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Inhibitory effects of *Tinospora cordifolia* and *Rubia cordifolia* Linn. on egg albumin-induced experimental allergic conjunctivitis in rats

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SUMMARY

Allergic conjunctivitis is the most common allergic disease. These diseases are severe & frequent which requires search of new treatments. The aim of the study was to investigate the effects of *Tinospora cordifolia* (TC), *Rubia cordifolia* Linn. (RC) on experimentally induced allergic conjunctivitis in rats. In this study, dried water soluble extracts of TC and RC. (250 and 500 mg/kg, p.o. for 7 days) were evaluated for their antiallergic activity in Wistar rats. They were tested for inhibition of egg albumin-induced vascular permeability, inhibition of histamine release from the rat conjunctiva as well as in histamine content in tears. TC and RC showed significant ($P < 0.05$) inhibition in vascular permeability, inhibition in histamine release from the rat conjunctiva which is reflected by reduced level of histamine content in tears. The activities were found to be comparable to azelastine hydrochloride. These results suggest that the inhibitory effect on egg albumin-induced experimental allergic conjunctivitis in rat may be due to the antihistaminic activity of TC and RC. Our studies provide evidence that TC and RC may be beneficial in the treatment of allergic conjunctivitis.

Key words: Mast cell; Allergic conjunctivitis; Histamine release; Vascular permeability; Antihistaminic

INTRODUCTION

Allergic conjunctivitis occurs when the eye is exposed to antigens in the environment (sensitization to allergens). Acute allergic conjunctivitis also known as hay fever conjunctivitis, Seasonal allergic conjunctivitis or simple conjunctivitis is triggered by allergens such as pollen (Abelson *et al*., 1993; McGill and Bacon, 1998). Exposure of appropriately sensitized IgE-coated mast cells to airborne allergen is the initiating stimulus. The allergen binds to separate IgE molecules, creating a dimmer formation that initiates the chain of reactions in the mast cell plasma membrane. The bridging of mast cell IgE molecules induces the activation of membrane-associated enzymes leading to an increase in uptake of calcium with the subsequent mobilization of intracellular calcium and initiation of the biochemical process of histamine release (Berdy, 1999). The early phase occurs when the mast cell degranulates with the immediate release of the preformed mediators such as histamine. The early phase lasts for about 20 to 30 min and occurs within

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minutes of exposure to an antigen. Histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity among the inflammatory substances released from mast cells (Petersen *et al*., 1996). The late phase response occurs approximately 6 to 12 h after the early phase and is marked by the activation of inflammatory cells. It is characterized by a chemotactic influx of immune cells such as eosinophils, neutrophils, lymphocytes, and basophils with eosinophils being the most active in promoting the inflammatory cascade (Epstein, 2002). The allergic conjunctivitis is thought to be driven by cross-linking of allergen-specific IgE bound to the surface of resident mast cells via the high-affinity IgE receptor, FcεRI. Thus, the mast cell is the key effector cell in immediate hypersensitivity reactions, releasing histamine, inflammatory cytokines, chemokines and platelet activating factor upon antigenic stimulation. Histamine stimulates nerve endings and dilates the blood vessels, causing itching and redness (Leonardi, 1999; Leonardi, 2000). *Tinospora cordifolia* (TC) is a large, glabrous, climbing shrub belonging to the family Menispermaceae. It is widely used in veterinary folk medicine/Indian system of medicine (Ayurvedic) for its general tonic, antispasmodic, anti-inflammatory, antiarthritic and anti-diabetic properties (Pendse *et al*., 1981; Singh *et al*., 2003). *Rubia cordifolia* Linn. (RC) is considered to be traditionally useful as an external application in inflammations, ulcers and skin diseases, to relieve the symptoms of pruritis, burning and exudation from skin (Jorapurkar *et al*., 2003a,b). The antioxidant, anti-inflammatory and immuno-modulatory properties of TC and RC has also been well documented (Tripathi *et al*., 1997; Kasture *et al*., 2001; Bishayi *et al*., 2002; Subramanian *et al*., 2002). In light of the aforementioned anti-inflammatory properties of TC and RC, it is hypothesized that these herbs may be helpful in reducing the antigen-induced conjunctivitis in rats via anti-histaminic activities since inflammatory mediators like histamine is also involved in allergy. Therefore it is aimed to study the effect of these two herbs against antigen-induced conjunctivitis in rats in allergic rat conjunctivitis models viz. vascular permeability of the conjunctiva, histamine release from the conjunctiva, histamine content in tears and histopathological examination (Minami and Kamei, 2004). The results were compared with topically active H1 anti-histamine azelastine hydrochloride as positive control (Ciprandi *et al*., 1997).

MATERIALS AND METHODS

Reagents

Egg albumin and aluminum hydroxide hydrate gel (alum) were purchased from S D Fine-Chem Limited, India. Evans blue and o-phthaldialdehyde (OPT) were purchased from HiMedia, India. *Bordetella pertussis* inactive microorganism suspension (*B. pertussis*) was a gift samples from The Serum Institute of India, Pune, India. Azelastine hydrochloride eye drops (Azelast eye drops, 0.05%) was purchased from Sun Pharmaceutical Industries Limited, India and was instilled at 30 min, before antigen challenge into the right eyes at 10 μl per eye. All other chemicals were of analytical grade.

Preparation of TC and RC extract

The stem parts of TC and RC were collected from the Himalaya. The plant specimens were certified (voucher number JVV1 for TC and voucher number Vidya 1 for RC) by Botanical Survey of India, Ministry of Environment and Forests Government of India, Pune. The plant samples were extracted with distilled water at 70 °C for 5 h. The extract was filtered through a 0.45 μm filter and the filtrate was lyophilized, and kept at 4 °C. The yield of dried extract of TC and RC from starting crude materials of TC, RC was about 10% and 12% respectively. The dried extracts were dissolved in saline before use.

Experimental protocol

All experiments and protocols described in the

present study were approved by the Institutional Animal Ethics Committee of M.S. University, Baroda, and with permission from the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. Eight to ten week-old male Wistar rats (200 ± 25 g) were housed in groups of 3 animals and maintained under standardized conditions (12 h light/dark cycle, 22 ± 2 °C and relative humidity of 55 ± 5%) and provided free access to pelleted Chakkan diet (Nav Maharashtra Oil Mills Pvt Ltd, Pune, India) and purified drinking water ad libitum. Rats were randomly divided into seven groups, each consisting of six animals for four models of the study. Animals were divided into following groups: Group-1 (UNIM-CON): Un-immunized control animals, Group-2 (IM-CON): Immunized control animals treated with vehicle by oral gavage from 8th day to 14th day, Group-3 (AZ HCL): Treated with 0.05% azelastine hydrochloride eye drops (10 µl/right eye on the 14th day, 30 min before the antigen challenge) and served as positive control. Group-4 (TC 250): Treated with extract of TC (250 mg/kg, p.o.) from 8th day to 14th day. Group-5 (RC 250): Treated with extract of RC (250 mg/kg, p.o.) from 8th day to 14th day. Group-6 (TC 500): Treated with extract of TC (500 mg/kg, p.o.) from 8th day to 14th day. Group-7 (RC 500): Treated with extract of RC (500 mg/kg, p.o.) from 8th day to 14th day. On day 14th, 2 h after the treatment of TC and RC, rats were subjected to the challenge with egg albumin.

Sensitization

The rats of the group two to seven were sensitized by injection of 0.6 ml of physiological saline containing egg albumin (1 mg), alum (2 mg) and 1×10^{10} *B. pertussis* into the four footpads on the first day. Five days later, they were boosted by subcutaneous injection of 1 ml of physiological saline containing egg albumin (0.5 mg) at 10 sites on the back. Then, local sensitization was performed on day 14th by instilling egg albumin in physiological saline (10 mg/ml, 10 µl per site) into the right eyes using a micropipette.

Conjunctivitis intensity score (CIS)/Wheal index

On the 14th day following antigen challenge, 0.4 ml of Evans blue (20 mg/ml) solution was intravenously injected by tail vein. 30 min later, the rats were sacrificed and right eyes were enucleated along with the conjunctiva and eyelids. Length (L) and breadth (B) of Evans blue extravasated conjunctiva was measured with the help of digital caliper (Mitytoyo, Japan). Color intensity (i) of conjunctiva was evaluated numerically according to an arbitrary 5-point graded scale from 0 to 4 that increased with severity (0, no Evans blue extravasation; 1, light blue color; 2, mild blue color; 3, moderate blue; 4, severe dark blue). CIS/Wheal index was calculated as L x B x i for each conjunctiva (Yanni *et al*., 1996).

Vascular permeability of the conjunctiva

After scoring the CIS, the extravasated Evans blue was extracted from the ocular tissues by immersing the tissues in an extracting solution of 3 ml sodium sulphate (0.5% w/v) and 7 ml acetone kept at room temperature and vigorously shaken. 24 h later, the solutions were centrifuged at 300 rpm for 10 min and the color intensity of the supernatant was evaluated by spectrophotometer (Model UV-1601, Shimadzu) at 620 nm. Standard curves were performed to transform absorbance units into µg Evans blue per ml of solution.

Histamine release from the conjunctiva

Thirty minutes after antigen challenge, the right conjunctiva was carefully excised and washed twice with saline. The tissues were homogenized with 0.4 N perchloric acid and placed in an ice-bath for 1 h. After centrifugation at 1000 rpm for 10 min at 4 °C, histamine content in the supernatant was determined by a fluorometric assay as % emission (Anton and Sayre, 1969). Briefly, samples were incubated in the presence of NaOH (1 M) and OPT (5 mg/ml) for exactly 4 min. The reaction was

quenched by the addition of 200 μl of citric acid (2 M) and the fluorescence measured on a Shimadzu RF-540 Spectrofluorophotometer, with λexcitation = 345 nm and λemission = 441 nm. Standard curves were performed to transform % emission units into μg histamine per ml of solution.

Histamine content in tears

Thirty minutes after antigen challenge, 50 μl saline was applied to right eyes. This procedure was repeated 4 times and a 200 μl sample was carefully collected. The sample and the same quantity of 0.8 N perchloric acid were then mixed together. After centrifugation at 1000 rpm for 10 min at 4 °C, the histamine content of the supernatant was determined by a fluorometric assay as % emission as described earlier. Standard curves were performed to transform % emission units into μg histamine per ml of solution.

Histopathological examination

Thirty minutes after the antigen challenge, the right bulbar conjunctiva with palpebral skin was carefully excised from all the animals of histopathology groups. The tissues were fixed in formaldehyde (10%) and were embedded in paraffin. Cross sections were stained with hematoxylin and eosin. Preparations were observed under a light microscope (400X; Olympus Optical, Tokyo, Japan). Pathological examination revealed edema in the conjunctiva and the palpebral skin. The edema was graded as follows: –, no remarkable abnormality; ±, very slight changes; +, slight changes; ++, moderate changes; and +++, severe changes.

Statistical analysis

All the data are expressed as mean ± S.E.M. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed Student's *t*-test as appropriate using a computer- based fitting program (Prism, GraphPad). Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Effect TC and RC treatment on conjunctivitis intensity score in allergen-induced conjunctivitis

There was a significant ($P < 0.001$) increase in conjunctivitis intensity score from the conjunctiva in the IM-CON group as compared with the UNIM-CON group which indicates the induction of allergic conjunctivitis. There was a significant ($P < 0.001$) reduction in conjunctivitis intensity score of TC and RC (500 mg/kg, for 7 days) treated rats as compared with the right eye treated with saline of IM-CON group. However TC and RC (250 mg/kg) did not show a significant reduction in conjunctivitis intensity score. Azelastine hydrochloride, a reference drug, treated rats showed a significant ($P < 0.001$) reduction in conjunctivitis intensity score from the conjunctiva as compared with the right eye treated with saline of immunized control rats (Fig. 1).

Effect TC and RC treatment on Evans blue extravasation in allergen-induced conjunctivitis

There was a significant ($P < 0.001$) increase in Evans blue extravasation from the conjunctiva in

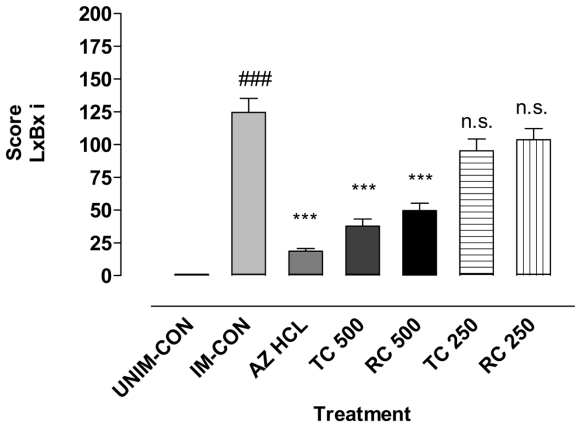

Fig. 1. The effect of TC (250 and 500 mg/kg per day, p.o.) and RC (250 and 500 mg/kg per day, p.o.) treatment for 7 days on conjunctivitis intensity score in allergen-induced conjunctivitis. Azelastine hydrochloride (10 μl per right eye) was instilled topically 30 min before the challenge. Values are expressed as mean ± S.E.M. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, n.s. = non significant compared to immunized control group, $^{###}P < 0.001$ = significant compared to UNIM-CON (n = 6).

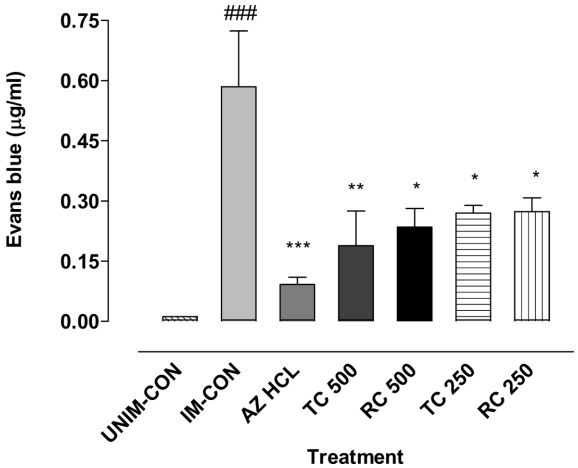

Fig. 2. The effect of TC (250 and 500 mg/kg per day, p.o.) and RC (250 and 500 mg/kg per day, p.o.) treatment for 7 days on vascular permeability of Evans blue in allergen-induced conjunctivitis. Azelastine hydrochloride (10 µl per right eye) was instilled topically 30 min before the challenge. Values are expressed as mean ± S.E.M. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, n.s. = non significant compared to immunized control group, $^{###}P < 0.001$ = significant compared to UNIM-CON (n = 6).

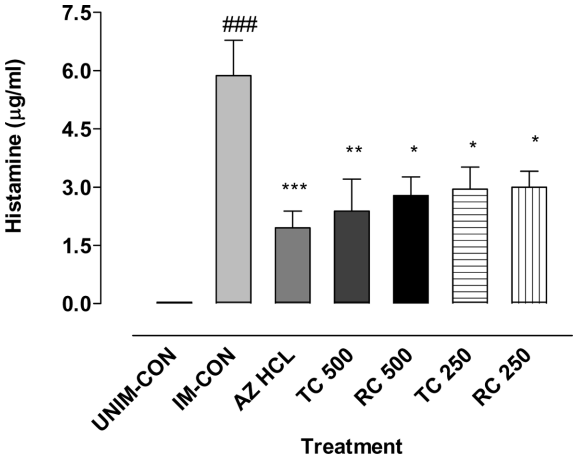

Fig. 3. The effect of TC (250 and 500 mg/kg per day, p.o.) and RC (250 and 500 mg/kg per day, p.o.) treatment for 7 days on allergen-induced histamine release in conjunctiva of rats. Azelastine hydrochloride (10 µl per right eye) was instilled topically 30 min before the challenge. Values are expressed as mean ± S.E.M. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, n.s. = non significant compared to immunized control group, $^{###}P < 0.001$ = significant compared to UNIM-CON (n = 6).

the IM-CON group as compared with the UNIM-CON group which indicates the induction of allergic conjunctivitis. TC and RC (500 mg/kg) treatment for 7 days significantly ($P < 0.01$, $P < 0.05$ respectively) reduced Evans blue extravasation from the ocular tissues as compared with the right eye treated with saline of IM-CON group. Also TC and RC (250 mg/kg) treatment for 7 days showed a significant ($P < 0.05$) reduction in Evans blue extravasation. While azelastine hydrochloride treated rats showed a significant ($P < 0.001$) reduction in Evans blue extravasation from the conjunctiva as compared with the right eye treated with saline of immunized control rats (Fig. 2).

Effect TC and RC treatment on histamine release from the conjunctiva in allergen-induced conjunctivitis

The immunized control rats of IM-CON group showed a significant ($P < 0.001$) increase (5.87 ± 0.91 µg/ml) in the histamine release from the conjunctiva as compared with the UNIM-CON group. Decrease in the histamine release from the conjunctiva of the rats treated with TC and RC (500 mg/kg, for 7 days) was found to be significant at 2.38 ± 0.82 µg/ml ($P < 0.01$) and 2.78 ± 0.48 µg/ml ($P < 0.05$) respectively as compared with the IM-CON group. TC and RC treatment (250 mg/kg per day, orally for the period of 7 days) showed a significant ($P < 0.05$) reduction (2.95 ± 0.57 µg/ml and 3.00 ± 0.41µg/ml respectively) in histamine release from the conjunctiva as compared with the immunized control rats. Azelastine hydrochloride treated rats showed a significant ($P < 0.01$) reduction in histamine release from the conjunctiva as compared with the right eye treated with saline of IM-CON group (Fig. 3).

Effect TC and RC treatment on histamine content in tears induced by antigen

There was a significant ($P < 0.001$) increase (2.19 ± 0.25 ng/ml) in the histamine content in tears of the immunized control rats as compared with the unimmunized control rats. TC (500 mg/kg, for 7 days) treated rats showed a significant ($P < 0.01$)

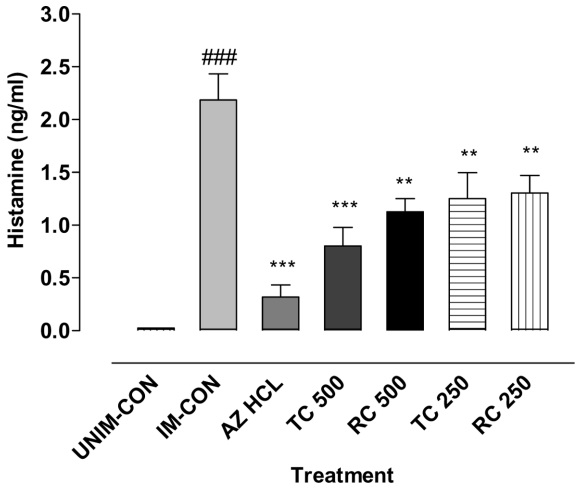

Fig. 4. The effect of TC (250 and 500 mg/kg per day, p.o.) and RC (250 and 500 mg/kg per day, p.o.) treatment for 7 days on allergen-induced histamine content in tears of rats. Azelastine hydrochloride (10 μl per right eye) was instilled topically 30 min before the challenge. Values are expressed as mean ± S.E.M. ***P < 0.001, **P < 0.01, *P < 0.05, n.s. = non significant compared to immunized control group, $^{###}P$ < 0.001 = significant compared to UNIM-CON (n = 6).

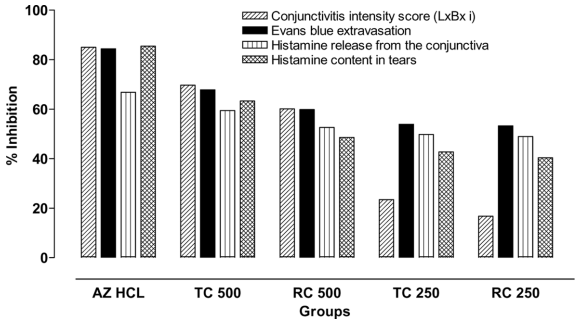

Fig. 5. The comparison of % inhibition of TC (250 and 500 mg/kg per day, p.o.) and RC (500 and 250 mg/kg per day, p.o.) treatment for 7 days treatment on different parameters in allergen-induced conjunctivitis. Azelastine hydrochloride (10 μl per right eye) was instilled topically 30 min before the challenge.

decrease (0.80 ± 0.18 ng/ml) in the histamine content in tears as compared with IM-CON group while RC (500 mg/kg, for 7 days) treatment showed a significant (P < 0.05) decrease (1.13 ± 0.13 ng/ml) in the histamine content in tears. However rats treated with TC and RC (250 mg/kg per day, orally for the period of 7 days) also showed a significant (P < 0.05) reduction in histamine release from the conjunctiva as compared with the immunized control rats (Fig. 4).

Histopathological evaluation

Histopathological examination revealed edema in the conjunctiva and the palpebral skin, irregular epithelium, destruction of most superficial epithelial layers and mast cells frequently infiltrating the epithelium in the IM-CON group. A heavy lymphocyte

Table 1. Effect of TC (250 and 500 mg/kg per day, p.o.) and RC (250 and 500 mg/kg per day, p.o.) treatment for 7 days on the morphology of the conjunctiva and palpebral skin score in rats with allergen-induced conjunctivitis

Site	Finding	Grade	No. of animals						
			UNIM-CON	IM-CON	AZ HCL	TC500	RC 500	TC 250	RC 250
Conjunc-tiva	Edema	–	6	1	4	2	1		
		±			2	3	4	4	4
		+		2		1	1	2	2
		++		3					
		+++							
Palpebral skin	Edema	–	6	2	4	3	1		
		±			2	3	4	6	3
		+					1		3
		++		4					
		+++							

Azelastine hydrochloride (10 μl per right eye) was instilled topically 30 min before the challenge. Tissue sections were stained with hematoxylin and eosin and graded as follows: –, no remarkable abnormality; ±, very slight change; +, slight change; ++, moderate change; and +++, severe change (n = 6).

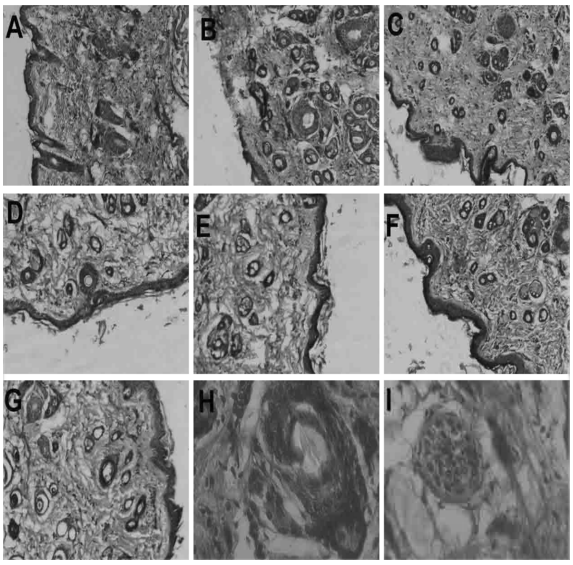

Fig. 6. The photographs show the effects of TC (250 and 500 mg/kg per day, p.o.) and RC (250 and 500 mg/kg per day, p.o.) treatment for 7 days on allergen-induced histopathological changes in conjunctiva of rats. Azelastine hydrochloride (10 μl per right eye) was instilled topically 30 min before the challenge. Tissue sections were stained with hematoxylin and eosin. (A) UNIM-CON, (B) IM-CON, (C) AZ HCL, (D) TC 500, (E) TC 250, (F) RC 500, (G) RC 250 (400 X), (H) degranulated mast cell, (I) non-degranulated mast cell (1000 X).

infiltration was found in the conjunctiva. There was a reduction in both edema and lymphocyte infiltration in azelastine hydrochloride treated eyes. TC and RC treated eyes showed an organized epithelium and no infiltrating mast cell. In addition, a marked reduction in mast cell count was observed (Table 1, Fig. 6).

DISCUSSION

The pathophysiology of allergic conjunctivitis involves the early-phase of type I hypersensitivity reactions with subsequent mast cell degranulation and release of preformed mediators. In severe allergic conjunctivitis, a late phase reaction occurs with stimulation of secondary mediators causing further mast cell degranulation and lymphocyte infiltration (Leonardi *et al*., 1990; Abelson *et al*., 1994; Donshik and Ehlers, 1994). In rats, egg albumin-induced edema and mast cell degranulation was reported to mimic closely human ocular conjunctivitis which is associated with vascular permeability and release of histamine from mast cells (Calonge *et al*., 1990; Minami and Kamei, 2004). Increase in vascular permeability is mainly due to histamine action and an increase in vascular permeability is generated by the contraction of endothelial cells after the binding of histamine H1 receptors located in these cells (Togias, 2003). Also vascular permeability in the conjunctiva in H1 receptors deficient mice suggested that vascular allergic conjunctivitis is regulated through H1 receptors and H1 receptor permeability is a parameter to study the allergic conjunctivitis (Nakahara *et al*., 2000). Allergen mediated cross linking of pairs of immunoglobulin IgE on the surface of conjunctival mast cells which leads to mast cell degranulation and release of mediators including histamine (Leonardi, 1999, 2000). Therefore, the objective of the present study was to investigate the antihistaminic effect and mechanism of action of TC and RC at the two dose levels viz. 250 and 500 mg/kg, p.o., for 7 days by studying parameters like change in vascular permeability and change in release of histamine from mast cells induced by antigen-antibody reaction. Vascular permeability in conjunctiva was assessed by CIS/wheal index and Evans blue extravasation method (spectrophotometric method). As a result, the treatment of TC at the dose of 500 mg/kg for 7 days showed 69.70% and 67.81% inhibition in conjunctivitis intensity score (L×B×i) and Evans blue extravasation respectively. Azelastine hydrochloride is well known anti-histamine. It reduces allergic conjunctival reaction and exerts anti-allergic activity through topical instillation (Ciprandi *et al*., 1997). In our study, reference drug azelastine hydrochloride showed 85% and 84.33% inhibition in conjunctivitis intensity score (L×B×i) and Evans blue extravasation respectively. While in both these parameters, RC at the dose of 500 mg/kg for 7 days showed 60.09%

and 59.83% inhibition respectively. Mast cell mediated diseases can be treated by immunomodulation, competitive inhibition of released mediators or prevention of release of mediators (mast cell stabilization) (Allansmith and Ross, 1986; Stock and Pendleton, 1993). In our study, treatment of TC showed dose dependent inhibition (59.41% and 63.31%) in histamine release from the conjunctiva and histamine content in tears respectively. While RC treatment also showed similar inhibition in histamine release from the conjunctiva and histamine content in tears but less than TC treated rats (Fig. 5). These results show inhibition of released histamine from mast cell. Azelastine hydrochloride has multiple pharmacologic actions, including antihistamine, mast cell stabilization (Chand *et al*., 1983; Bielory *et al*., 2004). From the histopathological studies, we found that ratio of degranulated to non-degranulated mast cells was less in the TC, RC and AZ HCl groups as compared to the ratio in IM-CON group which suggests mast cell stabilization action of these drugs. A marked reduction in ocular allergic reaction (invasion of mast cell and eosinophils) was demonstrated by histological findings which is the characteristic of ocular allergic reaction in humans (Allansmith and Baird, 1981). It was reported that eosinophils infiltrated into the organ in chronic allergic diseases (Tripathi YB *et al*., 2002; Hamid *et al*., 2003). As shown in the present study, the number of conjunctival eosinophils was also increased by topical sensitization of egg albumin in sensitized animals while TC and RC treatment showed reduction in the infiltration of eosinophils in the conjunctiva as compared with control. Both TC and RC were effective in reducing mast cell and mast cell infiltration in epithelium.

Based on produced results, our study demonstrates the effectiveness of TC and RC in decreasing early and late phase component of ocular reactions in egg albumin-induced conjunctivitis in rat. This is the first report of TC and RC which suggests that these two herbs could be used in the treatment of allergen-induced allergic conjunctivitis.

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