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Evaluation of Effect of *Taxus baccata* Leaves Extract on Bronchoconstriction and Bronchial Hyperreactivity in Experimental Animals

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Abstract: The present investigation was undertaken to evaluate the bronchodilating and bronchial hyperreactivity of aqueous extract of *Taxus baccata* Linn. leaves in experimental animals. Bronchodilator activity of aqueous extract of *T. baccata* studied on the histamine and acetylcholine aerosol induced bronchospasm in guinea pigs and bronchial hyperreactivity was studied on broncho alveolar lavage fluid (BALF) in the egg albumin sensitized guinea pigs and histopathological studies. *In vitro* mast cell stabilizing activity was studied using compound 48/80 as degranulatimg agent. Treatment with aqueous extract of *T. baccata* (200 and 400 mg/kg, p.o., for 7 days) showed significant protection against histamine and acetylcholine aerosol induced bronchospasm in guinea pigs. Significant decreased in the total leukocyte and differential leukocyte count in the BALF of the egg albumin sensitized guinea pigs was observed by administration of aqueous extract of *T. baccata* (200 and 400 mg/kg, p.o., for 15 days,). Aqueous extract of *T. baccata* dose dependently protected the mast cell disruption induced by compound 48/80. These results suggest that aqueous extract of the *T. baccata* has not only bronchodilating activity but also decreases bronchial hyperreactivity by decreasing the infiltration of inflammatory cells in the airway and inhibition of release of histamine like mediators from the mast cell by stabilizing it.

Key words: Bronchoconstriction · Bronchial hyperreactivity · *Taxus baccata* (taxaceae) · Aqueous extract

INTRODUCTION

Asthma is one of the most common disorders encountered in clinical medicine in both children and adults characterized by inflammation of the airway that is central to airway dysfunction. It is known that asthma can be triggered by various factors: allergens, drugs, respiratory infection, dust, cold air, exercise, emotions, occupational stimuli, chemicals, histamine etc. [1]. Histological examination of bronchial biopsies and cytology of broncho alveolar lavage fluid (BALF) have demonstrated infiltrating inflammatory cells in the tracheobronchial mucosa and airway lumen of patients with asthma, even those with mild disease [2-3]. The influx of inflammatory cells is accompanied by marked and characteristic pathophysiological changes to the airways, including thickening of the airway wall, which have been implicated in the restriction of airflow and the development of airway hyperresponsiveness [3]. The disease statistics clearly necessitates the increasing need for drugs targeting the mechanisms involved in eosinophil and neutrophil activation and accumulation, for the management of asthma. Glucocorticosteroids are the only drugs currently available that effectively reduce airway inflammation in asthma [4].

As a result, there is high prevalence of usage of complementary and alternative medicines for treatment of this disease [5]. Ayurveda, an ancient system of Indian medicine, has recommended a number of drugs from indigenous plant sources for the treatment of bronchial asthma and allergic disorders [6]. *Taxus baccata* Linn. (Taxaceae) is an evergreen tree, usually 6 m in height and 1.5-1.8 m in growth, found in the temperate Himalayas at an altitude between 1800 and 3300 m and in the hills of Meghalaya and Manipur at an altitude of 1500 m [7]. *T. baccata* has been used in the Ayurvedic system for the treatment of cancer, diarrhea, asthma, haemoptysis and also used as carminative, expectorant, stomachic etc. [8].

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T. baccata leaves are reported to be used in traditional medicine as abortificient, antimalarial, antirheumatic and for bronchitis [9-11] and dried leaves and barks are used against asthma [12]. Anticancer [13], anti-inflammatory and antinociceptive [14], antifungal [15], antimycobacterial [16] activity of *T. baccata* has been reported. Many Ayurvedic practitioners prescribed leaves of *T. baccata* for the treatment of asthma. However, no scientific studies are carried out to investigate anti-asthmatic effect of leaves of *T. baccata.* In present study, the anti-asthmatic activity of aqueous extract of leaves of *T. baccata* was evaluated in experimental animals by using various *in vivo* and *in vitro* models.

MATERIALS AND METHODS

Plant Material: Dried leaves of *T. baccata* were purchased from commercial supplier of Bombay, India. The plant was authenticated by Prof. Minoo Parabia, Head of Department of Bioscience, Veer Narmad South Gujarat University, Gujarat, India, where a plant specimen was deposited under the no. HMG/0404/2007.

Preparation of Extract: The leaves were reduced to coarse powder and macerated with distilled water for 48 h, Filtered and filtrate was evaporated under reduced pressure to obtained dried extract. The extract was stored in cool and dry place and used for pharmacological evaluation. (water extractive value 4.5 % w/w). After obtaining of dry extract qualitative preliminary phytochemical screening was performed to find out the presence of various phytochemicals. [17]. For pharmacological evaluation, extract was dissolved in saline prior to its use.

Chemicals: Histamine, acetylcholine, Ketotifen, compound 48/80 were purchased from Sigma-Aldrich Chemical Co., USA. Egg albumin and other chemicals were purchased from Himedia Laboratories Pvt. Ltd., India.

Experimental Animals: Wister rats (175-200 g) and guinea pigs (400-600 g) of either sex housed in standard conditions of temperature (22 ± 2°C), relative humidity (55 ± 5%) and light (12 hrs light/dark cycles) were used. They were fed with standard pellet diet and water *ad libitum*. In addition to pellet diet guinea pigs were supplemented with Lucerne. The experimental protocol was approved by Institutional Animal Ethical Committee as per the guidance of CPCSEA, Ministry of Social Justice and Empowerment, Government of India (Protocol No. Project 5005). A minimum of six animals were used in each group. Throughout the experiments, animals were processed according to the suggested ethical guideline for the care of laboratory animals.

Acute Toxicity Testing: Albino rats of either sex weighing 200-250 gm were used in the study. Acute oral toxicity study was performed as per Organisation for Economic Co-operation and Development (OECD)-425 guideline.

Histamine and Acetylcholine Aerosol Induced Bronchospasm in Guinea Pigs: Experimental bronchial asthma was induced in guinea pigs by exposing them to histamine and acetylcholine aerosol [18]. Guinea pigs were selected and divided into four groups each containing six animals out of which groups I and group III were exposed to 0.1% w/v of histamine dihydrochloride aerosol and another group II and group IV were exposed to 0.5% w/v of acetylcholine bromide aerosol. The animals exposed to histamine and acetylcholine aerosol showed progressive dyspnoea. The end point preconvulsion dyspnoea (PCD) was determined from the time of aerosol exposure to the onset of dyspnoea leading to the appearance of convulsion. As soon as PCD commenced, the animals were removed from chamber and placed in fresh air. This time of PCD was taken as day 0 value. Guinea pigs of group-I and group III were treated with the aqueous extract of the leaves of *T. baccata* 200 mg/kg, p.o. and group II and group IV were treated with 400 mg/kg, p.o. once a day for 7 days. On the 7th day 2 h after the last dose, the time for the onset of PCD was recorded as on day 0. The percentage increased in time of PCD was calculated using following formula [19].

$$\text{Percentage increased in time of PCD} = \left(1 - \frac{T_1}{T_2}\right) \times 100$$

Where: T_1= time for PCD onset on day 0, T_2= time for PCD onset on day 7

Studies on Broncho Alveolar Lavage Fluid (BALF) in Egg Albumin Sensitized Guinea Pigs [20]: Guinea pigs were selected and divided in four groups i.e. group I (control, saline 10 ml/kg), group II (sensitized), group III (sensitized + *T. baccata* 200 mg/kg, p.o.) and group IV (sensitized + *T. baccata* 400 mg/kg, p.o.) each containing six animals. The guinea pigs of group II, group III and group IV were sensitized with egg albumin (1 ml, 10% w/v, i.p.) on the 1st day. The animals of group III and group IV were dosed once daily for fifteen days with aqueous extract of leaves

of *T. baccata.* Two hour after the last dose of drug administration (on 15th day), all the animals of group II, group III and group IV were again challenged with egg albumin (0.5 ml, 2% w/v, i.v.) through saphenous vein. After 3 h of the challenged of the egg albumin or just prior to death of animals which ever was earlier. The trachea was immediately cannulated after anaesthetization and the airways lavaged with saline at 25°C (two aliquots of 1 mL/100 g body weight). Bronchoalveolar cells were collected in two successive lavages using saline and recovered through a tracheal cannula. The BALF was stored on ice and total WBC cell counts were performed using light microscope. Dilutions of lavage fluid (1 in 10) were made in saline and differential WBC were counted by light microscopy stained with Leishman's stain. At least 200 cells were counted on each slide. Cells were differentiated using standard morphological criteria. All differential cell counts were performed blind and in randomized order at the end of the study. The result obtained where compared with controlled with sensitized group and sensitized with treated groups.

Lung Histology: Same animals of the above model i.e. studies on the BALF were used for the histological study of the lungs. Left bronchi were tied before collection of BALF to avoid possible traumatic damage due to BALF. The lungs were removed and then fixed by slowly inflating with buffer formalin and subsequently embedded in paraffin. A transverse section (2-4 µm thick) was cut from the each collected lungs and stained with haematoxylin and eosin. Histopathology assessment under light microscope was performed on sections.

***In vitro* Mast Cell Degranulation by Compound 48/80:** Effect of *T. baccata* on *in vitro* mast cell degranulation by compound 48/80 was studied according to Gupta and Srimal [21]. Normal saline containing 5 units/ml of heparin was injected in the peritoneal cavity of male rats lightly anaesthetized with ether. After a gentle abdominal massage, the peritoneal fluid containing mast cells was collected in centrifuge tubes placed over ice. Peritoneal fluid of rats was collected and centrifuged at 2000 rpm for 5 min. Supernatant solution was discarded and the cells was washed twice with saline and resuspended in 1 ml of saline. All the solutions were prepared in normal saline.

The peritoneal cell suspension divided in six parts viz. –ve control, +ve control, reference standard (Ketotifen 10 µg/ml), aqueous extract of *T. baccata* of three concentration i.e. 500, 750, 1000 µg/ml each containing 0.1 ml of cell suspension and incubated in a constant temperature in water bath at 37°C for 15 min. Then 0.1 ml of compound 48/80 (10 ìg/ml) was added in all samples except in –ve control and the suspensions were further incubated for 10 min at 37°C. The cells were then stained with 10% of Toluidine blue solution and observed under the high power of light microscope. The percentage granulated and percentage degranulated mast cell were counted. In positive control group in which compound 48/80 was added without addition of test agents i.e. kitotifen and *T. baccata* and a negative control group in which neither compound 48/80 nor the test agents were added to correct for spontaneous degranulation of mast cells without any degranulating agent.

Statistical Analysis: The results of various studies were expressed as mean ± SEM and analyzed statistically using one way ANOVA followed by Student's *t*-Test to find out the level of significance. Data were considered statistically significant at minimum level of $p < 0.05$.

RESULTS

Phytochemical Screening: Preliminary qualitative phytochemical screening of aqueous extract of leaves of *T. baccata* showed the presence of lignans, flavonoids, glycosides, sterols, sugars, amino acids and triterpenoids.

Acute Toxicity Testing: No mortality and the sign of toxicity were observed at the dose of 2000mg/kg. Dose selected for pharmacological evaluation were 200 mg/kg and 400 mg/kg.

Effect on Histamine and Acetylcholine Aerosol Induced Bronchospasm in Guinea Pigs: Aqueous extract of *T. baccata* significantly and dose dependently increased the time of PCD following exposure to histamine ($p < 0.001$) and acetylcholine ($p < 0.01$) aerosols induced bronchospasm in guinea pigs (Table 1). Increased in the time of PCD was more against histamine aerosol as compared to acetylcholine aerosol following administration of *T. baccata* leaves extract.

Effect on Broncho Alveolar Lavage Fluid in Egg Albumin Sensitized Guinea Pigs: After fifteen days guinea pigs were again challenged with egg albumin, in the BALF significant increased in the total leukocyte count and differential leukocytes count were observed in sensitized i.e. group II ($p < 0.001$) as compared to the control i.e. group I. Aqueous extract of the *T. baccata* (200 and

Table 1: Effect of *Taxus baccata* (p.o., for 7 days) on histamine and acetylcholine aerosol induced bronchospasm in guinea pigs

	Preconvulsion Dyspnoea Time (sec)		
Groups	Before Treatment (control)	After Treatment	% increase in the time of PCD
Histamine Aerosol (0.1% w/v)			
I- *T. baccata* (200 mg/kg)	125.8 ± 18.56	447.2 ± 25.34 *	72.82 ± 3.14%
II- *T. baccata* (400 mg/kg)	127.4 ± 20.32	632.7 ± 53.47 *	80.67 ± 6.23%
Acetylcholine aerosol (0.5% w/v)			
III- *T. baccata* (200 mg/kg)	149.27 ± 27.2	354.5 ± 45.09 #	58.73 ± 3.09%
IV- *T. baccata* (400 mg/kg)	137.4 ± 23.6	438.57 ± 43.71 *	67.98 ± 4.61%

Values are expressed as mean ± SEM for 6 guinea pigs in each group,

* $p < 0.001$, # $p < 0.01$ when compared with control group

Table 2: Effect of *Taxus baccata* (p.o., for 15 days) on BALF in egg albumin sensitized guinea pigs

	Control	Sensitized	Sensitized + *T. baccata* (200 mg/kg)	Sensitized +*T. baccata* (400 mg/kg)
TLC/cmm	8,842 ± 429	14,740 ± 670.9 *	11,680 ± 313.5 @	9,680 ± 248.32 $
Neutrophil count/cmm	2,774 ± 304.2	4,100 ± 169.9 *	3,921 ± 287.25 #	3,081 ± 267.34 $
Lymphocyte count/cmm	4,472 ± 384.8	9,130 ± 235.2 *	6,772 ± 343.2 $	5,892 ± 327.82 $
Eosinophil count/cmm	184.8 ± 20.82	506.2 ± 36.71 *	420.3 ± 19.45 $	337.78 ± 13.26 $
Monocyte count/cmm	109.3 ± 19.28	263.1 ± 44.52 *	200.5 ± 21.08 $	153.6 ± 18.31 $

Values are expressed as mean ± SEM for 6 guinea pigs in each group,

* $p < 0.001$ when compared with control group,

@ $p < 0.05$, # $p < 0.01$, $ $p < 0.001$ when compared with sensitized group

BALF= broncho alveolar lavage fluid

TLC= total leukocyte count

Table 3: Effect of *Taxus baccata* on Compound 48/80 induced mast cell degranulation

		Mast cells	
Treatment	Concentration (µg/ml)	% Granulated	% Degranulated
-ve control	-	91.83 ± 0.654	8.167 ± 0.654
+ve control	-	26.5 ± 1.176	73.5 ± 1.176
Ketotifen	10	80.5 ± 0.957*	19.5 ± 0.957
Taxus baccata	500	41.17 ± 0.945*	58.83 ± 0.945
Taxus baccata	750	50.67 ± 0.666*	49.33 ± 0.666
Taxus baccata	1000	63.17 ± 0.945*	36.83 ± 0.945

Values are expressed as mean ± SEM, n=6 in each groups,

* $p < 0.001$ when compared with base line value i.e. +ve control

400 mg/kg, p.o., for 15 days) significantly and dose dependently decreased in the total leukocyte count ($p < 0.05$) and differential leukocytes count ($p < 0.001$) was observed in group III and group IV as compared to group II (Table 2).

Lung Histology: Histological analysis of the lungs from non-sensitized i.e. group I showed normal lung histology (Figure 1A). In contrast, similar to the BALF study, histological sections of lung tissue from group II guinea pigs exhibited airway inflammation, infiltration of eosinophils, lymphocytes and sub mucosal edema of the lungs, bronchoconstriction shown as lumen plugging by mucus and cells (Figure 1B). Treatment with *T. baccata* i.e. group III and group IV prevented the tissue edema, epithelial cell hypertrophy, infiltration of inflammatory cell and airway lumen plugging thereby decreasing inflammation and broncoconstriction which leads to normal lumen size (Figure 1C, 1D).

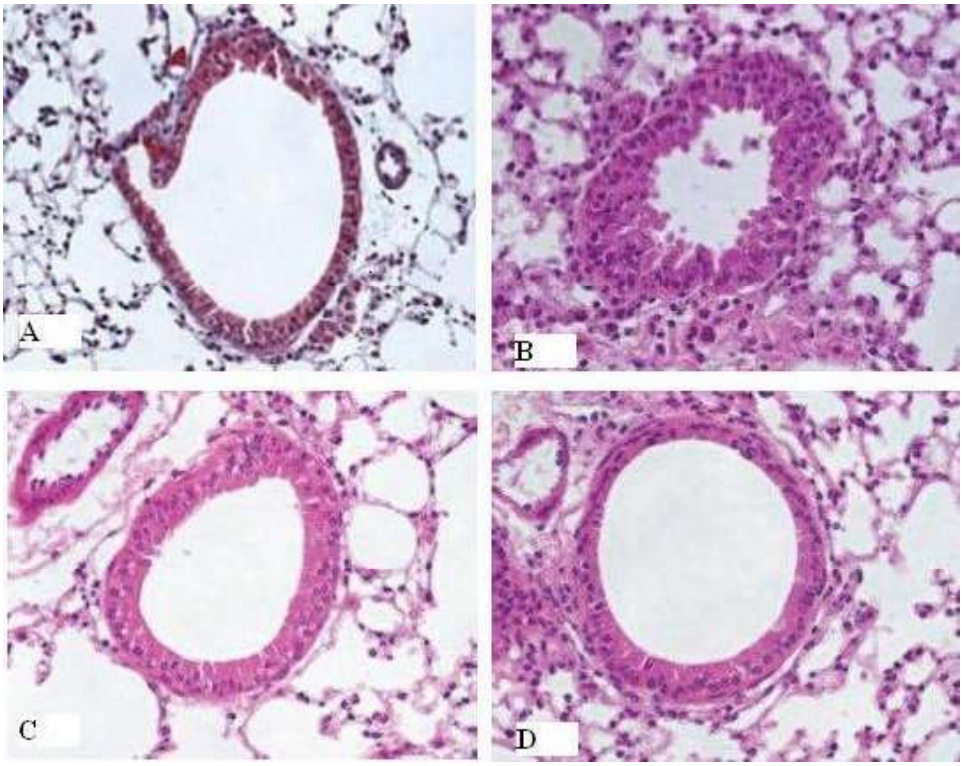

Fig. 1: Effect of *Taxus baccata* on the histology of lung tissue. (A) Control group I, (B) egg albumin sensitized group II, (C) egg albumin sensitized + *T. baccata* treated (200 mg/kg, p.o.) group III, (D) egg albumin sensitized + *T. baccata* treated (400 mg/kg, p.o.) group IV. *T. baccata* treatment was given for 15 days.

Effect on Compound 48/80 Induced Mast Cell Degranulation: Aqueous extract of *T. baccata* and ketotifen was found to significantly ($p < 0.001$) inhibit *in vitro* rat peritoneal mast cell degranulation induced by compound 48/80 as compared to base line value i.e. positive control group (Table 3).

DISCUSSION

Bronchial asthma is commonly characterized by increased airway reactivity to spasmogens. An initial event in asthma appears to be the release of inflammatory mediators like histamine triggered by exposure to allergens that directly cause acute bronchoconstriction [22-23]. In the present study, histamine and acetylcholine were used as spasmogens in the form of aerosol to cause immediate bronchoconstriction in the form of PCD in guinea pigs. Bronchodilating effect of aqueous extract of *T. baccata* was evaluated by observing its effects on the time of PCD. In our study we found that the time of occurrence of PCD was significantly increased that suggestive of bronchodilating activity following treatment with *T. baccata* against spasmogens.

Increasing evidence suggests that the frequently observed association between activated T lymphocytes and eosinophils play a major role in the development of airway inflammation and in the accompanying bronchial hyperreactivity [24-25]. Neutrophils and monocytes play a pivotal role in the disease process as they are source of variety of inflammatory mediators which are responsible for bronchial hyperresponsiveness and airway inflammation [26]. In association with asthma, elevated numbers of these inflammatory cells like eosinophils, neutrophils, lymphocytes, monocytes have been identified in various tissue compartments like blood, biopsies of lung tissue, in broncho alveolar lavage fluid and in sputum. In present study, sensitization using egg albumin (1 ml, 10 % w/v, i.p.) and then second exposure to same antigen i.e. egg albumin (0.5 ml, 2 % w/v) through saphenous vein causes acute anaphylactic shock resembling the acute asthmatic attack resulting in the release of various mediators and cellular infiltration. Antigen challenge resulted in significant increase in the number of eosinophil in the BALF. This was accompanied by intense eosinophil infiltration, accumulation and degranulation in the guinea pig lungs as evident of

histopathology which is consistence with human asthmatic lungs. In our study we found that treatment with *T. baccata* in antigen challenged animal significantly inhibited antigen induced hyper reactivity by preventing increase in infiltration of total leukocyte count, eosinophils count. After antigen challenge airway hyperresponsiveness is supported by inflammatory pathology suggesting involvement of other mediators in pathogenesis of asthma. Neutrophil numbers have also been reported to increase in bronchial lvage fluid in asthmatics, but neutrophilia is generally shorter duration than eosinophilia [27-28]. This was observed with our result that treatment with *T. baccata* resulted in significant inhibition of antigen induced bronchial hyperreactivity by decreasing neutrophil count. The participation of T lymphocytes in the pathogenesis of bronchial asthma and the accompanying bronchial hyperreactivity, has been widely demonstrated [25]. Indeed, activated $CD4^+$ T lymphocytes are found in the blood and bronchial lumen from asthmatics [29]. Recently, interest has been focused on the characterization of $CD4^+$ T lymphocytes based on their repertoire of secreted cytokines and its possible role in the pathogenesis of allergic disorders. Thus, $CD4^+$ T cells from asthmatics preferentially elaborate Th2-derived cytokines, such as IL-4 and IL-5, which have been shown to enhance IgE synthesis [30] and to act specifically on eosinophil survival, activation and secretion of proinflammatory mediators [31]. Large numbers of T lymphocytes, mainly of the $CD4^+$ subset, have been identified in the bronchial mucosa of antigen challenged guinea pigs [32]. In line of above the present finding show that treatment with *T. baccata* in sensitized animal produce significant decrease in lymphocyte count as compared to sensitized animals. The predominant cells in BALF recovered from unchallenged guinea-pigs were those of the monocyte. The numbers of these cells were increased after antigen challenge [33]. In the line of above context treatment with *T. baccata* significantly decreased monocyte as compared to sensitized guinea pigs. In our conclusion result of our study, suggest that in guinea pig airways antigen challenge induced eosinophil, neutrophil, monocyte and lymphocyte infiltration and activation is similar to that of reported in human asthmatics. These shows that protective effect of *T. baccata* is by preventing the infiltration of inflammatory cell, thereby decreasing the release of preformed inflammatory mediators, which can prevent the direct damage to airway, which in turn prevent airway hyperrescponsiveness.

Various processes involved in bronchial asthma such as inflammatory response can explain various histopathological alterations observed in biopsy of asthmatic patients. In asthma chronic inflammation is responsible for the bronchoconstriction which leads to airway narrowing and decrease in the lumen size of the bronchiole [34]. This can be clearly seen by the histopathological studies of the lung tissue by observing the cross section of bronchi. In the present study, the sections of the lung tissues of animals sensitized with egg albumin depicted marked bronchitis and severe bronchoconstriction. Treatment with *T . baccata* prevented the inflammation and bronchoconstriction which leads to normal lumen size and normal cellular structure compared to antigen sensitized guinea pigs.

Mast cell degranulation is important in the initiation of immediate responses following exposure to allergens [35]. Once binding of allergen to cell-bound IgE occurs, mediators such as histamine; eosinophil and neutrophil chemotactic factors; leukotrienes C4, D4 and E4; prostaglandins; platelet-activating factor; and others are released from mast cells which are responsible development of airway inflammation and bronchoconstriction. An attempt was made to find out whether aqueous extract of *T. baccata* has any effect on the rate of disruption of mast cells following exposure to compound 48/80, an agent which causes histamine release [36]. It has been assumed that the process leading to histamine secretion may be mediated by calcium release from an intracellular store of mast cells [37]. In this study, *T. baccata* offered significant protection against Compound 48/80 induced mast cell degranulation by stabilizing it, which is responsible for the decreasing airway inflammation by preventing release of various inflammatory mediators.

Phytochemical screening of *T. baccata* showed presence of lignans, flavanoids, steroids and sugar derivatives etc. [38-39]. Lignans are known to posses various biological activities including antibacterial, antioxidant, anticancer, spasmolytic and anti-inflammatory effects [40]. Flavonoids are known to possess various biological activities, including antibacterial, antifungal, spasmolytic, antiviral, anticancer and anti-inflammatory effects [41-43]. Anti-asthmatic activity of *T. baccata* may be due to presence of the above constituents. In conclusion, our data suggest that the aqueous extract of the leaves of *T. baccata* possesses significant anti-asthmatic activity and have beneficial effect in asthma.

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