Abstract
1. ABSTRACT

Asthma is an inflammatory disease of the airways that is characterized by airflow obstruction, acute or chronic inflammation, airway hyperresponsiveness (AHR) and structural remodeling. 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. Asthma affects approximately 8% of the world’s population. 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. The cost for asthma-related treatment is estimated to be $14.5 billion. In India the overall burden of asthma is estimated at more than 15 million patients.

The use of complementary and alternative medicines (CAM) for asthma has become an increasing appealing component of standard medical care. In this connection, we have selected a herbal drug *Moringa oleifera* Lam. belonging to the family, Moringaceae, reported to have beneficial effects in inflammation and allergic disorders. Our previous 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 in asthmatic patients. Further, in preclinical studies also ethanolic extract of seeds showed to possess bronchodilatory and antiarthritic activity. In addition, various reports in the literature suggested the presence of active compounds responsible for pharmacological activities of *M.oleifera*. Thus, the objective of the present study was to investigate into the mechanism of action of *M. oleifera* with special reference to its antiasthmatic activity.

The work carried out in the present study was divided into phytochemical and pharmacological studies. Phytochemical studies involved authentication of the seeds of *M. oleifera* used, extraction of the drug, fractionation of the extract with various solvents, standardization of extract and fractions for finger printing profile, quantitative estimation of some known marker compounds, isolation and characterization of the possible active compound (β-sitosterol).
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 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. 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%. Earlier reports from our laboratory showed that dried seed powder as well as ethanolic extract has greater bronchodilatory effect in both clinical and preclinical studies, so 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 in rat peritoneal mast cells, delayed type hypersensitivity reaction and humoral antibody responses were studied in mice using sheep’s red blood cells (SRBC) as antigen for immunomodulatory
activities. Further, antianaphylactic activity was studied with systemic anaphylactic reaction in mice and passive cutaneous anaphylaxis reaction (PCA) in Wistar rats.

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. In the present study, pretreatment with ethanolic extract and its all fractions were found to be significantly increased the preconvulsion time against histamine and acetylcholine aerosol as compared to control. Among various fractions tested n-butanol fraction produced significant increase in PCD time as compared to chloroform and ethyl acetate fraction. Significant protection of rat peritoneal mast cells was observed from disruption by Compound 48/80 and egg albumin by ethanolic extract and its fractions. 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.

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 CD4+ and CD8+ T cells and need 24 hr to 48 hr to develop. Upon recurrent contact with identical antigens, recruitment of CD4+ and CD8+ T cells cause inflammation and cytotoxic induced apoptosis in target cells as well as cytokine mediated leukocyte infiltration. 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. Humoral immune reactions are mediated by IgG and IgM antibodies which are directed against membrane associated antigens. 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. Treatment with ethyl acetate fraction
produced significant inhibition at higher dose only. Whereas, 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. In the present investigation, pre-treatment with ethanolic extract and n-butanol fraction 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. IgE-mediated local allergic reaction was also studied by using passive cutaneous anaphylaxis 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.

Standardization of the extract and fractions was carried out by HPTLC finger printing analysis to trace out the possible number of components present in each of them. Estimation of marker compounds; ascorbic acid, quercetin, benzylisothioeyanate and glycerol-1-(9-octadecanoate) was also performed by HPTLC methods and β-sitosterol content was estimated by HPLC analysis 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, benzylisothioeyanate 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, 0.38% 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 all 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.

Our results with ethanolic extract and n-butanol fraction showed significant bronchodilatory, mast cell stabilizing, immunosuppressive and antianaphylactic activities as compared to chloroform and ethyl acetate fraction. 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. Since, the concentration of β-sitosterol was found to be higher in n-butanol fraction, the isolation of β-sitosterol from n-butanol fraction was performed by preparative TLC. The isolated β-sitosterol was subjected to purification by column chromatography. The yield of the β-sitosterol isolated from n-butanol fraction was found to be 0.34% of the weight of the powder of the dried seeds of M. oleifera. TLC analysis of the isolated sample along with the reference standard β-sitosterol 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 13C-NMR spectral analysis. The results confirmed the identity of the isolated compound as β-sitosterol.

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, a low molecular weight compound widely used as an industrial chemical, has been associated with asthma. In this model of asthma at the end of the study, the airway hyperreactivity symptoms (sneezing, rhinorrhea and nasal obstruction) were observed, total and differential leukocytes were counted in blood and bronchoalveolar (BAL) fluid; serum and BAL TNF-α, IL-4, and IL-6 levels were assessed by ELISA method. In addition to this, oxidative stress parameters (MDA, SOD, CAT and GSH) were estimated and lung histological examination was carried out.
In the present study, the airway inflammation observed in the TDI-control rats showed respiratory hyperreactivity symptoms that were similar to asthma. 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 macrophages 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. 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. The levels of TNF-α, IL-4 and IL-6 were increased significantly in TDI-control rats as compared to non-sensitized controls 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. An increased level of lung tissue malondialdeyde (MDA) and decreased activites of selected antioxidant enzymes superoxide dismutase, (SOD), catalase (CAT) and level of reduced glutathione (GSH) were observed in TDI-control rats. Treatment with dexamethasone, ethanolic extract and n-butanol fraction showed significant decrease in level of MDA and significant increase in the level of SOD, CAT and GSH. Further, the protective effect of this extract/fraction was also confirmed by histopathological observations.

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. During the experimental period, body weight was taken regularly, the test drugs were administered by oral route 2.5 hr prior to challenge with aerosolized 0.5% OVA. Lung function parameters (i.e., tidal volume and respiratory rate) were measured on day 18, 21, 24 and 29 before and after acetylcholine (0.25% for 30sec) exposure using Biopac data acquisition system. At the end of experiment, blood and BAL fluid was collected from each animal to perform total and differential counts. TNF-α, IL-4, IL-5 and IL-6 are
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considered to play a pivotal role in immunologic airway inflammatory responses were evaluated by ELISA method in both serum and BAL fluid. Lung tissue histamine assay was performed using the homogenate of one lobe from each animal; a separate lobe and the trachea were subjected to histopathology. 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.

We found increase in number of eosinophils, neutrophils, monocytes/macrophages and lymphocytes in blood as well as in BAL fluid of OVA-controls 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. 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 those levels seen in OVA-control animals. 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. Further, 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.

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
The antiasthmatic activity of these extract/fraction 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 histopathological 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. Moreover, as mentioned earlier, the concentration of β-sitosterol in ethanolic extract and n-butanol fraction of *M. oleifera* correlate well with pharmacological activities. Thus we have isolated β-sitosterol and made an attempt to study its pharmacological activities. We have studied the effect of β-sitosterol using acute and chronic models of asthma. Acute bronchosapsm was induced by histamine and acetylcholine; chronic inflammation was induced by ovalbumin which produces features of allergic asthma in guinea-pigs. In latter model, lung function parameters (tidal volume, respiration rate; minute volume, inspiration time, expiration time and maximum expiratory flow) were measured before and after bronchoconstriction test (0.25% acetylcholine for 30sec as spasmogen) on day 18, 21, 24 and 29 using Respiromax Instrument. Other parameters include total and differential counts in blood and BAL fluid, levels of TNF-α, IL-4, IL-5, IL-6, histamine and histopathological analysis of lung tissue and trachea.

Histamine and acetylcholine challenge control animals showed dyspnoea and gasping; a sign of bronchospasm within short time. β-sitosterol treated animals produced significant increase in PCD time at higher doses against both the spasmogens as compared to control. However, it failed to show increase in PCD time significantly at a lower dose. 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, we found that β-sitosterol treated animals did not show any change in body weight. 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. The increase in the total and differential cellular count in blood as well as in BAL fluid of OVA-control animals 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, β-sitosterol treatment showed decrease in each cell count in blood and eosinophils and neutrophils count in BAL fluid as compared to OVA-control animals. Moreover, amelioration of inflammatory cell numbers in BAL fluid was confirmed by tracheal and lung tissue histology.

OVA-control animals showed significant increase in level of Th2 type cytokines; TNF-α, IL-4, IL-5 and IL-6 which is suggestive of chronic airway inflammation. Dexamethasone treatment suppressed the level of these mediators significantly. β-sitosterol significantly decrease the level of TNF-α, IL-5 and IL-4 in serum as well as in BAL fluid. However, it did not produce any significant inhibition in the IL-6 level in both serum and BAL fluid. The significant increase in histamine levels in OVA-control animals is also suggestive of lung tissue inflammation. Treatment with dexamethasone and β-sitosterol significantly decreased these levels as compared to OVA-control animals. These reductions in the level of the cytokines as well as histamine may be co-related with mast cell stabilizing activity of the n-butanol fraction. Further, bronchodilatory action of β-sitosterol might be due to its ability to inhibit the release of cytokine production and inflammatory mediator-like histamine. Our histopathological results also suggest that β-sitosterol treatment inhibited leukocyte infiltration and angiogenesis into the airway after ovalbumin challenge. Thus, β-sitosterol has shown bronchodilatory activity 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.

In conclusion our data suggest that seed extracts of M. oleifera produced beneficial effects in antigen induced bronchospasm, mast cell degranulation, immune and anaphylactic reactions. A good co-relation was found between the concentration of marker compounds and pharmacological activities. β-sitosterol appears to be the active principle responsible for the observed pharmacological activities. The antiasthmatic activity of n-butanol fraction and its active principle β-sitosterol might be due to the inhibition of infiltration of inflammatory cells, release/synthesis of cytokines; TNF-α, IL-4, IL-5, IL-6 and mediators like histamine.