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RESEARCH ARTICLE

The protective effect of *Tinospora cordifolia* on various mast cell mediated allergic reactions

Rishit Zalawadia, Chintan Gandhi, Vaibhav Patel, and Ramchandran Balaraman

Pharmacy Department, Faculty of Technology and Engineering, Kalabhavan, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India

Abstract

This study investigated the effect of an aqueous extract of *Tinospora cordifolia* (Willd.) Miers (Menispermaceae) stem on mast cell mediated allergic reactions *in vivo* and *in vitro* and studied its possible mechanism. *T. cordifolia* (125 to 1000 mg/kg) dose-dependently inhibited compound 48/80 induced lethality in rats, histamine induced paw edema in mice and histamine induced bronchial asthma in guinea pigs. *T. cordifolia* significantly ($p < 0.001$) inhibited the cutaneous anaphylaxis reaction activated by histamine in a rat model and compound 48/80 induced ear swelling response in mice. *T. cordifolia* (2.5–160 $\mu\text{g/mL}$) also showed significant ($p < 0.001$) inhibition of histamine induced contraction of guinea-pig ileum *in vitro* implying the H_1 antihistamine activity. *T. cordifolia* (0.01 to 10 mg/mL) significantly ($p < 0.001$) inhibited the histamine release from rat peritoneal mast cells activated by compound 48/80. In addition, *T. cordifolia* (0.01 to 10 mg/mL) significantly ($p < 0.001$) inhibited the secretion of tumor necrosis factor- α (TNF- α) in antidinitrophenyl (DNP) IgE-stimulated rat peritoneal mast cells. The level of cAMP in RPMC transiently and significantly increased compared with that of control cells when *T. cordifolia* was incubated with mast cells. *T. cordifolia* (0.01 to 10 mg/mL) showed concentration-dependent inhibition in compound 48/80 induced reactive oxygen species (ROS) generation. In addition, *T. cordifolia* decreased intracellular calcium levels of activated mast cells. These results show that *T. cordifolia* may be beneficial in the treatment of acute and chronic allergic disorders.

Keywords: Antihistaminic; cytokine; histamine release; mast cell; *Tinospora cordifolia*; vascular permeability

Introduction

Tinospora cordifolia (Willd.) Miers. (Menispermaceae) is a large, glabrous, climbing shrub. It is widely used in veterinary folk medicine/Indian system of medicine (Ayurvedic) for its general tonic, antispasmodic, anti-inflammatory, anti-arthritic, and antidiabetic properties. The antioxidant, antidiabetic, anti-inflammatory and immunomodulatory properties of novel polysaccharide and glucans from *T. cordifolia* have also been well documented (Nair et al., 2006; Pendse et al., 1981; Prince et al., 2004; Singh et al., 2003). In light of the aforementioned anti-inflammatory properties of *T. cordifolia*, it is hypothesized that the herb may be helpful in reducing the mast-mediated allergic reactions in rats via anti-histaminic activities since inflammatory mediators such as histamine are mainly involved in

allergic reactions. The allergic reactions are 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, Fc ϵ RI (Beaven & Metzger, 1993; Bryce & Oettgen, 2005). 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 (Kim & Lee, 1999). Mast cell activation by IgE-dependent and IgE-independent stimuli brings about the process of degranulation which results in the fusion of the cytoplasmic granule membranes with the plasma membrane. This is followed by the fast release of granule-associated stored mediators such as histamine, cytokines, chemotactic factors, neutral proteases, proteoglycans, etc., as well as by the generation and release of newly generated mediators (arachidonic acid metabolism products)

Address for Correspondence: Rishit Zalawadia, Research Scholar, Pharmacy Department, Faculty of Technology and Engineering, Kalabhavan, The Maharaja Sayajirao University of Baroda, Vadodara - 390001, Gujarat, India. Tel.: +91-265-2434187; Fax: +91-265-2423898/2418927; E-mail: rmzalawadia@gmail.com

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(Kuehn & Gilfillan, 2007). At the later stage, mast cells start production and release of an array of cytokines (Church & Levi-Schaffer, 1997). 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 (Robin et al., 2008).

Histamine stimulates nerve endings and dilates the blood vessels, causing itching and redness (Leonardi, 2000). Mast cell degranulation can be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells (Palomaki & Laitinen, 2006). Compound 48/80 is best known as a potent inducer of degranulation of mast cells and the release of chemical mediators of anaphylactic reactions (Nishikawa & Kitani, 2008). The secretory responses of mast cells can be also induced by aggregation of their cell surface-specific receptors for IgE by the corresponding antigen. Although mast cells also store small amounts of cytokines in their granules (Gordon & Galli, 1990), these cells dramatically increase their production of tumor necrosis factor- α (TNF- α) and other cytokines within 30 min after their surface Fc ϵ RI are cross linked with specific antigen (Burd et al., 1989). Therefore, the aim of our study was the effect of *T. cordifolia* on histamine or compound 48/84 or anti-DNP IgE induced *in vivo* and *in vitro* allergic reaction models. Further *in vitro* studies on measurement of cAMP levels, intracellular Ca²⁺ levels and intracellular reactive oxygen species were also performed to find out the mechanism of action of *T. cordifolia* on allergic disorders.

Materials and methods

Reagents

Compound 48/80, histamine diphosphate, anti-DNP IgE, DNP-human serum albumin (HSA), dichlorofluorescein diacetate (DCF-DA), Fura-2/AM and metrizamide were purchased from Sigma Chemical (St Louis, MO). Evans blue and o-phthaldialdehyde (OPT) were purchased from HiMedia, Mumbai, India. All other chemicals were of analytical grade.

Preparation of *T. cordifolia* extract

The stem parts of *T. cordifolia* were collected from the Himalayas during March and were authenticated by P. S. N. Rao, at the Botanical Survey of India, Ministry of Environment and Forests, Government of India, Pune, where the voucher specimen (no. JVV1) was deposited at the herbarium unit of the institute. The plant sample was extracted with distilled water at 70°C for 5 h. The extract was filtered through a 0.45 μ m filter and the filtrate was

lyophilized, and kept at 4°C. The yield of dried extract from starting crude materials was about 10%. The dried extract was 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 The Maharaja Sayajirao University of Baroda, and with permission from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Animals were housed in groups of three and maintained under standardized conditions (12 h light/dark cycle, 22° \pm 2°C and relative humidity of 55% \pm 5%) and provided free access to pelleted Chakkan diet (Nav Maharashtra Oil Mills, Pune, India) and purified drinking water *ad libitum*. All the experimental animals were fasted overnight before the experiment. For *in vivo* studies animals were randomly divided into six groups as follows: Control animals (CON) group 1 treated with vehicle by oral gavage. Groups 2, 3, 4, and 5 were treated with extract of *T. cordifolia* orally at the dose of 125, 250, 500, 1000 mg/kg, respectively (*TC* 125, *TC* 250, *TC* 500, *TC* 1000). Animals of group 6 (cetirizine) were treated orally with cetirizine (0.25/0.3 mg/kg) and served as positive control.

For the histamine induced contraction of guinea-pig ileum study, the drug under investigation (*T. cordifolia*) was studied individually. For all other *in vitro* efficacy studies, rat peritoneal mast cells (RPMC) were isolated and incubated in the absence or presence of the test sample (*T. cordifolia*, cetirizine) at different concentrations ranging from 0.01 to 10 mg/mL before the addition of compound 48/84 or anti-DNP IgE. For the histamine release assay, sodium chromoglycate was studied as the reference drug as a mast cell stabilizer (0.0625 to 0.5 mg/mL).

The following groups were involved in the *in vitro* assay.

- Group I: Control group (saline treated)
- Group II: RPMC treated with herbal drug (*T. cordifolia*) at the concentration of 0.01 mg/mL
- Group III: RPMC treated with herbal drug (*T. cordifolia*) at the concentration of 0.1 mg/mL
- Group IV: RPMC treated with herbal drug (*T. cordifolia*) at the concentration of 1 mg/mL
- Group V: RPMC treated with herbal drug (*T. cordifolia*) at the concentration of 10 mg/mL
- Group VI: RPMC treated with cetirizine at the concentration of 0.0625 mg/mL

Group VII: RPMC treated with cetirizine at the concentration of 0.125 mg/mL

Group VIII: RPMC treated with cetirizine at the concentration of 0.25 mg/mL

Group IX: RPMC treated with cetirizine at the concentration of 0.5 mg/mL

In vivo studies

Protective effect of *T. cordifolia* against compound 48/80 induced lethality in rats

The study of compound 48/80 induced lethality in rats was carried out as previously described (Pandey et al., 1978). Briefly, male Sprague-Dawley rats (200 ± 25 g) were given an intravenous injection of compound 48/80 (0.75 mg/kg) through the caudal vein. Mortality was observed for 4 h after induction of the systemic anaphylactic reaction. Test drugs were orally administered 1 h before the injection of compound 48/80. Percentage protection from lethality was recorded after 48/80 injection with reference to the control group (vehicle treated) animals. Percentage protection and dose, was converted into Probit and log dose, respectively. ED_{50} calculations were performed by plotting the graph of log dose versus Probit.

Inhibitory effect of *T. cordifolia* on histamine induced paw edema in mice

This study was carried out as previously described (Kreutner et al., 2000). Male CD-1 mice (25 ± 5 g) were administered with test drug at different doses. After 1 h of dosing, animals were injected with 30 μ g histamine diphosphate (in 25 μ L physiological saline) into the sub-plantar region of the right hind paw and the same volume of saline into the left hind paw. The difference in the weight of paws was the index of the edema. After 30 min of histamine injection, the animals were sacrificed and both the hind paws were cut immediately at the tarsal joint. Right and left paws were weighed separately and differences in the weight of paws were recorded. Percentage inhibition was calculated with reference to control group animals. ED_{50} calculations were performed by plotting the graph of dose versus percentage inhibition.

Inhibitory effect of *T. cordifolia* on anti-DNP IgE induced cutaneous reaction in rats

Male Sprague-Dawley rats (200 ± 25 g) were anesthetized and the rats were injected intradermally with 0.5 μ g (50 μ L) of anti-DNP IgE into a dorsal skin site that had been shaved earlier. After 48 h, each rat received an injection of 100 μ g of DNP-HSA in PBS containing 4% Evans blue (1:4) via the tail vein. The animals were

sacrificed after 30 min after the anti-DNP IgE injection and reaction sites were removed. Evans blue was extracted from tissues by immersing the tissues in an extracting solution of 3 mL 0.5% sodium sulfate and 7 mL acetone kept at room temperature and vigorously shaken. Twenty-four hours later, the solutions were centrifuged at 400 g 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 μ g of Evans blue/mL of solution. Test drugs were given orally 1 h before anti-DNP IgE injection. The inhibitory effects of test drugs were expressed as the percentage reduction in dye leakage compared with that determined in vehicle treated animals. ED_{50} calculations were performed by plotting the graph of dose versus percentage inhibition (Shin et al., 2005).

Inhibitory effect of *T. cordifolia* on compound 48/80 induced ear swelling response in mice

The study of compound 48/80 induced ear swelling response was carried out as previously described (Jin et al., 2002). Briefly, male CD-1 mice (25 ± 5 g) were treated with *T. cordifolia* at different doses as described earlier. After 1 h of dosing, animals were injected intradermally with 25 μ L of compound 48/80 (5 mg/mL) into the dorsal aspect of a mouse ear using a microsyringe with a 28-gauge hypodermic needle. Within a 40 min interval, ear swelling response evoked by physiologic saline returned nearly to baseline thickness. Therefore, ear swelling response was determined 40 min after the injection of compound 48/80. After 40 min of compound 48/80 injection the animals were sacrificed and both the ears were cut immediately. Right and left ears were weighed separately and differences in the weight of ears were recorded. Percentage inhibition was calculated with reference to control group animals. ED_{50} calculations were performed by plotting the graph of dose versus percentage inhibition.

Histamine induced experimental bronchial asthma

Either sex of Dunkin Hartley guinea pigs (350 ± 25 g) were kept on fasting for 12 h before the experiments, only drinking water was available *ad libitum*. The animals were treated orally with the vehicle/test samples/reference 2 h before bronchial challenge in an inhalator chamber (Dolphin Instruments, Mumbai, India). The appearance of the first evidence of symptoms of asphyxia (the bronchospastic reaction) was monitored and the time for onset of convulsions (preconvulsion) was recorded. In the control groups, an aerosol of histamine (0.5% histamine diphosphate in 0.9% NaCl solution) provoked a bronchospastic reaction in 100% of the animals within three minutes. The delay in the appearance of the bronchospastic reaction was considered as

a protective effect. The mean preconvulsion time of animals treated with the test compounds, was compared to the control and is expressed in terms of percentage protection (Czarnecki et al., 2001).

$$\text{Percentage protection} = [1 - (T_1/T_2)] \times 100$$

where T_1 is the preconvulsive time of control and T_2 is the preconvulsive time of test sample.

In vitro studies

Inhibition of histamine induced contraction of guinea pig ileum in vitro for evaluation of the H₁ antihistamine activity of *T. cordifolia*

Dunkin Hartley guinea-pigs of either sex weighing 350 ± 50 g were fasted overnight before the experiment. The abdomen was cut open after sacrificing the animal and a good length of the ileum was placed on a Petri dish containing Tyrode solution at 37°C. A 2.5 cm long piece of the distal part of the ileum was taken for the study. Experiments were performed in 24 mL organ bath containing Tyrode solution at 37°C and bubbled with a mixture of 95% O₂ and 5% CO₂. The contractions of the ileum strips to histamine were recorded using a two-channel Gemini recorder (UGO-BASILE, Comerio VA, Italy) and isotonic force displacement transducer and 1 g tension. The preparation was allowed to equilibrate for 30 min during which the Tyrode solution was changed at intervals of 10 min. The non-cumulative stimuli with submaximal dose histamine (5.5×10^{-7} M) were given. The contraction in the absence and presence of (10 min incubation) of the test compounds was recorded. The responses of the *T. cordifolia* (2.5-160 µg/mL) or cetirizine (0.061-1.22 µg/mL) were recorded. The percentage inhibition of the test drugs on contractions induced by histamine was calculated (Harish et al., 2001). EC₅₀ calculations were performed by plotting the graph of log dose versus percentage inhibition. In another set of experiments, dose-response curves for histamine were obtained in the absence and presence of *T. cordifolia* at various concentrations (2.5 to 20 mg/mL). Data were analyzed using the method of Schild slope to find out the pA₂ value.

Assay of histamine release from rat peritoneal mast cells (RPMC)

RPMC were isolated as previously described (Jippo-Kanemoto et al., 1993). In brief, rats were anesthetized by ether and injected with 20 mL of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO₃, 2.7 mM KCl, 0.3 mM NaH₂PO₄ and 0.1% gelatin), into the peritoneal cavity and the abdomen was gently massaged for about 90 sec. The peritoneal cavity was carefully opened and the fluid containing peritoneal

cells was aspirated by a Pasteur pipette. Thereafter, the peritoneal cells were sedimented at 150 g for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells, i.e., macrophages and small lymphocytes, according to the method previously described (Jippo-Kanemoto et al., 1993). In brief, peritoneal cells suspended in 1 mL of Tyrode buffer B were layered on 2 mL of metrizamide (22.5% w/v) and centrifuged at room temperature for 15 min at 400 g. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 mL Tyrode buffer A. Purified RPMC were resuspended in Tyrode buffer A for the treatment of compound 48/80. RPMC suspensions (2×10^5 cells/mL) were preincubated for 10 min at 37°C before the addition of compound 48/80 (5 mg/mL). The cells were preincubated without or with the *T. cordifolia* (0.01 to 10 mg/mL) or sodium chromoglycate (0.0625 to 0.5 mg/mL) preparations and then incubated (10 min, 37°C) with the compound 48/80. The cells were separated from the released histamine by centrifugation at 400 g for 5 min at 4°C. Residual histamine in cells was released by disrupting the cells with perchloric acid and centrifugation at 400 g for 5 min at 4°C. After centrifugation, histamine content in the supernatant was determined by a fluorometric assay as % emission (Anton & 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 $\lambda_{\text{excitation}} = 345$ nm and $\lambda_{\text{emission}} = 441$ nm. Standard curves were performed to transform percentage emission units into µg histamine per mL of solution. The percentage inhibition of histamine release was calculated using the following equation:

$$\% \text{ Inhibition} = (a - b) \times 100/a$$

where "a" is histamine release without test drug and "b" is histamine release with test drug.

Assay of TNF-α production from rat peritoneal mast cells (RPMC)

TNF-α production was measured with the quantitative enzyme immunoassay technique, using a commercial kit (Endogen, Pierce Biotechnology, Inc., Rockford, IL, USA). RPMC (3×10^5 cells/mL) were sensitized with anti-DNP IgE (1 mg/mL) and incubated for 18 h in the absence or presence of *T. cordifolia* (0.01 to 10 mg/mL) or cetirizine (0.0625 to 0.5 mg/mL) preparations before the DNP-HAS (0.1 mg/mL) challenge. TNF-α production was measured by ELISA. The ELISA was

performed in an anti-mouse TNF- α precoated 96-well strip plate. Fifty μ L standard, controls, and samples were pipetted into the wells in duplicate. An aliquot of 50 μ L biotinylated antibody reagent was added to each well. The plate was covered and incubated at room temperature for 2 h. After incubation the plate was washed five times with wash buffer. An aliquot of 100 μ L dilute Streptavidin-HRP concentrate was added to each well. The plate was covered and incubated at room temperature for 30 min. After incubation the plate was washed five times. Thereafter, 100 μ L premixed TMB substrate solution was added to each well. The plate was developed in the dark at room temperature for 30 min and the reaction was stopped by adding 100 μ L stop solution to each well. Absorbance was measured on a plate reader at 450 minus 550 nm. The intensity of the color measured was in proportion to the amount of TNF- α bound in the initial step. The sample values were then read off the standard curves.

Measurement of cAMP level

The cAMP level was measured as previously described (Peachell et al., 1988) In brief, purified mast cells were resuspended in prewarmed (37°C) Tyrode buffer A. Typically, an aliquot of cells (2×10^5 cells) was added to an equivalent volume (50 μ L) of prewarmed buffer containing the *T. cordifolia* (1 mg/mL) in a micro-centrifuge tube. The reaction was allowed to proceed for discrete time intervals, terminated by the addition of ice-cold acidified ethanol (0.9 mL of 86% ethanol/1M HCl, 99:1) with brief vigorous vortexing and was then snap frozen in liquid nitrogen. The sample was later thawed and vortexed, then the debris was sedimented in a centrifuge (400 g at 4°C, for 5 min), and an aliquot (0.9 mL) of the supernatant was removed and evaporated to dryness under reduced pressure. The dried sample was reconstituted in assay buffer (150 mL) and stored frozen. The cAMP level was determined by enzyme immunoassay kit (Cayman).

Intracellular Ca²⁺ measurement

The RPMC (2×10^5 cells/mL) were preincubated with Fura-2/AM (2 μ M) for 30 min at 37°C. After washing the dye from the cell surface, *T. cordifolia* was pretreated 10 min prior to the compound 48/80 treatment. Cells were incubated for another 10 min at 37°C with compound 48/80 (5 mg/mL). Fluorescence was measured from the supernatant on a Shimadzu RF-540 spectrofluorophotometer, with $\lambda_{\text{excitation}} = 340$ nm and $\lambda_{\text{emission}} = 500$ nm.

Measurement of intracellular reactive oxygen species

The measurement of intracellular reactive oxygen species was carried out as previously described (Lebel & Bondy, 1990). Briefly, purified RPMC suspensions (2×10^5 cells/mL) were preincubated for 10 min at 37°C

before the addition of compound 48/80 (5 mg/mL). The cells were preincubated without or with the *T. cordifolia* (0.01 to 10 mg/mL) preparations and then incubated (10 min, 37°C) with the compound 48/80. After the challenge of cells with compound 48/80, the cells were incubated with dichlorofluorescein diacetate (DCF-DA; 1.25 μ M final concentration) in 0.1% methanol for 15 min at 37°C. The cells were separated from the released ROS by centrifugation at $400 \times g$ for 5 min at 4°C. Basal ROS generation was measured from cells incubated in vehicle alone. Fluorescence was measured from supernatant on a Shimadzu RF-540 spectrofluorophotometer, with $\lambda_{\text{excitation}} = 488$ nm and $\lambda_{\text{emission}} = 525$ nm and intracellular ROS generation was expressed as the percentage ROS generated above the basal.

Statistical analysis

All the data are expressed as mean \pm SEM. 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 of *T. cordifolia* treatment on compound 48/80 induced lethality in rats

Compound 48/80 induced 100% mortality in an orally administered vehicle-treated control group of animals, indicating degranulation of mast cells by compound 48/80. There was a dose dependent protection in compound 48/80-induced lethality in TC (125-1000 mg/kg)-treated groups as compared with the CON group which indicates the protective effect of *T. cordifolia* against mast cell degranulation. The maximal protective effect shown by TC 1000 was 80%. ED₅₀ of *T. cordifolia* was found to be 517.81 mg/kg, while cetirizine, a known H₁ antihistaminic, showed 70% protection in the dose of 0.3 mg/kg (Table 1).

Effect of *T. cordifolia* treatment on histamine induced paw edema in mice

T. cordifolia treatment showed significant ($p < 0.001$) reduction in the paw volume which was increased after histamine injection as compared with the CON group. ED₅₀ of *T. cordifolia* was found to be 672.32 mg/kg while cetirizine-treated mice showed 73.58% inhibition in histamine induced edema at the dose of 0.25 mg/kg (Table 2).

Table 1. The effect of TC (125-1000 mg/kg, p.o.) treatment on compound 48/80 induced lethality in rats.

Group	Dose (mg/kg)	Log dose	Compound 48/80 (0.75 mg/kg)	% Protection	ED ₅₀ (mg/kg)
Control	-	-	+	0	-
TC 125	125	2.097	+	10	517.81
TC 250	250	2.398	+	20	
TC 500	500	2.699	+	40	
TC 1000	1000	3.000	+	80	
Cetirizine	0.3	-	+	70	-

Groups of rats (n=10 per group) were treated with the test drugs 1 h before the compound 48/80 injection. Protection (%) within 1 h following compound 48/80 injection was represented as the number of survived rats × 100/total number of experimental rat.

Table 2. The effect of TC (125-1000 mg/kg, p.o.) treatment on histamine induced paw edema in mice.

Group	Dose (mg/kg)	Mean % increase in paw volume	% Inhibition	ED ₅₀ (mg/kg)
Control	-	27.2 ± 2.09		
TC 125	125	21.43 ± 2.57 ^{n.s.}	21.22	672.32
TC 250	250	18.07 ± 3.39 ^{n.s.}	33.56	
TC 500	500	14.48 ± 2.49 ^{**}	46.78	
TC 1000	1000	12.12 ± 2.67 ^{**}	55.45	
Cetirizine	0.25	7.19 ± 0.39 ^{***}	73.58	

Groups of mice (n=10 per group) were treated with the test drugs 1 h before the compound 48/80 injection. Percentage increase in volume of paw was represented as the (left paw weight in mg - right paw weight in mg) × 100/ right paw weight in mg. Values are expressed as mean ± SEM. ***p < 0.001; **p < 0.01; *p < 0.05; n.s., not significant compared to control group.

Effect of *T. cordifolia* on anti-DNP IgE induced cutaneous reaction in rats

In this experiment, the effects of *T. cordifolia* on anti-DNP IgE induced cutaneous reactions in rats were evaluated by extravasation of intravenously administered Evans blue. In the control group, mean Evans blue extravasation was 3.11 ± 0.44 µg/mL, which was found to be maximal among all groups. This reaction was inhibited dose-dependently by oral administration of *T. cordifolia* (ED₅₀ = 520.07 mg/kg) as compared with CON group. The maximal inhibitory effect shown by *T. cordifolia* was 67.61%. Cetirizine showed a 76.82% inhibition on anti-DNP IgE induced cutaneous reactions at the dose of 0.3 mg/kg (Table 3).

Effect of *T. cordifolia* on compound 48/80 induced ear swelling response

The pretreatment of TC 125 to 1000 mg/kg showed dose-dependent inhibition in ear swelling response. The inhibition was significant (p < 0.001) at the doses of 500 and 1000 mg/kg as compared to CON group. ED₅₀ of *T. cordifolia* was found to be 423.93 mg/kg. Cetirizine also significantly (p < 0.001) inhibited compound 48/80

Table 3. The effect of TC (125-1000 mg/kg, p.o.) treatment on anti-DNP IgE induced cutaneous reaction (vascular permeability of Evans blue) in rats.

Group	Dose (mg/kg)	Mean Evans blue concentration (µg/mL)	% Inhibition	ED ₅₀ (mg/kg)
Control	-	3.11 ± 0.44		
TC 125	125	2.65 ± 0.04 ^{n.s.}	14.64	520.07
TC 250	250	2.16 ± 0.06 [*]	30.45	
TC 500	500	1.61 ± 0.12 ^{***}	48.01	
TC 1000	1000	1.01 ± 0.06 ^{***}	67.61	
Cetirizine	0.3	0.72 ± 0.06 ^{***}	76.82	

Groups of rats (n=6 per group) were treated with the test drugs 1 h before the anti-DNP IgE injection. Values are expressed as mean ± SEM. ***p < 0.001; **p < 0.01; *p < 0.05; n.s., not significant compared to control group.

Table 4. The effect of TC (125-1000 mg/kg, p.o.) treatment on compound 48/80 induced ear swelling response in mice.

Group	Dose (mg/kg)	Mean % increase in ear volume	% Inhibition	ED ₅₀ (mg/kg)
Control	-	50.48 ± 5.93		
TC 125	125	40.93 ± 1.5 ^{n.s.}	18.93	423.93
TC 250	250	33.16 ± 3.49 ^{n.s.}	34.31	
TC 500	500	22.9 ± 0.84 ^{***}	54.63	
TC 1000	1000	13.62 ± 2.95 ^{***}	73.02	
Cetirizine	0.25	23.39 ± 2.94 ^{***}	53.68	-

Groups of mice (n=10 per group) were treated with the test drugs 1 h before the compound 48/80 injection. % increase in volume of ear was represented as the (left ear weight in mg - right ear weight in mg) × 100/ right ear weight in mg. Values are expressed as mean ± SEM. ***p < 0.001; **p < 0.01; *p < 0.05; n.s., not significant compared to control group.

induced ear swelling response at the dose level of 0.25 mg/kg (Table 4).

Effect of *T. cordifolia* on histamine induced experimental bronchial asthma

The effect of *T. cordifolia* on guinea pigs exposed to histamine spray is shown in Table 5. The extract produced a dose-dependent prolongation of pre-convulsive breathing and showed significant (p < 0.05) 69.97% inhibition in allergic bronchoconstriction at 1000 mg/kg. ED₅₀ of *T. cordifolia* was found to be 374.33 mg/kg. Cetirizine was found to exhibit 68.91% inhibition at 0.3 mg/kg.

Inhibition of histamine induced contraction of guinea-pig ileum in vitro

Histamine (5.5 × 10⁻⁷ M) produced sub-maximal contractile responses on guinea-pig ileum. Addition of *T. cordifolia* (2.5-160 µg/mL) resulted in concentration-dependent significant (p < 0.001) inhibition in the contractions as compared to control (sub-maximal response of histamine) value. *T. cordifolia* in these

concentration ranges did not show any effect on the ileum when given alone. Cetirizine (1-625 nM) significantly ($p < 0.001$) inhibited histamine induced contraction of guinea pig ileum. EC_{50} was found from the plot of log dose versus percentage inhibition of histamine response. EC_{50} of *T. cordifolia* and cetirizine was found to be 13.48 and 0.04 $\mu\text{g}/\text{mL}$, respectively (Table 6). Dose-response curves for histamine induced contraction of guinea-pig ileum are shown in Figure 1. Schild plot analysis showed a pA_2 value of 7.5.

Effect of *T. cordifolia* on histamine release from RPMC

The inhibitory effects of *T. cordifolia* on compound 48/80 induced histamine release from RPMC are shown in Figure 2. *T. cordifolia* (0.01-10 mg/mL) and sodium chromoglycate (0.0625-0.5 mg/mL) showed concentration-dependent inhibition in compound 48/80 induced mediated histamine release from RPMC as compared to saline value. At the highest concentration, *T. cordifolia* and sodium chromoglycate

showed 72.33% and 98.73% inhibition, respectively, in histamine assay.

Effect of *T. cordifolia* on $TNF-\alpha$ secretion from RPMC

T. cordifolia showed significant 54.35% ($p < 0.01$) and 66.77% ($p < 0.001$) inhibition at a concentration level of 1 and 10 mg/mL respectively, in anti-DNP IgE induced $TNF-\alpha$ production in RPMC as compared with mast cell + anti-DNP IgE + DNP-HSA group. The cetirizine (0.0625-0.5 mg/mL) treatment had no significant activity on $TNF-\alpha$ release from RPMC as compared with mast cell + anti-DNP IgE + DNP-HSA group (Figure 3).

Table 5. The effect of TC (125-1000 mg/kg, p.o.) or cetirizine (0.3 mg/kg) alone on allergic bronchoconstriction in guinea pigs.

Group	Dose (mg/kg)	Preconvulsive Breathing (min)	% Inhibition	ED_{50} (mg/kg)
CON	-	3 ± 0.26		
TC 125	125	3.60 ± 0.2 n.s.	16.67	374.33
TC 250	250	4.88 ± 0.37 n.s.	38.52	
TC 500	500	9.55 ± 0.61 *	68.59	
TC 1000	1000	9.99 ± 1.04 **	69.97	
CTZ 0.3	0.3	9.65 ± 1.97 *	68.91	-

Groups of guinea pigs ($n = 3$ per group) were treated with the test drugs 1 h before the exposure of histamine aerosol. Values of preconvulsive breathing (min) are expressed as mean \pm SEM. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s., not significant compared to control group.

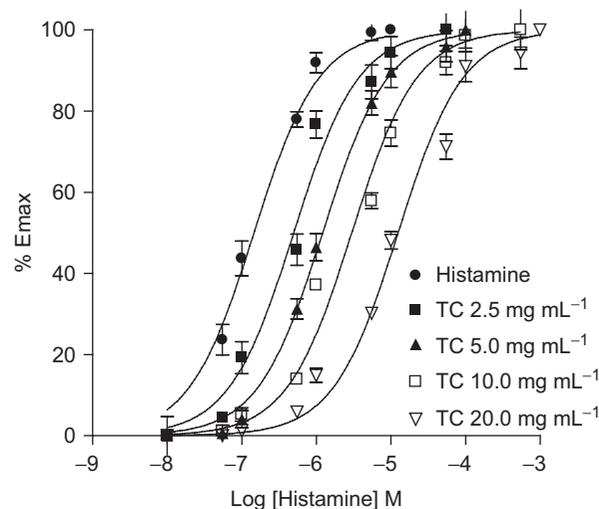


Figure 1. Effect of TC on dose-response curves for histamine-induced contraction of guinea pig ileum. Dose-response curves for histamine-induced contraction of guinea pig ileum were obtained in the absence of (●) or presence of 2.5 (■), 5 (▲), 10 (□) and 20 (▼) mg/mL TC. Each point is the mean percentage of response ($n = 4-5$) at a given concentration of histamine with respect to the maximum contraction obtained in the control curve (●). $pA_2 = 7.5$.

Table 6. The effect of TC (2.5-160 $\mu\text{g}/\text{mL}$) treatment on histamine induced contraction of guinea pig ileum *in vitro*.

Sr No.	Treatment	Dose	Mean contraction (mm)	% Inhibition of histamine response	EC_{50} ($\mu\text{g}/\text{mL}$)
1	Histamine	5.5×10^{-7} M	45 ± 1.22	-	-
2	TC	2.5 $\mu\text{g}/\text{mL}$	32 ± 1 ***	28.89	13.48
3	TC	5 $\mu\text{g}/\text{mL}$	28 ± 0.92 ***	37.78	
4	TC	10 $\mu\text{g}/\text{mL}$	25 ± 0.99 ***	44.44	
5	TC	20 $\mu\text{g}/\text{mL}$	23 ± 0.8 ***	48.89	
6	TC	40 $\mu\text{g}/\text{mL}$	18 ± 0.41 ***	60.	
7	TC	80 $\mu\text{g}/\text{mL}$	10 ± 0.2 ***	77.78	
8	TC	160 $\mu\text{g}/\text{mL}$	3 ± 0.2 ***	93.33	
9	Cetirizine	1 nM	40 ± 1.11 *	11.11	0.04
10	Cetirizine	5 nM	33 ± 0.9 ***	26.67	
11	Cetirizine	25 nM	25 ± 0.43 ***	44.44	
12	Cetirizine	125 nM	10 ± 0.8 ***	77.78	
13	Cetirizine	625 nM	2 ± 0.2 ***	95.56	

Each datum represents the mean \pm SEM of 3 independent experiments. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s., not significant compared to control (Submaximal response of histamine) value.

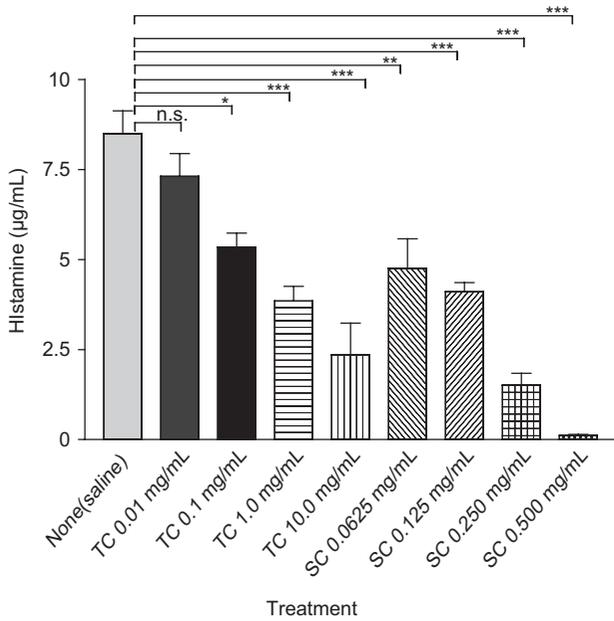


Figure 2. The effect of TC (0.01-10 mg/mL) and sodium chromoglycate (SC) treatment on compound 48/80-induced histamine release from mast cells. RPMC (2×10^5 cells/mL) were preincubated with test samples at 37°C for 10 min prior to incubation with compound 48/80. Each data represents the mean \pm SEM of 3 independent experiments. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s., not significant compared to saline value.

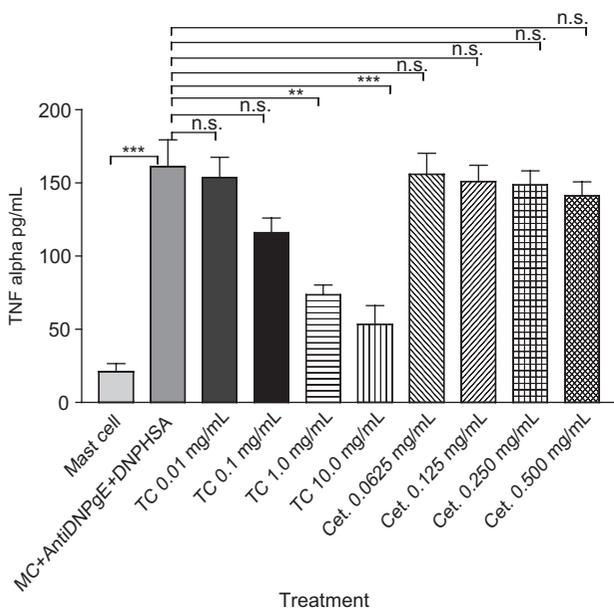


Figure 3. The effect of TC (0.01-10 mg/mL) and cetirizine (Cet.) treatment on anti-DNP IgE antibody-induced TNF- α secretion from mast cells (MC). RPMC (3×10^5 cells/mL) were sensitized with anti-DNP IgE (1 mg/mL) and incubated for 18 h in the absence or presence of test samples before the challenge with DNP-HSA (0.1 mg/mL). Each data represents the mean \pm SEM of 3 independent experiments. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s., not significant compared to mast cell+ anti-DNP IgE+DNP-HSA group.

Effect of *T. cordifolia* on cAMP level of RPMC

To understand the mechanism of inhibition of histamine secretion from mast cells, we studied the effect of *T. cordifolia* incubation on the cAMP content. Incubation of *T. cordifolia* (1 mg/mL) with RPMC significantly ($p < 0.001$) increased the cAMP content at 2 min and decreased the cAMP content to basal value at 10 min (Figure 4).

Effect of *T. cordifolia* on intracellular Ca^{2+}

To investigate the mechanisms of *T. cordifolia* on the reduction of histamine release, we analyzed the intracellular Ca^{2+} . Figure 5 shows the increased fluorescent intensity when the RPMC are treated with compound 48/80 (5 mg/mL). Preincubation of *T. cordifolia* (10 mg/mL) with RPMC, decreased compound 48/80 induced intracellular Ca^{2+} levels (Figure 5).

Effect of *T. cordifolia* on generation of intracellular ROS

Unstimulated RPMC were found to generate a low level of basal ROS, and this was used as a baseline to which all other results were compared. Compound 48/80 caused maximal of $333.23\% \pm 44.96\%$ generation of ROS over basal ($n = 6$) at 5 mg/mL. *T. cordifolia* (0.01-10 mg/mL) showed significant ($p < 0.001$) inhibition of ROS generation over basal level (Figure 6).

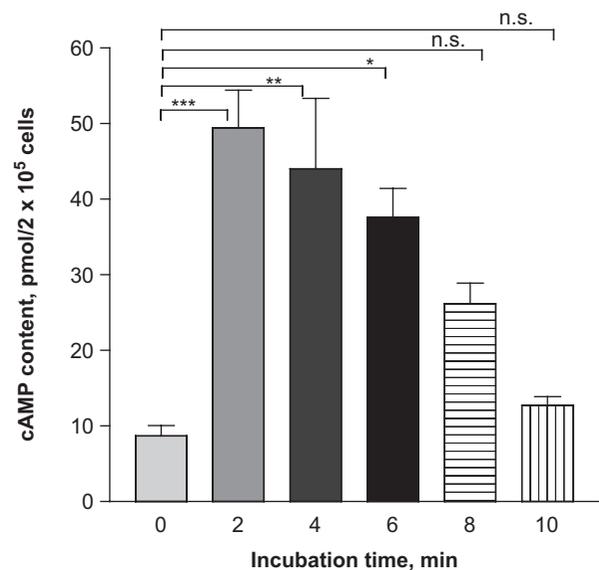


Figure 4. Time-dependent effect of TC (1 mg/mL) on cAMP level of RPMC. RPMC (2×10^5 cells/mL) were preincubated with test samples at 37°C for 10 min prior to incubation with compound 48/80 (5 mg/mL). Each datum represents the mean \pm SEM of three independent experiments. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s., not significant compared to saline value.

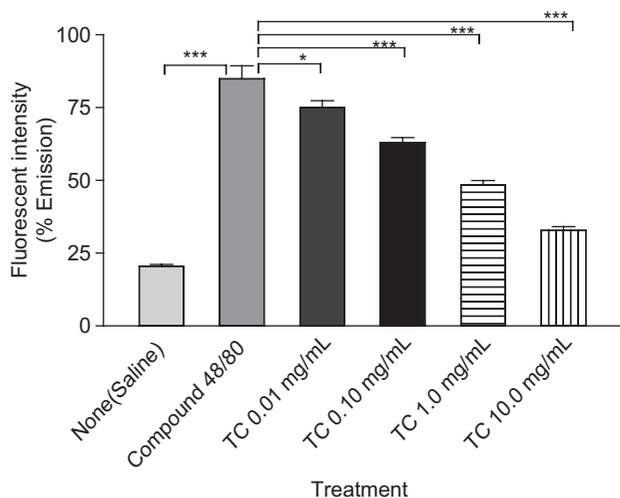


Figure 5. Effect of TC on the intracellular Ca²⁺ levels in RPMC. RPMC (2×10^5 cells/mL) were preincubated with test samples at 37°C for 10 min before adding compound 48/80 (5 mg/mL) and then another 10 min with compound 48/80. Each data represents the mean \pm SEM of 3 independent experiments. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s., not significant for the compound 48/80 value.

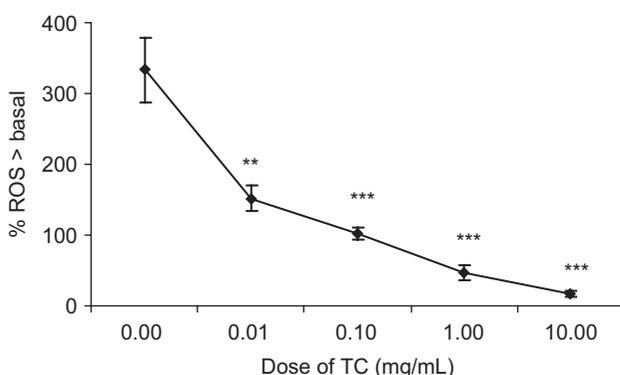


Figure 6. Effect of TC on concentration-response relationships for intracellular reactive oxygen species. Concentration-response relationships for intracellular reactive oxygen species generation in the absence and presence of TC (0.01–10 mg/mL) from rat peritoneal mast cells (2×10^5 cells/mL) in response to compound 48/80. Each point is the mean \pm SEM of six experiments. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s., not significant compared to saline value.

Discussion

Mast cell mediated diseases can be treated by immunomodulation, competitive inhibition of released mediators or prevention of release of mediators (mast cell stabilization) (Dubuske, 2007; Hisbergues et al., 2007). Compound 48/80 has been used as direct secretagogues and the compound 48/80 induced increase in membrane permeability is an essential trigger for the release of the mediator from mast cells (Shin & Kim, 2000). From the present study in animal models, *T. cordifolia* showed an inhibitory effect on compound 48/80 induced lethality (systemic anaphylaxis) and

compound 48/80 induced ear swelling response while *T. cordifolia* stabilized the lipid bilayer membrane in the *in vitro* assay of histamine release from rat peritoneal mast cells. Histamine is known to cause edema and has also been a well-known inflammatory mediator for a long time (Grega et al., 1972; Rowley & Benditt, 1956). In the early phase of allergic response, histamine initiates edema formation (White, 1990). Supporting these observations, treatment with *T. cordifolia* displayed an inhibitory effect on edema induced by histamine. These results suggest that the *T. cordifolia* can be much more effective in inhibiting reactions whose mechanism depends on the release of histamine. In addition, anti-DNP IgE induced local allergic cutaneous reaction is a well-known animal model for anaphylaxis in rats. Our results suggest the inhibitory effect of *T. cordifolia* in anaphylaxis. The role of histamine in asthma disease is well established (Nelson, 2003). The present study indicates that administration of *T. cordifolia* can reduce the histamine induced bronchospasm. Since bronchospasm occurs after H1-receptor activation of the PLC-IP3-DAG cascade and intracellular calcium mobilization (Smit et al., 1999), many possibilities of antagonistic intervention for the plant extracts may apply.

T. cordifolia also attenuated histamine induced contraction of guinea-pig ileum and TNF- α secretion from RPMC *in vitro*. Reduction of pro-inflammatory cytokines from mast cells is one of the key indicators of reduced allergic symptom (Thomas, 2001). Thus, from the *in vitro* studies it can be proved that *T. cordifolia* can competitively inhibit the released mediators as well as it can prevent the release of mediators. The role of cAMP and calcium on histamine release is controversial but the cAMP and Ca²⁺ pathways to the degranulation of mast cells are critical. The agents that induce elevations in intracellular cAMP attenuate the stimulated release of mediators from mast cells (Norn et al., 2007). The study results showed that the incubation of *T. cordifolia* increased the intracellular cAMP content of the mast cells from that of basal cell cAMP level. The present study observations showed an attenuation of compound 48/80 induced intracellular Ca²⁺ in mast cells with *T. cordifolia* treatment and proved that the decreased intracellular Ca²⁺ is involved in the inhibitory effect of *T. cordifolia* on histamine release. Further, cAMP inhibits the secretion of mediators by reducing the membrane permeability to Ca²⁺ (Alfonso et al., 2000). It means that inhibitory action of *T. cordifolia* is by elevation of cAMP as well as by decrease in intracellular Ca²⁺. Mast cells are known to generate intracellular ROS in response to antigen challenge, which may be involved in histamine release. It could also be suggested that the anti-allergic action of *T. cordifolia* is partly due to antioxidant potential of *T. cordifolia* as anti-oxidants can reduce ROS levels in antigen-IgE activated mast cells and concomitantly

inhibited histamine release (Chen et al., 2000; Matsui et al., 2000).

It is also confirmed from our study that cetirizine a well known antiallergic drug, possesses only H₁ antihistamine activity and it has no effect on mast cell stabilization, while *T. cordifolia* showed H₁ antihistamine activity as well as mast cell stabilization.

Conclusion

It can be concluded that *T. cordifolia* has mast cell stabilizing effect as well as H₁ antihistamine effect on allergic disorders. From all these observations of the study involving different models of allergic reactions with distinct mechanism of action, it can be said that effects of herbal drugs alone on allergic disorders is encouraging. Herbal drug treatment provides a unique opportunity to develop a therapy to reduce the side effects of currently marketed antiallergenics and can achieve better management of patients suffering from allergic disorders.

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