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The plant extract *Isatis tinctoria* L. extract (ITE) inhibits allergen-induced airway inflammation and hyperreactivity in mice

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ABSTRACT

Background: The herbal *Isatis tinctoria* extract (ITE) inhibits the inducible isoform of cyclooxygenase (COX-2) as well as lipoxygenase (5-LOX) and therefore possesses anti-inflammatory properties. The extract might also be useful in allergic airway diseases which are characterized by chronic inflammation.

Methods: ITE obtained from leaves by supercritical carbon dioxide extraction was investigated in ovalbumin (OVA) immunised BALB/c mice given intranasally together with antigen challenge in the murine model of allergic airway disease (asthma) with the analysis of the inflammatory and immune parameters in the lung.

Results: ITE given with the antigen challenge inhibited in a dose related manner the allergic response. ITE diminished airway hyperresponsiveness (AHR) and eosinophil recruitment into the bronchoalveolar lavage (BAL) fluid upon allergen challenge, but had no effect in the saline control mice. Eosinophil recruitment was further assessed in the lung by eosinophil peroxidase (EPO) activity at a dose of 30 µg ITE per mouse. Microscopic investigations revealed less inflammation, eosinophil recruitment and mucus hyperproduction in the lung in a dose related manner. Diminution of AHR and inflammation was associated with reduced IL-4, IL-5, and RANTES production in the BAL fluid at the 30 µg ITE dose, while OVA specific IgE and eotaxin serum levels remained unchanged.

Conclusion: ITE, which has been reported inhibiting COX-2 and 5-LOX, reduced allergic airway inflammation and AHR by inhibiting the production of the Th2 cytokines IL-4 and IL-5, and RANTES.

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Introduction

Isatis tinctoria L. (woad, Brassicaceae) is an ancient European dye and medicinal plant which was used as astringent and for treating skin inflammation and ulcers (*Isatis tinctoria*, monograph 2002). Lipophilic extracts from *Isatis tinctoria* (ITE) significantly inhibit cyclooxygenase 2 (COX-2), 5-lipoxygenase (5-LOX), inducible NO synthase (iNOS), leucocytic elastase, and histamine and serotonin release from stimulated mast cells (Hamburger 2002). ITE showed anti-inflammatory activity in carageenan-induced paw oedema and in tetradecanoylphorbol acetate (TPA)-induced mouse ear oedema (Recio et al. 2006a, b). In a clinical pilot study the anti-inflammatory activity of topical administered ITE was confirmed in skin irritation models (Heinemann et al.

2004). Based on this data it can be suggested that ITE may also be useful in allergic airway inflammations.

There is general consensus that allergic inflammation is driven by the activation of T-helper (Th) type 2 cells. They are known to produce IL-4 and IL-13 contributing to IgE production, mucus hypersecretion, airway hyperresponsiveness (AHR), and IL-5 promoted eosinophilic inflammation (Ahern and Robinson 2005; Robinson 2005). Therefore, several attempts are being made to reduce the inappropriate Th2 response to reduce allergic airway diseases. Therapeutic concepts include Th2 cytokine inhibitors, neutralising antibodies directed towards B-cell IgE, histamine and leukotriene blockers, as well as other targets (Barnes 2008; Holgate and Polosa 2008; Wills-Karp 1999). The aim of the present investigation was to test the ability of the ITE to inhibit Th2 response.

Murine models can be used for such investigations, since systemic responses as well as airway responses are well defined (Kips et al. 2003). It was previously demonstrated that intranasally applied allergens are equally distributed within the upper and

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lower airway systems, and that repeated allergen challenges induced airway hyperresponsiveness (AHR) (Wang and McCusker 2005).

Therefore, Th2 activation, allergic tissue reactions, inflammatory cell recruitment in the bronchoalveolar lavage (BAL), and airway hyperresponsiveness to methacholine were studied in the OVA antigen-induced experimental model in mice, and then compared with negative and positive controls. We report that orally administered ITE, which inhibits COX-2 and 5-LOX, effectively inhibits an established Th2 response in the lung.

Methods

Animals and immunization

Balb/c mice, aged 6–8 weeks, were immunised twice subcutaneously in groups of 6 mice at weekly intervals with a 0.4 ml saline solution containing 1 µg OVA and 1.6 mg alums. One week after the second immunisation, on day 14, intranasal challenge was performed under light i.v. ketamine anaesthesia by applying 50 µl OVA in alum-free saline solution (10 µg) or saline alone as control. The intranasal challenges were given on three consecutive days (Couillin et al. 2004). All experimental studies were approved by the local ethical animal research committee.

Plant extraction and administration

The ITE extract was manufactured using supercritical carbon dioxide with leaves of cultivated *Isatis tinctoria*. Fresh leaves were dried on a band drier operating at 60 °C and coarsely powdered with an SK 100 cross-beater mill (Retsch; Haan, Germany) equipped with a 2 mm sieve. The supercritical CO₂ (SFE) extract was prepared at the Adalbert-Raps-Zentrum, Technical University München-Weihenstephan, in a pilot-plant extractor by extraction with CO₂ at 800 bar and 50 °C for 3 h. The extraction yield was 0.85%. The extract corresponded to the SFE extract in tryptanthrin, indolin-2-one, indirubin, indigo and alpha-linolenic acids at 0.23%, 0.012%, 1.51%, 0.21% and 9.09%, respectively (Hamburger 2002, Recio et al. 2006a, b).

The ITEs extract was given intranasally at a dose of 10, 30, and 100 µg (in 40 µl) per mouse before saline or OVA challenge in OVA sensitised Balb/c mice under light ketamine anaesthesia (Fig. 1).

Airway hyperresponsiveness (AHR)

Airway resistance was evaluated by whole-body plethysmography as described before (Couillin et al. 2004). Bronchial hyperreactivity to aerosolised methacholine was investigated 24 h after the last saline or OVA challenge in OVA sensitised mice as described before (Couillin et al. 2004). Unrestrained conscious mice were placed in whole-body plethysmography chambers (Buxco Electronic, Sharon, CO, USA). Methacholine at 100 mM is aerosolized for 1 min and mean airway bronchoconstriction readings, as assessed by Enhanced Respiratory Pause (PenH), were obtained over 15 min periods. PenH can be conceptualised as the phase shift of the thoracic flow and the nasal flow curves; where an increased phase shift correlates with increased respiratory system resistance. PenH is calculated by the formula $\text{PenH} = (\text{Te}/\text{RT} - 1) \times \text{PEF}/\text{PIF}$, where Te is the expiratory time, RT the relaxation time, PEF the peak expiratory flow, and PIF the peak inspiratory flow.

Bronchoalveolar lavage (BAL)

BAL was performed 24 h after the last antigen challenge by cannulating the trachea under ketamine anaesthesia and washing with 0.5 ml of ice-cold phosphate-buffered saline (PBS) (Couillin et al. 2004). The first 500 µl of the lavage fluid was centrifuged and the supernatant frozen for subsequent cytokine determinations and the trachea was further lavaged with 3 × 0.5 ml of PBS and centrifuged. The cell pellet was resuspended in PBS, counted by a haemocytometer chamber and cytospin preparations were made using a Shandon cytocentrifuge. The cells were analysed after differential staining with May–Gruenwald–Giemsa.

Lung histology

After bronchoalveolar lavage, the mice were sacrificed 24 h after the last saline or OVA challenge. The whole lung was removed and fixed in 4% buffered formaldehyde for standard microscopic analysis with haematoxylin and eosin (H&E) and periodic acid Schiff reagent (PAS) staining. Peribronchial inflammation, eosinophilic recruitment, and mucus hypersecretion was assessed using a semi-quantitative score (a scale from 0 to 5, absent; mild, slight, moderate, distinct, severe) by two independent observers as described before (Couillin et al. 2004).

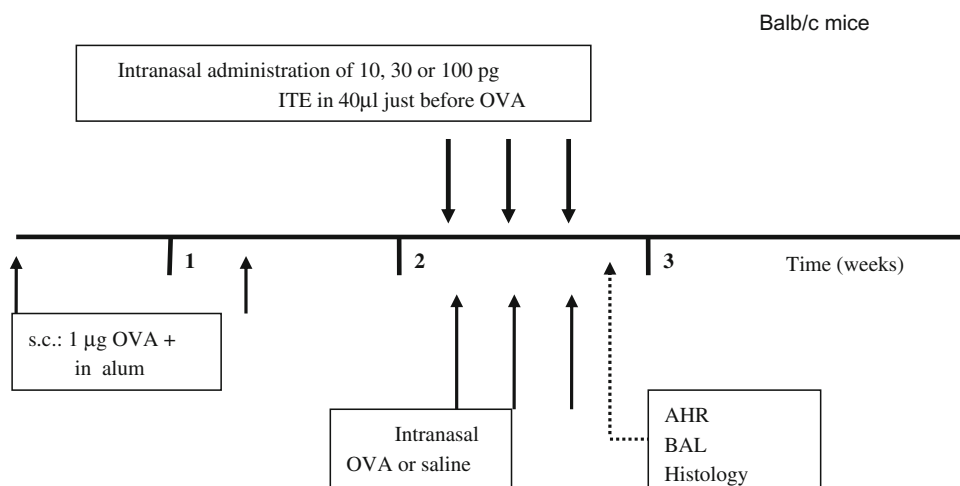


Fig. 1. Scheme of allergen OVA sensitization (days 0 and 7) and intra-nasal instillation of ITE followed by OVA challenge (days 14, 15, 16) and analysis on day 17 (AHR, BAL, cytokines, IgE and histology).

Eosinophil peroxidase (EPO) activity

Fresh lung tissues were frozen and homogenised in buffer and substrate was added to assess EPO activity as described before (Couillin et al. 2004).

Cytokines in BAL and plasma

IL-4, IL-5, IL-10, IL-12, IL-13, IFN γ , and the chemokines RANTES and eotaxin in the first volume of 500 μ l of the BAL fluid, and eotaxin in plasma were evaluated by enzyme-linked immunosorbent assays (ELISA) from Pharmingen and R&D following the instructions of the manufacturer.

Serum IgE levels

Blood was taken 24 h after the last saline or OVA challenge and plasma was prepared. Total and specific IgE levels were measured in plasma by ELISA as described before (Couillin et al. 2004).

Statistical analysis

The Student-*t* test was used to compare the differences between experimental groups ($p < 0.05$).

Results

Isatis tinctoria extract reduces airway hyperresponsiveness (AHR)

ITE was given intranasally at doses of 10, 30, and 100 μ g per mouse immediately before each antigen challenge in OVA sensitised Balb/c mice. ITE inhibited partially methacholine-induced

AHR, which was assessed by whole body plethysmography. PenH is an indirect measure of airway resistance and reflects inflammatory changes in the airway system (Couillin et al. 2004). The PenH values after methacholine exposure are shown over a period of 15 min (Fig. 2A). The inhibitory effect was more prominent in the later phase of the response which is after 5 min. The dose effect was calculated by the area under the curve (AUC) of PenH over the 15 min time period as shown in Fig. 2B. ITE caused a dose related, significant reduction of PenH at 30 μ g ($p < 0.05$) and 100 μ g ($p < 0.01$). Importantly, ITE had no effect on PenH in saline challenged, sensitized mice. Therefore, the data suggest that the ITE may inhibit AHR as reported for leukotriene inhibitors.

Diminished eosinophil recruitment in the lung by Isatis extract

A hallmark of allergic asthma is the recruitment of eosinophils into the lung. Therefore, we investigated the BAL fluid obtained 24 h after the last antigen challenge, counted the cells and prepared cytospin preparations for the differential cell counts. ITE inhibited in a dose related manner eosinophil recruitment into the BAL fluid (Fig. 3A). The recruitment of eosinophils was further evaluated in lung tissue homogenate followed by the determination of eosinophil peroxidase (EPO) activity expressed as optical absorbance (OD) as described (Couillin et al. 2004). ITE inhibited EPO activity at the dose of 30 μ g ($p < 0.05$, Fig. 3B). Therefore, ITE given together with the antigen challenge reduces eosinophil recruitment in the lung.

Reduced IL-4, IL-5, and RANTES production in BAL fluid

Since eosinophilic inflammation was reduced, we asked whether typical mediators of a Th2 response are affected by ITE. The antigen challenge induced a significant increase in IL-4, IL-5,

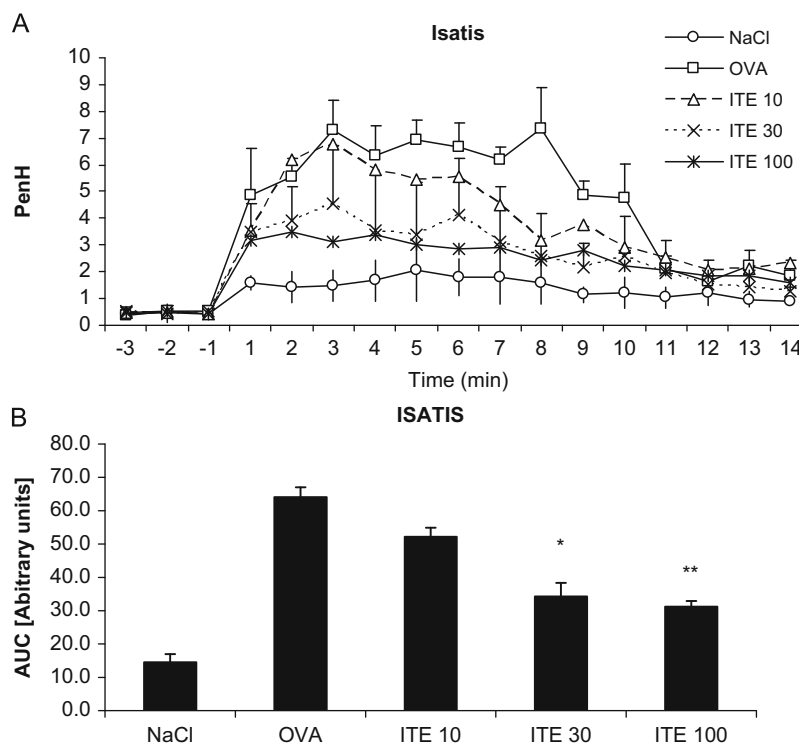


Fig. 2. Methacholine-induced airway hyperresponsiveness (AHR) at 24 h after the last antigen challenge (day 17). **A.** Methacholine (100 μ M)-induced AHR in saline controls (NaCl), OVA (OVA) in the absence or presence of ITE in OVA sensitized mice. ITE was given at doses of 10 μ g (ITE 10), 30 μ g (ITE 30), and 100 μ g (ITE 100) per mouse or saline to OVA challenged mice (Figure OVA data only). Mean $\pm$ SD of PenH values and SD are shown ($n=4$ mice per group). **B.** Dose response effect of ITE (10–100 μ g) peron PenH values expressed as mean $\pm$ SD of the area under curve, (AUC; $n=4$ mice per group, * $p < 0.05$; ** $p < 0.01$).

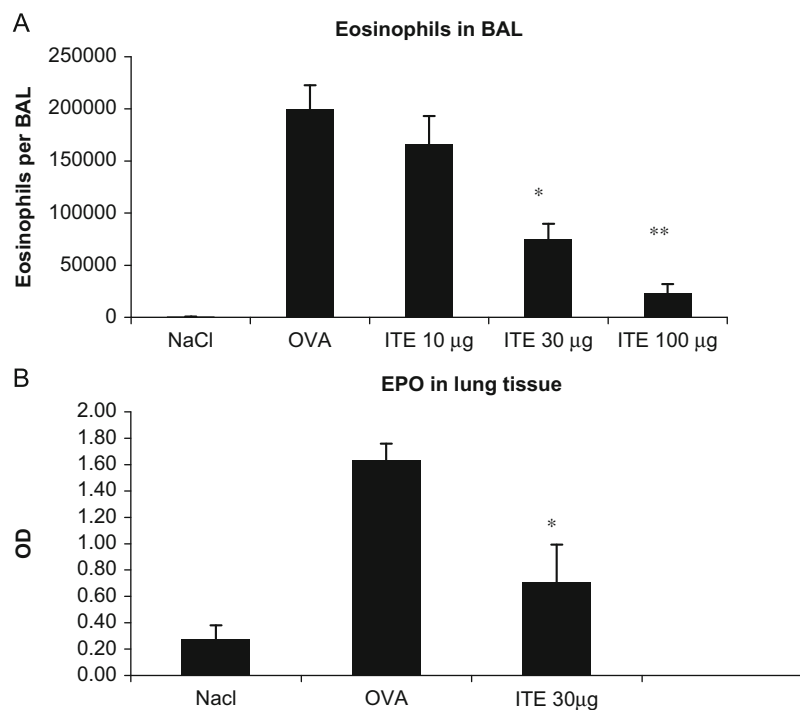


Fig. 3. Reduced recruitment of eosinophils in BAL and lung at 24 h after antigen challenge (day 17). **A.** Effect of ITE doses (10–100 µg) on eosinophil recruitment in BAL (day 17). Total count of eosinophils in BAL of saline controls (NaCl), OVA challenged mice in the absence of ITE (OVA) and OVA challenged mice with ITE at doses of 10 µg (ITE 10), 30 µg (ITE 30), and 100 µg (ITE 100). **B.** Effect of PET on lung eosinophil peroxidase (EPO) activity in the homogenized lung tissue after BAL lavage (day 17). OD values of EPO in homogenized lung tissue after BAL lavage of saline controls (NaCl), OVA challenged mice in the absence of ITE (OVA) and OVA challenged mice with 30 µg ITE. Graph shows mean values $\pm$ SD and indicated p -values ($n=6$ mice per group, * $p < 0.05$; ** $p < 0.01$).

and RANTES levels in the BAL fluid in immunised mice when compared to saline controls. The levels of IL-4, IL-5, and RANTES were reduced in the BAL fluid from mice treated with 30 µg ITE ($p < 0.05$, Fig. 4A–C), the others doses of ITE were not tested.

Furthermore, we measured IL-10 levels in the BAL. IL-10 was detectable upon OVA challenge, but was not modified by ITE administration at the 100 µg dose. IFN γ , eotaxin, or IL-13 in the BAL fluid were not detected in any experimental group (data not shown). Furthermore, plasma eotaxin levels were determined as an indicator of the systemic allergic response. OVA immunisation induced an increase in eotaxin plasma levels, which however were not influenced by ITE (Fig. 4D).

Finally, total and OVA specific IgE levels were determined in the serum at 24 h after the last saline or OVA challenge. OVA administration had a slight effect on total IgE and OVA specific IgE levels. ITE given at a dose of 100 µg had no effect on IgE levels (data not shown).

Therefore, ITE inhibits the production of IL-4, IL-5, and RANTES, which are critical mediators of the allergic response.

Allergic lung inflammation and mucus hypersecretion is diminished by *Isatis* extract

Microscopic investigations were performed after the antigen challenge to investigate lung morphology. Typical representative microscopic pictures are shown in Fig. 5. While the antigen-challenged immunised mice displayed impressive inflammation, eosinophilic infiltration and mucus production (B), saline-challenged immunised mice had normal lung architecture (A). ITE treated mice (C) showed a distinct reduction of inflammation and mucus production.

The lung sections were further analysed semi-quantitatively by a severity score (0–5). The extent of peribronchial inflammation, infiltration by eosinophils, and mucus hyperproduction were recorded. We found that ITS dose related inhibited the recruitment of eosinophils and the production of mucus (Table 1). Therefore, the morphological findings corroborated functional data and in particular the reduced eosinophil recruitment in the BAL.

Discussion

The ITE extract prepared from leaves of *Isatis tinctoria* by supercritical carbon dioxide showed activities in murine model of allergic asthma using OVA challenge of OVA sensitized BALB/c mice. The typical Th2 cytokine profile response to an allergen challenge was seen in the present investigation, which consisted of a prevailing IL-4 and IL-5 production and relatively low or undetectable levels of IFN- γ and IL-10 (Ohkawara et al. 1997; Wegmann et al. 2005). This cytokine profile reflects an allergen-induced release by activation of Th2 cells and therefore may be related to extrinsic eosinophilic disorders (Simon and Simon 2007). There was no evidence that Th1 cells are activated to balance Th2 stimulation, since neither IFN- γ nor IL-12 was found in BAL. The absence of IL-13 in BAL could be due to the limited sensitivity of the ELISA detection system, as IL-13 clearly participates in the allergic response and neutralizing IL-13 antibody diminishes allergy (Wang and McCusker 2005). Interestingly an extract from green tea *Camellia sinensis* increases expression of Th1 cell specific bio-markers, which counteracts the dominance of Th2 cells markers typically seen in the ovalbumin-induced asthmatic model (Heo et al. 2008a).

The Th2 cytokines IL-4 and IL-5 were significantly reduced in response to ITE when given just before intranasal OVA challenge,

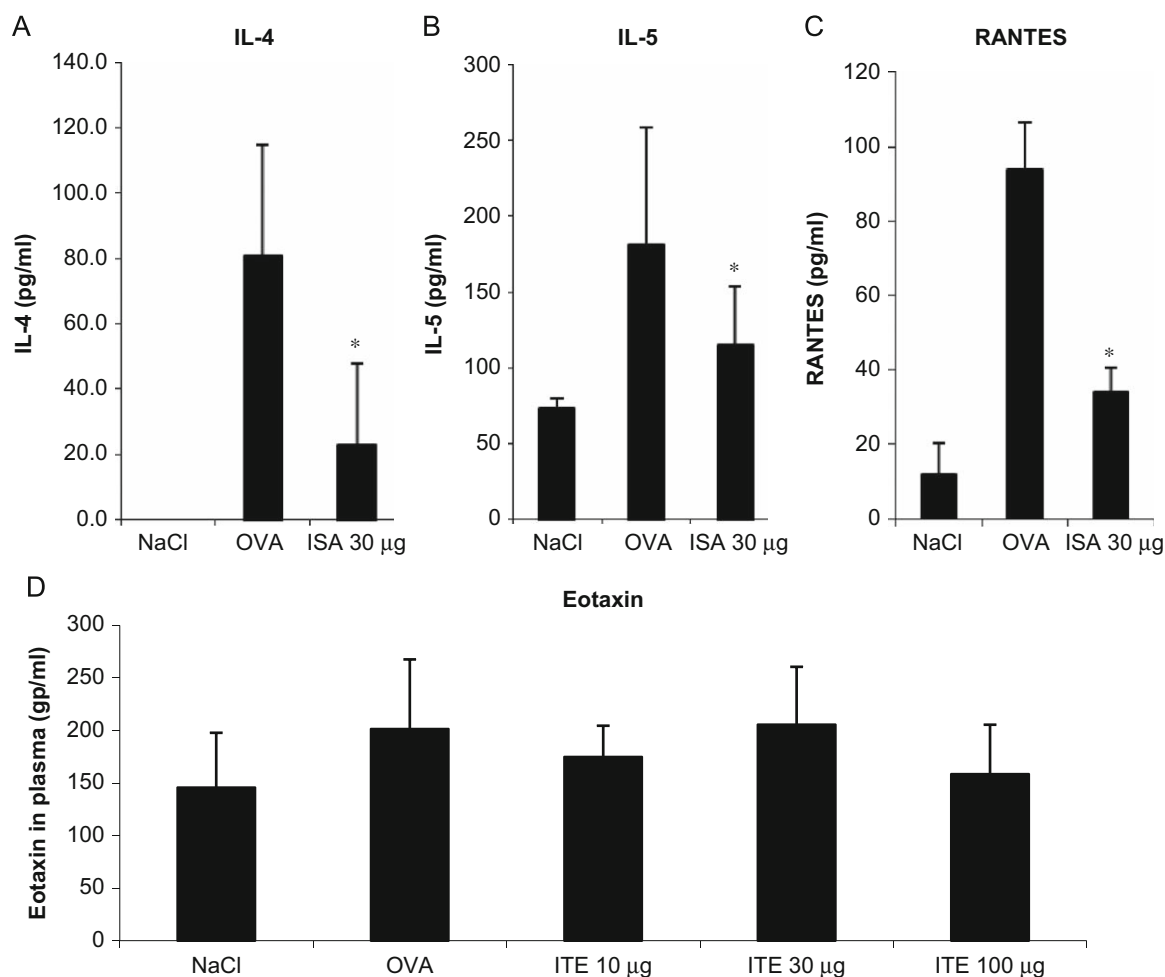


Fig. 4. Inhibition of cytokine secretion in BAL fluid and eotaxin in serum at 24 h after last antigen challenge as analysed on day 17. Determination by ELISA of IL-4 (A), IL-5 (B), and RANTES (C), concentrations in the first 500 µl BAL fluid of saline controls (NaCl), OVA challenged mice in the absence of ITE (OVA) and OVA challenged mice with ITE at doses of 30 µg (ITE 30). (D) eotaxin concentration in plasma of saline controls (NaCl), OVA challenged mice in the absence of ITE (OVA) or in the presence of ITE at doses of 10 µg (ITE 10), 30 µg (ITE 30), and 100 µg (ITE 100). Graphs shows mean $\pm$ SD and indicated *p*-values ($n=4-6$ mice per group).

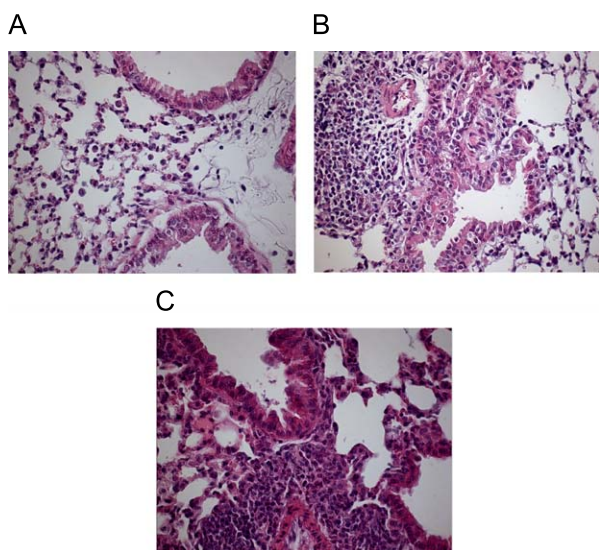


Fig. 5. Representative microscopic plates of lung tissue demonstrating reduced infiltration by inflammatory cells, eosinophil recruitment and mucus hypersecretion one day after the last OVA challenge and 30 µg ITE administration (day 17). (A) Saline controls, (B) OVA challenged in the absence of ITE, (C) OVA challenged with 30 µg ITE.

but had no effect in saline challenged mice. The dose related reduction of AHR, as well as mucus hypersecretion, may be due to a reduced IL-4 production by ITE. The reduced activity of EPO within lung tissue indicates a diminished recruitment of eosinophils into the lung parenchyma. Moreover, accumulation of eosinophils into lung tissue and migration into BAL fluid were significantly inhibited. On the one hand this observation may be linked to the reduced IL-5 secretion in BAL. On the other hand, leukotriene B₄ may play a pivotal role in mediating the selective recruitment of eosinophils in a model of chronic eosinophilic inflammation driven by allergen challenge of CD4⁺ cells (Cheraim et al. 2008).

Extracts from *Saururus chinensis* (Lee et al. 2006), semen *Armeniaea amarum* (Do et al. 2006), *Helianthus annuus* (Heo et al. 2008b), and *Petasites hybridus* (Brattström et al. 2009) like ITE alleviates similarly asthmatic symptoms in the ovalbumin sensitized asthmatic mouse model by suppressing Th2 biomarkers.

Total and OVA specific IgE levels were augmented upon allergen challenge. It has been shown that IL-4-induced IgE production by normal PBMCs was enhanced by leukotriene B₄, whereas by itself leukotriene B₄ was not effective for IgE production (Yamaoka et al. 1994). However, our data demonstrate that intranasal ITE administration during the antigen challenge

Table 1

Quantification of the effects of ITE on allergic inflammation, eosinophil infiltration and mucus hypersecretion in the lung (day 17).

Isatis reduced inflammation, eosinophil recruitment and mucus hyperproduction				
Group	#	Inflammation	Eos infiltration	Mucus hyperproduction
NaCl	1	1	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	0	0	0
Mean		0.2	0.0	0.0
SD		0.4	0.0	0.0
OVA	11	3	3	4
	12	3	4	2
	13	4	3	4
	14	2	3	3
	15	3	4	3
Mean		3.0	3.4	3.2
SD		0.7	0.5	0.8
ITE 10 µg	51	2	2	3
	52	2	2	2
	53	1	3	3
	54	3	2	3
	55	2	3	2
Mean		2.0	2.4	2.6
SD		0.7	0.5	0.5
ITE 30 µg	61	1	1	2
	62	2	2	3
	63	1	1	1
	64	1	3	3
	65	2	2	2
Mean		1.4	1.8	2.2
SD		0.5	0.8	0.8
ITE 100 µg	71	1	1	1
	72	2	2	2
	73	2	1	1
	74	1	3	2
	75	2	2	2
Mean		1.6	1.8	1.6
SD		0.5	0.8	0.5
Score: 0, no changes; 1–5 increasing extent of parameter				

Microscopic evaluation of lung tissue sections stained with haematoxylin and eosin and PAS (mucus) was performed by a semi-quantitative score (0=no change, 1–5 with increasing severity as described in Methods. Mean scores $\pm$ SD are given ($n=5$ mice per group).

does not reduce IgE production. The reduced airway inflammation and hyperreactivity are related to the whole extract since purified components were not tested.

The ITE extract exerts multifaceted activities including inhibition of COX-2, 5-LOX, iNOS, histamine and serotonin release, and of leukocyte elastase (Hamburger 2002). These activities are related to different components within the ITE extract and can be explained, such as tryptanthrin and indirubin. However additional compounds may contribute to the *in-vivo* activity of ITE (Recio et al. 2006a, b), and numerous minor alkaloids. Therefore, the anti-inflammatory effect of ITE is due to several pharmacological active components, which need to be further identified.

In conclusion, when given during an antigen challenge, ITE effectively inhibits allergen-induced inflammation and airway hyperreactivity by reducing the Th2 immune response in a murine model of asthma.

Declaration of conflicts of interest

The authors declare no financial conflicts of interest with this publication.

Acknowledgments

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