

## ***In vivo* Evaluation of Antiasthmatic Activity of the Essential Oil of *Zanthoxylum armatum***

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Sharma, *et al.*: Antiasthmatic Activity of *Zanthoxylum armatum* Oil

*Zanthoxylum armatum* DC. is used in traditional medicines as a therapy of many diseases including bronchial asthma. The antiasthmatic effect of *Zanthoxylum armatum* essential oil was studied using histamine aerosol-induced bronchoconstriction in guinea pigs and ovalbumin-sensitized albino mice. Absolute eosinophils count in blood, total immunoglobulin E, eosinophils and neutrophils in bronchoalveolar lavage fluid and histopathology of lung tissue were investigated. Treatment with *Zanthoxylum armatum* essential oil significantly increased the time of preconvulsive dyspnoea in histamine-induced guinea pigs. Oral treatment of *Zanthoxylum armatum* oil showed significant decrease in absolute eosinophils count, serum level of immunoglobulin E and the number of eosinophils, neutrophils in bronchoalveolar lavage fluid. Histopathological examination of lungs showed that essential oil rescinded bronchial asthma. Results obtained in the present investigation provided evidence that essential oil of *Zanthoxylum armatum* caused bronchorelaxation and showed antiasthmatic properties. The traditional use of *Zanthoxylum armatum* essential oil against asthma could be attributed to the bronchorelaxation and antiasthmatic activity.

**Key words:** *Zanthoxylum armatum*, bronchorelaxation, antiasthmatic activity, essential oil

Asthma is a chronic disease involving the airways inflammation of the lungs. These airways or bronchial tubes allow air to come in and out of the lungs. Asthma can be classified as allergic asthma, caused by exposure to an allergen and non-allergic asthma caused by stress, exercise, illnesses like a cold or flu or exposure to extreme cold weather, irritants in the air or by some medications<sup>[1]</sup>. Sometimes asthma is called bronchial asthma or reactive airway disease. Asthma is characterized by inflammation of the bronchial tubes with increased production of sticky secretions inside the tubes. Common asthma symptoms include, coughing especially at night, wheezing, shortness of breath, chest tightness, pain or pressure<sup>[2]</sup>. The burden of asthma is immense, with more than 300 million individuals currently suffering from asthma worldwide, about a tenth of those living in India. The prevalence of asthma has been estimated to range 3-38 % in children and 2-12 % in adults, being the commonest chronic disorder among children<sup>[3]</sup>. Although currently available treatments for asthma include  $\beta$ -2 agonists, anticholinergics, methylxanthines, mast cell stabilizers, leukotriene antagonists, glucocorticoids, antiimmunoglobulin E (antiIgE) antibody like omalizumab, which are administered for long duration. Moreover, these treatments are associated with several adverse effects, like muscle tremors, restlessness, hypotension, hyperglycaemia, tachycardia, flushing, convulsions, mood changes and adrenal crisis<sup>[4]</sup>. To minimize and possibly prevent

these side effects alternative and complementary medicine is being sought. Some essential oils obtained from plants have been traditionally used in respiratory tract infections. Essential oils inhalation therapy has been used to treat bronchitis as it has antiinflammatory effect on trachea<sup>[5]</sup>.

*Zanthoxylum armatum* DC. (Rutaceae) is an important medicinal plant, which is commonly known as Indian prickly ash, Nepal pepper or toothache tree, *Tejphal* (Hindi), *Tejowati* (Sanskrit), and *Mukthruhi* (Manipuri and Nepal)<sup>[6]</sup>. In traditional medicine *Z. armatum* is used in treatment of asthma, bronchitis, colic, cough, convulsions, cardiac debility, diabetes, diarrhoea, dyspepsia, fever, goitre, difficult micturition, eye and ear disease, helminthiasis, hepatopathy, leprosy, leucoderma, paralysis, skin disease, stomach disorder, tumours, ulcers and wounds<sup>[7,8]</sup>. The *Bhotiya* tribes of India use this plant to treat cough and colds<sup>[9]</sup>. The major essential oil constituents such as 3-borneol, isobornyl acetate, dihydrocarveol<sup>[10]</sup>, linalool,  $\alpha$ -limonene diepoxide,  $\alpha$ -pinene, myrcene, D-limonene<sup>[11]</sup>, have been reported as the major constituent of the *Z. armatum* essential oil. Various biological

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Accepted 15 February 2018

Revised 07 July 2017

Received 04 June 2016

Indian J Pharm Sci 2018;80(2):383-390

activities *viz.* antioxidant activity<sup>[12]</sup>, anticonvulsive, antinociceptive activity<sup>[13]</sup> and antifungal, antispasmodic, antibacterial<sup>[11]</sup> have been reported from essential oil of *Z. armatum*. The essential oil of *Z. armatum* has not been explored for its effect related to medicinal use as asthma disorder. Therefore, the aim of present study was to evaluate antiasthmatic activity of the essential oil of *Z. armatum* using *in vivo* models.

The following reagents were commercially purchased and used: ovalbumin (OVA, Sigma-Aldrich, USA), aluminium hydroxide, histamine dihydrochloride, Tween 80 (HiMedia, Mumbai, India), dexamethasone (Centaur Pharmaceuticals, Goa), Turk solution (Nice Chemical Pvt Ltd., Cochi), ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) and *Z. armatum* oil (Kshipra Biotech Private Limited, Indore, India).

Female guinea pigs (400-600 g) purchased from J. N. Medical College, Belagavi, India and housed in standard conditions of temperature (22±2°), relative humidity (55±5 %) and light (12 h light/dark cycles) were used for bronchoconstriction activity. They were fed with vegetables, fruits, grass and water *ad libitum*. Female albino mice (18-25 g) were purchased from Sri Venkateshwara enterprises, Bengaluru, India and housed in standard conditions of temperature (22±2°), relative humidity (55±5 %) and light (12 h light/dark cycles) were used for bronchial asthma activity. They were fed with standard pellet diet and provided water *ad libitum*. The Institutional Animal Ethics Committee (IAEC) approved the experimental protocol (KLECOP/IEAC/Res.22-10/10/2015). All experiments were conducted in strict compliance with the ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals. As per the IAEC advice, since *Z. armatum* is used in traditional medicine determination of toxicity of *Z. armatum* oil was not performed. However, as per literature available, the acute toxicity of rat and mice was determined to be safe therapeutic at a dose of 2000 mg/kg (limit dose)<sup>[13,14]</sup>.

The chemical composition of the essential oil (1 % solution of essential oil in equal ratio of n-hexane:dichloromethane) was analysed using a gas chromatograph (GC; Varian 450 fitted with a fused silica capillary column TG-5, 5 % diphenyl-95 % dimethyl polysiloxane; Thermo Scientific, 30 m×0.25 mm i.d. 0.25 µm film thickness) under the experimental conditions reported earlier<sup>[15-17]</sup>. The oven

temperature was programmed from 60 to 220° at 3°/min, using nitrogen as carrier gas. The injector and the flame ionization detector temperature were set at 230 and 240°, respectively. Gas chromatography-mass spectrometer (GC-MS) analysis was employed a Thermo Scientific Trace Ultra GC interfaced with a Thermo Scientific ITQ 1100 mass spectrometer fitted with a BP-1 (100 % dimethyl polysiloxane; SGE Analytical Science) fused silica capillary column (30 m×0.25 mm; 0.25 µm film thickness). The oven temperature range was programmed from 60 to 220° at 3°/min, and helium was used as carrier gas at 1.0 ml/min for analysis. The injector temperature was set at 230°, and the injection volume was 0.1 µl in n-hexane, with a split ratio of 1:50. MS was taken at 70 eV with a mass range of m/z 40-450 and other parameters used were those reported earlier<sup>[18-20]</sup>. The major constituent of the essential oil of *Z. armatum* was identified and confirm (co-injection of commercial sample from Sigma-Aldrich, India (≥98 % purity).

Experimental bronchial asthma was induced in guinea pigs by exposing them to 0.1 % w/v histamine aerosol under constant pressure in an aerosol chamber (24×14×24 cm<sup>3</sup>) made of perplex glass. Each animal was placed in a chamber with histamine aerosol and pre-convulsive time (PCT; time of histamine aerosol exposure to guinea pig for initiating dyspnoea leading to the appearance of convulsions) was noted. On day 0 animals were kept in the histamine chamber and PCT was recorded as a baseline value. Day 0, PCT was taken as before treatment value. As per pre-convulsion dyspnoea (PCD) was recorded, the animals were removed from the chamber and exposed to fresh air for recovery. After development of PCD, the animals were divided into 4 groups (n= 6 in each group): group-I negative control, received histamine aerosols 0.1 % w/v; group-II received intraperitoneal injection of dexamethasone 2 mg/kg once daily for 7 d, group-III and IV received orally *Z. armatum* oil at the dose of 200 and 400 µl/kg once daily for 7 d. On the d 7, 2 h after the last dose, the time for the onset of PCT was recorded<sup>[21]</sup>. The percent increase in the time of PCT was calculated using following formula. Percent increase in PCT =  $(1 - T_1/T_2) \times 100$ , where, T<sub>1</sub> = time for PCT onset on day 0, T<sub>2</sub> = time for PCT onset on day 7.

Female mice were divided into four groups (n= 6 in each group), normal, negative control, standard and treatment group. Except normal mice other groups were sensitized by intraperitoneal injection of 50 µg OVA and 1 mg aluminium hydroxide in 200 µl

phosphate buffer saline (PBS) on day 1 and day 7. Group-I was given normal saline and feed for 14 d. After sensitization, from 8 to 14 d group-II was given OVA aerosols (1 % w/v) in PBS for 30 min. Group-III was given dexamethasone (2 mg/kg) intraperitoneal injection daily for 7 d along with OVA aerosols. *Z. armatum* oil was given orally to group-IV at a dose of 200 µl/kg for 7 d along with OVA aerosols. On day 15, 24 h after the final allergen lung lavage was performed for preparation of bronchoalveolar lavage fluid (BALF), trachea was aspirated (three time) with PBS until 2 ml of BALF was taken. The suspension of BALF was centrifuged and the supernatant collected and stored at -80°. Blood was collected in blood collecting tube containing disodium EDTA and absolute eosinophils were determined by direct microscopic counting with a haemocytometer. Blood serum was collected to estimate IgE level; lungs were collected for histopathological examination<sup>[22]</sup>. The accumulation of inflammatory cells in BALF was examined to evaluate airway inflammation. Briefly, 24 h after the final inhalation of antigen (day 15), animals were sacrificed by over dose diethyl ether inhalation, the left bronchus was tied for histological examination. Then, the right air lumen was washed four times with 0.5 ml PBS containing. BALF from each animal was pooled in a plastic tube, cooled in ice, and centrifuged (5000 g) at 4° for 10 min. Cell pellets were re-suspended in the same buffer (1 ml). A portion of the cell suspension was mixed with Turk solution and nucleated cells were counted in a haemocytometer.

Twenty-four hours after the last OVA challenge, mice were anesthetized with diethyl ether and blood was drawn. Differential cell counts were performed after staining with a modified Giemsa stain and cells with red cytoplasmic granules were counted as eosinophil's to calculate absolute eosinophil's count. The serum level of OVA-specific IgE was measured using an ELISA kit with commercially available reagents, according to the manufacturer's instructions. The process of measurement was same as cytokine analysis. The detection limit was 0.1 ng/ml for IgE.

The lungs were harvested after dissection and fixed in 10 % buffered formalin for 24 h, dehydrated, embedded in paraffin, sectioned into thin slices, stained with haematoxylin-eosin and observed by light microscopy, The degree of peribronchial and perivascular inflammation was observed.

Results were expressed as mean±SEM where n= 6. Differences among data were determined using one-way ANOVA followed by Dunnett's multiple comparison test (GraphPad Prism software, version 5.01). P≤0.05 was considered statistically significant.

The GC-MS analysis of essential oil of *Z. armatum* revealed that the main compound was identified as linalool (75.7 %). The essential oil of *Z. armatum* was significantly and dose-dependently increased the latent period of PCD. Percent increase in PCT in histamine-induced bronchoconstriction at the dose of 200 and 400 µl/kg body weight was found to be 61.20±0.29 and 69.48±0.99 %, respectively. Hence, the increase of PCT at a dose of 400 µl/kg was observed maximum protection compared to standard drug (dexamethasone)-treated group (Table 1).

Control group showed maximum increase in eosinophil count 24 h after the final exposure of OVA blood was drawn in blood collecting tube containing disodium EDTA. *Z. armatum* oil-treated group at the dose of 200 µl/kg significantly inhibited the increased inflammatory cell (absolute eosinophils count) in blood (fig. 1A).

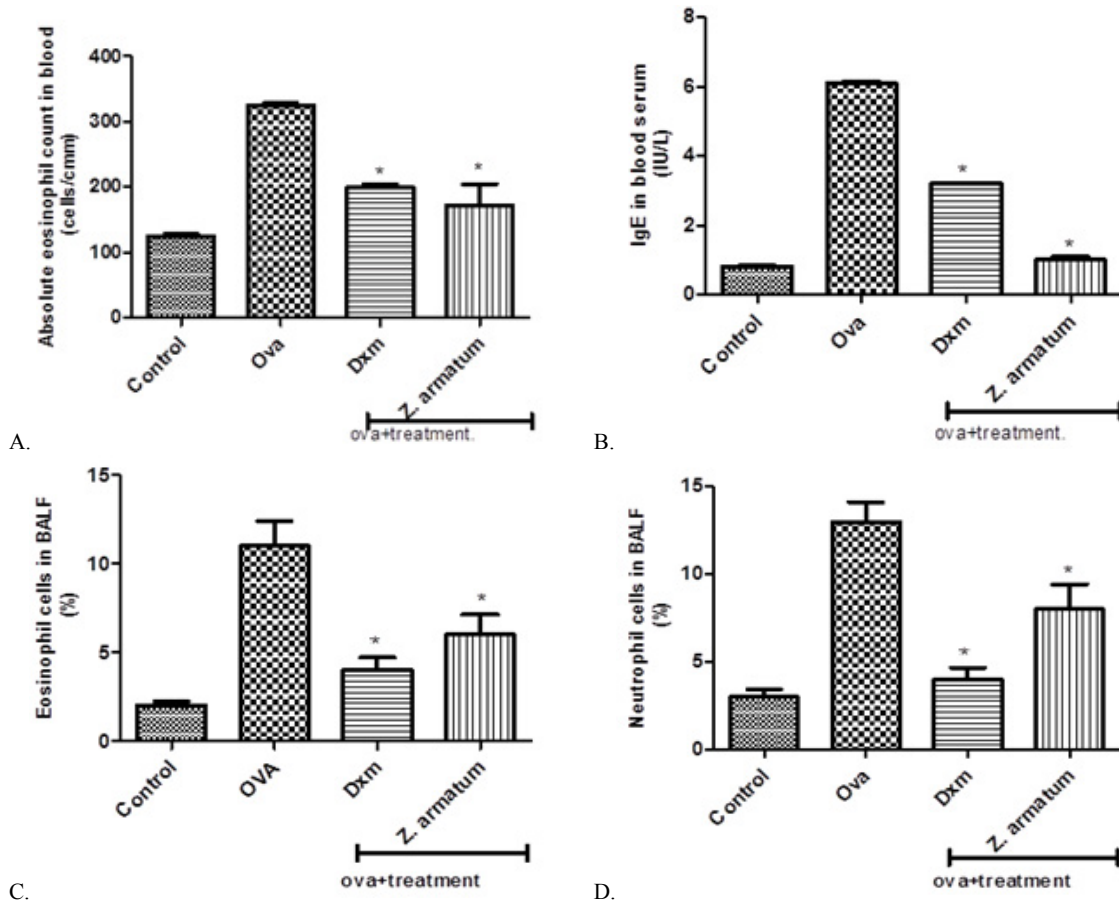
Antigen-specific Th2 responses are known to induce antigen-specific IgE antibody production. Repeated OVA inhalation significantly increased the number of inflammatory cells in blood serum. On the day 15, immediately after the final OVA challenge, significantly increased the total serum level of IgE. The treatment with essential oil of *Z. armatum* significantly inhibited the increased level of serum IgE at a dose of 200 µl/kg body weight (fig. 1B).

After challenging with OVA, the levels of inflammatory cells including eosinophil's and neutrophils were

**TABLE 1: EFFECT OF *Z. ARMATUM* OIL ON HISTAMINE-INDUCED BRONCHOCONSTRICTION IN GUINEA PIGS**

Grouping	Before treatment	After treatment	% Increase in pre-convulsive dyspnoea time PCD
Negative control group	108	120	6.17±2.49
Dexamethasone	90	180	50.53±3.77*
<i>Z. armatum</i> 200 µl/kg	249	642	61.20±0.29*
<i>Z. armatum</i> 400 µl/kg	83	271	69.48 ±0.99*

Each value was expressed as mean±SEM; where n= 6 in each group: \*p≤0.05 as compared with control by one-way analysis of variance, followed by Dunnett's test



**Fig. 1:** Effect of essential oil on absolute eosinophils count in blood of mice Cells/cubic mm (A); effect of *Z. armatum* essential oil on Immunoglobulin E in blood serum of mice, IU/l (B); effect of *Z. armatum* oil on inflammatory cells: eosinophils in bronchoalveolar lavage fluid of mice, % (C); effect of *Z. armatum* oil on inflammatory cells: neutrophils in bronchoalveolar lavage fluid of mice, % (D); values were expressed as mean $\pm$ SEM, where n= 6 in each group, \*p $\leq$ 0.05 compared with ovalbumin-treated group

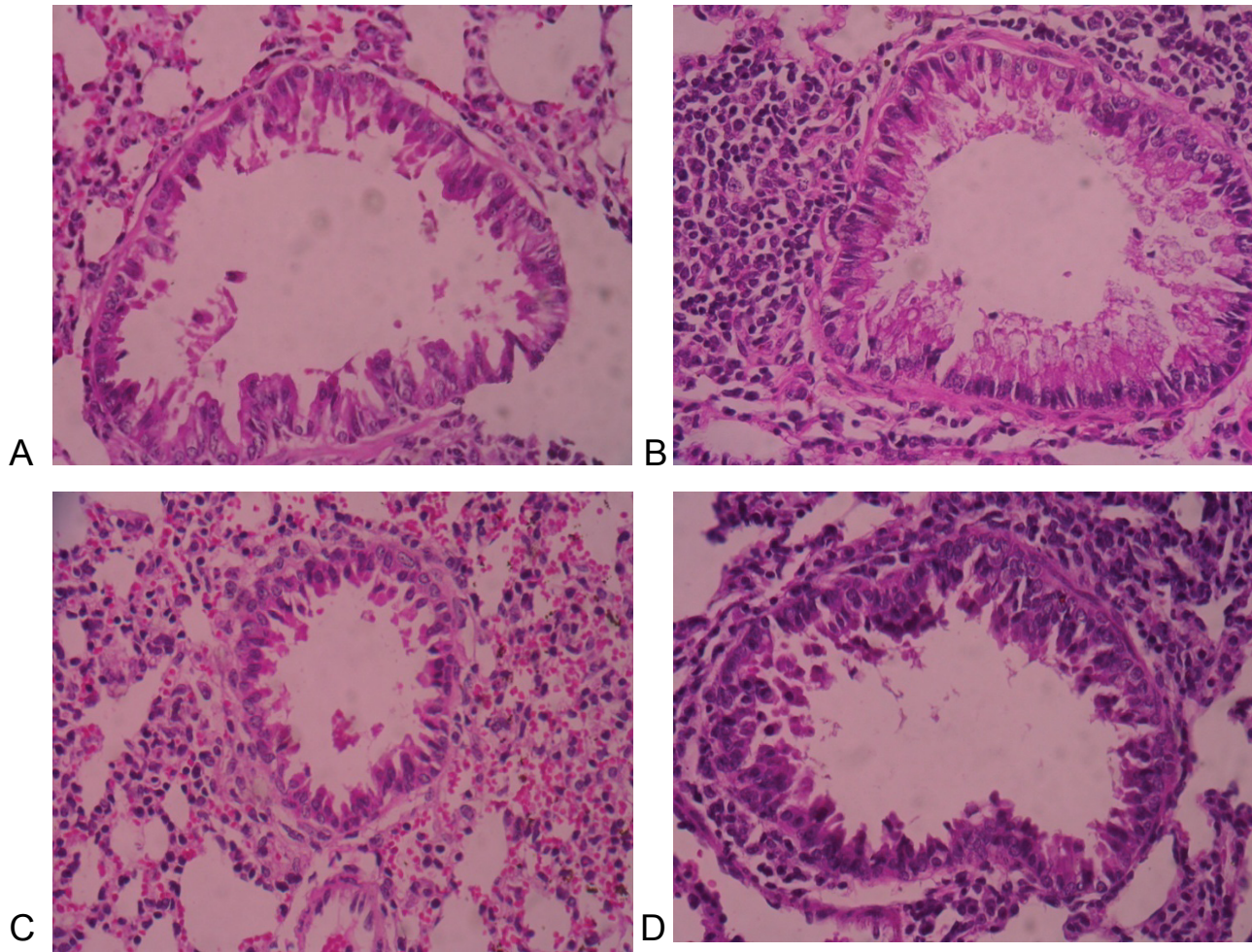
significantly increased in OVA-treated group as compared with normal group. But, sensitization of OVA along with the essential oil treatment, the levels of inflammatory cells were significantly decreased as compared with asthma-induced OVA group. The accumulation of inflammatory cells in BALF was significantly inhibited by 200  $\mu$ l/kg body weight of essential oil (fig. 1C and 1D).

The microscopic images of tissue sections of each group stained with haematoxylin and eosin. The histopathological examination of lungs of mice exposed to OVA showed significant inflammatory alteration in peribronchial area and also increased bronchial muscles thickening, epithelial hyperplasia (fig. 2). In contrast, *Z. armatum* oil treatment at a dose of 200  $\mu$ l/kg body weight showed significant changes (fig. 2) as compared to normal (fig. 2) and dexamethasone-treated groups (fig. 2).

Asthma is an allergic and respiratory disease commonly characterized by increased airway reactivity to

different spasmogens. An initial attack of asthma was triggered by the release of inflammatory mediators like histamine, acetylcholine, leukotriene, prostaglandins or specific exposure of allergens, which reflected the signals of acute bronchoconstriction<sup>[23,24]</sup>. Histamine and other inflammatory mediators causes a host of changes in bronchial tissue by increasing the mucous secretion and simultaneous rapid constriction of bronchial smooth muscle, which narrows the bronchial tube and reduce the amount of air passes through them. Bronchodilating effect of *Z. armatum* oil was evaluated by observing its effects to increases the latent period of PCT in guinea pigs. The study revealed that the time of occurrence of PCT was significantly increased that suggests bronchodilating activity of *Z. armatum* oil against spasmogens.

Ovalbumin-induced model of allergic airway inflammation demonstrates that IgE levels in blood and eosinophilic infiltration in the lungs are markedly increased in asthmatic condition. Eosinophil count



**Fig. 2: Histopathology (40 x) of lungs**  
 (A) Normal control; (B) ovalbumin; (C) dexamethasone; (D) *Zanthoxylum armatum* oil (200  $\mu$ l/kg) treated

is elevated in response to the inflammation due to the OVA exposure. The pathogenesis of asthma is associated with increased infiltration of inflammatory cells and excessive mucus secretion into airway. OVA-induced asthma is recognized as a disease that results from chronic airway inflammation characteristically associated with the infiltration of lymphocytes, eosinophil's, macrophages and neutrophils into the bronchial lumen.

An absolute eosinophil count is a blood test that measures the number of white blood cells called eosinophils. Eosinophil's become active at a time of certain allergic diseases, infections, and other medical conditions. Treatment of the essential oil of *Z. armatum* significantly reduced the total absolute eosinophils in the blood. Asthma is almost always associated with some type of IgE-related reaction and therefore has an allergic basis. Numerous epidemiologic studies have shown a highly significant relationship between asthma and sensitization to various allergens as demonstrated by skin tests or the presence of specific IgE in the

serum. IgE initiates the allergic response by causing mast cells to release inflammatory mediators and by recruiting eosinophils. Thus, blocking the effects of IgE is a promising strategy for preventing or ameliorating allergic symptoms<sup>[25]</sup>. This study showed significantly reduction in serum IgE level by *Z. armatum* oil-treated group. Eosinophils mediator secretion in asthma has been confirmed by BAL fluid analysis, which shows increased concentrations of granule-derived basic proteins<sup>[26]</sup>.

Once established, the repetitive cycle of tissue damage and inflammatory cell recruitment becomes chronic. Even in the absence of sustained allergen, the chronic inflammation persists. Eosinophil level in sputum is associated with the degree of chronic airway obstruction<sup>[26]</sup>. In the present studies eosinophil cells in BALF significantly lower in *Z. armatum* oil treated groups as compared to the group treated by standard drug dexamethasone. Beside eosinophils, neutrophils also have an important role in the late-phase asthmatic reaction. Neutrophil products can cause airway

narrowing, increased mucus secretion and increased antigen-presenting cells responsiveness. In this studies essential oil of *Z. armatum*-treated group reduced the elevated neutrophils in BALF. The elevated numbers of the inflammatory cells reflects the sign of asthma. The results of this study showed that the treatment of *Z. armatum* oil (200 µl/kg) with OVA- sensitized mice significantly reduced the levels of eosinophils, neutrophils, and total inflammatory cells in the BALF as compared with OVA-sensitized mice. In this study linalool, a monoterpene was identified as the major constituent of *Z. armatum* essential oil and a number of linalool and its acetate-producing species are used in traditional medicine systems to relieve symptoms and cure a variety of ailments, both acute and chronic. Linalool was evaluated for its psychopharmacological activity in mice, revealing marked dose-dependent sedative effects on the central nervous system<sup>[27,28]</sup> as well as protection against pentylenetetrazol, picrotoxin and transcorneal electroshock-induced convulsions, hypnotic and hypothermic properties<sup>[29,30]</sup>.

Moreover, linalool modulates glutamate activation expression *in vitro* to competitive antagonism of L-[3H] glutamate binding and *in vivo* model to delayed subcutaneous N-methyl-D-aspartates-induced convulsions and blockade of intra cerebroventricular quinolinic acid-induced convulsions<sup>[31-33]</sup>. Furthermore, presence of linalool in essential oil exhibited potential antiinflammatory activity in *in vivo* models<sup>[27]</sup>. Hence, obtained results of essential oil of *Z. armatum* provides evidence as bronchodilator and antiasthmatic properties in histamine and OVA-induced allergens in guinea pigs and mice. In conclusion, the essential oils obtained from the plants have been used traditionally for the treatment of respiratory tract infections. The present investigation provides evidence that essential oil of *Z. armatum* has bronchorelaxation and antiasthmatic properties. The traditional uses of *Z. armatum* against asthma could be attributed to their antiasthmatic activity as observed in present study.

### Acknowledgements:

The authors are grateful to Director-in-Charge Regional Medical Research Centre (ICMR), Belagavi for encouraging the study. The authors are indebted to Kshipra Biotech Private Limited, Indore, India for providing essential oil of *Z. armatum*.

### Conflicts of interest:

There are no conflicts of interest.

### Financial support and sponsorship:

Nil.

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