

Effect of *Moringa oleifera* Lam. Seed Extract on Ovalbumin-Induced Airway Inflammation in Guinea Pigs

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To determine the therapeutic potential of herbal medicine *Moringa oleifera* Lam. family: *Moringaceae* in the control of allergic diseases, the efficacy of the ethanolic extract of the seeds of the plant (MOEE) against ovalbumin (OVA)-induced airway inflammation in guinea pigs was examined. During the experimental period, the test drugs (MOEE or dexamethasone) were administered by oral route prior to challenge with aerosolized 0.5% OVA. Bronchoconstriction tests were performed and respiratory parameters (i.e., tidal volume and respiratory rate) were measured. At the end of experiment, blood was collected from each animal to perform total and differential counts and serum was used for assay of IL-4, IL-6, and TNF α . Lung lavage fluid (BAL) was collected for estimation of cellular content and cytokine levels. Lung tissue histamine assays were performed using the homogenate of one lobe from each animal; a separate lobe and the trachea were subjected to histopathology to measure the degree of any airway inflammation. The results suggest that in OVA-sensitized control animals that did not receive either drug, tidal volume (V_t) was decreased, respiration rate (f) was increased, and both the total and differential cell counts in blood and BAL fluid were increased significantly. MOEE-treatment of sensitized hosts resulted in improvement in all parameters except BAL TNF α and IL-4. Moreover, MOEE-treatment also showed protection against acetylcholine-induced broncho-constriction and airway inflammation which was confirmed by histological observations. The results of these studies confirm the traditional claim for the usefulness of this herb in the treatment of allergic disorders like asthma.

Allergic asthma is a chronic inflammatory disease associated with predominant Th2 response and immunoglobulin (Ig) E synthesis (Barnes et al., 1998) and affects an estimated 100 million individuals worldwide (Cohn & Ray, 2000). Asthma is characterized by airway inflammation, excess reversible constriction of airway smooth muscles, and airway hyperresponsiveness (AHR) to a wide variety of spasmogens, viz. histamine, acetylcholine, and 5-hydroxytryptamine derived from mast cells (Saria et al., 1983). Pathophysiological features of asthma also show infiltration of inflammatory cells like eosinophils (Wardlaw et al., 1988), neutrophils (Sur et al., 1993), lymphocytes (Sergio, 2000), and macrophages/monocytes (Bousquet et al., 2000). These cells release various inflammatory mediators, including histamine (Liu et al., 1991), cytokines (Chung & Barnes, 1999), leukotrienes (LT), prostaglandins (PG), and thromboxane (TX) (Wenzel et al., 1997; Devillier & Bessard, 1997).

Several studies have also demonstrated elevated levels of histamine in the plasma of patients with asthma (Ind et al., 1983);

similar effects have been noted in the plasma (Masahiro et al., 2002) and lung tissues (Bartosch et al., 1932) of guinea pigs. Many cytokines have been detected in increased quantity in bronchial biopsies from asthmatic subjects (Broide et al., 1992). Specifically, elevated levels of tumor necrosis factor (TNF)- α (Coker & Laurent, 1998), interleukin (IL)-4 (Gharaee-Kermani et al., 2001), and IL-6 (Elias et al., 1997) have been noted in the airways of patients with asthma (Robinson et al., 1992). Furthermore, recent studies have showed the presence of these mediators in bronchoalveolar lavage fluid (BAL) and urine in asthmatic patients after allergen challenge (Hui et al., 1991).

In our previous studies, we have investigated the activity of *Moringa oleifera* ethanolic extract (MOEE) in chemical (i.e., toluene diisocyanate-TDI)-induced immune-mediated inflammatory responses in rats (Mahajan et al., 2007b). Further, to find out the mechanism of action of antiasthmatic drugs, ovalbumin-sensitized guinea pigs have been preferred as an animal model in studying human allergic asthma. Ovalbumin challenge in sensitized animals evoked anaphylactic reactions, which result from the action of various mediators such as histamine, leukotrienes, prostaglandins, thromboxane A_2 , and platelet-activating factor from inflammatory cells (Ogunbiyi & Eyre, 1985). To this goal, we have selected this model in the present study to reveal the

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potential treatment of immune-mediated airway inflammation in guinea pigs with an herbal extract, MOEE, which is a product we have previously investigated. We found that the extract has inhibiting activity for cytokines and also prevents eosinophilia and neutrophilia.

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 & 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, 1991) and is also used for the treatment of ascites, rheumatism, venomous bites, and for enhancing cardiac function (Nadkarni & Nadkarni, 1976; Chaudhri, 1996). The leaves exhibit strong hypotensive, diuretic, and spasmolytic effects, and have been seen to be useful against inflammation, in anaphylaxis (Mahajan et al., 2006), in helminthiasis, and in scurvy (Caceres et al., 1992). 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).

The seeds of the plant have been used as purgatives, antipyretics, and as anti-inflammatory agents (Varier, 1997). We have previously reported on the anti-arthritic (Mahajan et al., 2007a), anti-asthmatic (Mahajan et al., 2007b), and anti-anaphylactic (Mahajan & Mehta, 2007) activity of the ethanolic extract of these seeds in rats. Guevara et al. (1999) noted the antigenotoxic activity of these extracts. Ayurvedic practitioners used to administer 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 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 has been seen to be useful in the treatment of asthma; alkaloid *Moringine* relaxes bronchioles (Kirtikar & Basu, 1975).

MATERIALS AND METHODS

Plant Material and Extract Preparation

Seed kernels of *M. oleifera* were obtained from a commercial supplier of Ahmedabad and were identified and authenticated by Department of Pharmacognosy, L. M. College of Pharmacy, Ahmedabad, India. Voucher specimens were deposited at the Department of Pharmacognosy, Ahmedabad.

The coarse powder (500 g) of the dried seed kernels was defatted using petroleum ether (60–80°C) and then it was exhaustively extracted using 95% ethanol (2 L) in a Soxhlet extractor at 55°C for 6 h. The extract was concentrated under reduced pressure to

yield a syrupy mass. The extract was stored in airtight container in cool place and used throughout the study. Freshly prepared solution of extract was used for the treatment of animals.

Phytochemical Analysis

Preliminary phytochemical studies of the extract were performed for alkaloids, flavanoids, saponins, glycosides, phenols, steroids, tannins, and terpenoids according to published standard methods (Peach & Tracey, 1955).

Drugs and Chemicals

All solvents and chemicals were obtained from the S.D. Fine Chemicals Private Limited (Mumbai, India) and all were of analytical grade. *o*-Phthalaldehyde (OPT), acetylcholine, histamine, ovalbumin, and dexamethasone were obtained from Sigma-Aldrich (St. Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kits for TNF α (BD Biosciences, San Diego, CA), IL-4 (Immunotech, Marseille Cedex, France), and IL-6 (R & D Systems, Minneapolis, MN) were purchased for cytokine assay.

Animal Husbandry and Feeds

Specific-pathogen-free male Dunkin-Hartley guinea pigs (300–600 g) were housed in a room maintained at 22 $\pm$ 1°C with a relative humidity of 55 $\pm$ 5% and a 12-h light–dark cycle. Animals had free access to standard pellet diet (certified Amrut brand rodent feed, Pranav Agro Industries, Pune, India) and filtered tap water. All experiments were carried out with strict adherence to ethical guidelines and were conducted as per protocols approved by the Institutional Animal Ethics Committee (IAEC) and as per Indian norms laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi. Throughout the entire study period, the animals were monitored for growth, health status, and food intake capacity to be certain that they were healthy.

Sensitization and Treatment of Animals

Guinea pigs used in the study were divided into four different groups of six animals each: group I, nonsensitized controls provided distilled water; group II, OVA-sensitized controls provided distilled water; group III, OVA-sensitized and then received dexamethasone (DXM; 2.5 mg/kg body weight [BW] per os) as reference standard; and group IV, OVA-sensitized and then received MOEE (ethanolic extract of seeds of *M. oleifera*; 200 mg/kg per os). All the drugs were administered daily by oral route and doses were selected based upon our previous report (Mahajan et al., 2007a).

All animals (except group I) were sensitized by the method of Duan et al. (2003) using a subcutaneous injection of 100 μ g ovalbumin (that had been adsorbed onto aluminum hydroxide in saline) on day 0 as the first sensitization. Boosting was then carried out using the same dose of antigen two weeks later (i.e., on day 14). The daily doses of drug or vehicle were initiated on day 18 and continued until day 29.

Ovalbumin Exposure

On days 18 through 29, the animals were challenged with aerosolized 0.5% OVA for 10 min at 2.5 h after receiving their drug or vehicle treatment. For the challenge, conscious animals were placed into a plastic circular chamber (diameter = 70 cm, height = 40 cm) connected to a nebulizer (CX4-Omron Healthcare Company Ltd., Kyoto, Japan). Animals in the nonsensitized group (group I) were exposed to aerosolized saline using the same protocols.

Measurement of Lung Function

On days 18, 21, 24, and 29, all guinea pigs were subjected to lung function parameter measurements 2 h after the 10 min of OVA exposure. Tidal volume and respiration rate in the animals were measured (in the conscious state) using a data acquisition system (model MP-34, Biopac System, Inc., Santa Barbara, CA).

Bronchoconstriction Test

Immediately after the measurement of the lung function parameters, all animals were subjected to a bronchoconstriction test. All OVA-sensitized hosts were exposed (in conscious state) to 0.25% acetylcholine (ACH) solution for 30 s using a nebulizer (CX4-Omron Healthcare Company Ltd., Kyoto, Japan) connected to the animal holder. Guinea pigs in the nonsensitized control group were exposed to normal saline in place of ACH. In each case, tidal volume and respiration rate of each animal were noted before and after the exposure.

Cellular Count and Serum Preparation

At the end of experiment (i.e., day 30), blood (3 ml) was collected from each animal under light ether anesthesia. Each sample was then divided into two portions. The first aliquot (2.5 ml) was placed in a nonheparinized tube for serum separation; the isolated serum was stored at -80°C for later quantitative determinations of levels of TNF α , IL-4, and IL-6. The second portion (0.5 ml) was placed in a heparinized tube and used for total and differential leukocyte counts. Total leukocytes were counted after the blood was diluted at 1:10 with 1% acetic acid to lyse red blood cells. Detailed procedures for total and differential leukocyte counts were the same as those outline for the BAL fluid (see next subsection).

Bronchoalveolar Lavage (BAL)

For the collection of BAL fluid, ketamine (30 mg/kg) and xylazine (20 mg/kg) were subcutaneously administered to each animal to induce anaesthesia. A polypropylene cannula was then inserted into the trachea and 0.9% (w/v) normal saline solution (10 ml) at 37°C was introduced into lungs via a 10-ml syringe and then recovered 5 min later. The trachea was subsequently dissected out for histological analyses. The recovered lavage fluid (5 ml) was centrifuged at $500 \times g$ for 10 min at 4°C ; the resultant supernatant was collected and stored at -80°C until used

in cytokine determinations. The cells in the pellet were washed in 0.5 ml saline and total cell counts were then performed in an automated cell counter (Cell Dyne 3500, Abbott Laboratories, New York). In order to perform differential analyses, aliquots of the cells were placed onto slides and then stained with Field's stain. After drying, 300 cells/slide were counted using a compound microscope (Optima X5Z-H) at $400\times$ magnification and cells identified as eosinophils, lymphocytes, macrophages, or neutrophils using standard morphologic determinants.

Measurement of Cytokines in Serum and BAL Fluid

The levels of TNF α , IL-4, and IL-6 in recovered serum and BAL samples were measured using enzyme-linked immunosorbent assay (ELISA) kits. After following the manufacturer's protocol, all plates were analyzed on an automated plate reader (Lab System Multiscan model 51118220, Thermo Bioanalysis Co., Helsinki, Finland). The detection limit of the kits for TNF α , IL-4, and IL-6 were <15 pg/ml, <5 pg/ml, and <10 pg/ml, respectively.

Histamine Assay in Lavaged Lung Tissue

Lung tissue lobes from each animal were dissected out separately immediately following BAL fluid collection. One lobe was utilized for assay of nonlavagable histamine and another for histology of lavaged tissue. With the former, lung tissue (200 g) was placed in 2.5 ml normal saline and then 2.5 ml of 0.8 *N* perchloric acid was added. After mixing and then centrifugation ($4000 \times g$, 7 min at 4°C), 2 ml of the resulting supernatant was transferred to a test tube containing 0.25 ml of 5 *N* NaOH, 0.75 g NaCl, and 5 ml *n*-butanol. The mixture was vortexed for 5 min to localize histamine into the butanol, then centrifuged. The aqueous phase was discarded by aspiration and the organic phase was washed with 2.5 ml salt-saturated 0.1 *N* NaOH solution to remove any residual histidine. The mixture was recentrifuged and the butanol relocated to a test tube containing 2 ml of 0.1 *N* HCl and 5 ml *n*-heptane. The mixture was again centrifuged and the presence of histamine determined fluorometrically (SL-174, Elico, India) by the method of Shore et al. (1959).

Histological Examination

Histopathologic analyses of the lavaged lung tissues and trachea obtained from each guinea pig were carried out to study the effect of extract on the immune-mediated airway inflammation brought about by sensitization with OVA. Dissected lungs and trachea were washed with normal saline and then placed in 10% formaldehyde solution for 1 wk. After the tissues were fixed, specimens of lungs and trachea were embedded in paraffin wax, and 5- μm sections were cut and stained with hematoxylin and eosin dye for morphology. Images of selected sections were captured at $10\times$ and $40\times$ magnifications using a zoom digital camera (model C763, Eastman Kodak Company, Rochester, NY).

Statistical Analysis

Results were reported as mean $\pm$ SEM. Statistical analysis was performed using a one-way analysis of variance (ANOVA). If the overall F value was found to be statistically significant (i.e., $p < .05$), further comparisons among groups were made using the post hoc Tukey's test. Though data was considered statistical significant at $p < .05$. When data were found to be very (i.e., $p < .01$) or highly (i.e., $p < .001$) significant, this was indicated in the results. All statistical analyses were performed using Graph Pad software (San Diego, CA).

RESULTS

Phytochemical Study

The preliminary phytochemical screening of the ethanolic extract of *M. oleifera* showed the presence of alkaloids, flavanoids, steroids, glycosides, tannins, and terpenoids (Tables 1 and 2).

Effect of MOEE on Body Weight

All animals in the control OVA-sensitized (group II) and drug regimen groups (groups III and IV) did not display any significant differences in body weight as compared to those of the non-sensitized controls during the experimental period (data not shown). Furthermore, there were no apparent effects upon appetite/water consumption or upon the outward appearance (i.e., fur coat, eyes) of these various animals in each treatment group.

TABLE 1

Preliminary phytochemical study on *M. oleifera* seed extract

Tests	Observation
Alkaloids	
Wagner's test	++
Mayer's test	++
Flavonoids	
Dilute HCl	+ + +
Glycosides	
Bromine test	++
Cupraloin test	++
Saponins	
Sodium bicarbonate	—
Polyphenols	
Ferric chloride test	—
Lead acetate test	—
Steroids	
Liebermann–Burchard's test	+
Salkowski test	+
Tannins	
Ferric chloride test	+
Lead acetate test	+
Triterpenoids	
Acetic anhydride test	+ + +

Note. +, Opaque; ++, turbidity; + + +, precipitation; —, absent.

Effect of MOEE on Lung Function Parameters

During the experimental period (i.e., specifically, on days 18, 21, 24, and 29), lung function parameters in each guinea pig were measured. While no significant effects were observed on days 18, 21, or 24, it was noted on day 29 that the tidal volume was decreased significantly ($p < .01$) and respiration rates increased very significantly ($p < .001$) in OVA-sensitized animals as compared to values in nonsensitized controls. In contrast, on this same day, the DXM-, and MOEE-treated animals presented values for each of these parameters that were comparable to those of the non-sensitized hosts (Tables 3 and 4).

Effect of MOEE on Circulating Cellular Count

Compared to levels in nonsensitized controls, the number of total leukocytes ($p < .001$), eosinophils ($p < .001$), and neutrophils ($p < .01$) were increased significantly, while those of lymphocytes ($p < .001$) and monocytes ($p < .01$) were decreased significantly, in OVA-sensitized control hosts (group II). As with several of the other parameters noted earlier, the treatments with MOEE or DXM resulted in values for these endpoints not significantly different from the nonsensitized controls, i.e., the numbers of circulating eosinophils and neutrophils were significantly decreased, and those of lymphocytes and monocytes significantly increased, in MOEE- and DXM-treated animals as compared to levels in the OVA-sensitized controls (Table 5).

Effect of MOEE on Inflammatory Cellular Responses in the BAL Fluid

As demonstrated in Table 6, analysis of the BAL fluid from the OVA-sensitized controls indicated that levels of total cells, eosinophils, and neutrophils were all significantly elevated ($p < .001$, $p < .001$, and $p < .01$, respectively), and those of lymphocytes and macrophages significantly decreased as compared to values in the BAL of group I nonsensitized controls. However, treatment with DXM or MOEE induced significant reductions in these counts relative to values seen in the vehicle-treated OVA-sensitized guinea pigs (Table 6).

Effect of MOEE on Cytokine Production in Serum

Select cytokines were assayed to ascertain whether MOEE treatment modulated cytokine production after OVA sensitization. TNF α , IL-4, and IL-6 levels (Figure 1) in the serum of OVA-sensitized control animals were all highly significantly ($p < .001$) increased compared to those in nonsensitized hosts. Interestingly, treatment with DXM and MOEE caused significant reductions in the levels of each of these cytokines relative to those in the group II hosts.

Effect of MOEE on Cytokine Production in BAL Fluid

The concentrations of BAL TNF α , IL-4, and IL-6 (Figure 2) in OVA-sensitized guinea pigs were highly significantly ($p < .001$) greater than those in the nonsensitized controls. The DXM

TABLE 2
List of major phytochemical constituents of the seeds of *M. oleifera*

Active principle/s (isolated/reported from seed extract)	Reported activity	Reference
<i>Alkaloid</i> : Moringine, moringinine	Relaxes bronchioles	Kirtikar and Basu, 1975
<i>Flavonoids</i> : Tocopherols, vitamin C and different polyphenolic compounds	Antioxidant	Laandrault et al., 2001
<i>Glycosides</i> : Moringyne, benzylisothiocyanate, its derivatives (a glycoside of mustard oil) 4 (4'-acetyl-L-L-rhamnosyloxy)-benzoylthiocyanate	Antibiotic External counterirritant property	Memon et al., 1985 Das et al., 1958
4-[α -L-Rhamnosyloxy], phenylacetone nitrile, 4-hydroxyl phenylacetone nitrile and 4-hydroxyphenylacetamide	As mutagens	Villasenor et al., 1989 Villasenor et al., 1990 Villasenor, 1994
4-[α -L-Rhamnosyloxy]	Antibiotic	Dayrit et al., 1990
4-[α -L-Rhamnosyloxy] phenyl acetone nitrile β -carotene, β -sterols and lecithin	Antimicrobial	Njoku and Adikwu, 1997
<i>O</i> -Ethyl-4-(α -L-rhamnosyloxy)benzylcarbamate together with seven known compounds like niazimicin, niazirin, β -sitosterol, glycerol-1-1-(9-octadecanoate), 3- <i>O</i> -(6'- <i>O</i> -oleoyl- β -D-glucopyranosyl)- β -sitosterol, and β -sitosterol-3- <i>O</i> - β -D-glucopyranoside	Antitumor promoting activity	Guevara et al., 1999
Thiamine, riboflavin, nicotinic acid, folic acid, pyridoxine, ascorbic acid, and α -tocopherol	As nutritive, antioxidant	Dahot and Memon, 1985
Monopalmitic acid, oleic acid, tri-oleic triglycerides were isolated from seeds and oil	—	Memon and Khatri, 1987

and MOEE treatment regimen did cause significant ($p < .05$) reductions in IL-6 levels compared to the OVA-sensitized controls, but there was no effects from the MOEE on TNF α and IL-4 levels.

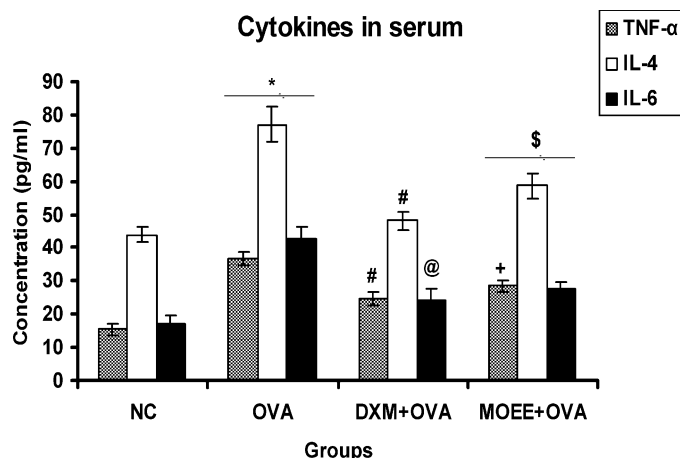


FIG. 1. Effect of treatments on serum cytokine levels of guinea pigs. Value is significantly different from non-sensitized control (* $p < .001$; + $p < .05$). Value different from Group II OVA-sensitized vehicle-treated controls (@ $p < .001$; # $p < .01$; \$ $p < .05$). All bars represent the mean $\pm$ SEM from $n = 6$ guinea pigs/treatment group.

Effect of MOEE on Histamine Levels

To confirm the role that effects from the treatments upon nonlavagable mast cells and their associated release of histamine might have in the observed responses, histamine levels in lung homogenates of the earlier lavaged lungs were assayed (Figure 3). Treatment with MOEE yielded a significant ($p < .05$) decrease in the degree of elevated histamine release that was seen in the group II OVA-sensitized control guinea pigs. These findings suggest that oral MOEE administration might exert anti-allergic and bronchodilatory activities by causing reductions in the release of histamine (which, in turn, affords protection against acetylcholine-induced bronchoconstriction).

Effect of MOEE on Acetylcholine-Induced Bronchoconstriction

When all OVA-sensitized animals were exposed to the aerosol of 0.25% ACH for 30 s on days 18, 21, 24, and 29, bronchospasm in the form of preconvulsion dyspnea (PCD) was evinced. However, significant changes in tidal volume ($p < .01$) and respiration rate ($p < .001$) were observed on day 29 only (data not shown) in OVA-sensitized animals. The graphical presentation shows the change in the pattern of lung function in OVA-sensitized control animals (Figure 4).

Histopathology Results

For histopathological observations two sections from each slide were observed showing maximum features of asthma.

TABLE 3
Effect of MOEE on tidal volume of guinea pigs

Groups	Tidal volume (Vt), ml/s			
	Day 18	Day 21	Day 24	Day 29
Nonsensitized control (distilled water)	3.00 ± 0.22	2.80 ± 0.19	2.81 ± 0.15	2.70 ± 0.16
OVA-control (distilled water)	3.28 ± 0.18	2.32 ± 0.22	2.29 ± 0.25	1.81 ± 0.15*
OVA+ DXM (2.5 mg/kg)	3.35 ± 0.36	2.85 ± 0.18	3.32 ± 0.20 ^{\$}	3.00 ± 0.20 [@]
OVA+ MOEE (200 mg/kg)	3.03 ± 0.13	2.36 ± 0.25	2.91 ± 0.26	2.91 ± 0.12 [@]

Note. Values are expressed as mean ± SEM ($n = 6/\text{regimen}$). Value is significantly different from nonsensitized control (* $p < .01$). Value different from group II OVA-sensitized vehicle-treated controls ([@] $p < .001$; ^{\$} $p < .05$).

TABLE 4
Effect of MOEE on respiration rate of guinea pigs

Groups	Respiration rate (f), breaths/min			
	Day 18	Day 21	Day 24	Day 29
Nonsensitized control (distilled water)	94.10 ± 5.20	99.50 ± 4.60	93.66 ± 6.00	101.80 ± 4.56
OVA-control (distilled water)	103.50 ± 4.73	92.23 ± 5.25	82.61 ± 3.60	122.00 ± 8.10*
OVA+ DXM (2.5 mg/kg)	93.73 ± 4.75	100.18 ± 5.15	99.30 ± 6.31	97.71 ± 5.20 [#]
OVA+ MOEE (200 mg/kg)	92.10 ± 5.80	106.90 ± 6.81	103.20 ± 6.05	90.16 ± 4.20 ^{\$}

Note. Values are expressed as mean ± SEM ($n = 6/\text{regimen}$). Value is significantly different from non-sensitized control (* $p < .01$). Value different from group II OVA-sensitized vehicle-treated controls ([#] $p < .01$; ^{\$} $p < .05$).

TABLE 5
Effect of MOEE on total cells and differential leukocyte count in blood ($\times 10^3$ cells/ml)

Groups	Total cells	Eosinophils	Neutrophils	Lymphocytes	Monocytes
Nonsensitized control (distilled water)	4.260 ± 0.499	0.012 ± 0.002	0.038 ± 0.002	0.030 ± 0.002	0.018 ± 0.002
OVA-control (distilled water)	6.550 ± 0.691 ^{*c}	0.030 ± 0.002 ^{*c}	0.054 ± 0.002 ^{*b}	0.007 ± 0.002 ^{*c}	0.007 ± 0.002 ^{*b}
OVA + DXM (2.5 mg/kg)	4.793 ± 0.823 [#]	0.017 ± 0.003 [#]	0.030 ± 0.002 [@]	0.027 ± 0.002 [@]	0.024 ± 0.003 [@]
OVA+ MOEE (200 mg/kg)	4.940 ± 0.903 [#]	0.020 ± 0.002 ^{\$}	0.037 ± 0.003 [#]	0.022 ± 0.003 [@]	0.017 ± 0.002 ^{\$}

Note. Values are expressed as mean ± SEM ($n = 6/\text{regimen}$). *Value is significantly different from non-sensitized control (^b $p < .01$; ^c $p < .001$). Value different from group II OVA-sensitized vehicle-treated controls ([@] $p < .001$; [#] $p < .01$; ^{\$} $p < .05$).

TABLE 6
Total cells and differential leukocyte counts in BAL fluid ($\times 10^3$ cells/ml)

Groups	Total cells	Eosinophils	Neutrophils	Lymphocytes	Macrophages
Nonsensitized control (distilled water)	1.206 ± 0.075	0.012 ± 0.001	0.019 ± 0.003	0.032 ± 0.003	0.036 ± 0.002
OVA-control (distilled water)	2.268 ± 0.191 ^{*c}	0.028 ± 0.003 ^{*c}	0.033 ± 0.003 ^{*b}	0.022 ± 0.003 ^{*a}	0.022 ± 0.002 ^{*a}
OVA + DXM (2.5 mg/kg)	1.458 ± 0.198 [#]	0.017 ± 0.002 [#]	0.021 ± 0.002 ^{\$}	0.033 ± 0.005 ^{\$}	0.028 ± 0.002
OVA + MOEE (200 mg/kg)	1.615 ± 0.123 ^{\$}	0.019 ± 0.001 [#]	0.023 ± 0.002	0.029 ± 0.004	0.028 ± 0.004

Note. Values are expressed as mean ± SEM ($n = 6/\text{regimen}$). *Value is significantly different from non-sensitized control (^a $p < .05$; ^b $p < .01$; ^c $p < .001$). Value different from group II OVA-sensitized vehicle-treated controls ([#] $p < .01$; ^{\$} $p < .05$).

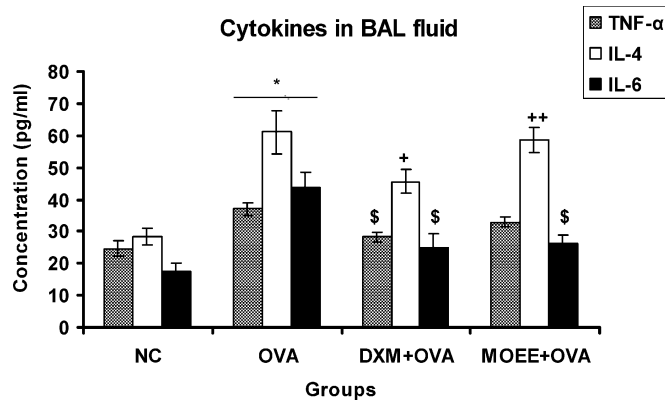


FIG. 2. Effect of treatments on BAL fluid cytokine levels of guinea pigs. Value is significantly different from non-sensitized control (* $p < .001$; ** $p < .01$; + $p < .05$). Value different from Group II OVA-sensitized vehicle-treated controls (\$ $p < .05$). All bars represent the mean $\pm$ SEM from $n = 6$ guinea pigs/treatment group.

In the group II OVA-sensitized vehicle-treated guinea pigs, a prominent infiltration of inflammatory cells, reduced lumen size, epithelial shedding, and angiogenesis were detected in their lavaged lung tissues and tracheal sections. Both drug treatment regimens appeared to provide an improvement in that these hosts only displayed greatly reduced or only mild pathological changes in their lung tissue and tracheal sections (Figure 5).

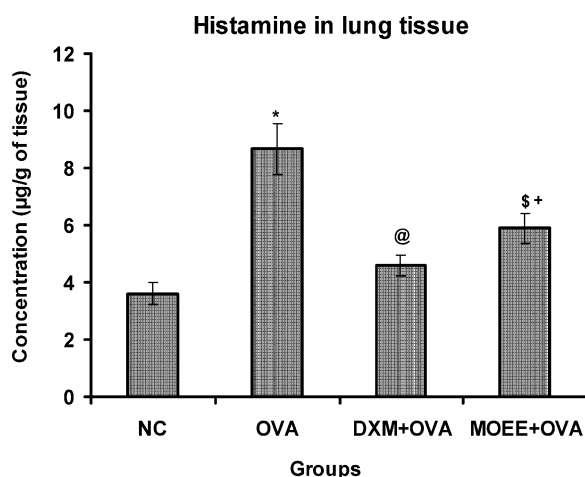


FIG. 3. Effect of treatments on lung tissue histamine levels of guinea pig. Value is significantly different from non-sensitized control (* $p < .001$; + $p < .05$). Value different from Group II OVA-sensitized vehicle-treated controls (@ $p < .001$, \$ $p < .05$). All bars represent the mean $\pm$ SEM from $n = 6$ guinea pigs/treatment group.

DISCUSSION

Complementary and alternative medicine (CAM) has been used to treat asthma for hundreds of years (Chung & Adcock, 2000). 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, 1997b). Some Ayurvedic drugs of interest for consideration in asthma include *Datura* plants (Ziment & Tashkin, 2000), *Albizia lebbek* (Tripathi & Das, 1977), *Tinospora asthmatica* (Nayampalli et al., 1986), *Solanum xanthocarpum* (Chitravan-shi et al., 1990), *Vitex negundo* (Nair & Saraf, 1995), *Tephrosia purpurea* (Gokhale et al., 2000), *Cissampelos sympodialis* (Bezerra-Santos et al., 2004), 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 the light of the facts just listed, as a part of our continuing search for biologically active anti-allergic agents from the medicinal resources, to amplify our knowledge on the suitability of the use of *Moringa oleifera* extract for the treatment of an allergic diseases, we evaluated the safety and efficacy of MOEE in ovalbumin-sensitized guinea pigs. To study the safety of the extract, we carried out pilot toxicity studies; MOEE showed no toxic effect at doses up to 1.6 g/kg. This report here is the first to examine the effects of this herbal plant in OVA-induced airway inflammation in guinea pigs to reveal its potential usefulness in the prevention and/or amelioration of symptoms of allergic asthma.

In diseased animals, a significant decrease in the tidal volume is suggestive of bronchoconstriction due to airway inflammation that resembles asthmatic conditions. All the animals in the OVA-sensitized group exhibited irritability, sneezing, and hyper-rhino-rhea. The significant increases in respiration rate observed in diseased animals were indicative of exertional breathing, a symptom of asthma. Further, protection against acetylcholine-induced bronchoconstriction in drug-treated animals shows the bronchodilatory effect of the extract. The nonsensitized control and DXM- and MOEE-treated animals did not show any signs of airway abnormality. Treatment with MOEE shows a protective effect by normalizing the level of these parameters. We showed that orally treated animals did not show any change in loss in body weight, suggesting that the extract did not interfere with the normal growth of the animals and toxic effects.

Various inflammatory mediators, released through the activation of many inflammatory and structural cells, result in the typical pathophysiologic changes of asthma (Barnes, 1996; Barnes et al., 1998). Eosinophilic inflammation of the airways, with an increase in activated and degranulated eosinophils, is the key feature of both allergic and nonallergic asthma (Bousquet et al., 1990). Activated eosinophils can release a variety of cationic proteins, including major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and eosinophil derived neurotoxin (EDN); there is evidence from

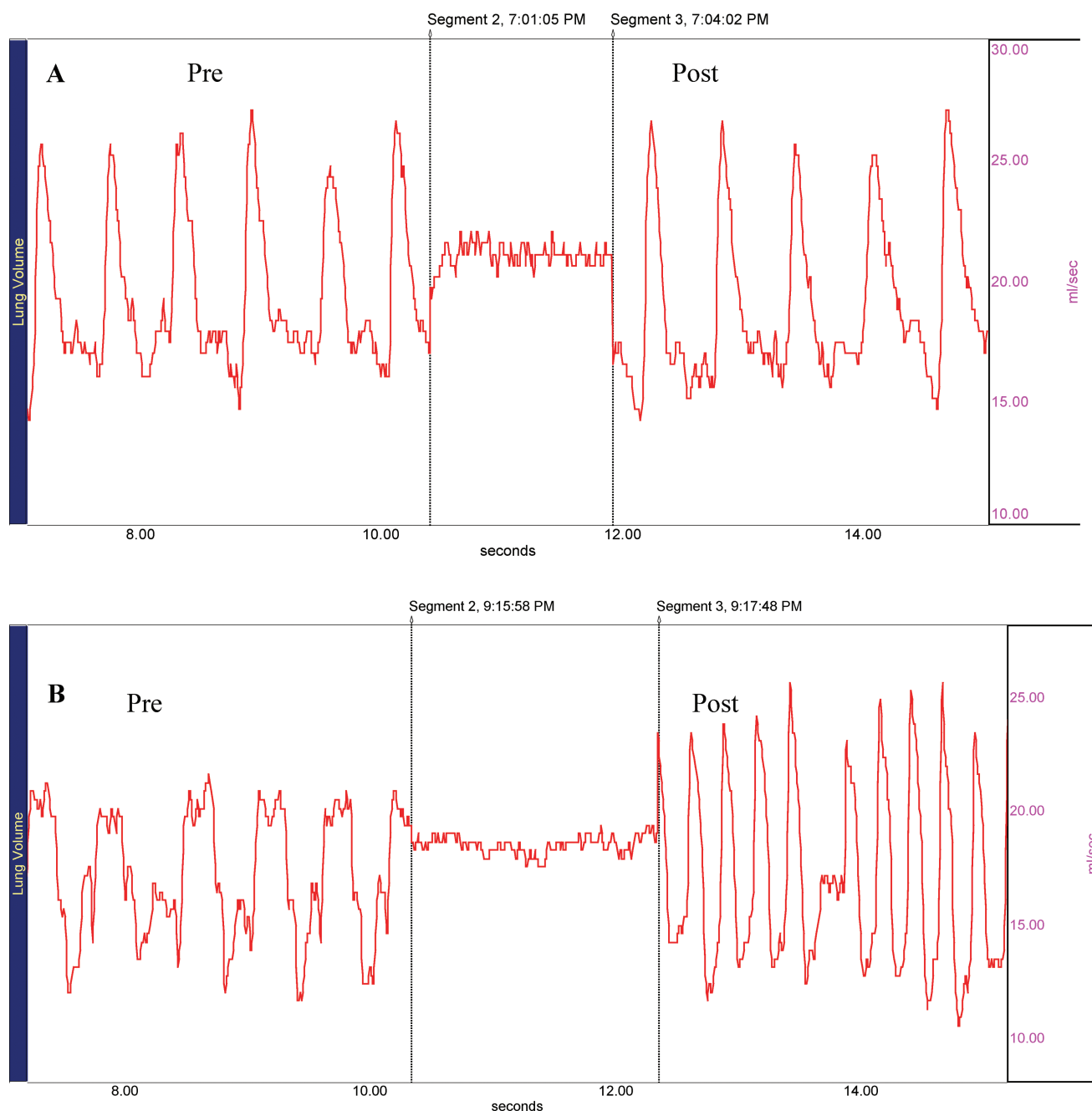


FIG. 4. Effect of MOEE on acetylcholine induced bronchoconstriction in guinea pigs. Representative tracings showing the changes in pre- and posttidal volume (V_t) and respiration rate (f) on bronchoconstriction induced by acetylcholine in guinea pigs: (A) nebulization with saline aerosol to nonsensitized control animals, (B) OVA-sensitized control animals, (C) DXM-treated animals, and (D) MOEE-treated animals sensitized with OVA. All animals present in these groups (except group I) were challenged with 0.5% of OVA followed by 0.25% acetylcholine for 30 s, which produces bronchoconstriction. (Continued)

a number of studies that these proteins can damage the respiratory epithelium (Motojima et al., 1989; Frigas et al., 1980). Recent evidence has indicated a possible correlation between activation status of eosinophils and immune-mediated responses (Filipovic & Snezana, 2001). It has been shown in guinea pigs

(Pretolani et al., 1994), nonhuman 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. Further, neutrophilic inflammation in the airway is also increasingly

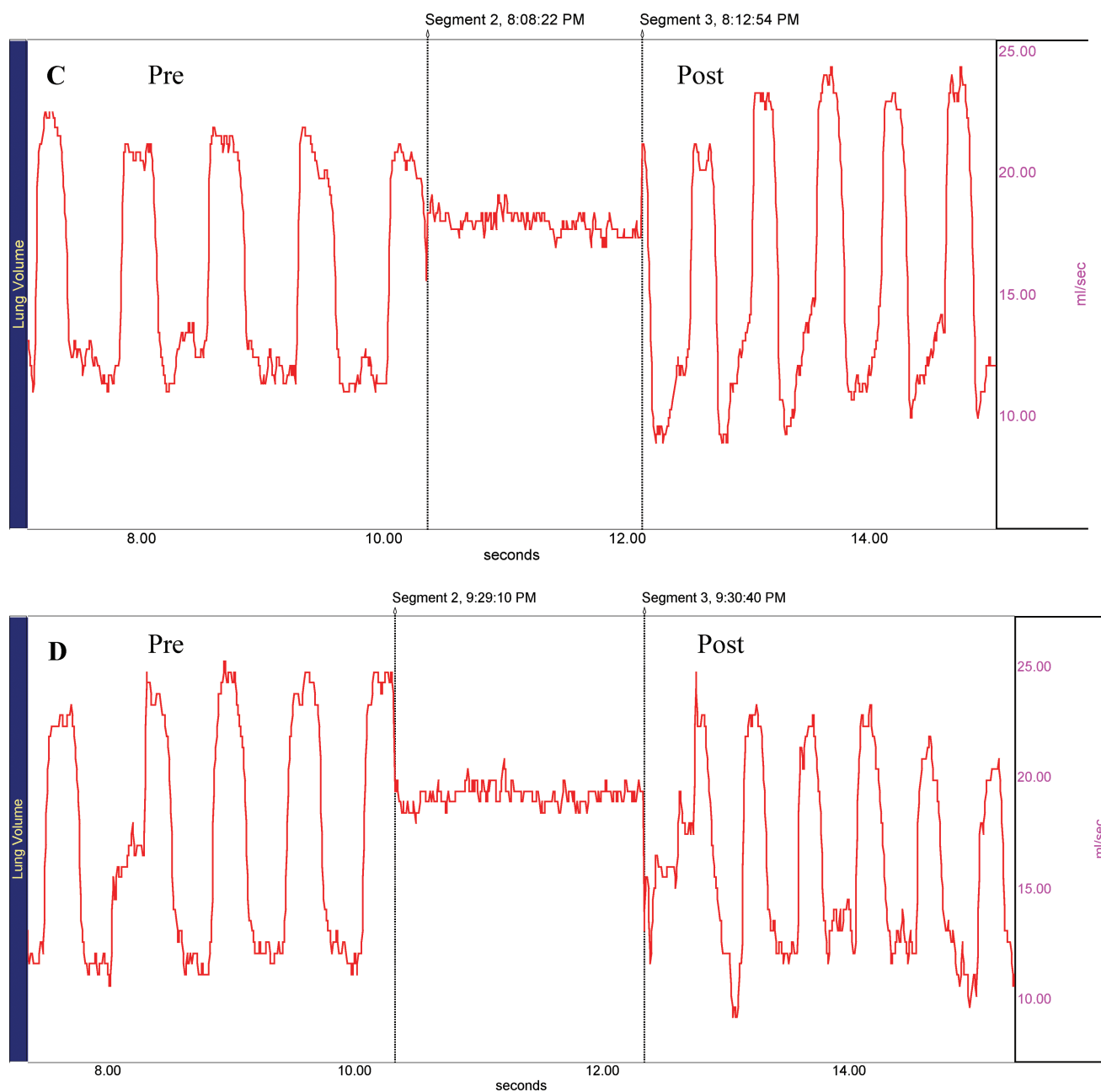


FIG. 4. (Continued)

recognized in acute exacerbations of asthma and in status asthmaticus (Sur et al., 1993; Ordonez et al., 2000). In our study, we found the significant increase in number of these cells in blood as well as in BAL fluid of OVA-sensitized diseased control animals. However, these cell numbers, especially eosinophils and neutrophils, decrease significantly; lymphocytes and monocytes/macrophages were found to be increased significantly by MOEE treatment. Further, the attenuation of inflammatory cell numbers in BAL fluid was mirrored by tracheal and lung tissue histology. Therefore, these results provide the evidence that

MOEE treatment may be useful in controlling the activation of the inflammatory processes underlying the exacerbation of allergic asthma.

Cytokines play a critical role in orchestrating and perpetuating inflammation in asthmatic airways, and several specific cytokine and chemokine inhibitors are now in development for the treatment of asthma (Chung & Barnes, 1999). Among the various cytokines, T-lymphocyte-derived $\text{TNF}\alpha$, IL-6, and Th2-type cytokine IL-4, in particular, have significant roles in the asthmatic state (Ricci et al., 1994). The multifunctional

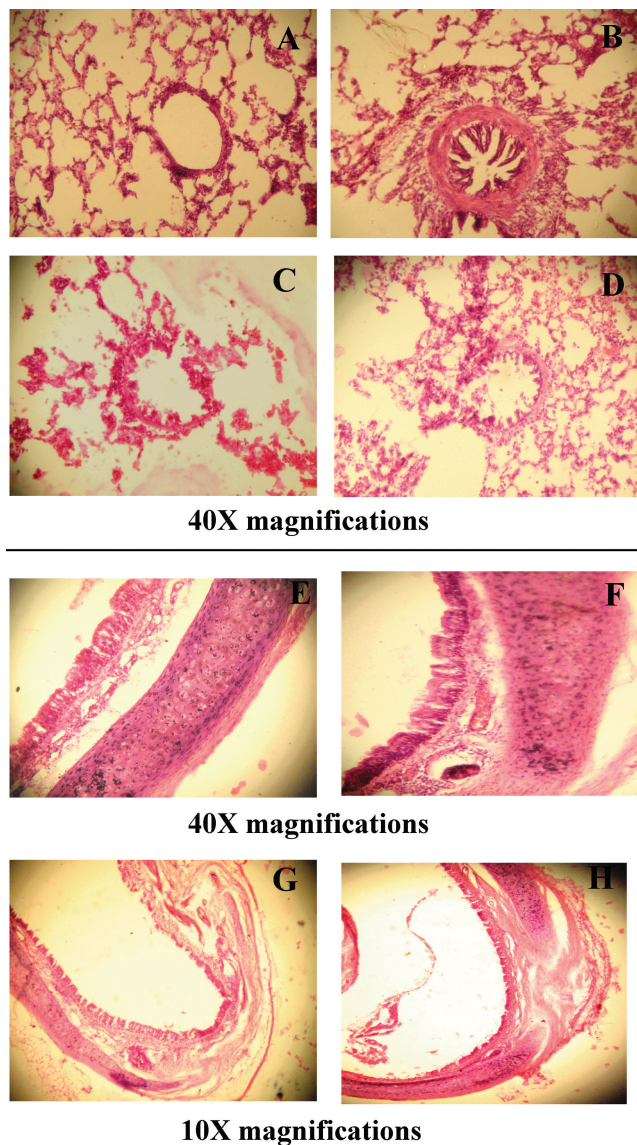


FIG. 5. Histopathological effects from the various treatments in guinea pigs lung and trachea. Lavaged lung tissue and tracheal sections of treated and untreated OVA-sensitized animals stained with hematoxylin-eosin. Top panels show sections from lung tissue while lower panels show sections of trachea. (A) and (E): Normal lung and trachea. (B) and (F): typical damaged lung and tracheal tissue from OVA-control group with total and differential leukocyte infiltration (eosinophils), reduced lumen size, endothelial shedding and angiogenesis. (C) and (G): sections from DXM-treated animals. (D) and (H): sections from MOEE-treated animals.

proinflammatory $\text{TNF}\alpha$ may have an important amplifying effect in immune-mediated inflammation (Kips et al., 1992). There is evidence for increased $\text{TNF}\alpha$ expression in the airways of asthmatics (Bradding et al., 1994), and IgE triggering in sensitized lungs leads to increased $\text{TNF}\alpha$ expression in epithelial

cells in both guinea pig and human lungs (Ohno et al., 1990; Ohkawara et al., 1992). Elevated levels of $\text{TNF}\alpha$ have also been detected in the sputum, bronchoalveolar lavage fluid, and biopsy samples from asthmatics (Broide et al., 1992). By activating the secretion of cytokines from various cells in the airways, $\text{TNF}\alpha$ may also be an important mediator in the initiation of chronic inflammation (Hughes et al., 1995). Recent studies indicate a predominance of Th2 cell-derived cytokines such as IL-4 in the pathogenesis of bronchial asthma (Anderson & Coyle, 1994). IL-4 was reported to be released by basophils; mast cells and eosinophils exert a wide range of effects 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. IL-6 has been reported to have effects 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. There is also evidence for increased release of IL-6 from alveolar macrophages from asthmatic patients after allergen challenge, as compared to cells from 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 & Firestein, 1991).

A result of our study suggests that levels of all inflammatory cells and cytokines that are increased predominantly in OVA-sensitized animals were suppressed significantly by drug treatment. 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 IL-6. In contrast, it seemed that MOEE did not have an ability to significantly reduce the levels of $\text{TNF}\alpha$ and IL-4 in BAL fluid, even though the TNF levels in the MOEE-treated hosts were much closer to those seen in DXM-treated guinea pigs than the sensitized controls.

The mast cells are thought to play a major role in the development of many physiologic changes during allergic responses (Theoharidesa & Cochraned, 2004). 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) were the first to report that histamine is released from guinea pig lung during anaphylaxis. Acute release of histamine following an allergic or nonallergic insult may lead to bronchoconstriction, which can be attenuated by selective H_1 -receptor antagonists (Holgate & Finnerty, 1989).

With regard to effects on inflammatory cells that increase in numbers during the host response to the antigen, histamine has been shown to activate eosinophils (Raible et al., 1994). In the present study, the expected increase in histamine levels and number of eosinophils due to sensitization with ovalbumin itself were in fact significantly decreased in the MOEE-treated animals. This suggests that the extract might have some

efficacy in inhibiting the release of inflammatory mediator-like histamine, in part by causing a downregulation in the levels of histamine-producing cells.

The results of this study were consistent with the work of several other investigators in determining the effectiveness of selected natural products against inflammation-related pathologies in the lungs. For example, Singh et al. (2000) demonstrated that *Elaeocarpus sphaericus* fruits possessed both mast-cell stabilizing activity and an efficacy against bronchial asthma, it shows the presence of glycoside, steroids, alkaloid and flavanoids, and β -sitosterol. *Ganoderma tsugae* supplementation alleviates bronchoalveolar inflammation in an airway sensitization and challenge mouse model (Lin et al., 2006). Further, Ishimitsu et al. (2001) reported an effect of a traditional Japanese medicine, Bu-zhong-yi-qi-tang, on allergic asthma in mice. Recently, Christofidou-Solomidou and Muzykantov (2006) proved that ascorbic acid is the most extensively investigated antioxidant for effects on asthma and that it 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 on exercise-induced bronchoconstriction have shown a protective effect (Cohen et al., 1997).

In this report, we describe the potential mode of action used by a crude ethanolic extract of *M. oleifera* seeds to bring about anti-inflammatory activity in situ by investigating its efficacy against T-cell-derived cytokine production and subsequent cytokine-induced cellular infiltration, its bronchodilatory potential (counteraction against acetylcholine-induced broncho-constriction), and its capacity to block mediator release into the local tissues of lungs and trachea. Phytochemical investigation of MOEE here and by other investigators has shown the presence of various active components (Tables 1 and 2). In addition, we have also succeeded in isolating benzyl isothiocyanate from the extract in our laboratory. The active principle(s) listed in the tables might be responsible for one or more mechanisms for such activities. These results, using an antecedent approach, provide evidence that the consumption of certain common plants may have the potential to inhibit the antigen-induced release of cytokines. Thus the evidence justifies the rationale behind the use of *Moringa oleifera* in the treatment of respiratory-tract infection, rhinitis, and bronchial relaxation.

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