Acute oral toxicity and *in vitro* leukotriene inhibitory property of hydroalcoholic extract of *Nicotiana tabacum* in guinea pig lung strips

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Abstract

Objective: The present study investigates acute toxicity and leukotriene inhibitory property of *Nicotiana tabacum.* **Materials and Methods:** Leaves of *N. tabacum* were extracted with aqueous ethanol (1:1) by cold percolation method. The extract was administered orally at the limit dose of 2000 mg/kg to evaluate the toxic potential of the plant. *In vitro* leukotriene inhibitory property of extract was examined on isolated lung strips of guinea pigs contracted with leukotriene D4 (10 µMol). The relaxation response was observed at six cumulative concentrations (6.25, 12.5, 25, 50, 100, and 200 mg/ml) of extract and standard, Montelukast, (0.15, 0.3, 0.45, 0.6, 0.75, and 0.9 mM). One-way ANOVA followed by Tukey's test was used for statistical analysis of data using GraphPad Prism 7. **Results:** Crude extract *of N. tabacum* administered orally at the dose of 2000 mg/kg did not cause any mortality indicating safety of the plant. The LD_{50} of the plant was found to be >2000 mg/kg. Increasing concentrations of *N. tabacum* showed a decrease in muscle tone induced by LTD_4 in a dose-dependent manner ($105.12 \pm 1.03\%$ at 200 mg/kg and $EC_{50} = 22.22$ mg/ml). However, the relaxant effect of extract at highest concentration was non-significant ($P > 0.05$) compared to Montelukast. The relaxant effects of all concentrations of montelukast were significantly higher ($P < 0.001$) than saline. On the other hand, at the lowest concentration, the relaxant effect of *N. tabacum* was not significant ($P > 0.05$) compared to saline. Montelukast showed maximum relaxation of $112.82 \pm 2.16\%$ at highest concentration ($EC_{50} = 0.20$ mM).

Key words: Asthma, leukotriene inhibition, *Nicotiana tabacum,* oral toxicity

INTRODUCTION

Traditional medicine has gained a lot of attention and importance in the treatment of many diseases. Plants and their extracts contain diverse phytoconstituents which have proved to be effective and are responsible for their medicinal properties. Herbal medicines approach the root cause of health problems, and their emphasis is to prevent and treat the underlying problem rather than provide a symptomatic relief.

Asthma is a serious health problem which has globally affected millions of people.[1-3]

Several host and environmental factors are responsible for triggering the symptoms of asthma.[4-6] In asthma, there is an inflammation of airways which causes narrowing of the bronchial tubes. There are many inflammatory cells present in asthmatic airways which release mediators causing bronchoconstriction.[1] Some of these include the mast cells, eosinophils, T-lymphocytes, dendritic cells, macrophages, and neutrophils.[7-13]

Some of the important inflammatory mediators involved in bronchoconstriction include chemokines, cysteinyl

leukotrienes, cytokines, histamine, and prostaglandin D2.[14-19]

Cysteinyl leukotrienes (leukotrienes C4, D4, and E4) play a crucial role in the pathology of asthma. They bind to the receptors CysLT1 and CysLT2, cause bronchoconstriction, and mimic the pathologic changes of airways in asthma. Therefore, leukotriene receptor antagonists are emerging as a novel approach in the treatment and management of asthma.[20-22] Many plants have been reported to have lipoxygenase inhibitory property as well as leukotriene receptor antagonist activity.[23,24]

Nicotiana tabacum Linn., Solanaceae, is an annually grown herbaceous. The leaves are commercially grown in many countries to be processed into tobacco. It is originated in tropical Americas, but now it is cultivated all over the world. The plant is known to possess a wide variety of biological activity. In Ayurveda medicine, it finds its use in the treatment of joint pain, cough, snake bite poisoning, swelling, dental caries, gingivitis, strychnine poisoning, headache, sinusitis, and nerve stimulant.[25] Previous studies have reported many pharmacological properties of this plant including antibacterial and antioxidant properties[26-28] anti-inflammatory and analgesic properties,[29] antiviral property,[30,31] anti-microbial activity[32,33] hypoglycemic property,[34] anthelmintic activity,[35] and anti-HIV activity.[36] Literature study reveals that the extract of various parts of this plant has been used traditionally in India and other parts of the world for the treatment of many ailments such as skin diseases, rheumatic swelling, painful piles, stings, inflammation, bacterial infection, sedation, antispasmodic, vermifuge, antiseptic, tuberculosis, and cough.[37] The pharmacological activities of medicinal plants are due to the presence of wide variety of phytochemical constituents in them. Extracts of *N. tabacum* in various solvents have shown the presence of a wide spectrum of secondary metabolites such as alkaloids, flavonoids, glycosides, steroids, phenols, and tannins.[26]

Many studies have been performed to evaluate the biological properties of *N. tabacum,* but there are no scientific reports about its effect on the contractility of airway smooth muscles. Therefore, this study was initiated with an aim to evaluate the acute toxicity and the potential of hydroalcoholic extract of this plant to inhibit the spasmogen-induced contraction in isolated lung strips of guinea pigs.

METHODS

Animals

Animals used in this experiment were treated in accordance with the guidelines provided by the Committee for the Purpose of Controlled and Supervision of Experiment on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethics Committee (1275/PO/Re/09/CPCSEA) of School of Pharmacy, CEC, Bilaspur, C.G. Animals were maintained under controlled environmental conditions, with room temperature at 18–22°C, an alternating 12-h/12-h light/dark cycle, and *ad libitum* water, fed with standard pellet diet supplied by Golden Feeds Ltd., India.

Preparation of Extract

Collection of plant sample

The fresh leaves of *N. tabacum* were collected from the local regions of Bilaspur, Chhattisgarh, in the months from January to March.

Authentication of plant sample

The sample was identified and authenticated by Dr. V Rama Rao, Research officer (Scientist -2), Botany, Regional Ayurveda Research Institute for Metabolic Disorders, Bengaluru, Karnataka, India. (Ref: RRCBI-mus36)

Processing of plant sample

The plant material was thoroughly washed with tap water and then rinsed with distilled water to remove the undesirable material. The rinsed leaves were dried on filter paper and then kept under shade for further drying for 3 weeks. The dried material of the plants was ground to a coarse powder using a sterile electric blender. The powdered material was stored in well-labeled airtight glass containers, protected from direct sunlight until required for further use.

Preparation of Plant Extract

The dried plant material was exhaustively extracted by cold percolation technique. For this, 200 g of powdered material was extracted with 500 ml of aqueous ethanol (1:1) at room temperature for 72 h with subsequent filtration. The extract was filtered using a Whatman filter paper no. 1. The filtrate obtained was evaporated to dryness using a rotary evaporator (BUCHI, Switzerland) at 40°C temperature under reduced pressure. The crude extract of *N. tabacum* was dried in freeze drier, kept in a bottle sealed with parafilm, labeled as HAENT, and preserved at 4°C.

The percentage yield of the hydroalcoholic extract of *N. tabacum was* determined using the following formula:

Percentage Yield = Weight of the extract × 100/Weight of the powder sample

Acute Toxicity Study

15 healthy young adult female Swiss albino mice, non-pregnant, nulliparous, 8–12 weeks old, and weighing 25–30 g

were used for the study. The study was carried out according to the OECD guidelines for testing of chemicals, number 420.[38]

Preparation of Animals

Animals were randomly selected and marked for individual identification. Mice were divided into two groups of five mice each. The treatment given to the two groups was designed as follows:

Group I - Control treated with distilled water (2 ml/kg, bw).
Group II - *N. tabacum* (2000 mg/kg, bw).

Preparation and of Doses

The extract was suspended in distilled water, and the dose of the test substance was prepared shortly before the administration.

Procedure

Animals were fasted before dosing. Food was withheld for 3–4 h before the administration of test substance. Animals had free access to water. Following the fasting period, body weights of the animals were recorded and the dose to be administered was calculated as the volume of the extracts solution given to the mice was 10 mL/kg [Table 1].

2 h after the administration of the test substance, animals were provided with food.

OBSERVATIONS FOR THE SIGNS OF TOXICITY AND MORTALITY

After dosing, animals were observed individually, once during the first 30 min and periodically during the first 24 h. Special attention was given to the first 4 h. Animals were observed for 2 weeks for any signs of acute toxicity. Observations included physical or behavioral changes such as skin and fur, eyes and mucus membranes, respiration, circulation, autonomic nervous system (ANS), central nervous system, somatomotor activity, and behavior pattern. Observations were also recorded for the presence of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma. Individual records of the animals were maintained.

Body Weight

The animals were weighed individually before the commencement of the study and weekly thereafter. The changes in the body weights were recorded and compared.

Food and Water Consumption

The amount of feed and water consumed by the mice was measured daily on the basis of the quantity of feed and water supplied and the quantity remaining after 24 h.

Leukotriene Inhibitory Property

Isolation of lungs from guinea pigs

Female albino guinea pigs weighing 300–450 g were sacrificed by a blow on the neck and pinned on a polystyrene plate. The chest cavity was opened, and the lungs were carefully excised and placed in a Petri dish containing a freshly prepared Krebs–Henseleit solution, aerated with carbogen (95% O_2 and 5% CO_2), and maintained at pH 7.4 ± 0.05 at temperature 37°C.

Preparation of Lung Strips

Sections of about 5 cm in width were cut from the lower part of the right lobe of the lung. The tissue was mounted in a 10 ml organ bath containing aerated Krebs–Henseleit solution at 37°C. One end of the tissue was tied to the aerator tube and the other one to a light frontal lever to the Kymograph paper on Sherrington Rotating Drum. The tissue was suspended under an isotonic tension of 1 g and left to equilibrate for 1 h. The solution in the organ bath was replaced several times at an interval of 15 min.

The response (relaxation) due to the extract *N. tabacum* at six cumulative concentrations (6.25, 12.5, 25, 50, 100, and 200 mg/ml) and the standard drug Montelukast at six cumulative concentrations (0.15, 03, 0.45, 0.6, 0.75, and 0.9 mM) was measured in lung tissues pre-contracted with 10 μMol leukotriene D_4 (LTD_4). The dose of 10 μMol of LTD_4 was optimized from a cumulative concentration-response curve (CCRC) ranging from 1×10^{-6} M to 3×10^{-3} M. Animals were randomly divided into the following groups:

Group I: Normal saline (negative control).
Group II: CCRC of Montelukast (standard drug, positive control).
Group III: CCRC of hydroalcoholic extract of *N. tabacum*.

Cumulative relaxation was measured in Groups II and III following a 10 μMol dose of LTD4 in an organ bath.

Procedure

Each dose-response experiment was done on a set of six strips. Concentration-response curves were plotted by the treatment of precontracted lung strips (10 μMol LTD_4) with cumulative concentrations of an extract of *N.*

tabacum or Montelukast, at 5 min intervals. The cumulative concentration of the extract was created by adding 0.5 ml of the first concentration (6.25 mg/ml). After that at 5 min intervals, 6.25, 12.5, 25, 50, and 100 mg/ml concentrations were added to the organ bath to obtain 6.25, 12.5, 25, 50, 100, and 200 mg/ml. For the positive control, Montelukast, first concentration (0.15 mM) was added, and after 5 min intervals, five 0.15 mM solutions were added to create 0.15, 0.3, 0.45, 0.6, 0.75, and 0.9 mM concentrations. For negative control, the effect of treatment with 0.1 ml saline on pre-contracted lung strips was evaluated.

The effect of six cumulative concentrations of extract or standard drug or saline on contracted lung strips was measured after exposure of tissue to the solution for 5 min. A decrease in tone was considered as a relaxant effect and expressed as positive percentage change in proportion to the maximum contraction, and an increase in tone was considered as a contractile effect, which was expressed as negative percentage change.[39]

Statistical Analysis

The data were expressed as mean ± standard deviation (SD). Differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $P < 0.001$ (***), 0.01 (**), or 0.05 (*)was considered to be statistically significant.

RESULTS AND DISCUSSION

Yield Extract

The extraction yield is presented in Table 2.

Mortality

In the present investigation, animals were administered hydroalcoholic extract of *N. tabacum* in a single dose level of 2000 mg/kg bw. All the animals were monitored daily for 14 days. No mortality or symptoms of toxicity were observed in any of the animals treated with the extract. Table 3 shows the toxic effect of an extract of leaves of *N. tabacum* on mice.

Behavioral Analysis

Animals of the treated group were analyzed for their behavioral pattern and compared with the animals of the control group. It was observed that the animals in both the groups showed normal behavior and did not display any change in their general behavior. Visual observations showed no signs of change in skin and fur, eyes, mucus membrane, or sleep. Tremors, convulsions, diarrhea, or coma was not observed in any of the groups (control and treated). Table 4 summarizes the general behavioral pattern of the animals treated with the two extracts.

LD$_{50}$

Data from acute oral toxicity study of the hydroalcoholic extract of leaves of *N. tabacum* show that the extract is safe as neither did it cause the death of any animal nor did it show any signs of toxicity. Therefore, the median lethal dose LD$_{50}$ value for the extracts of *N. tabacum* is greater than the limit dose of 2000 mg/kg. The extract is safe at this dose and all the other doses below 2000 mg/kg.

Body Weight

Before the experiment, animals were weighed and the mean of their body weights was measured weekly for 2 weeks. Statistically, no significant difference ($P > 0.05$) was found in the body weights of mice in the treatment group when compared to control group [Figure 1]. All the animals in both the groups showed a normal increment in the body weight, and no drastic difference was observed between the control group and the treated group [Table 5]. This justifies the safety of the extract.

Food Intake and Water Consumption

Determination of food consumption is important in the study of the safety of a product with a therapeutic purpose, as proper intake of nutrients is essential to the physiological status of the animals and to measure the right response of the drug tested.[40-42] The consumption of food and water of all the animals was normal which indicates that the administration of a hydroalcoholic extract of leaves of *N. tabacum* did not affect the food and water consumption. Neither did the extract of the plant induce the suppression of appetite nor

Table 1: The dose of the extract and frequency of administration				
Treatment Group	Dose	Vehicle	Route of administration	Frequency of administration
Group I, Control	2 ml/kg	Distilled water	Orally using an intubation cannula	Single dose
Group II, Extract of *N. tabacum*	2000 mg/kg	Distilled water	Orally using an intubation cannula	Single dose

did it show any deleterious effect. This shows that there was no disturbance in the metabolism of protein, fat, or carbohydrates.[43]

Airway Smooth Muscle Contraction by LTD4: Determination of EC_{50}

Increasing concentration of LTD_4 (10^{-6} M to 3×10^{-3} M) caused concentration-dependent contraction of airway smooth muscles [Figure 2]. LTD_4 caused 100% contraction at the dose of 3×10^{-5}M with an EC_{50} value of 1.01 µMol. A dose of 10 µMol LTD_4 was selected to attain submaximal response.

Table 2: Percentage yield of hydroalcoholic extract of leaves of *N. tabacum*

Extract	Color	Odor	Yield (%)
Hydroalcoholic extract	Dark green	Characteristic	56

Table 3: *Mortality in mice treated with extract N. tabacum*

S. No.	Group	Number of mice dead/ Number of mice used
1.	I, Control	0/5
2.	II, extract of *N. tabacum*	0/5

Relaxant Effects

CCRC of relaxation was expressed as a percentage of the maximal response measured for each test drug. A dose-dependent relaxation was observed for the extract and the standard, Montelukast, in lung strips of guinea pigs precontracted with 10 µMol doses of LTD4.

The results reveal that increasing concentrations of hydroalcoholic extract of *N. tabacum* (6.25, 12.5, 25, 50, 100, and 200 mg/ml) showed a decrease in muscle tone induced by LTD_4 in a dose-dependent manner with a maximum value of $105.12 \pm 1.03\%$ ($n = 6$) obtained at the highest concentration of 200 mg/ml ($EC_{50} = 22.22$ mg/ml) as shown in Figure 3. However, the relaxant effect of *N. tabacum* at the highest concentration (200 mg/ml) was non-significant ($P > 0.05$) when compared to positive control, Montelukast. The relaxant effects of all concentrations of montelukast (0.15, 0.3, 0.45, 0.6, 0.75, and 0.9 mM) were significantly higher ($P < 0.001$) than saline. On the other hand, the relaxant effects of last four concentrations of the extract were significantly higher ($P < 0.01$) than saline. At the lowest concentration (6.25 mg/ml), the relaxant effect of *N. tabacum* was not statistically significant ($P > 0.05$) compared to saline. Montelukast showed a decrease in the muscle tone induced by LTD_4 in a concentration-dependent manner, with a maximum value of $112.82 \pm 2.16\%$ ($n = 6$) observed at the highest concentration of 0.90 mM ($EC_{50} = 0.20$ mM) [Figure 3].

Table 4: General appearance and behavioral pattern in mice treated with a crude extract of Nicotiana tabacum (2000 mg/kg bw)

Observations	30 min		4 h		12 h		24 h		48 h		7th Day		14th Day	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II
Skin and fur	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Mucus Membrane	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Lethargy	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Sleep	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Coma	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Tremors	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Convulsions	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Behaviour pattern	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Diarrhoea	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO

I: Control group treated with distilled water, II: Group treated with a crude extract of *N. tabacum,* N: Normal, NO: Not observed

Table 5: Changes in the body weights of mice treated with extracts of control and *N. tabacum*
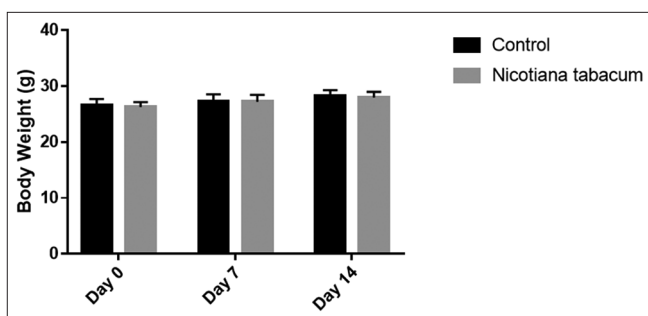
Treatment	Dose	Day 0 BW (g)	Day 7 BW (g)	Day 14 BW (g)
Vehicle, Distilled water	2 ml/kg	26.46 ± 0.46^{ns}	26.66 ± 0.69^{ns}	27.318 ± 0.18^{ns}
N. tabacum	2000 mg/kg	26.52 ± 1.04^{ns}	27.1 ± 0.98^{ns}	27.42 ± 0.87^{ns}

Data were expressed as mean±SD, $n=5$, $P<0.05$ was considered to be statistically significant, ns: not significant

Figure. 1: Effects of a hydroalcoholic extract of *N. tabacum* on body weight in acute toxicity study in mice. Data are expressed as mean ± standard deviation, *n* = 5. No statistical difference between control and extract-treated groups
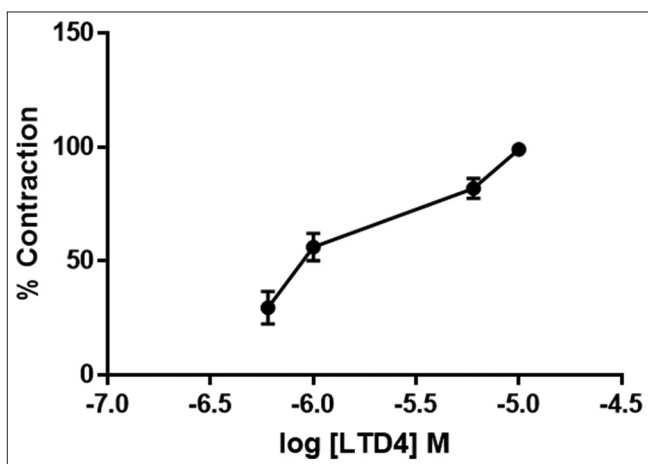
Figure 2: Dose-response curve of lung strips of guinea pigs contracted by LTD_4 (10^{-6} M to 3×10^{-3} M). Maximum contraction (100%) was observed at a concentration of 3×10^{-5} M. Data are represented as mean ± standard deviation (n=3)
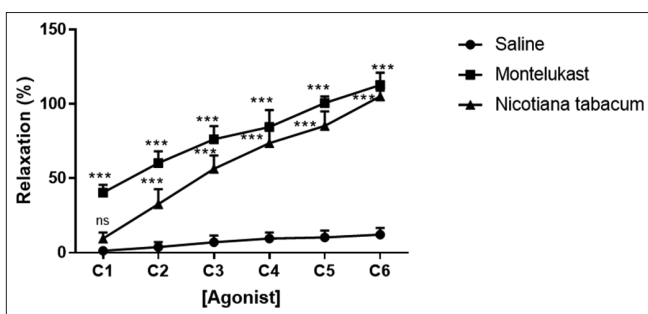
Figure 3: Concentration-response relaxant effect (mean ± standard deviation [SD]) of different concentrations of *N. tabacum* extract on LTD4 (10 µMol)-induced contraction of lung strips of guinea pig. Concentrations of *N.tabacum* C1, 6.25 mg/ml; C2, 12.5 mg/ml; C3, 25 mg/ml; C4, 50mg/ml; C5, 100 mg/ml; and C6, 200 mg/ml. Concentrations of Montelukast C1, 0.15mM; C2, 0.30mM; C3, 0.45mM; C4,0.6mM; C5,0.75mM; and C6,0.9 mM. Symbols and vertical bars represent mean and SD (n=6). Data with different superscript symbols differ significantly among groups: **$P < 0.01$; *** $P < 0.001$, ns: not significant ($P > 0.05$)

DISCUSSION

As there is a surge in the use of natural medicines and herbal medicines for the treatment of many diseases, there is a need to screen the toxicity of the medicinal plants to ensure the safety and effectiveness of their use. In the present study, oral acute toxicity was investigated by observing the toxic effects of hydroalcoholic extract of leaves of *N. tabacum* in Swiss albino mice. The route of administration of the test compound is important while studying the acute toxicity effects. This depends on the dosage form in which the test substance is available. The most preferred route for studying acute toxicity is the oral route because it is convenient, costs less, and is painless to the animals. According to a research, mice were found to give a better prediction for acute lethal dose in humans compared to rats.[43] Animals are fasted overnight before administering the extract otherwise it might interfere with the food present in the digestive tract. The study was carried out according to the OECD guidelines 420.[38]

In our study, the animals in control and treated groups were administered with the vehicle and the crude extract, respectively. The mice were monitored for any signs of toxicity or mortality, daily for a period of 14 days. During this period, animals in the treated group, which received a single dose of 2000 mg/kg, did not show any signs of distress. There were no symptoms of toxicity or lethality and none of the animals died. There were no changes in the behavior of the animals, and the physical appearance was found to be normal. There was no significant change in the body weights of the mice in the treated group, and the food and water consumption remained unaffected. Changes in the body weight after the administration of the test compound are an indication of toxicity.[44] If the body weight loss is more than 10% of the initial weight, then it is a sign of the adverse effect.[45,46]

The extract of leaves of *N. tabacum* did not cause any toxicity, and the LD_{50} value is >2000 mg/kg. According to the Globally Harmonized System of Classification and Labeling of Chemicals recommended by the OECD, the crude extract of leaves of *N. tabacum* was assigned Category 4 status (LD_{50} > 2000 mg/kg). Thus, the limit test method is a way of classifying the crude extract based on the observation and expectation of the dose level at which the animals would survive.[47]

In the evaluation of leukotriene inhibitory property of the crude extract of *N. tabacum*, the concentration-dependent relaxant effect of the extract and the positive control, Montelukast, was seen on the lung strips contracted by the spasmogen LTD4. The relaxant effect of the plant on lung strips of guinea pig might be due to different mechanisms such as inhibition of leukotriene receptors,[48] stimulation of β-adrenergic receptors,[49,50] inhibition of histamine H1 receptors,[51] or anticholinergic property.[52] Since the extract showed relaxant effect on LTD4-induced contraction, the

most possible mechanism of relaxant effect of *N. tabacum* might be due to its inhibitory effects on leukotriene receptors. Leukotrienes are powerful bronchoconstrictors which act on smooth muscles through specific receptors. These are a family of lipid mediators which play an important role in the pathogenesis of inflammation. Leukotrienes are synthesized from arachidonic acid by the action of the enzyme 5 lipo-oxygenase. The action of LTs on smooth muscles can be blocked either by inhibiting the synthesis of LTs, i.e., by inhibiting the enzyme which would not be able to synthesize LT further or by blocking the LT receptors. In this experiment, the second approach of blocking the LT receptors was used to study the leukotriene inhibitory property of the plant. LTD4, a cysteinyl LT, was used as the spasmogen to induce contraction, and the relaxation response of the crude extract was measured. The extract showed a significant decrease in the contraction of the smooth muscle compared to the saline. However, there was a significant difference in the relaxation brought about by the extract when compared to the standard drug at all the concentrations. The relaxant effect of this plant may be due to the contribution of diverse phytoconstituents present in it. In a study, flavonoids showed potent bronchorelaxant property on guinea pigs trachea.[53] Further study is needed to establish the molecular mechanism of relaxation caused by the extract of *N. tabacum* and to find the active principle responsible for the relaxant activity.

This study supports the traditional use of this plant in the treatment of various inflammatory diseases.

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