



## Immunopharmacology and Inflammation

Suppression of ovalbumin-induced Th2-driven airway inflammation by  $\beta$ -sitosterol in a guinea pig model of asthma

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## ABSTRACT

In the present study, the efficacy of  $\beta$ -sitosterol isolated from an n-butanol extract of the seeds of the plant *Moringa oleifera* (Moringaceae) was examined against ovalbumin-induced airway inflammation in guinea pigs. All animals (except group I) were sensitized subcutaneously and challenged with aerosolized 0.5% ovalbumin. The test drugs,  $\beta$ -sitosterol (2.5 mg/kg) or dexamethasone (2.5 mg/kg), were administered to the animals (p.o.) prior to challenge with ovalbumin. During the experimental period (on days 18, 21, 24 and 29), a bronchoconstriction test (0.25% acetylcholine for 30 s) was performed and lung function parameters (tidal volume and respiration rate) were measured for each animal. On day 30, blood and bronchoalveolar lavaged fluid were collected to assess cellular content, and serum was collected for cytokine assays. Lung tissue was utilized for a histamine assay and for histopathology.  $\beta$ -sitosterol significantly increased the tidal volume ( $V_T$ ) and decreased the respiration rate ( $f$ ) of sensitized and challenged guinea pigs to the level of non-sensitized control guinea pigs and lowered both the total and differential cell counts, particularly eosinophils and neutrophils, in blood and bronchoalveolar lavaged fluid. Furthermore,  $\beta$ -sitosterol treatment suppressed the increase in cytokine levels (TNF $\alpha$ , IL-4 and IL-5), with the exception of IL-6, in serum and in bronchoalveolar lavaged fluid detected in model control animals. Moreover, treatment with  $\beta$ -sitosterol protected against airway inflammation in lung tissue histopathology.  $\beta$ -sitosterol possesses anti-asthmatic actions that might be mediated by inhibiting the cellular responses and subsequent release/synthesis of Th2 cytokines. This compound may have therapeutic potential in allergic asthma.

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## 1. Introduction

Allergic asthma, which affects an estimated 100 million individuals worldwide (Cohn and Ray, 2000), is caused by chronic airway inflammation associated with IgE- synthesis and subsequent Th2 (T-helper type-2 cell)-responses (Barnes et al., 1998). Asthma is characterized by airway inflammation and airway hyper-responsiveness to the spasmogens such as histamine, acetylcholine and 5-hydroxytryptamine (5-HT) (Saria et al., 1983). The pathophysiological hallmark of asthma is the infiltration of inflammatory cells, including eosinophils (Wardlaw et al., 1988), neutrophils, lymphocytes and macrophages (Bousquet et al., 2000). These cells release various inflammatory mediators, including histamine (Liu et al., 1991) and cytokines (Chung and Barnes, 1999).

Numerous studies have also found elevated levels of histamine in the plasma of patients with asthma (Ind et al., 1983); similar effects have been noted in the lung tissues (Bartosch et al., 1932) of guinea

pigs. Elevated levels of tumor necrosis factor (TNF)- $\alpha$  (Coker and Laurent, 1998), interleukin (IL)-4 (Gharaee-Kermani et al., 2001), IL-5 (Egan et al., 1996) and IL-6 (Elias et al., 1997) have been noted in bronchoalveolar lavaged fluid from asthmatic patients after allergen challenge.

Phytosteroids possesses interesting medicinal and pharmacological activities (Dinan et al., 2001). Chemically, these compounds' structures are steroid-like, and modern clinical studies have shown that plants containing such steroids are anti-inflammatory agents. Among the phytosteroids,  $\beta$ -sitosterol is found in a variety of plants, including *Moringa oleifera* Lam. (Moringaceae). In our previous pre-clinical studies, we reported the anti-arthritis (Mahajan et al., 2007a), anti-anaphylactic (Mahajan and Mehta, 2007) and immunosuppressive (Mahajan and Mehta, 2010) activity of ethanolic extract from seeds of the plant. Furthermore, we evaluated the efficacy of ethanolic extract in chemical-induced, immune-mediated inflammatory responses in rats (Mahajan et al., 2007b) and in ovalbumin-induced airway inflammation in guinea pigs (Mahajan and Mehta, 2008). We established that the extract inhibits cytokines and subsequently prevents eosinophilia and neutrophilia. Furthermore, to obtain a potent extract, we fractionated the ethanolic extract using n-butanol as a solvent and again confirmed the extract's

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activity in the ovalbumin-induced guinea pig model of allergic asthma, where it significantly lowered cytokine and histamine levels (Mahajan et al., 2009). Our preliminary clinical studies also showed a decrease in the severity of asthma symptoms and improvement in peak expiratory flow rate in patients with asthma (Agrawal and Mehta, 2008).

Collectively, results from our preceding studies demonstrated that the individual extract(s) could significantly downregulate the synthesis and/or the release of cytokines and histamine but did not alter the lung function parameters. Furthermore, to determine the extract components, the quantitative estimation was carried out for marker compounds present in the plant including  $\beta$ -sitosterol. The efficacy of  $\beta$ -sitosterol was evaluated against histamine- and acetylcholine-induced bronchospasm in guinea pigs.  $\beta$ -sitosterol produced a significant increase in pre-convulsion dyspnea time against both the spasmogens compared to control animals, indicating the possible bronchodilatory activity of  $\beta$ -sitosterol. Therefore, to verify our previous results and to determine the constituent of the extract/fraction responsible for the anti-asthmatic activity, we conducted the present study using a compound;  $\beta$ -sitosterol.

## 2. Materials and methods

### 2.1. Reagents

All solvents used in the study were of analytical grade. Diethyl ether, ethyl acetate, n-butanol, petroleum ether (60–80 °C), hexane, hydrochloric acid, n-heptane, methanol and toluene were purchased from Rankem (New Delhi, India). Chloroform and carbón tetra chloride ( $\text{CCl}_4$ ) were purchased from Finar Chemicals Pvt. Ltd. (Ahmedabad, India). Silica gel (60–120 mesh), formaldehyde solution and aluminium hydroxide gel were obtained from S. D. Fine Chemicals (Mumbai, India).  $\beta$ -sitosterol, acetylcholine, histamine and ovalbumin (Grade V) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dexamethasone was obtained as a gift sample from Zydus Research Pvt. Ltd. (Ahmedabad, India). Perchloric acid, NaOH and NaCl were purchased from Ranbaxy Fine Chemicals Ltd. (New Delhi, India). Thin layer chromatography (TLC) plates silica gel (GF254) was purchased from Merck (Darmstadt, Germany). Ketamine was purchased from Themis Medicare Ltd. (Goregaon, India). Xylazine was obtained from Five Star Pharmaceuticals (Ahmedabad, India). Kits for TNF $\alpha$  and IL-5 were purchased from Pro Lab Marketing Pvt. Ltd. (New Delhi, India), and for IL-4 and IL-6 from Cusabio Biotech Co., Ltd. (Newark, DE, USA).

### 2.2. Plant material

Seeds of *M. oleifera* were obtained from a commercial supplier in Ahmedabad and were identified and authenticated by the Department of Pharmacognosy, L. M. College of Pharmacy, Ahmedabad, India. A voucher specimen was deposited in the herbarium of the same department.

### 2.3. Extraction and isolation of compound

One kilogram of course powder of dried seeds of *M. oleifera* was defatted using petroleum ether (60–80 °C), and then, it was exhaustively extracted using 95% (v/v) ethanol (500 ml) in a soxhlet extractor at 55 °C for 6 h. The resulting extract was further fractionated using the solvent n-butanol. The n-butanol fraction was filtered, and the solvent was removed under vacuum. The remaining n-butanol fraction was then partitioned with  $\text{CCl}_4$ . The  $\text{CCl}_4$  fraction (25 g) was loaded on a preparative TLC plate of silica gel ( $\text{F}_{254}$ ) using the solvent system methanol–toluene–ethyl acetate (1:8:1). The fraction band was scraped, collected from TLC plates and dissolved in methanol concentrated to dryness (yield 4.21 g). The powder (1 g)

was chromatographed for purification on a silica gel and eluted with a hexane–ethyl acetate solvent system. The solvent system was employed starting with hexane (100%) and then increasing the polarity of the elution solvent with ethyl acetate by 10% (v/v) increments until pure isolates were obtained. Fractions of 20 ml were collected. The progress of separation for  $\beta$ -sitosterol was monitored by TLC using the solvent system of methanol–toluene–ethyl acetate (1:8:1). Fractions of hexane and ethyl acetate elutants containing  $\beta$ -sitosterol were pooled and concentrated to dryness, and the presence of  $\beta$ -sitosterol was confirmed by co-chromatography with standard  $\beta$ -sitosterol. The yield of pure  $\beta$ -sitosterol was 0.82 g; hence, the total yield from the n-butanol fraction was 0.35% (w/w) of the weight of starting material (Guevara et al., 1999).

### 2.4. Characterization of the isolated compound

The melting point of the isolated compound was measured on Model II/III (Veego Instruments Corporation, Mumbai, India). The UV absorption spectrum of the isolated sample in methanol was recorded on a UV/Vis spectrophotometer [UV 1601, Shimadzu (Asia Pacific) Pvt. Ltd., Sydney, Australia]. Infrared (IR) (Spectrum GX Perkin-Elmer, USA) and mass spectra (Shimadzu LCMS model 2010, Columbia, USA) were recorded. The isolated compound was dissolved in  $\text{CDCl}_3$ , and  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  spectra were also obtained for the structure elucidation of the compound (Bruker Advance II 400 NMR Spectrometer, Billerica, MA, USA).

### 2.5. Animals

Specific pathogen-free male Dunkin–Hartley guinea pigs (300–500 g) were housed in a climate-controlled room (temperature  $22 \pm 1$  °C; relative humidity  $55 \pm 5\%$ ) on a 12-h light–dark cycle. Animals had access to standard pellet diet (certified Amrut brand rodent feed, Pranav Agro Industries, Pune, India) and filtered tap water *ad libitum*. All experiments were carried out with strict adherence to ethical guidelines and were conducted according to the protocol approved by the Institutional Animal Ethics Committee (IAEC) and according to Indian norms set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. Throughout the entire study period, the animals were monitored for growth, health status, and food intake capacity to be certain that they were healthy.

### 2.6. Sensitization and treatment of animals

Animals were divided into four groups ( $n=6/\text{group}$ ). Group I, non-sensitized controls, received distilled water (2.5 ml/kg); group II, the model control group, was ovalbumin sensitized and then received distilled water (2.5 ml/kg) supplemented with dimethyl sulphoxide (DMSO; vehicle used for dexamethasone [DXM] and  $\beta$ -sitosterol treatments); group III, the reference standard group, was ovalbumin sensitized and then received DXM (2.5 mg/kg); group IV, the experimental group, was ovalbumin sensitized and then received  $\beta$ -sitosterol (2.5 mg/kg). All animals (except group I) were sensitized and challenged as previously described (Duan et al., 2003). Briefly, animals were injected, s.c., with 100  $\mu\text{g}$  of ovalbumin (which had been adsorbed onto 100 mg of 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; they were administered orally.

### 2.7. Ovalbumin exposure

On days 18–29, 2.5 h after receiving the appropriate drug or vehicle treatment, the animals were challenged with 0.5% (w/v) of aerosolized

ovalbumin for 10 min. For the challenge, conscious animals were placed into a plastic circular chamber (diameter = 70 cm, and height = 40 cm) connected to a nebulizer (CX4-Omron Healthcare Company Ltd., Kyoto, Japan). Animals in the non-sensitized group (group I) were exposed to aerosolized saline using the same protocol.

### 2.8. Lung function and bronchoconstriction test

On days 18, 21, 24, and 29, 2 h after a 10-min ovalbumin exposure, the tidal volume (ml/s) and respiration rate (breaths/min) of the animals were measured with a Respiromax (Model no.070613-1, Columbus Instruments, OH, USA) before and after an acetylcholine-induced bronchoconstriction test. All ovalbumin-sensitized hosts were exposed (in a conscious state) to a 0.25% (w/v) acetylcholine solution for 30 s using a nebulizer connected to the animal holder. Guinea pigs in the non-sensitized control group were exposed to normal saline in place of acetylcholine.

### 2.9. Cellular count and serum preparation

On 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 non-heparinized tube for serum separation; the isolated serum was stored at  $-80^{\circ}\text{C}$  until quantitative determination of cytokines. The second portion (0.5 ml) was placed in a heparinized tube and used for leukocyte counts. Each sample was centrifuged at  $500\times g$  for 10 min at  $4^{\circ}\text{C}$ ; 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 X400 magnification and cells were identified as eosinophils, lymphocytes, macrophages, or neutrophils using standard morphologic determinants.

### 2.10. Bronchoalveolar lavaged fluid

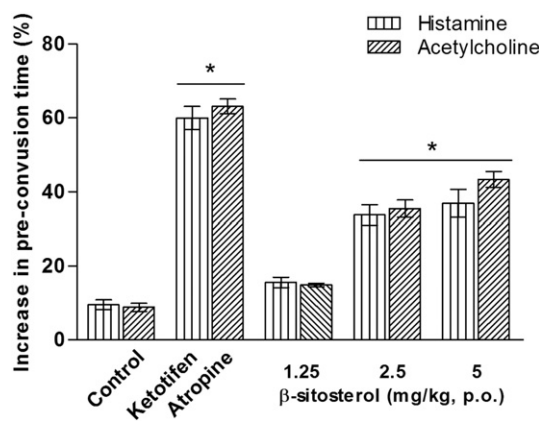
At the end of the experiment (i.e., day 30), bronchoalveolar lavaged fluid was collected from each animal. An overdose of ketamine (30 mg/kg) and xylazine (20 mg/kg) was administered s.c. A polypropylene cannula (24G) was inserted into the trachea, and then, 0.9% (w/v) normal saline solution (10 ml) was introduced into the lungs via a 10-ml syringe at  $37^{\circ}\text{C}$  and then recovered 5 min later. The recovered lavaged fluid (5 ml) was centrifuged at  $500\times g$  for 10 min at  $4^{\circ}\text{C}$ ; the resulting supernatant was collected and stored at  $-80^{\circ}\text{C}$  for cytokine determination. The cells in the pellet were washed in 0.5-ml saline, and the total and differential cell counts were performed as described for blood analysis (refer to Section 2.9).

**Table 1**

Effect of treatments on the tidal volume of guinea pigs.

Day	Values before and after acetylcholine exposure	Tidal volume ( $V_t$ ) in ml/s			
		I Non-sensitized control (distilled water)	II Model control (vehicle)	III OVA + DXM (2.5 mg/kg)	IV OVA + $\beta$ -sitosterol (2.5 mg/kg)
18	Before	$2.78 \pm 0.26$	$3.11 \pm 0.18$	$3.30 \pm 0.25$	$3.04 \pm 0.20$
	After	$2.53 \pm 0.21$	$2.97 \pm 0.16$	$3.16 \pm 0.23$	$2.99 \pm 0.17$
21	Before	$2.99 \pm 0.19$	$2.15 \pm 0.14^a$	$2.94 \pm 0.23^d$	$2.74 \pm 0.20^d$
	After	$2.79 \pm 0.17$	$2.00 \pm 0.13^a$	$2.81 \pm 0.23^d$	$2.59 \pm 0.13$
24	Before	$2.73 \pm 0.14$	$1.90 \pm 0.17^a$	$3.01 \pm 0.13^e$	$2.68 \pm 0.25^f$
	After	$2.58 \pm 0.18$	$1.73 \pm 0.15^b$	$2.93 \pm 0.11^f$	$2.66 \pm 0.20^e$
29	Before	$2.96 \pm 0.10$	$1.61 \pm 0.10^c$	$2.92 \pm 0.20^f$	$2.87 \pm 0.13^f$
	After	$2.92 \pm 0.11$	$1.46 \pm 0.07^c$	$2.89 \pm 0.19^e$	$2.77 \pm 0.14^f$

Values shown are the mean  $\pm$  S.E.M. ( $n=6$ ). <sup>a</sup> $P<0.05$ , <sup>b</sup> $P<0.01$ , and <sup>c</sup> $P<0.001$  compared to the non-sensitized control. <sup>d</sup> $P<0.05$ , <sup>e</sup> $P<0.01$ , <sup>f</sup> $P<0.001$  compared to the OVA (ovalbumin)-sensitized vehicle-treated model control.



**Fig. 1.** Effect of treatments on histamine and acetylcholine-induced bronchospasm in guinea pigs. Group I: control (received distilled water), group II: treated with ketotifen fumarate (1 mg/kg) or atropine sulphate (2 mg/kg), and groups III, IV and V: treated with  $\beta$ -sitosterol (1.25, 2.5 and 5 mg/kg, respectively). \* $P<0.001$  compared to the control. All bars represent the mean  $\pm$  S.E.M. from  $n=6$  guinea pigs per treatment group.

### 2.11. Cytokines in serum and bronchoalveolar lavaged fluid

The levels of TNF $\alpha$ , IL-4, IL-5 and IL-6 in each sample of recovered serum (400  $\mu$ l) and bronchoalveolar lavaged fluid (4.5 ml) were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol. All plates were analyzed on an automated plate reader (Lab System Multiscan Model-51118220, Thermo Bioanalysis Co., Helsinki, Finland).

### 2.12. Histamine assay on lavaged lung tissue

Lung tissue lobes from each animal were separately dissected out immediately following bronchoalveolar lavaged fluid collection. One lobe was used for non-lavagable histamine measurements and the other for the histology of lavaged tissue. For the former, lung tissue ( $200 \pm 20$  mg) was placed in 2.5-ml normal saline for the preparation of homogenate, and then 2.5-ml, 0.8-N perchloric acid was added. After mixing and centrifugation ( $4000\times g$ , 7 min at  $4^{\circ}\text{C}$ ), 2 ml of the resulting supernatant was transferred to a test tube containing 0.25-ml, 5-N NaOH, 0.75-g NaCl and 5-ml n-butanol. The mixture was vortexed for 5 min to partition histamine into the butanol and 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 histamine. The mixture was re-centrifuged and the butanol was transferred to a test tube containing 2-ml, 0.1-N HCl and 5-ml n-heptane. The

**Table 2**  
Effect of treatments on the respiration rate of guinea pigs.

Day	Value before and after acetylcholine exposure	Respiration rate (f) in breaths/min			
		I Non-sensitized control (distilled water)	II Model control (vehicle)	III OVA + DXM (2.5 mg/kg)	IV OVA + $\beta$ -sitosterol (2.5 mg/kg)
18	Before	103.0 $\pm$ 1.5	103.7 $\pm$ 4.7	108.4 $\pm$ 2.2	110.3 $\pm$ 5.7
	After	110.7 $\pm$ 4.2	111.3 $\pm$ 4.1	116.3 $\pm$ 3.0	118.9 $\pm$ 3.7
21	Before	108.5 $\pm$ 1.1	127.5 $\pm$ 4.8 <sup>c</sup>	107.7 $\pm$ 1.3 <sup>f</sup>	111.1 $\pm$ 1.9 <sup>e</sup>
	After	113.5 $\pm$ 2.8	135.9 $\pm$ 10.6	112.1 $\pm$ 2.4 <sup>d</sup>	116.5 $\pm$ 2.5
24	Before	105.9 $\pm$ 1.4	129.4 $\pm$ 1.0 <sup>c</sup>	110.7 $\pm$ 1.0 <sup>f</sup>	105.0 $\pm$ 1.2
	After	110.6 $\pm$ 3.9	148.9 $\pm$ 7.2 <sup>c</sup>	112.4 $\pm$ 3.0 <sup>f</sup>	108.9 $\pm$ 3.6 <sup>f</sup>
29	Before	106.5 $\pm$ 1.3	130.2 $\pm$ 2.0 <sup>c</sup>	114.9 $\pm$ 0.9 <sup>f</sup>	107.3 $\pm$ 1.5 <sup>f</sup>
	After	115.3 $\pm$ 2.1	154.1 $\pm$ 6.6 <sup>c</sup>	116.2 $\pm$ 2.5 <sup>f</sup>	110.5 $\pm$ 3.5 <sup>f</sup>

Values shown are the mean  $\pm$  S.E.M. (n = 6). <sup>c</sup>P < 0.001 compared to the non-sensitized control. <sup>d</sup>P < 0.05, <sup>e</sup>P < 0.01, and <sup>f</sup>P < 0.001 compared to the OVA (ovalbumin)-sensitized vehicle-treated model control.

mixture was again centrifuged, and the presence of histamine was determined fluorometrically (SL-174, Elico, India) as previously described (Shore et al., 1959).

### 2.13. Histological examination

Dissected lung tissues were washed with normal saline (5 ml) and then placed in 10% (v/v) formaldehyde solution for 1 week. After fixation, lung specimens were embedded in paraffin wax, and 5- $\mu$ m sections were cut and stained with hematoxylin and eosin dye for morphology. Images of selected sections were captured at X10 magnification using a zoom digital camera (Model C763, Eastman Kodak Company, Rochester, NY, USA).

### 2.14. Statistical analyses

Results are reported as mean  $\pm$  S.E.M. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's test; differences were considered statistically significant at P < 0.05. All statistical analyses were performed using the Graph Pad software (San Diego, CA, USA).

## 3. Results

### 3.1. Characterization and structure elucidation of the isolated compound

The melting point was obtained at 138–140 °C. The UV absorption spectrum of the isolated sample in methanol was scanned and showed maximum absorbance at 292.56 nm. The different peaks of mass spectra were obtained as M-18 (414.3-18), 397.3, H<sub>2</sub>O; M-3 (414.3-3), 411.3, 3H; M-70 (414.3-70), 344.4, C<sub>24</sub>H<sub>40</sub>O; and M-84 (414.3-84), 330.6, C<sub>23</sub>H<sub>38</sub>O. The isolated compound was identified as  $\beta$ -sitosterol based on IR, <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectroscopic data and comparison with those reported in the literature (data not shown).

**Table 3**  
Effect of treatments on total cells and differential leukocyte count in blood ( $\times 10^6$  cells/ml).

Groups	Total cells	Eosinophils	Lymphocytes	Monocytes	Neutrophils
I Non-sensitized control (distilled water)	8.22 $\pm$ 0.50	0.44 $\pm$ 0.005	6.20 $\pm$ 0.038	0.44 $\pm$ 0.005	0.22 $\pm$ 0.005
II Model control (vehicle)	20.96 $\pm$ 2.55 <sup>c</sup>	0.93 $\pm$ 0.01 <sup>c</sup>	12.56 $\pm$ 0.34 <sup>c</sup>	0.93 $\pm$ 0.01 <sup>c</sup>	0.36 $\pm$ 0.005 <sup>c</sup>
III OVA + DXM (2.5 mg/kg)	12.92 $\pm$ 0.41 <sup>e</sup>	0.70 $\pm$ 0.03 <sup>e</sup>	8.96 $\pm$ 0.24 <sup>e</sup>	0.70 $\pm$ 0.03 <sup>e</sup>	0.25 $\pm$ 0.01 <sup>f</sup>
IV OVA + $\beta$ -sitosterol (2.5 mg/kg)	16.94 $\pm$ 0.75 <sup>d</sup>	0.83 $\pm$ 0.03 <sup>d</sup>	10.83 $\pm$ 0.45 <sup>e</sup>	0.83 $\pm$ 0.03 <sup>d</sup>	0.32 $\pm$ 0.006 <sup>e</sup>

Values shown are the mean  $\pm$  S.E.M. (n = 6). <sup>c</sup>P < 0.001 compared to the non-sensitized control. <sup>d</sup>P < 0.05, <sup>e</sup>P < 0.01, and <sup>f</sup>P < 0.001 compared to the OVA (ovalbumin)-sensitized vehicle-treated model control.

### 3.2. Effect of treatments on histamine and acetylcholine-induced bronchospasm in guinea pigs

A pilot study was conducted with three different doses of  $\beta$ -sitosterol (1.25, 2.5, or 5 mg/kg) to determine the dose dependent effect in histamine and acetylcholine-induced bronchospasm. It was observed that  $\beta$ -sitosterol post-treatment at doses of 2.5 and 5 mg/kg significantly (P < 0.05) increased pre-convulsion dyspnea time compared to the control animals. Hence, a lower dose was chosen for our subsequent chronic studies (Fig. 1).

### 3.3. Effect of treatments on body weight

All animals present in the model control (group II) and drug regimen (groups III and IV) groups did not show any significant difference in body weight during the experimental period compared to the animals in the non-sensitized control group (group I). Furthermore, there were no apparent effects on the appetite/water consumption or on the outward appearance (i.e., fur coat, and eyes) of animals in each treatment group (data not shown).

### 3.4. Effect of treatments on lung function parameters in the acetylcholine-induced bronchoconstriction test

Lung function parameters were measured by Respiromax during the experimental period on days 18, 21, and 24 and on day 29 before and after exposure to acetylcholine (0.25% for 30 s). Tidal volume (Table 1) was decreased and respiration rate (Table 2) was increased significantly (P < 0.05) before and after exposure to acetylcholine in model control animals compared to non-sensitized animals from days 21 to 29. However, dexamethasone- and  $\beta$ -sitosterol-treated animals showed significant increase in tidal volume [before (P < 0.001, P < 0.001) and after (P < 0.01, P < 0.001), respectively] and decrease in respiratory rate [before (P < 0.001, P < 0.001) and after (P < 0.001, P < 0.001), respectively, of acetylcholine exposure] compared to the model control animals, suggesting improvement in these parameters on day 29.



**Table 4**  
Effect of treatments on total cells and differential leukocyte counts in bronchoalveolar lavaged fluid ( $\times 10^5$  cells/ml).

Groups	Total cells	Eosinophils	Lymphocytes	Macrophages	Neutrophils
I Non-sensitized control (distilled water)	8.51 $\pm$ 0.17	0.40 $\pm$ 0.012	6.4 $\pm$ 0.66	0.40 $\pm$ 0.012	0.24 $\pm$ 0.017
II OVA-control (vehicle)	17.64 $\pm$ 0.93 <sup>c</sup>	0.83 $\pm$ 0.037 <sup>c</sup>	11.68 $\pm$ 0.65 <sup>b</sup>	0.83 $\pm$ 0.037 <sup>c</sup>	0.39 $\pm$ 0.009 <sup>c</sup>
III OVA + DXM (2.5 mg/kg)	12.74 $\pm$ 0.23 <sup>e</sup>	0.62 $\pm$ 0.025 <sup>f</sup>	8.04 $\pm$ 0.10 <sup>d</sup>	0.62 $\pm$ 0.025 <sup>f</sup>	0.27 $\pm$ 0.007 <sup>f</sup>
IV OVA + $\beta$ -sitosterol (2.5 mg/kg)	13.19 $\pm$ 0.30 <sup>d</sup>	0.68 $\pm$ 0.017 <sup>e</sup>	8.21 $\pm$ 0.14 <sup>d</sup>	0.68 $\pm$ 0.017 <sup>e</sup>	0.30 $\pm$ 0.008 <sup>f</sup>

Values shown are the mean  $\pm$  S.E.M. (n = 6). <sup>b</sup>P < 0.01 and <sup>c</sup>P < 0.001 compared to the non-sensitized control. <sup>d</sup>P < 0.05, <sup>e</sup>P < 0.01, and <sup>f</sup>P < 0.001 compared to the OVA (ovalbumin)-sensitized vehicle-treated model control.

### 3.5. Effect of treatments on circulating cellular counts

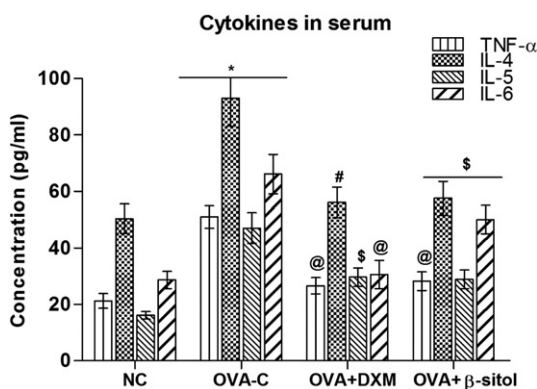
The total number of leukocytes and each differential count in blood samples recovered from the model control animals were markedly increased (P < 0.001) compared to the non-sensitized controls. However, the numbers of circulating eosinophils (P < 0.01 and P < 0.05), lymphocytes (P < 0.01), monocytes (P < 0.01 and P < 0.05) and neutrophils (P < 0.001 and P < 0.01) in the blood were significantly decreased in dexamethasone- and  $\beta$ -sitosterol-treated animals, respectively, compared to those numbers seen in the model control guinea pigs (Table 3).

### 3.6. Effect of treatments on inflammatory cellular counts in bronchoalveolar lavaged fluid

The model control animals showed a significant increase in the total cell count and differential cellular count in bronchoalveolar lavaged fluid compared to the non-sensitized controls. Dexamethasone and  $\beta$ -sitosterol treatment significantly decreased these counts from the model control levels [total cells (P < 0.01 and P < 0.05), eosinophils (P < 0.001 and P < 0.01), lymphocytes (P < 0.05) macrophages (P < 0.001 and P < 0.01) and neutrophils (P < 0.001)] (Table 4).

### 3.7. Effect of treatments on cytokine production in serum

The model control animals showed significant (P < 0.001) increases in levels of TNF- $\alpha$ , IL-4, IL-5 and IL-6 compared to the non-sensitized controls. These elevated levels of TNF- $\alpha$  (P < 0.001), IL-4 (P < 0.05) and IL-5 (P < 0.05) were significantly decreased in guinea pigs that received  $\beta$ -sitosterol treatment compared to those levels seen in the model controls. However, this treatment did not correlate with any significant reductions in the level of IL-6 (Fig. 2).



**Fig. 2.** Effect of treatments on serum cytokine levels of guinea pigs. \*P < 0.001 compared to the non-sensitized controls. @P < 0.001, #P < 0.01, and \$P < 0.05 compared to the OVA (ovalbumin)-sensitized vehicle-treated model controls. All bars represent the mean  $\pm$  S.E.M. from n = 6 guinea pigs per treatment group.

### 3.8. Effect of treatments on cytokine levels in bronchoalveolar lavaged fluid

The significant (P < 0.001) increase in cytokine levels in bronchoalveolar lavaged fluid from the model control animals was not present in  $\beta$ -sitosterol-treated animals [TNF- $\alpha$  (P < 0.01), IL-4 (P < 0.05), and IL-5 (P < 0.05)]. Dexamethasone caused a significant (P < 0.05) reduction in IL-6 levels compared to the model controls. In contrast, there was no change in IL-6 levels resulting from  $\beta$ -sitosterol treatment (Fig. 3).

### 3.9. Effect of treatments on histamine levels

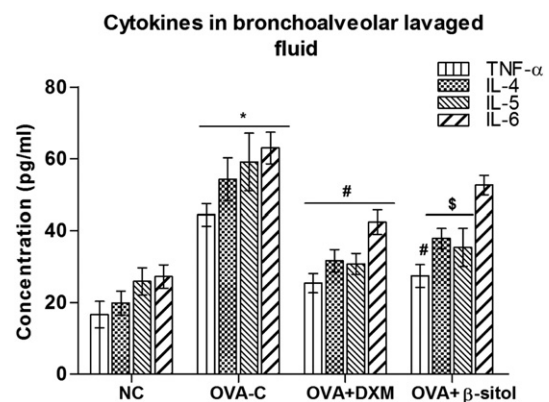
The level of histamine measured in lung tissues from the model control animals was significantly higher (P < 0.01) than the level in the non-sensitized controls. Compared to the model control group, treatment group IV showed a significant (P < 0.05)  $\beta$ -sitosterol-induced normalization of elevated histamine levels; this effect was approximately equal in magnitude to the normalization-induced by dexamethasone treatment (Fig. 4).

### 3.10. Effect of treatments on histopathology of lung tissue

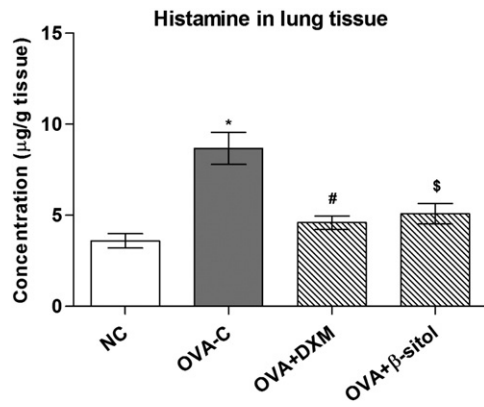
The histological examination of lung tissue from the model control guinea pigs showed a massive inflammatory infiltration of the peribronchial tissues, reduced lumen size, epithelial desquamation and angiogenesis. Treatment with dexamethasone and  $\beta$ -sitosterol showed a protective effect, as evidenced by the presence of milder or less pathological features (Fig. 5).

## 4. Discussion and conclusion

Herbal medicines have been used to treat asthma for hundreds of years (Chung and Adcock, 2000). However, so far, very few compounds have been isolated from such herbal plants and subjected to clinical studies based on their anti-asthmatic effects in experimental studies.



**Fig. 3.** Effect of treatments on bronchoalveolar lavaged fluid cytokine levels of guinea pigs. \*P < 0.001 compared to the non-sensitized controls. #P < 0.01 and \$P < 0.05 compared to the OVA (ovalbumin)-sensitized vehicle-treated model controls. All bars represent the mean  $\pm$  S.E.M. from n = 6 guinea pigs per treatment group.



**Fig. 4.** Effect of treatments on lung tissue histamine levels of guinea pigs. \* $P < 0.001$  compared to the non-sensitized controls. # $P < 0.01$  and \$ $P < 0.05$  compared to the OVA (ovalbumin)-sensitized vehicle-treated model controls. All bars represent the mean  $\pm$  S.E.M. from  $n = 6$  guinea pigs per treatment group.

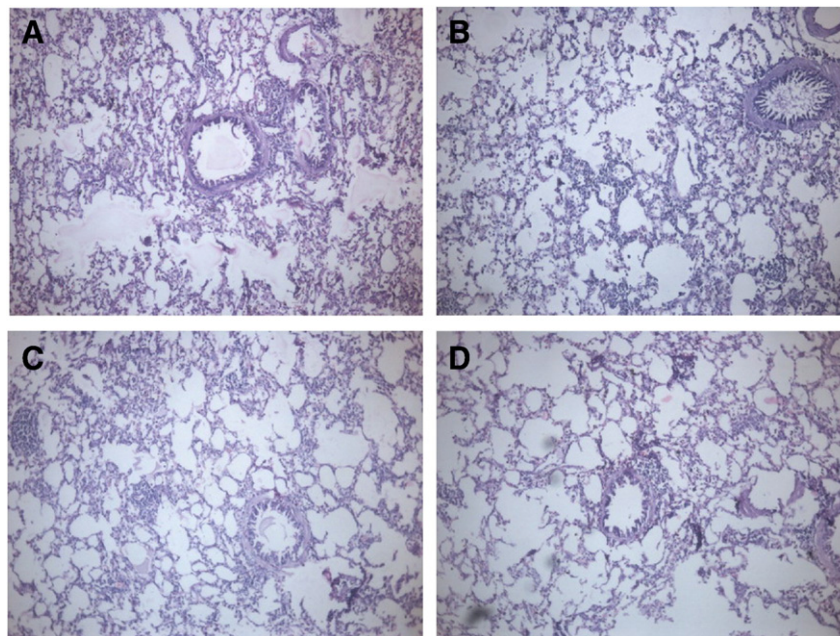
The exceptions include ephedrine from the plant *Ephedra* (Berger and Dale, 1910), theophylline from *tea* (Macht and Ting, 1921) and cromolyn sodium (sodium cromoglycate) from *Khellin* (Cox, 1967); these drugs have been used for the treatment of asthma for several years. Furthermore, the scientific literature is replete with reports of the biological activities of sterols or their glycosides in various animal models of asthma. The possible efficacy of  $\beta$ -sitosterol as a therapeutic drug for immune-mediated disorders has been reported (Bouic and Lambrecht, 1999).  $\beta$ -sitosterol and its glycoside have been shown to reduce carcinogen-induced colon cancer in rats (Raicht et al., 1980) and to have anti-inflammatory activity through cytokine inhibition (Aherne and O'Brien, 2008). Moreover, *in vitro* studies showed that  $\beta$ -sitosterol increased Th1 while dampening Th2-cell activities (Chen et al., 2009).

In this study, no animals in the model control or drug-treated groups showed any significant difference in body weight during the experimental period compared to the non-sensitized control animals, suggesting that  $\beta$ -sitosterol treatment did not interfere with the normal growth of the animals. All animals in the model control

group exhibited irritability, sneezing and hyper-rhinorrhea, indicative of the severity of disease. Furthermore, tidal volume in the model control animals was decreased significantly before and after exposure to acetylcholine from days 21 to 29, demonstrative of bronchoconstriction due to chronic airway inflammation, which resembles an asthmatic condition. Similarly, the significant increase in respiration rate observed in these animals was indicative of exertional breathing—a symptom of asthma. Treatment with dexamethasone and  $\beta$ -sitosterol had a significant protective effect; both drugs improved tidal volume and respiratory rate. This defensive effect might be due to the indirect decrease in resistance resulting from reduction in airway inflammation.

The late-phase airway response in asthma is associated with the infiltration of inflammatory cells to the site of the response (Williams, 2004). In the present study, the model control animals had increased total and differential cellular counts in blood and in bronchoalveolar lavaged fluid; these increases correlated with the level of cellular infiltration. Guinea pigs that received dexamethasone and  $\beta$ -sitosterol treatment had significantly decreased the numbers of total cells in both blood and bronchoalveolar lavaged fluid. However, in the differential cell count,  $\beta$ -sitosterol decreased each cell count in blood but only the eosinophil and neutrophil count in bronchoalveolar lavaged fluid compared to the model control animals. Furthermore, the amelioration of inflammatory cell numbers in bronchoalveolar lavaged fluid was confirmed by lung tissue histology. Therefore, these results suggest that  $\beta$ -sitosterol treatment could possibly be useful to control the activation of the inflammatory processes underlying 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 is associated with Th2 cytokine expression (Boynton and Altmann, 2004). Mast cells are most likely an important source of TNF- $\alpha$ . Furthermore, the localization of cytokines to mast cell subsets reveals preferential IL-4 with prominent IL-5 and IL-6 expression (Chung and Barnes, 1999). In the present study, we confirmed the existence of the prominent Th2 type cytokines—TNF- $\alpha$ , IL-4, IL-5 and IL-6—in the model control



**Fig. 5.** Effect of treatments on the histopathological changes in lung tissue. Representative hematoxylin- and eosin-stained sections of the lung tissue (X10). A shows a typical normal lung histology. B shows a typical damaged lung tissue from a model control group animal with total and differential leukocyte infiltration, reduced lumen size, endothelial shedding and angiogenesis. C shows a section from a dexamethasone-treated animal. D shows a section from a  $\beta$ -sitosterol-treated animal.

animals, suggesting persistent airway inflammation.  $\beta$ -sitosterol treatment decreased the level of TNF- $\alpha$ , IL-4, and IL-5 in bronchoalveolar lavaged fluid and in serum. This reduction in the level of cytokines correlates with the inhibition of inflammation (as determined by decreased histamine levels) by  $\beta$ -sitosterol.

Furthermore, ongoing chronic inflammation is associated with mast cell degranulation as evidenced by the increased levels of mast cell mediators in lung tissues (Bartosch et al., 1932; Foresi et al., 1990). In this study, a significant increase in histamine levels in model control animals was indicative of the inflammation of lung tissues and the release of mediators. Treatment with dexamethasone and  $\beta$ -sitosterol significantly decreased histamine levels compared to the diseased control animals. These data suggest that  $\beta$ -sitosterol might inhibit the release of inflammatory mediators such as histamine. In addition, atopic asthma has been extensively investigated and involves structural changes in the airways (Amin et al., 2000). The results of histopathology study suggest that  $\beta$ -sitosterol treatment inhibited angiogenesis, epithelial shedding and leukocyte infiltration into the airway after ovalbumin challenge. In spite of the results presented in this study, we still do not know how  $\beta$ -sitosterol attenuates the airway inflammation allied with asthma; hence, we intend to clarify the precise mechanism underlying the antiasthmatic function of  $\beta$ -sitosterol in future studies.

In conclusion,  $\beta$ -sitosterol exerted anti-inflammatory effects in allergen-induced airway inflammation. We described the potential mode of action of  $\beta$ -sitosterol by investigating its efficacy against Th2-cell-derived cytokine production and subsequent cytokine-induced cellular infiltration (eosinophils and neutrophils), its protective potential (counteraction of acetylcholine-induced bronchoconstriction and improvement in lung functions) and its capacity to block the release of inflammatory mediators, such as histamine, into the local lung tissues. Lastly, the results of our study suggest that  $\beta$ -sitosterol may be a valuable therapy for asthma; however, a well-designed clinical trial is warranted, which includes persistent, mild or moderate asthmatic patients.

### Conflict of interest statement

The authors state no conflict of interest.

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