthma include traditional Indian and Chinese medicinal herbs, homeopathy, acupuncture, aromatherapy, reflexology, relaxation therapy, massage and even prayer. Medicinal plants play a major role and constitute the
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backbone of the traditional medicine. They have yielded many useful compounds and plant-derived ingredients like morphine, atropine, papaverine and codeine, which are an important component of modern pharmaceuticals (Miller and Gereau, 2000). Moreover, medicinal plants contain a wide range of chemical compounds that could serve as 'leads' for the development of novel agents. Plant cells are now considered to be the chemical factories synthesizing a large variety of chemical compounds and thus serve as a very important renewable source for the production of novel drugs. According to the World Health Organization (WHO) survey, 80% of the populations living in the developing countries rely almost exclusively on traditional medicine for their primary health care needs. Thus, WHO has approved the use of traditional medicines as a part of health programme. In a wider context, there is a growing demand for plant based medicines, health products, pharmaceuticals, food supplements, cosmetics etc. International market of medicinal plant is over US$ 60 billion per year, which is growing at the 7% rate (Huntley and Ernst, 2000).

Some of the historical theories, techniques, and treatments that have been used in the management of breathing disorders and chest diseases have persisted over thousands of years (Ziment, 1986). The favored drugs for asthma that were used in the second half of the twentieth century had their origins in folk remedies discovered by our ancestors. Ephedrine was developed from ma huang, a favorite Chinese herbal remedy in use for thousands of years (Berger and Dale, 1910). Ancient asthmatic subjects have breathed in the smoke of heated henbane leaves, which released anticholinergic drugs, as did the stramonium cigarettes that were introduced into Europe from India in the nineteenth century (Sakula, 1988; Mann, 1984). Asia also provided the herbal origin of theophylline, which is found in tea leaves. Interestingly, the related herbal products caffeine and its congeners in coffee offered a favorite asthma remedy during the same century (Hirsch, 1922). Cromolyn was a derivative of the chromones found in Ammi visnaga, the source of the ancient Middle Eastern bronchodilator khella (Cox, 1967). Even steroids have a historical precedent, such as the use of placentas or pubescent boys' urine in treating asthma, whereas in the first half of the nineteenth century, ground adrenal glands were used. Some of these ancient sources of therapy are still made available today (Ziment, 2000).
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Universally popular respiratory remedies include eucalyptus, menthol, anise, fennel, tolu balsam, and camphor; some of these are incorporated in products such as Vicks VapoRub and Tiger Balm (Gupta et al., 1998a). These aromatic agents, when inhaled as vapors, can soothe the inflamed nasal mucosa and seem to benefit the tracheobronchial tree. Other soothing remedies of the throat include menthol, marshmallow, Iceland moss, mullein, plantain, and slippery elm. It would be expected that honey, candies, or other nonspecific throat drops may be just as effective as the mucilaginous contents of these phytomedicines. In contrast to herbs, it is possible that some foods (onion, garlic, pungent spices, antioxidants, omega-3 fatty acids, and essential oils from citrus fruits) and vitamins are of physiologic value in helping improve natural body defenses (Baker et al., 1999; Bodner et al., 1999; Villani et al., 1998).

Most of the drugs used in traditional system of medicines in China and India are from herbal origin. During the last two to three decade's herbal medicine have been attracting the attention of not only common public but also the scientific community including the pharmacy and medical professionals of Asia, Europe and the USA for safer treatments (Patwardhan et al., 2004). India has about 45,000 medicinal plant species and at least hundred chemical substances of known structures have been extracted from plants that are being used as drugs throughout the world. Efforts are being made by various government agencies and research laboratories to maintain the quality of herbal drugs by proper identification, detailed pharmacognostical and phytochemical investigations and standardization. A large number of manufacturing units, some with multicrore investment and some multinationals have also entered the area of production of Ayurvedic drugs and pharmaceuticals (Shrikumar and Ravi, 2007).

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, Tibetan and the Indosyunic system of Pakistan, are likely to remain obscure (Ziment, 1997a, Ziment, 1997b) Some Ayurvedic drugs of interest for consideration in asthma include Datura plants (the historical source of atropine); Tylophora asthmatica, which is used for asthma, and the Malabar nut, from which the European mucokinetic agent bromhexine was derived. Coleus forskohlu is a plant from which an interesting β-sympathomimetic drug has been obtained; forskolin (colforsin)
enters cells and directly stimulates the production of 3, 5-adenosine monophosphate (Bauer, 1993). One study has shown that *Coleus forskohlii* is superior to placebo and equivalent to fenoterol in protecting against metahcholine-induced bronchoconstriction (Kaik and Witte, 1986). *Ginkgo biloba* has also been investigated; it contains several unique terpene molecules which in themselves antagonize platelet activating factors, hence limiting the immune response and subsequent bronchial reactivity. Studies on ginkgolides extracted directly from the herb show that oral administration improves pulmonary function and protects against exercise-induced asthma (Wilkens, 1990). Other agents used for asthma and cough include spices, frankincense, jaggery, Indian gooseberry, costus and myrobalm. Studies on frankincense, which contains *boswellic acid*, have demonstrated that it can inhibit 5-lipoxygenase (Gupta et al., 1998a).

Thus ‘Ayurveda’ is an example of a long standing tradition in India that offers a unique insight into comprehensive approach to asthma management. The potential of plant as a source for drug is yet to be explored systematically. To pursue research in traditional systems of medicine, several USA agencies and institutions such as FDA and National Institute of Health (NIH) have setup separate wings. Ayurveda has recommended number of drugs from indigenous plants sources for the treatment of bronchial asthma and other allergic disorders and have been successful in controlling the disease as well. Large numbers of medicinal plants and plant preparations have been reported to possess anti-asthmatic effects and are classified in a systematic way on the basis of their mechanism of action (Mehta et al., 2008). Majority of herbal products found to be useful in the management of asthma in the experimental studies have yet to undergo clinical trials.

*Moringa oleifera* Lam. belongs to the family *Moringaceae* (genus *Moringa*). It is a shrub and small deciduous tree of 2.5-10 m in height. It is grown throughout the tropics and subtropics of Asia and Africa (Michael and Horst, 1998). Numerous varieties of *M oleifera* have been developed to meet the tastes of local populations (Rajan, 1986). The medicinal values of the seeds and the different parts of the plant have long been recognized in folklore medicine (Faizi et al., 1995). The plant has been well positioned in Ayurvedic, Unani, and even allopathic systems of medicine (Mughal et al., 1999). The whole plant possesses antimicrobial activity (Caceres et al., 1991) and is also used for the
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treatment of ascites, rheumatism, venomous bites, and for enhancing cardiac function (Nadkarni and Nadkarni, 1976; Chaudhri, 1996). The leaves exhibit strong hypotensive, diuretic, and spasmyltic effects, in helminthesis, and in scurvy (Caceres et al., 1992). Further, we have studied the antiinflammatory and antianaphylactic activity of hydroalcoholic extract of leaves (Mahajan et al., 2006). Stem bark of this plant also showed promising antiinflammatory activity against acute and chronic inflammation (Mahajan et al., 2007a). The roots of the plant have been used as carminatives, anthelmintics, diuretics, and in the treatment of intermittent fever, epilepsy, and chronic rheumatism (Karadi et al., 2006).

*M. oleifera* is incorporated in various marketed formulations, such as Rumalaya and Septilin (The Himalaya Drug Company, Bangalore, India), Orthoherb (Walter Bushnell Ltd., Mumbai, India), Kupid Fort (Pharma Products Pvt.Ltd., Thayavur, India) and Livospin (Herbals APS Pvt. Ltd., Patna, India), which are available for a variety of disorders (Mehta et al., 2003). It has been reported that the seeds of the plant have been used as purgatives, antipyretics, and as anti-inflammatory agents (Varier, 1997). We have previously reported the anti-arthritic activity of the ethanolic extract of seeds in rats (Mahajan et al., 2007b). Guevara et al. (1999) reported the anti-genotoxic activity of seed extracts. Ayurvedic practitioners used the seed extract nasally in diseases like rhinitis, and the dried seeds were used as an anti-allergic medication (Chopra et al., 1938). Treatment with the powder of whole plant is also reported to elicit good clinical responses in children suffering from upper respiratory tract infections as well as skin infections. It has been reported that one of the alkaloids from the plant closely resembles ephedrine in action and relaxes bronchioles (Kirtikar and Basu, 1975). Our preliminary clinical study 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, in preclinical studies also ethanolic extract of seeds produced bronchodilatory effect after oral administration against histamine and acetylcholine induced bronchospasm in guinea pigs (Mehta and Agrawal, 2008). The crude extract of *M. oleifera* has been shown to possess bronchodilatory activity however, its mechanism of action in the light of better understanding of asthma pathophysiology and its associated conditions like allergy and anaphylaxis has not been
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Thus, the first objective of the present investigation was to study bronchodilatory, mast cell stabilizing, immunomodulatory and antianaphylactic activities of ethanolic extract of seeds of M. oleifera and its different fractions. On the basis of results of these activities, further we have made an attempt to investigate the mechanism of action for antiasthmatic activity of effective extracts (ethanolic extract and n-butanol fraction) using chemical induced immune mediated inflammatory responses in rats and ovalbumin induced airway inflammation in guinea pigs.

Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening, chemoprofiling and marker compound analysis using modern analytical techniques like HPTLC and HPLC. Although several formulations are available for asthma, very limited attempts have been made to evaluate their pharmacological aspects and verify therapeutic efficacy. Moreover, because of resurgence of interest on herbal drugs, it is also important to ensure that only quality products enter the market. Efforts are being made by various government agencies and research laboratories to maintain the quality of herbal drugs by proper identification and detailed pharmacognostic, phytochemical investigations and standardization. However, in spite of the continuing efforts, there are very few standard methods available for quality control of herbal drugs, which is considered as the biggest hurdle for India to enter into the multi-million dollar international market. Further, the composition of plant material can vary and it is known to be influenced by the place of origin, soil, climate, season, time of collection, post harvesting conditions, temperature changes, moisture which affect tremendously the quality and therapeutic efficacy of the drug. Therefore, the quality and efficacy of the herbal drugs need to be established through systematic pharmacognostic, phytochemical and pharmacological evaluation and standardization of the drug. In herbal research, it is also essential to authenticate the plant and to establish phytochemical standardization with help of reliable instruments like HPTLC and HPLC. We have carried out phytochemical standardization of M. oleifera extract and its all fractions using HPTLC fingerprinting and HPLC analysis. We have also standardized ethanolic extract and its each fraction used in the investigation with respect to concentration of ascorbic acid, quercetin, benzylisothiocyanate, glycerol-1-(9-octadecanoate) and β-sitosterol, marker compounds present in M.oleifera plant.
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*M. oleifera* is rich in compounds containing the simple sugar, rhamnose and a fairly unique group of compounds called glucosinolates and isothiocyanate glycosides (Fahey et al., 2001; Bennett et al., 2003). The plant is also reported to contain various polyphenolic compounds (Sidduraju and Becker, 2003), tannins (Santos et al., 2005), flavonoids like quercetin (Lako et al., 2007), amino acids, fatty acids, vitamins and antioxidants (Nesamani, 1999). β-sitosterol, chemically α-dihydrofucosterol is a major constituent of many medicinal plants including *Glycyrrhiza glabra*, *Elaeocarpus sphaericus*, *Sideritis foetens*, *Bennica hispida* etc. The scientific literature is replete with reports of the biological activities of sterols or their glycosides in various animal models. Moreover, β-sitosterol is one of the major compounds reported in *M. oleifera* (Anwar et al., 2007; Faizi et al., 1998; Makonnen et al., 1997). In this connection and on the basis of pharmacological and phytochemical studies, we have isolated (0.34%) and characterize the compound β-sitosterol from n-butanol fraction. Further, we have also made an attempt to investigate its molecular mechanism of action.
OBJECTIVES OF THE STUDY

1. Preparation and preliminary phytochemical analysis of ethanolic extract of seeds of *M. oleifera* and its different fractions.

2. To perform pharmacological studies of ethanolic extract of seeds of *M. oleifera* and its different fractions using bronchodilatory, mast cell stabilizing, immunomodulatory and antianaphylactic activities.

3. To study the effective extract/fraction using chemical induced immune mediated inflammatory responses in rats and ovalbumin induced airway inflammation in guinea pigs.

4. Phytochemical standardization of extract/ fractions and quantitative estimation for their chemical constituents using standard marker compounds by HPTLC and HPLC analytical techniques.

5. Isolation and characterization of the isolated compound.

6. Pharmacological activity of isolated compound to find out the molecular mechanism of action