

SHORT COMMUNICATION

Inhibition of Early and Late Phase Allergic Reactions by *Euphorbia hirta* L.

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A 95% ethanol extract from whole aerial parts of *Euphorbia hirta* (EH A001) showed antihistaminic, antiinflammatory and immunosuppressive properties in various animal models. EH A001 inhibited rat peritoneal mast cell degranulation triggered by compound 48/80. It significantly inhibited dextran-induced rat paw edema. EH A001 prevented eosinophil accumulation and eosinophil peroxidase activity and reduced the protein content in bronchoalveolar lavage fluid (BALF) in a 'mild' model of asthma. Moreover, the CD4/CD8 ratio in peripheral blood was suppressed. EH A001 attenuated the release of interleukin-4 (IL-4) and augmented interferon- γ (IFN- γ) in ovalbumin-sensitized mouse splenocytes. The results were compared with the effects of known compounds, ketotifen, cetirizine and cyclophosphamide. These findings demonstrated that *Euphorbia hirta* possessed significant activity to prevent early and late phase allergic reactions. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: *Euphorbia hirta* L.; allergy; compound 48/80; peritoneal mast cells; histamine; interleukin-4; interferon- γ ; eosinophils; eosinophil peroxidase; edema; immunosuppression.

INTRODUCTION

Euphorbia hirta L. (Euphorbiaceae) is a herbaceous wild plant which grows in the hotter parts of India and has been employed as a traditional cure for several indications: gastrointestinal disorders (diarrhea, dysentery, intestinal parasitosis etc.) and bronchial and respiratory diseases (asthma, bronchitis, hay fever etc.) (Mhaskar *et al.*, 2000). In Ayurveda it is documented as a drug for the treatment of coughs and asthma (Shastri, 1952). Several pharmacological properties of *Euphorbia hirta* L. are reported such as: sedative, anxiolytic, analgesic, antipyretic and antiinflammatory (Lanthers *et al.*, 1990, 1991), antidiarrheic (Galvez *et al.*, 1992), antibacterial (Vijaya *et al.*, 1995) and diuretic (Johnson *et al.*, 1999). This plant has also been shown to inhibit prostaglandin biosynthesis, platelet aggregation and carragenan-induced edema (Hiermann and Bucar, 1994). Although *Euphorbia hirta* is regarded as an asthma weed, no reports are available for its possible effects on the immune system. The aim of the present investigation was to investigate the interaction of a 95% alcohol extract of *Euphorbia hirta* L. with early and late phase allergic reactions.

MATERIALS AND METHODS

Reagents. The biochemicals including drugs were procured from Sigma Chemical Co, (St Louis, MO, USA).

ELISA kits for IL-4 and IFN- γ were obtained from R & D Systems Inc, (Minneapolis, MN, USA). FITC labeled anti-mouse CD4 and CD8 antibodies were purchased from BD Pharmingen, USA. All other chemicals were of high purity reagent grade.

Preparation of plant extract. Authenticated samples of whole plant were collected from sub-Himalayan terrain during May 2005 and a voucher specimen (accession no: 19517) deposited in the herbarium of this Institute. Shade dried aerial parts of the plant were finely ground and percolated in 95% ethanol for 16 h at room temperature. This procedure was repeated four times and the combined ethanol extract was evaporated to dryness under reduced pressure at $50 \pm 1^\circ\text{C}$ in a rotavapour. The yield of dried residue (EH A001), was 13.5%. EH A001 was solubilized in 0.05% gum acacia and administered orally as mg/kg body weight of the animals at the dosages indicated in various experiments. EH A001 was given to animals (both mice and rats) in the dose range of 0.1–3.0 mg/g and no mortality or adverse effects were observed.

Animals. Male BALB/c mice (20–25 g) and Wistar rats (150–175 g) were kept in the Animal House and maintained under standard husbandry conditions (temperature $24 \pm 2^\circ\text{C}$; humidity 50%–80%; 12 h light/dark cycle). Animals received pelleted rodent feed (Ashirwad Industries, Chandigarh, India) and water *ad libitum*. The Institution Animal Ethics Committee approved the animal experiments and the Guidelines for Animal Care were adhered to as recommended by the Indian National Science Academy, New Delhi (1992).

Preparation of rat peritoneal mast cells (RPMC) and determination of histamine release. RPMC were

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isolated from rat peritoneal fluid and purified according to the procedure described earlier by Shin and Lee (2003). Mast cell preparations were about 95% pure as assessed by toluidine blue staining. The viability of the cells was >97% as determined by the trypan blue exclusion test. Purified mast cells were resuspended in a buffer containing 10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.0 mM MgCl₂, 1.4 mM CaCl₂, 5.6 mM glucose. Pre-incubation of the mast cells (10⁶ cells/mL) was done in the presence of test materials for 10 min at 37 °C. Compound 48/80 (10 µg/mL) was added and the cells were further incubated for 20 min. The reaction was stopped by cooling the tubes in ice. The cells were centrifuged (400 × g for 5 min) and histamine released in supernatants was measured as described by Shore *et al.* (1959).

Ovalbumin (OVA)-induced sensitization and determination of biochemical parameters. BALB/c mice were treated for 8 successive days (days 0–7) with 10 µg OVA (in PBS), once per day (i.p.) to develop mild asthma (Schmitz *et al.*, 2003). The animals were intranasally challenged with 100 µg ovalbumin for 4 days (days 18–21). The animals were kept segregated in six groups (6 mice/group): group A, control (vehicle only); group B (OVA challenged); groups C–E, treated with EH A001; group F, treated with cetirizine. Test drugs were administered, p.o., once daily on days 18–21. Twenty four hours after the last challenge, the mice were anesthetized, the trachea cannulated with a 22-gauge needle and bronchoalveolar fluid (BALF) collected. After centrifugation of BALF (600 × g for 10 min), the supernatant was used for the assay of eosinophil peroxidase (EPO) (Trifilieff *et al.*, 2002) and protein (Lowry *et al.*, 1951). The pellet was used for determining eosinophil counts. Analysis of lymphocytes surface markers CD4 and CD8 was done by flow cytometry in peripheral whole blood samples.

Estimation of IL-4 and IFN- γ . Splenocytes were isolated from a pool of five spleens removed from untreated sensitized mice (but not challenged) as described previously (Fitzpatrick *et al.*, 1994). The splenocytes were suspended in RPMI-1640 supplemented with 12 mM HEPES, (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 kU/L benzyl penicillin, 0.1 mg% streptomycin and 10% fetal calf serum. Splenocytes (3 × 10⁶ cells with >95% viability) were seeded in a 96-well plate and cultured for 18 h in a CO₂ incubator without (control) or with EH A001 (10–100 µg/mL) or cetirizine (5 µg/mL) in the presence of 50 µg/mL OVA. Cytokine levels were assayed in the supernatants by ELISA kits.

Dextran induced edema. Thirty six rats were divided into six groups (6 animals/group). The control group (A) received vehicle only. Group B rats received EH A001. Groups C and D animals were administered with ibuprofen and cetirizine, respectively. Forty five minutes later freshly prepared 0.1 mL dextran (6% w/v, in normal saline) was injected in the sub-plantar region of left hind paw (Gupta *et al.*, 2003) and the increase in paw volume was measured 1, 2 and 3 h later with the help of a volume differential meter (model 7101, Ugo Basile, Italy).

Humoral antibody response. Thirty six mice were immunized by injecting i.p., with 0.2 mL of 5 × 10⁹ fresh sheep red blood cells (SRBC) on day 0 and challenged 7 days later by injecting an equal volume of SRBC. Blood samples were collected on day +7 (before challenge) for primary antibody titer and on day +14 (7 days after challenge) for secondary antibody titer. The animals were kept segregated in six groups (6 animals/group): group A, control (0.1% BSA-saline); group B, treated with EH A001 once daily on 0–7 days; group C, treated with cyclophosphamide. Hemagglutination antibody titers were determined using the microtitration technique as reported previously (Kaul *et al.*, 2003).

RESULTS

Antihistaminic effect of EH A001

The effect of EH A001 on the inhibition of compound 48/80 induced histamine release from RPMC is shown in Fig 1. The compound 48/80 induced release was 74.1 ± 1.1 µg/10⁶ cells, which was 4-fold higher compared with the spontaneous release of histamine. EH A001 produced a concentration dependent inhibitory effect; 100–125 µg of EH A001 showed 100%–120% inhibition. Ketotifen, a known mast cell stabilizer, produced an 85% inhibitory effect at a concentration of 3.75 µg (the concentration dependent effect of ketotifen not shown).

Effect of EH A001 on eosinophil count, EPO activity and protein level in BALF from lungs of ovalbumin treated mice

Results are summarized in Table 1. A 4.4-fold increase of eosinophils was recorded in the BALF of challenged mice. EH A001 at 0.1 and 0.3 mg/g dose suppressed the accumulation of eosinophils by 76% and 100%, respectively. The specific activity of EPO after ovalbumin challenge increased by 56%. EH A001 showed a dose-dependent effect: at doses of 0.1, 0.3 and 0.6 mg/g, EPO

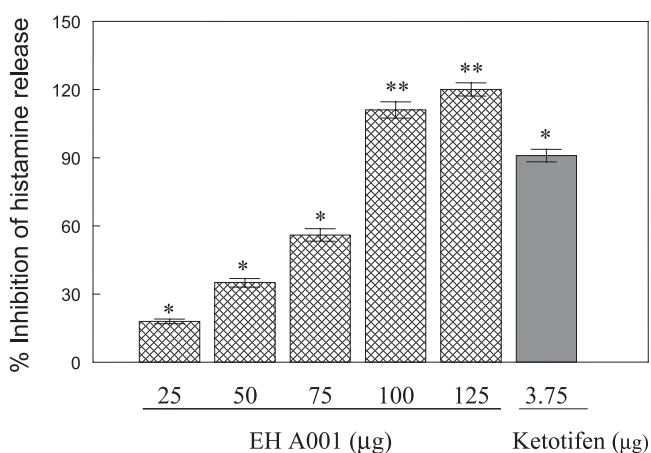


Figure 1. Effect of EH A001 on histamine release from RPMC. RPMC were treated with test materials as described in Materials and Methods section. Each datum represents the mean ± SEM of six independent determinations. * $p < 0.05$; ** $p < 0.001$ vs control (spontaneous release of histamine) (Student's t -test).

Table 1. Effect of EH A001 on BALF parameters in OVA challenged Balb/c mice

Treatment group	Dose (p.o.)	Eosinophil counts	Eosinophil peroxidase (Sp. act.)	Protein ($\mu\text{g/mL}$)
A. Control	–	05	1.55 ± 0.2	3.73 ± 0.66
B. OVA challenged	–	22 ^a	2.42 ± 0.8^a	7.45 ± 0.55^c
C. OVA challenged + EH A001	0.1 mg/g	09 ^b (76%)	1.36 ± 0.2^b (121%)	5.94 ± 0.55^b (40%)
D. OVA challenged + EH A001	0.3 mg/g	05 ^b (100%)	1.29 ± 0.2^b (129%)	5.34 ± 0.45^d (56%)
E. OVA challenged + EH A001	0.6 mg/g	05 ^b (100%)	1.13 ± 0.1^b (148%)	4.66 ± 0.50^e (75%)
F. OVA challenged + cetirizine	0.2 $\mu\text{g/g}$	04 ^b (70%)	1.16 ± 0.2^b (144%)	4.95 ± 0.29^e (67%)

For details of Experimental Groups refer to Materials and Methods section. Sp. act. EPO is shown as change in OD/mg protein/min. Figures in parenthesis show % restoration calculated as follows: $100 - [(OVA \text{ challenged} + \text{test material}) - \text{control}/(OVA \text{ challenged} - \text{control})] \times 100$. Data are presented as mean $\pm$ SEM ($n = 6$). ^a $p < 0.01$ vs group A; ^b $p < 0.001$ vs group B; ^c $p < 0.001$ vs group A; ^d $p < 0.05$ vs group B; ^e $p < 0.01$ vs group B (Student's *t*-test).

Table 2. Effect of EH A001 on composition of blood lymphocytes

Treatment group	Dose (p.o.)	% CD4	% CD8
A. Control	–	20.5 ± 1.0	10.7 ± 1.1 (1.92)
B. OVA challenged	–	44.8 ± 1.6^a	16.94 ± 0.8 (2.64)
C. OVA challenged + EH A001	0.1 mg/g	28.1 ± 2.2^b	15.1 ± 0.7 (1.85)
D. OVA challenged + EH A001	0.3 mg/kg	19.0 ± 1.1^b	13.8 ± 0.9 (1.44)
E. OVA challenged + EH A001	0.6 mg/g	20.5 ± 2.0^b	14.54 ± 1.1 (1.40)
F. OVA challenged + Cetirizine	0.2 $\mu\text{g/g}$	31.5 ± 1.0^c	11.6 ± 1.0 (2.68)

For details of experimental groups refer to Materials and Methods section. Two-color flow cytometric analysis was performed on FACS LRS (Becton Dickinson, USA) by analysing at least 10 000 cells. Electronic gating of forward versus side scatter was used to eliminate cellular fragments and debris. Isotype-matched antibodies were used as negative controls. Fluorescence histograms were created in FL-1 channel and the percentage of positive cells was analysed with Cell Quest software. Data are presented as mean $\pm$ SEM ($n = 6$). ^a $p < 0.001$ vs control; ^b $p < 0.001$ vs group B; ^c Non-significant vs group B (Student's *t*-test) ($n = 6$). (Figures in parenthesis show CD4/CD8 ratio).

activity was reduced by 121%, 129% and 148%, respectively. Cetirizine (0.2 mg/g) produced a 144% suppression. Ovalbumin challenge also caused an elevation of BALF protein levels by 2-fold. Doses of EH A001 at 0.1, 0.3 and 0.6 mg/g reduced the protein content by 40%, 56% and 75%, respectively. Cetirizine (0.2 mg/g) reduced the protein levels by 67%.

Effect of EH A001 on lymphocyte surface markers

Immunophenotypic studies of peripheral blood lymphocytes are summarized in Table 2. In the control group the CD4/CD8 ratio was 1.92 which increased to 3.13 after OVA challenge. EH A001 treatment altered the CD4/CD8 ratios due to its effect on both CD4 and CD lymphocyte cell population. EH A001 at doses of 0.1 and 0.3 mg/g reduced the CD4/CD8 ratio to 1.85 and 1.53, respectively. Cetirizine did not alter the CD4/CD8 ratio.

Effect of EH A001 on the release of cytokines in OVA-challenged splenocytes

Results are summarized in Fig. 2a–b. EH A001 was found to modify the cytokine production by splenocytes. IFN- γ release was stimulated by 1.3- to 2.0-fold in the presence of 10–100 μg of EH A001 compared with control. Cetirizine brought about a 1.4-fold increase (Fig. 2a). IL-4 production was suppressed by 0.7–0.52 fold with 10–100 μg of EH A001. Cetirizine inhibited IL-4 production by 0.4 fold (Fig. 2b).

Effect of EH A001 on histamine dominating phase of dextran induced edema in rats

Table 3 shows that EH A001 inhibited dextran induced inflammation in a dose dependent manner. During the first hour (histamine dominating phase) the inhibition was 22%, 30% and 48%, respectively, with 0.1, 0.3

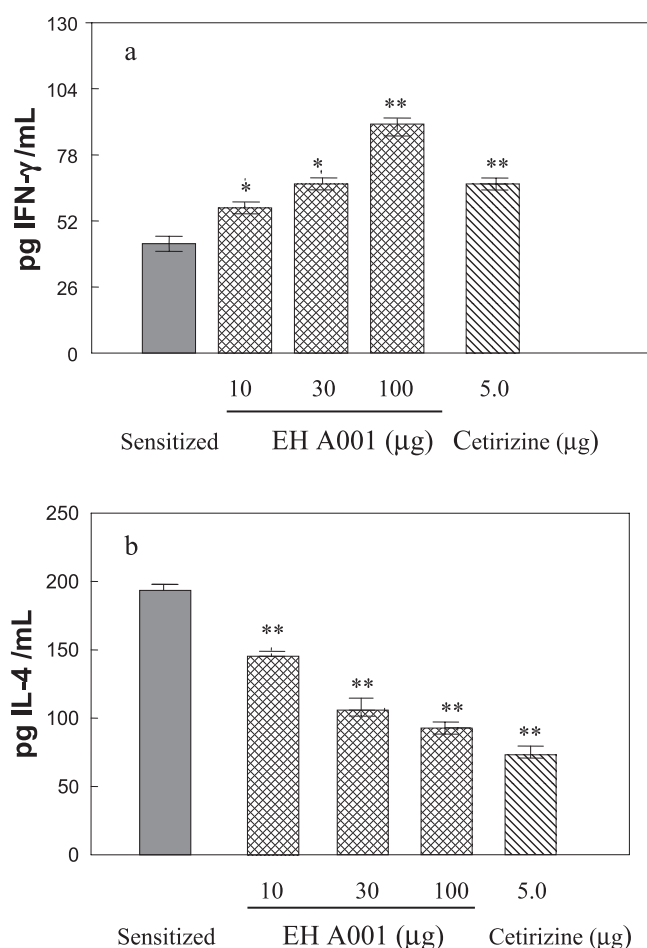


Figure 2. (a) Effect of EH A001 on IFN- γ production by spleen cells. Splenocytes were treated with test materials as described in Material and Methods section. Each datum represents the mean $\pm$ SEM of six independent determinations. * $p < 0.05$; ** $p < 0.001$ vs sensitized control (Student's t -test). (b) Effect of EH A001 on IL-4 production by spleen cells. Splenocytes were treated with test materials as described in Material and Methods section. Each datum represents the mean $\pm$ SEM of six independent determinations * $p < 0.05$; ** $p < 0.001$ vs sensitized control (Student's t -test).

and 0.6 mg/g doses of EH A001. Cetirizine (0.01 mg/g) produced 27% inhibition. This effect gradually declined during the later hours. On the other hand, ibuprofen (0.1 mg/g) produced only the late effect showing 21% and 33% inhibition after 2 and 3 h, respectively.

Immunosuppressive activity of EH A001

EH A001 produced a dose-related suppression in both primary and secondary antibody syntheses (Table 4). EH A001 at doses of 0.025, 0.05 and 0.1 mg/g, respectively produced 13%, 28% and 38% suppression of primary antibody (7 days) syntheses and 8%, 11% and 18% suppression of secondary antibodies syntheses (14 days). Cyclophosphamide (0.25 mg/g) showed 38% suppression of primary antibodies and 32% suppression of secondary antibodies syntheses.

DISCUSSION

In the present study, the effect of EH A001 on Type I hypersensitivity reactions represented by early and late-phase responses were evaluated in different experimental models. Type I hypersensitivity is mediated by mast cells which store several vasoactive mediators, of which histamine remains the most prominently implicated in the acute phase of immediate allergic reaction (Shin, 2004). The significant antiallergic activity of EH A001 is shown by its inhibitory effect on the release of histamine from rat peritoneal mast cells triggered by compound 48/80. Since compound 48/80, a well-known secretagogue of mast cells, induces a nonspecific anaphylactic reaction via alteration in membrane permeability (Mousli *et al.*, 1990), it may therefore seem possible that EH A001 possesses a potent membrane stabilizing activity to prevent histamine release. An antiinflammatory action of EH A001 is also revealed as these mediators (histamine, serotonin etc.) play a pivotal role in the onset and development of inflammation (Dai *et al.*, 2002). This is evident by the observation that EH A001 reverses acute inflammation caused by dextran which is known to be mediated both by histamine and serotonin (Gupta *et al.*, 2003). Although the early immediate-type reaction results largely from the release of histamine and other mediators from mast cells, the late phase reaction also depends on an altered T cell response, resulting in the B cell isotype switching to recruiting eosinophils and several others of a wide range of inflammatory mediators. Of the many effector cells involved in immediate type hypersensitivity reactions, eosinophils have possibly the most important and potentially a pivotal role in generating airway inflammation (Cui *et al.*, 2003). Its granular constituent EPO

Table 3. Effect of EH A001 on dextran-induced pedal edema

Treatment group	Dose (p.o.)	Paw volume (mL)		
		1st h	2nd h	3rd h
A. Control	–	0.57 $\pm$ 0.06	1.17 $\pm$ 0.04	1.22 $\pm$ 0.02
B. EH A001	0.1 mg/g	0.45 $\pm$ 0.02 (22%)	0.95 $\pm$ 0.10 (19%)	1.07 $\pm$ 0.11 (12%)
	0.3 mg/g	0.40 $\pm$ 0.02 (30%)	0.87 $\pm$ 0.08 (25%)	1.00 $\pm$ 0.07 (18%)
	0.6 mg/g	0.33 $\pm$ 0.04 (48%)	0.80 $\pm$ 0.07 (32%)	0.95 $\pm$ 0.09 (24%)
C. Ibuprofen	0.1 mg/g	0.54 $\pm$ 0.02 (5%)	0.92 $\pm$ 0.04 (21%)	0.82 $\pm$ 0.04 (33%)
D. Cetirizine	0.01 μ g/g	0.42 $\pm$ 0.03 (27%)	0.93 $\pm$ 0.05 (21%)	1.10 $\pm$ 0.06 (10%)

For details of experimental groups refer to Materials and Methods section. Figures in parenthesis show % inhibition vs control. Data are represented as mean $\pm$ SEM ($n = 6$).

Table 4. Effect of EH A001 on SRBC induced humoral response in mice

Treatment group	Dose (p.o.) (mg/g)	Humoral response	
		Primary antibody titre (day +7)	Secondary antibody titre (day +14)
A. Control	–	6.50 ± 0.25	6.33 ± 0.21
B. EH A001	0.025	5.66 ± 0.21 ^a (13%)	5.83 ± 0.16 ^a (8.0%)
	0.05	4.66 ± 0.21 ^c (28%)	5.66 ± 0.21 ^b (11.0%)
	0.1	4.00 ± 0.25 ^c (38%)	5.16 ± 0.16 ^b (18%)
	0.2	4.12 ± 0.33 ^b (36%)	5.10 ± 0.22 ^b (19%)
C. Cyclophosphamide	0.25	4.00 ± 0.25 ^c (38%)	4.33 ± 0.21 ^c (32%)

For details of experimental groups refer to Materials and Methods section. Figures in parenthesis show % inhibition vs control. ($n = 6$). ^a $p < 0.02$; ^b $p < 0.05$; ^c $p < 0.001$ vs control. (Student's t -test).

causes damage to the respiratory tract and therefore prevention of eosinophil infiltration and activation is considered an important target for the treatment of allergic inflammatory disorders (Whitney *et al.*, 2002). In the murine ovalbumin model used in the present investigation EH A001 prevented the recruitment of eosinophils, inhibited the activity of its granular constituent EPO, and prevented the increase in airway plasma protein accumulation. This model mimics the pathophysiology of allergic asthma which represents a breakdown in the normal tolerance to inhaled antigens, and several other features such as airway hyperreactivity and inflammation (Herz *et al.*, 1996). The cytokines produced by activated CD4 T helper (Th) cells determine their differentiation into Th1 and Th2 cells. IFN- γ is a major cytokine produced by Th1 cells, contributing to cell mediated inflammatory response, while IL-4 secreted by Th2 cells, is strongly associated with allergic reactions and airway hypersensitivity (Glimcher and Murphy, 2000). Atopic hypersensitivities are associated with an increase in the number of Th2 cells and Th2 cytokines and a decrease in the number of Th1 cells and Th1 cytokines. EH A001 preferentially decreased CD4 T cells with concomitant suppression of the IL-4 production and increased CD8 T cells leading to enhanced levels of IFN- γ . From the viewpoint of cooperation of cytokines and chemokines, our data showed that EH A001 promoted the Th1 dependent pathway. The dominance of a Th1 type response would contribute to a low responsiveness of eosinophils which is also one of the key sources of some of these mediators (Cui *et al.*, 2003). Th2 cells also mediate the humoral responses. Humoral immunity includes primarily B lymphocytes and is related to antigen/antibody reactions, which are predominantly involved in Type I hypersensitivity. Considerable research is now being focused on the induction of immune suppression to redirect the allergen-mediated Th2 cell response to a beneficial clinical outcome (Romagnani, 2003). EH A001 with its prominent immunosuppressive activity might be

expected to selectively alter the Th1/Th2 cytokine pattern as immunosuppressive agents block T-cell activation and cytokine secretion. Allergic inflammation is considered to be mediated mostly by mast cells, eosinophils and antigen specific CD4 cells. In this respect, the EH A001 has revealed a promising profile being antihistaminic, antiinflammatory and immunosuppressive, compared with ketotifen, cetirizine and cyclophosphamide in different models. Ketotifen, a known prophylactic agent used in the management of bronchial asthma and allergic disorders, is reported to inhibit mast cell degranulation, activated eosinophils and T-cell recruitment (Hoshino *et al.*, 1997). Cetirizine has antiinflammatory and antihistaminic properties which plays a role in asthma management (Portnoy, 2004). Cyclophosphamide is a known immunosuppressive agent (Sulkowski *et al.*, 1998). Taken together, the results obtained in the present study provide evidence that EH A001 interacts with multiple targets of Type I allergic reactions. Currently, allergic diseases are treated symptomatically with the use of antihistamines, steroids or immunosuppressants that produce detrimental side effects on prolonged use. On the other hand, traditional preparations that are widely used in India, south-east Asia and Africa as alternative medicines for a variety of ailments are effective drugs and sources of drugs, but also have a long tradition of human use and have been found to be safe. Ayurveda, an ancient treatise on Indian medicinal herbs, describes a number of such plants and plant based medicines that have proved to be beneficial against a variety of clinical indications. The present study demonstrates that one such herb, *Euphorbia hirta*, interacts with identified targets and could be developed as an effective remedy for Type I allergic disorders.

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