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Antiasthmatic potential of *Zizyphus jujuba* Mill and Jujuboside B. – Possible role in the treatment of asthma

Prafull Bandopant Ninave*, Savita Dattatray Patil

R.C. Patel Institute of Pharmaceutical Education & Research, Shirpur, Dhule, 425405, India

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ABSTRACT

Zizyphus jujuba Mill, a famous oriental traditional medicine, has been reported to exhibit diverse activities in biological systems including the respiratory system. However, a little information is available on its antiasthmatic activity. Jujuboside B (JB) is a natural saponin and one of the active constituent of fruits of *Zizyphus jujuba*. In the present investigation, JB was isolated from ethanolic extracts of fruits of *Zizyphus jujuba* (EZJF). EZJF and JB were then evaluated for anti-asthmatic activity using various screening methods. JB was additionally evaluated using ovalbumin (OVA) -induced allergic asthma in mice. Results obtained in the present study showed that EZJF and JB significantly inhibited clonidine-induced catalepsy, milk-induced leucocytosis and eosinophilia, clonidine-induced mast cell degranulation, and passive paw anaphylaxis. The number of inflammatory cells in bronchoalveolar lavage (BAL) fluid was considerably lowered and the severity of pulmonary inflammation was alleviated in the mice pretreated with JB. The high-level expression of T-helper type 2 (TH2) cytokines was markedly reduced in the serum, BAL fluid, and lung homogenates. Thus EZJF and JB showed potent anti-asthmatic activity. Hence EZJF and JB possess a potential role in the treatment of asthma.

1. Introduction

Allergic asthma is a persistent inflammatory disease of the bronchial airways in which various characteristics are seen such as recurrent reversible obstacle of the airflow, eosinophilia, airways hyperreactivity, overproduction of mucus, and airway remodeling (Lee et al., 2010). Liberation of inflammatory mediators in allergic asthma occurs due to the activation of many inflammatory and structural cells (Barnes and Drazen, 2002). About 300 million people across the world are currently suffering from asthma and the count goes on increasing day by day (Sahota and Robinson, 2018) and it has a deadly and costly impact on the nation. Although the manifestations of asthma may resolve instantly with the use of prescribed treatments in many patients, exposure of allergens may further worsen the condition (Genwa et al., 2017). Various categories of medications are presently utilized in the treatment of asthma but none of them seems to be perfect medication (Verstraelen et al., 2008). In addition, they fail to target important features of asthma pathology and are ineffective in some patients (Shaik et al., 2015) hence the search of new, effective and safe anti-asthmatic drug is still the need of the day (Genwa et al., 2017).

TH2 cells have significant contribution in worsening the symptoms of asthma (Wang et al., 2017). In pulmonary tissue, TH2 cells produce various cytokines including IL4, IL5, and IL13, which boost the chemotaxis, maturation, growth, activation, and endurance of eosinophils (Corren, 2013; Greenfeder et al., 2001). Production of IL4, IL5, and IL13, after activation of TH2 lymphocytes, are also responsible for IgE production by β cells, and overproduction of mucus (Deo et al., 2010). Both IL4 and IL5 are found abundantly in the BAL fluid and airway mucosa of persons with allergic asthma (Braun et al., 1998) and play crucial parts in activating and maintaining inflammation of the airway. IL13 has been found to signify various important pathophysiologic features of asthma independently, hence it is considered as a vital mediator of asthma (Corren, 2013). Eosinophil infiltration in the lung preferentially stimulates TH2 cell responses via activated antigen-presenting cells. Therefore, TH2 cells are leading in the lungs, and TH2 cytokines particularly IL4, IL5, and IL13 have a fundamental significance in the pathophysiologic features of asthma (Lee et al., 2008). In addition, anti-cytokine therapy studies in non-human primate are ongoing and will provide important insights for future human interventions to regulate inflammation (Manickam et al., 2018).

Zizyphus jujuba Mill is a tree belonging to family Rhamnaceae and is observed in almost all parts of India. The plant has different pharmacological activities, for example, anthelmintic (Veeresh, 2010), cytotoxic (Ahmad et al., 2011), anti-inflammatory (Al-Reza et al., 2010), antiplatelet (Seo et al., 2013), antinociceptive and anticonvulsant (Acharya et al., 1994), Antipyretic (Balakrishnan et al., 2012),

* Corresponding author at: R.C. Patel Institute of Pharmaceutical Education & Research, Near Karwand Naka, Shirpur, Dist: Dhule, 425405, India.
E-mail address: prafull.ninave@gmail.com (P.B. Ninave).

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Hypoglycemic & hypolipidemic (Anbarasi and Brindha, 2013) activity. The fruits of *Zizyphus jujuba* (FZJ) known to stimulate bile production, prevent allergies, and promote blood circulation (Seo et al., 2013). Traditionally the FZJ were used in asthma, anaphylaxis, and allergy. Furthermore, FZJ were also used as antispasmodic, anti-inflammatory, antitussive, expectorant, and as an immunostimulant. Fruits contain a number of ingredients including cyclic nucleotides (guanosine 3, 5-cyclic monophosphate and adenosine 3, 5-cyclic monophosphate), Zizyphus saponins I, II and III, *p*-coumaroylates of alphitolic acid, and JB (Duke, 2001; Khare, 2011; Rastogi, 1993). The antiallergic action of FZJ is because of elevated levels of both c-AMP and c-GMP in the fruit extract (Khare, 2011; Cyong and Hanabusa, 1980; Cyong and Takahashi, 1982).

JB, a natural saponin triterpenoid, is one of the active constituents of *Zizyphus jujuba* Mill. JB is known for its antiplatelet and antitumor effects (Xu et al., 2014; Seo et al., 2013). A recent study has shown that JB promotes nitric oxide (NO) generation and increases endothelial nitric oxide synthase activity (Zhao et al., 2016). NO is known to exhibit a considerable bronchorelaxant effect in asthma. Besides its bronchodilator effect, NO may also inhibit proliferation of airway smooth muscles, which is considered as the main feature of airway thickening in asthma and offer protection against airway remodeling (Hamad et al., 2003). However, the protective effect of JB against allergic inflammatory responses remains indistinct and hence needs to be studied. Therefore, we examined the effect of JB against the inflammation of airways in an OVA-induced murine model of allergic asthma.

Therefore, by considering the prior literature about conventional claims, reported pharmacological activity and chemical constituents exhibit in FZJ; the need was felt to assess the anti-asthmatic potential of ethanolic extract of FZJ (EZJF) and JB in laboratory animals. Hence the present study was undertaken to examine the beneficial effects of EZJF and JB in mice and rats using various experimental models of asthma.

2. Material and methods

2.1. Drug and chemicals

Clonidine (Unichem, India), Chlorpheniramine maleate (Research Lab Fine Chem. Industries, India), Dexamethasone (Cadila Healthcare Ltd., India), Egg Albumin (Burgoyne Burdidges Company, India), Sodium cromoglycate (Cipla Ltd., India) and RPMI buffer medium (Himedia Lab. Pvt. Ltd., India) were acquired from commercial sources. Analytical grade solvents and chemicals were used in the present study.

2.2. Plant material

FZJ were collected from the local area of Jalgaon district (M.S.) and identified as well as authenticated by J. Jayanti, Scientist 'D', Botanical Survey of India, Pune (Voucher specimen no: BSI/WRC/ 100-1./Tech./ 2017/7) where plant & fruit specimen has been deposited.

2.3. Experimental animals

Healthy Swiss Albino mice (20–25 g) and Wistar rats (150–250 g) were used depending upon suitability in selected screening methods. They were kept in opaque plastic (polypropylene) cages at 24 ± 2 °C and were fed with a commercially available pelleted diet (Nutrivet Life Sciences, Pune, India.) with free assess of water. They were kept on the standard biological clock of 12/12 h light/dark cycle. The protocol of the study was duly approved by the Institutional Animal Ethical Committee (IAEC) for animal care (Resolution No. IAEC/RCPIPER/ 2016-17/09) in accordance with the CPCSEA guidelines, Government of India.

2.4. Extraction and isolation

The extraction and isolation procedure was initiated by drying FZJ under shade. After complete drying, the fruits were crushed and passed through a sieve of 40 mesh size. 1 kg of powder so obtained was defatted by extraction with petroleum ether in a soxhlet extractor. Residue left after defatting was refluxed with 70% ethanol. The solvent in the filtrate of above reflux was removed using a rotary vacuum evaporator under reduced pressure to give a dry crude extract. Then concentrated ethanolic extract was successively extracted using 90% methanol and hexane to obtain a methanol and hexane extract respectively. The methanol extract was then sequentially extracted with ethyl acetate and butanol. For the isolation of JB, a saponin type of glycoside, the butanol extract (21.05 g) was eluted on a column, packed with silica gel as a stationary phase, using n-hexane: methanol (10:0, 9:1, 8:2, 7:3, 6:4) as mobile phase and fractions were collected (B1–B46). All fractions were subjected to TLC. As per their TLC patterns, fractions B31–B39 were combined (F1), evaporated to dryness and chromatographed on a silica gel column using Chloroform:Glacial acetic acid:Methanol:Water (64:32:10:10 → 64:32:12:08 → 64:32:14:06) yield subfractions (SF1-SF20). All subfractions were again analyzed using TLC. As per Rf values, subfractions SF8-SF13 were combined. The solvent was removed by vacuum evaporator and were recrystallized using ethanol. Finally, dried from the solvent, JB (0.17 g) was isolated. JB isolated from column chromatography was identified by comparison of the physical and spectroscopic data (MP, UV, IR, and NMR) of the compounds with those given in the literature (Otsuka et al., 1978; Kawai et al., 1974) and with the authentic compound.

2.5. Phytochemical analysis of EZJF

Presence of various phytoconstituents in EZJF were analyzed using Dragendorffs, Mayer's, Hager's, and Wagner's tests for alkaloid, Molish and Selivonff's tests for carbohydrate, Shinoda and zinc hydrochloride tests for flavonoids, Borntraggers, Legal's and Baljet tests for glycosides, Benedicts and bromine water tests for saponins, Salkowaski and Libermann–Burchard tests for steroid and triterpenoids, and ferric chloride test, lead acetate test and potassium permanganate tests for tannins. All tests were carried out as per the reported methods (Khandelwal, 2017; Harborne, 2008).

2.6. HPTLC analysis

1 mg/ml solution each of isolated JB, B31–B39 combined fraction (F1), and EZJF was prepared using methanol as solvent. HPTLC silica gel aluminum plate ($60F_{254}$ plate, 10 cm × 10 cm) was used as stationary phase. After prewashing with methanol, the plate was activated at 105 °C for 5 min and the sample of JB, F1, and EZJF, 100 µl each, was applied on it using Camag Linomate 5 automatic sample applicator. The chromatogram was run by ascending development in the Camag twin chamber presaturated for 20 min with Chloroform: Glacial acetic acid: Methanol: Water (6.4:3.2:1.2:0.8, v/v/v/v) as mobile phase at room temperature (25 ± 2 °C). The post chromatographic derivatization was carried out using anisaldehyde-sulfuric acid reagent followed by heating at 80 °C for 15 min. Finally, depending upon the resolved bands, R_f values were calculated.

2.7. Acute toxicity study

It has been reported that the methanolic extract of FZJ did not produce any mortality and found to be safe at 2000 mg/kg when given orally to mice (Kumar et al., 2009). Hence, in the present study, 1/5th, 1/10th, and 1/20th of the safe dose corresponding to 400, 200, and 100 mg/kg orally were selected for EZJF as high, medium and low doses respectively in mice. In the case of rats, the corresponding doses (280, 140, and 70 mg/kg orally) were calculated using the conversion

factor (Ghosh, 2008). A pilot study was also carried out to check whether the selected doses of extract show a beneficial effect or not. 1/3rd of EZJF doses, corresponding to 132, 66, and 33 mg/kg orally in mice and 93.33, 46.66, and 23.33 mg/kg orally in rats, were selected for isolated JB.

2.8. Effect on clonidine-induced catalepsy in mice

Mice were divided randomly into eight groups (6 animals/group): a control group, a chlorpheniramine maleate (CPM) (10 mg/kg, i.p.) group, three EZJF (100, 200, and 400 mg/kg, p.o.), and three JB (33, 66, and 132 mg/kg, p.o.) treatment groups. To study the effect of EZJF and JB on clonidine-induced catalepsy, a horizontal bar (1 cm in diameter, 3 cm above the table) was set as per reported method (Ghaisas et al., 2008; Ferre et al., 1990). Initially, all animals were administered with the assigned treatment. Control group mice were given the equal volume of distilled water. After one hour, mice were injected with 1 mg/kg clonidine subcutaneously and by placing the forepaws of mice on the horizontal bar, the length of catalepsy was measured at 15th, 30th, 60th, 90th, 120th, 150th, and 180th min.

2.9. Effect on milk-induced leukocytosis and eosinophilia in mice

Puncturing retro-orbital plexus, blood samples were collected from each mouse, under light ether anesthesia. Total and differential leukocytes were counted as per conventional method using hemocytometer set and Leishman's stain respectively under a light microscope. Mice were then randomly divided into eight groups (6 animals/group): a control group, a milk (10 ml/kg, s.c.) group, three EZJF (100, 200, and 400 mg/kg, p.o.), and three JB (33, 66, and 132 mg/kg, p.o.) treatment groups. All animals were administered with the assigned treatment. Control group mice were given the equal volume of distilled water. Then after one hour, mice of all groups, except mice of the control group, were subcutaneously received 4 ml/kg milk, which was boiled and cooled earlier. After 24 h, blood samples were collected once again and total and differential leukocytes were counted immediately as described earlier. The obtained results before and after milk administration were noted and the difference was calculated (Andhare et al., 2012; Vadnere et al., 2006).

2.10. Effect on mast cell degranulation in rats

The method was as described by Tripathi and Das, and Nirmal et al (Tripathi and Das, 1977; Nirmal et al., 2012) with some modifications. Eight groups were made keeping six rats in each group and were administered for 7 consecutive days with 5 ml/kg distilled water orally (control group), 50 mg/kg sodium cromoglycate (SCG) intraperitoneally (standard group), 70, 140 and 280 mg/kg EZJF orally (three EZJF groups), and 23.33, 46.66 and 93.33 mg/kg orally (three JB groups). 2 h after the last treatment, each animal was injected with 4 ml/kg saline solution (0.9%) intraperitoneally. The abdomen was massaged gently for 5 min. Peritoneal fluid containing mast cells were collected by exposing the peritoneal cavity. The collected peritoneal fluid was then moved into tubes filled with 7–10 ml RPMI 1640 buffer medium (pH 7.2–7.4). After centrifugation of the resultant solution at 400–500 rpm, the supernatant was discarded and using the same buffer medium, obtained pellets of mast cells were washed two times. The mast cells were challenged by exposing to 50 ug/ml clonidine followed by incubation at 37 °C for 10 min. Clonidine-challenged mast cells were stained using 1% toluidine blue. Stained mast cells were taken on a clean glass slide and examined using a light microscope (45×). By observing different visual areas, mast cells were screened for the intactness or degranulation against exact 100 cells.

2.11. Passive paw anaphylaxis in rats

On days 1, 3, and 5, rats were sensitized by giving egg albumin (100 mcg s.c.) adsorbed on aluminum hydroxide gel (12 mg) prepared in saline (0.5 ml) to increase the levels of anti-serum to egg albumin. On day 10, puncturing retro-orbital plexus, blood samples were collected from each rat, under light ether anesthesia. Serum samples were obtained by centrifugation (1500 rpm) for 20 min. The collected serum (0.1 ml) was administered into the left rear paw of each animal for passive sensitization. The contralateral hind paw received the same volume of saline. After 24 h, animals were divided into 8 groups (6 animals/group) and received 10 ml/kg of distilled water orally (control group), 0.5 mg/kg dexamethasone intraperitoneally (standard group), 70, 140 and 280 mg/kg EZJF orally (three EZJF groups), and 23.33, 46.66 and 93.33 mg/kg orally (three JB groups). In the left rear paw, animals were challenged by giving egg albumin (10 µg) prepared in 0.1 ml of saline, 1 h after treatment. Plethysmometer (UGO Basile, 7140) were calibrated using different probes and the paw volume was noted before and after antigen challenge. The obtained results, before and after antigen challenge, were noted and the difference was calculated as it signifies the edema volume (Pungle et al., 2003; Gokhale and Saraf, 2000). Percent inhibition of edema was calculated by using the following formula.

$$\%\,\text{inhibition} = \left\{1 - \frac{\text{Mean relative change in paw volume(Test)}}{\text{Mean relative change in paw volume(Control)}}\right\} \times 100$$

2.12. OVA-induced allergic asthma in mice

2.12.1. Experimental design

The experimental protocol was as described by Wang et al (Wang et al., 2017), with some modifications. In brief, the mice were divided randomly into six groups (5 animals/group): a control, an OVA, three JB (33, 66, and 132 mg/kg, p.o.), and a dexamethasone (DEX) (2 mg/kg, i.p.) group. Initially, the mice were injected with OVA (20 µg) emulsified with alum (2 mg) in phosphate buffer solution (PBS) (200 µl, pH 7.4) intraperitoneally and considered as day 0. The control group mice were administered PBS only. The same treatment was given on the 14th day. Subsequently, from 15th to 26th days, JB and DEX were administered to JB groups and DEX group respectively. On 27th, 28th, and 29th days, mice were challenged intranasally with OVA (100 µg) in PBS (50 µl) once daily. The control group mice were administered the corresponding quantity of PBS. Finally, to collect blood, BAL fluid, lung homogenates and to reveal the histopathological alterations, the mice were euthanized 24 h following the last OVA challenge.

2.12.2. Collection of blood and BAL fluid

From all the euthanized mice, blood and BAL fluid were collected by puncturing vena cava and by cannulating the trachea respectively. The collected blood was subjected to centrifugation (3000 rpm, 10 min, 4 °C) for serum separation and then kept at −40 °C until analysis. BAL fluid was collected by inserting a 24-gauge catheter into the trachea, and lavage was carried out with 0.5 ml aliquots of PBS twice. The same lavage fluid was pooled back and immediately subjected to centrifugation (1500 rpm, 10 min, 4 °C). The obtained supernatants were separated and stored at −40 °C until cytokine measurements while the remnant cells were allowed to suspend in additional 1 ml of cold PBS and utilized for counting total and differential cells. For the estimation of a total number of inflammatory cells per milliliter, the staining of BAL fluid was performed by trypan blue and cells were counted as per usual procedure using a hemocytometer under a light microscope. For differential cell counting, 150 µl of BAL fluid was stained on a clean glass slide with modified Leishman stain, and cells were counted by observing the stained slide under a light microscope.

2.12.3. Preparation of lung homogenate

To obtain clear lung homogenate, initially, 0.05% Triton X-100 and protease inhibitors were dissolved into 1 ml of PBS and lung tissues were taken in the resulting solution. PBS containing lung tissues were homogenized for 30 s using homogenizer. The obtained homogenate was then centrifuged (10,000 rpm, 15 min) and the subsequent debris-free supernatant was collected and stored at −40 °C until use (Shaik et al., 2015).

2.12.4. Histopathological analysis of lung tissue

Lung tissues were fixed with formalin solution (10%, pH 7.0) and implanted in paraffin. Thin section of 4 μm in thickness was taken and positioned on clean glass slides, and deparaffinized. Hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) were used to stain the deparaffinized sections and inflammatory cells infiltration and production of mucus were examined using light microscopy. To determine the infiltration of inflammatory cells in H&E-stained sections and percentage of goblet cells in PAS-stained sections, scores were given as described by Wang et al (Wang et al., 2017). The scores were assigned by a blinded eyewitness. The inflammatory cell infiltration and goblet cells score ranged from 0 to 4. For scoring the inflammatory cell infiltration, 0 denotes no inflammatory cells, 1 denotes a few inflammatory cells, 2 denotes a ring of cells 1–2 cell layer deep, 3 denotes a ring of cells 2–4 cell layer deep, and 4 denotes a ring of cells > 4 cell layers deep. For scoring the goblet cells, 0 denotes no goblet cells, 1 denotes < 25%, 2 denotes 25–50%, 3 denotes 50–75% and 4 denotes > 75% goblet cells. Three different visual areas were assessed for each lung section.

2.12.5. Assessment of cytokine levels

Levels of specific cytokines particularly IL4, IL5, and IL13 were quantified in serum, BAL fluid, and lung homogenate using mouse enzyme-linked immunosorbent assay (ELISA) kits (KinesisDx, Los Angeles, USA, and Krishgen Biosystems, Mumbai, India) as per the assay procedure of the manufacturer.

2.13. Statistics

The data were expressed as the mean ± SEM. The experimental groups were statistically compared using one-way ANOVA followed by Dunnett's test. Results were considered statistically significant when $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Preliminary phytochemical analysis

Following analysis, EZJF showed the occurrence of various phytoconstituents such as alkaloids, carbohydrates, flavonoids, saponins, steroids, and triterpenoids.

3.2. Characterization of JB

JB was obtained as white crystals; m. p. 221–223 °C. Physical and spectroscopic data (FT-IR, UV, and ^{1}H-NMR) of isolated JB were well correlated with reported literature. $R_f = 0.62$ on TLC using Chloroform: Glacial acetic acid: Methanol: Water (64:32:12:08). FT-IR (V_{max} at KBr) cm^{-1}: 1629 (C=C stretching), 2933 (C—H stretching), 3398 (O—H stretching), 1448 (C—H bending), 1236 (C—O stretching), and 1870 (C—O stretching). UV (λmax in Ethanol): no absorbance above 200 nm. ^{1}H-NMR (MeOD): 0.73, 1.09, 1.11, 1.17, 1.389, 1.70 (3H, each, all s, 19, 18, 28, 29, 21, 26-H_3), 1.67 (6H, br s, 27-H_3, Rha-6″-H_3), 2.48 (1H, m, 13-H), 5.13 (1H, m, 23-H), 5.37 (1H, d, 24-H), 5.99 (1H, br s, Rha-1″-H). Based on the data and by correlating the obtained data with reported literature, the compound was identified as JB (Fig. 1).

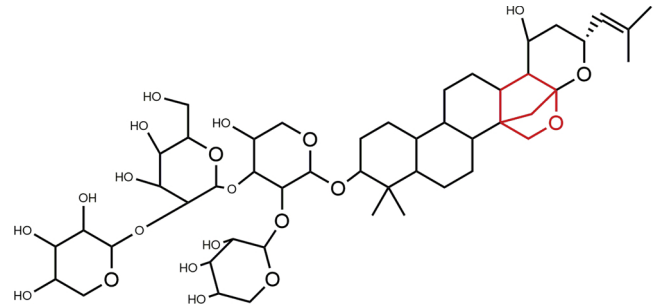

Fig. 1. Structure of Jujuboside B.

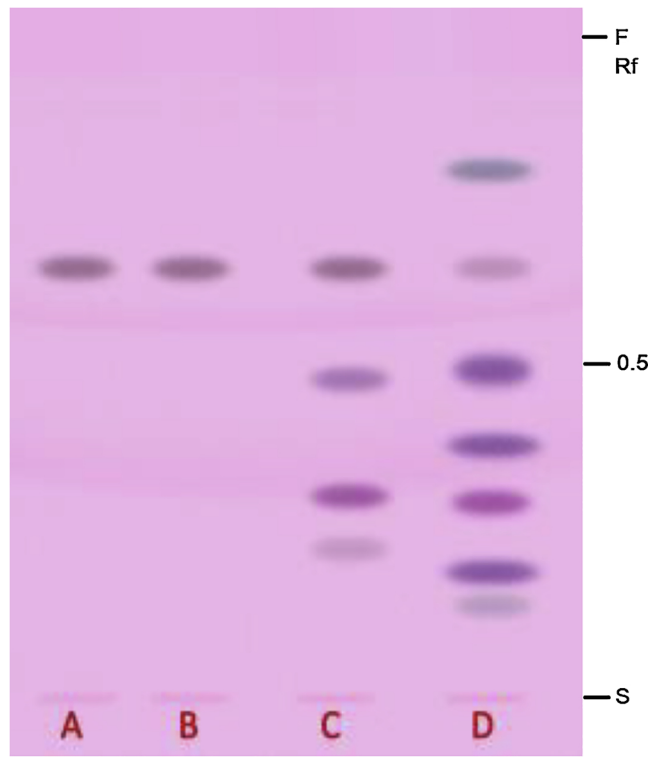

Fig. 2. HPTLC chromatogram of A) Standard JB B) Isolated JB C) Combined fractions (F1) D) EZJF. EZJF and F1 showed the presence of total seven and four bands respectively in the R_f range of 0.2–0.8. Chromatogram of isolated JB exhibits only one band (R_f - 0.62). The specific band, each of EZJF, F1, and isolated JB (at R_f - 0.62) exactly corresponds to the band of standard JB suggesting the presence of JB.

3.3. HPTLC

Analysis of HPTLC chromatogram of EZJF and F1 revealed the presence of total seven and four bands respectively in the R_f range of 0.2–0.8. Chromatogram of isolated JB exhibits only one band ($R_f = 0.62$). The specific band, each of EZJF, F1, and isolated JB (at R_f 0.62) exactly corresponds to the band of standard JB suggesting the presence of JB (Fig. 2).

3.4. Effect of EZJF and JB on clonidine-induced catalepsy in mice

Subcutaneous administration of clonidine (1 mg/kg) exhibited immobility in mice, which continued for 2 h. After clonidine administration, the extent of immobility (catalepsy) was found to be highest (202.16 ± 4.377) in control group mice at 120 min. EZJF and JB treated mice exhibited a dose-dependent reduction in the duration of catalepsy ($p < 0.01$) when compared with the control group mice (Table 1).

Table 1
Effect of EZJF and JB on clonidine-induced catalepsy in mice.

Treatment (n = 6)	Duration of catalepsy (sec) at (Mean ± SEM)						
	15 min	30 min	60 min	90 min	120 min	150 min	180 min
Control	25.33 ± 3.575	99.33 ± 4.645	145.66 ± 5.596	181.0 ± 5.247	202.16 ± 4.377	199.5 ± 2.405	165.16 ± 4.989
CPM (10 mg/kg, i.p.)	15.50 ± 0.846#	26.83 ± 2.626#	58.66 ± 5.296#	72.83 ± 2.358#	93.83 ± 5.564#	91.5 ± 4.493#	98.66 ± 7.013#
EZJF (100 mg/kg, p.o.)	18.16 ± 2.372	67.33 ± 14.047#	101.16 ± 6.600#	139.66 ± 4.356#	170.16 ± 4.438#	150.33 ± 3.283#	135.5 ± 2.790#
EZJF (200 mg/kg, p.o.)	15.16 ± 1.621#	67.5 ± 3.019#	91.16 ± 3.321#	114.66 ± 4.609#	130.00 ± 5.483#	119.66 ± 4.318#	93.00 ± 4.619#
EZJF (400 mg/kg, p.o.)	13.66 ± 2.319#	40.5 ± 1.708#	72.00 ± 3.246#	91.33 ± 2.883#	101.5 ± 3.658#	98.16 ± 3.208#	90.5 ± 5.162#
JB (33 mg/kg, p.o.)	15.16 ± 1.352#	44.83 ± 3.478#	83.16 ± 3.468#	105.00 ± 2.129#	114.00 ± 3.307#	99.33 ± 2.060#	89.66 ± 1.944#
JB (66 mg/kg, p.o.)	15.16 ± 0.600#	43.00 ± 2.633#	78.66 ± 3.730#	85.33 ± 2.753#	100.00 ± 3.907#	94.83 ± 2.056#	86.33 ± 2.275#
JB (132 mg/kg, p.o.)	13.00 ± 1.366#	31.16 ± 2.372#	61.00 ± 3.967#	76.33 ± 3.018#	96.5 ± 3.819#	93.5 ± 2.446#	88.00 ± 3.109#

All values are given as mean ± SEM, n = number of mice per group, # $p < 0.01$ vs. control.

3.5. Effect on milk-induced leukocytosis and eosinophilia in mice

The number of leukocytes and eosinophils was significantly increased ($p < 0.001$) upon injection of milk (4 mg/kg) in the mice administered with milk only when compared with control group mice. EZJF and JB treatment significantly attenuated ($p < 0.05$ and $p < 0.01$) the increase in a number of leukocytes and eosinophils induced by milk (Fig. 3). However, pretreatment with EZJF (100 mg/kg) attenuated the increase in a number of eosinophils non-significantly.

3.6. Effect on mast cell degranulation in rats

In the rats of the control group, the clonidine challenge induces substantial degranulation of mast cells (74.83%). Compared with the control group, pretreatment with EZJF and JB, at all the studied doses, dose-dependently protected ($p < 0.01$) the rats from clonidine-induced mast cell degranulation (Table 2). Compared to EZJF, SCG (50 mg/kg, i.p.) and JB (132 mg/kg, p.o.) treatment showed the somewhat smaller number of degranulated mast cells. The protection exhibited by JB at 132 mg/kg is comparable with that of SCG.

Table 2
Effect of EZJF and JB on clonidine-induced mast cell degranulation in rats.

Treatment (n = 6)	Mast cells %		% Protection
	Intact	Disrupted	
Control	25.16 ± 3.188	74.83 ± 3.188	–
SCG (50 mg/kg, i.p.)	77.33 ± 3.879@	22.66 ± 3.879@	69.71%
EZJF (70 mg/kg, p.o.)	59.66 ± 2.642@	40.33 ± 2.642@	46.1%
EZJF (140 mg/kg, p.o.)	61.83 ± 2.892@	38.16 ± 2.892@	49%
EZJF (280 mg/kg, p.o.)	67.33 ± 2.603@	32.66 ± 2.603@	56.35%
JB (23.33 mg/kg, p.o.)	61.16 ± 2.242@	38.83 ± 2.242@	48.1%
JB (46.66 mg/kg, p.o.)	70.00 ± 2.129@	30.00 ± 2.129@	67.92%
JB (93.33 mg/kg, p.o.)	76.00 ± 1.966@	24.00 ± 1.966@	

All values are given as mean ± SEM, n = number of rats per group, @ $p < 0.01$ vs. control.

3.7. Passive paw anaphylaxis in rats

Challenge with egg albumin markedly augmented the process of anaphylaxis (increased paw edema volume) in the sensitized rats of the control group and was found to be consistent up to 4 h. However, treatment with EZJF and JB, at all the studied doses, significantly ($p < 0.01$) reduced the paw edema volume dose-dependently (Table 3). Significant prevention of increased paw edema volume was seen at all the time periods. Maximum inhibition was found at 4 h i.e. 68.46.89% and 88.11% by EZJF (280 mg/kg) and JB (93.33 mg/kg) respectively.

3.8. OVA-induced allergic asthma in mice

3.8.1. Effect of JB on the recruitment of inflammatory cells in BAL fluid

Total and differential inflammatory cell counts in the BAL fluid were performed to observe the influence of JB on the recruitment of inflammatory cells into the lung. As expected, the mice challenged with OVA showed a significant increase in the total inflammatory cells count in the BAL fluid when compared with the control mice ($p < 0.01$). However, at all the studied doses, JB dose-dependently prevented the increase in inflammatory cells compared with the OVA-challenged group (Fig. 4). Study on differential cell count revealed that the number of different cells such as eosinophils, macrophages, lymphocytes, and neutrophils was abnormally increased in BAL fluid of mice challenged with OVA when compared with the number of these cells in control

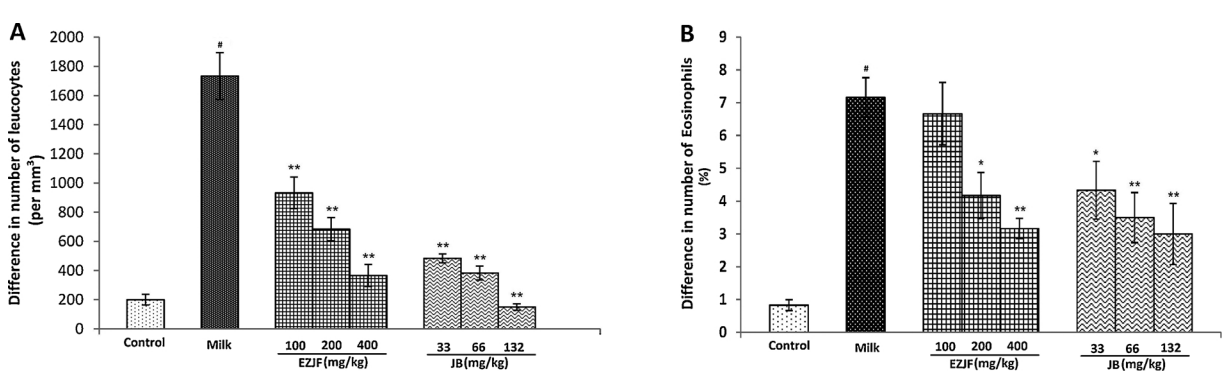

Fig. 3. Effect of EZJF and JB against milk-induced A) leukocytosis and B) eosinophilia in mice. Blood samples were collected from the retro-orbital plexus of each mouse. Total and differential leukocytes were counted as per conventional method. All animals were administered with the assigned treatment and after one hour boiled and cooled milk (4 ml/kg, s.c), except to the control group mice. After 24 h, blood samples were collected once again and total and differential leukocytes were counted. The difference in the cell count before and 24 h after milk administration was noted. All values were given as mean ± SEM. # $p < 0.001$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs. induced control (Milk) group.

Table 3
Effect of EZJF and JB on passive paw anaphylaxis in rats.

Treatment (n = 6)	Paw edema volume in ml (% inhibition)				
	0.5 h	1 h	2 h	3 h	4 h
Control	0.662 ± 0.014	0.670 ± 0.019	0.668 ± 0.030	0.677 ± 0.029	0.687 ± 0.024
DEX (0.5 mg/kg, i.p.)	0.260 ± 0.012@ (60.73%)	0.200 ± 0.008@ (70.15%)	0.130 ± 0.009@ (80.54%)	0.105 ± 0.006@ (84.49%)	0.060 ± 0.003@ (91.27%)
EZJF (70 mg/kg, p.o.)	0.495 ± 0.007@ (25.23%)	0.463 ± 0.010@ (30.85%)	0.417 ± 0.011@ (37.62%)	0.383 ± 0.013@ (43.38%)	0.333 ± 0.009@ (51.48%)
EZJF (140 mg/kg, p.o.)	0.468 ± 0.007@ (29.25%)	0.450 ± 0.008@ (32.84%)	0.395 ± 0.005@ (40.87%)	0.348 ± 0.016@ (48.55%)	0.277 ± 0.009@ (59.73%)
EZJF (280 mg/kg, p.o.)	0.397 ± 0.015@ (40.08%)	0.330 ± 0.014@ (50.75%)	0.278 ± 0.018@ (58.33%)	0.293 ± 0.023@ (56.67%)	0.217 ± 0.014@ (68.46%)
JB (23.33 mg/kg, p.o.)	0.393 ± 0.008@ (40.58%)	0.350 ± 0.016@ (47.76%)	0.237 ± 0.013@ (64.57%)	0.190 ± 0.008@ (71.94%)	0.163 ± 0.010@ (76.23%)
JB (46.66 mg/kg, p.o.)	0.360 ± 0.013@ (45.62%)	0.283 ± 0.010@ (57.71%)	0.198 ± 0.010@ (70.31%)	0.167 ± 0.006@ (75.38%)	0.125 ± 0.007@ (81.80%)
JB (93.33 mg/kg, p.o.)	0.290 ± 0.009@ (56.19%)	0.230 ± 0.009@ (65.67%)	0.150 ± 0.007@ (77.54%)	0.117 ± 0.006@ (82.77%)	0.082 ± 0.007@ (88.11%)

All values are expressed as mean ± SEM, n = number of rats per group, @ $p < 0.01$ vs. control.

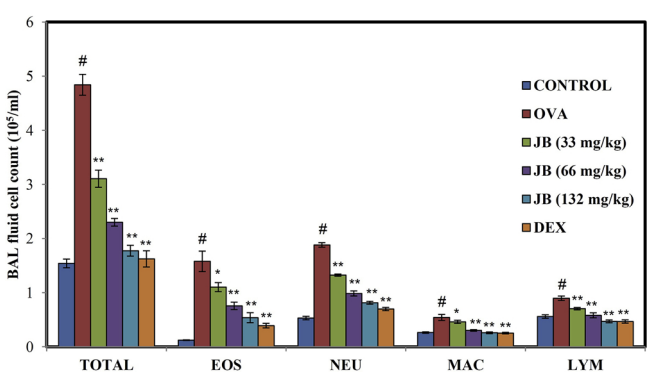

Fig. 4. Effect of Jujuboside B (JB) on total and differential cell count in BAL fluid. For total count, cells were stained with trypan blue and for the differential count; cells were stained with modified Leishman stain and counted using a light microscope. The data generated were expressed as the mean ± SEM of 5 mice. # $p < 0.01$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs. ovalbumin (OVA)-challenged group. EOS, eosinophil; NEU, neutrophil; MAC, macrophages; LYM, lymphocyte; and DEX, dexamethasone.

mice. Administration of JB and DEX in the mice showed significant inhibition ($p < 0.05$ and $p < 0.01$) of the rise in the number of these inflammatory cells compared with the mice challenged with OVA.

3.8.2. Effect of JB on cytokine levels

Following assay procedure, analysis of microplates showed an increase in the levels of IL4, IL5, and IL13 in serum, BAL fluid, and lung tissue homogenates of mice belonging to an OVA-challenged group as compared to mice of the control group (Fig. 5). Treatment with JB (66 and 132 mg/kg) or DEX significantly reduced the levels of these cytokines. But treatment with JB (33 mg/kg) reduced the levels of IL4 in BAL fluid and lung homogenates, IL5 in serum and lung homogenates, and IL13 in BAL fluid non-significantly.

3.8.3. Effect of JB on histopathological changes in lung tissue

The H&E-stained lung tissue sections of the OVA-challenged group showed the presence of an abnormal number of inflammatory cells into the peribronchial tissue. The airway epithelium was thickened, and there was a loss of parenchymal structure compared with the control group. The PAS-stained sections from the lungs of the OVA-challenged mice showed overproduction of mucus and increase in the number of goblet cells. However, the lung tissues of the mice pretreated with JB and DEX showed little inflammatory infiltration. The parenchymal architecture was normal, the number of goblet cells was found to be reduced, and the overproduction of mucus was inhibited (Fig. 6).

4. Discussion

Asthma, a multifactorial in origin, is basically an inflammatory disorder pertaining to airways and characterized by recruitment of many inflammatory cells such as eosinophils, neutrophils, and lymphocytes to the lung, severe bronchospasm, mucus overproduction, IgE production, and release of various inflammatory mediators. So far, more than 50 diverse mediators have been involved in the pathogenesis of asthma. Despite our improved understanding of the pathophysiology and available remedies, asthma is still one of the few chronic diseases that are increasing in prevalence. Even if various characteristics are implicated in asthma, currently asthma is broadly recognized as a disease of inflammation predominantly (Mehta and Mahajan, 2006). Two broad categories of drugs, bronchodilators and anti-inflammatory agents, are currently used to treat asthma. However, the drugs acting through a specific mechanism can be subcategorized into these broad categories. As per the National Heart, Lung, and Blood Institute endorsements, asthmatic patients need at least one drug from each pharmacologic category (Houglum, 2000).

In the present study, we have carried out a systematic investigation to examine the antiasthmatic potential of EZJF and JB, through possible modes, using various animal models.

Catalepsy is a state in which extensive inhibition of movement seen and animals are unable to correct their abnormal postures (Sukul et al., 1988). Clonidine is an alpha-adrenoceptor agonist which induces significant cataleptic condition in mice. This sustained state of immobility is intervened by histamine through H_1 receptors only (Jadhav et al., 1983). As histamine can be discharged by clonidine from mast cells (Lakdawala et al., 1980) and presence of such mast cells is now evident in the brain (Theoharides, 1990), it is obvious that the clonidine-induced catalepsy may be due to the release of histamine from brain mast cells. In our study, it was evident that the duration of catalepsy was significantly reduced in the mice pretreated with ESZJ and JB when compared with the control group mice where the duration of catalepsy was found to be augmented up to the time period of 2 h (Table 1). This reduction in the catalepsy possibly due to the prevention of release of histamine from brain mast cells demonstrating antihistaminic potential by EZJF and JB.

It is reported that the severity of functional lung impairment in the patients with asthma have a strong correlation with blood leukocyte and eosinophil counts. Therefore peripheral blood leukocyte counts and eosinophil counts are useful as an indirect marker of severity of functional lung impairment such as asthma (Devi et al., 2014). Various studies established that after 24 h of subcutaneous administration, milk elevates the number of leukocytes and eosinophils in mice (Bhargava and Singh, 1981; Taur and Patil, 2011). In view of this, in our study, we examined the efficacy of EZJF and JB against milk-induced leukocytosis and eosinophilia in mice. As expected, we observed a significant reduction in the leukocyte and eosinophil count in mice pretreated with EZJF and JB as compared to increased count in the vehicle-treated

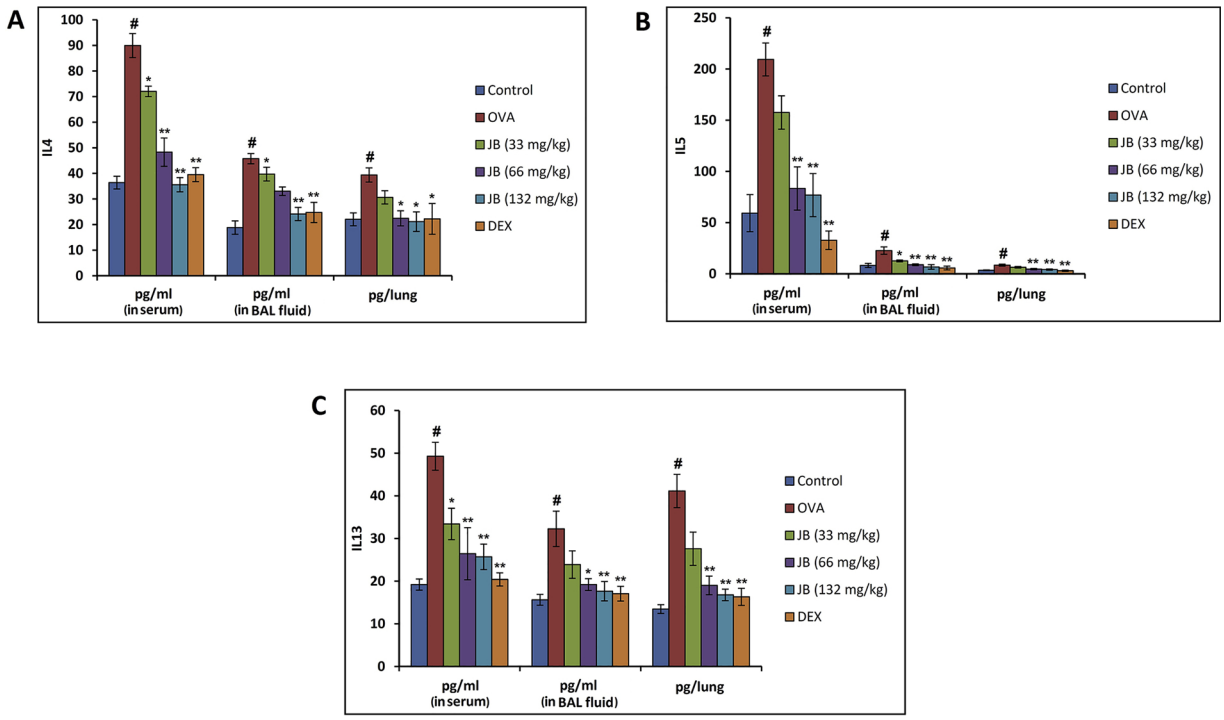

Fig. 5. Effect of JB on A) IL4, B) IL5, and C) IL13 levels in serum, BAL fluid, and lung homogenates. Twenty-four hours after the final challenge, serum, BAL fluid, and lung homogenates were collected and the levels of TH2 cytokines were estimated by ELISA. The data generated were expressed as the mean ± SEM of 5 mice. # $p < 0.01$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs. OVA-challenged mice.

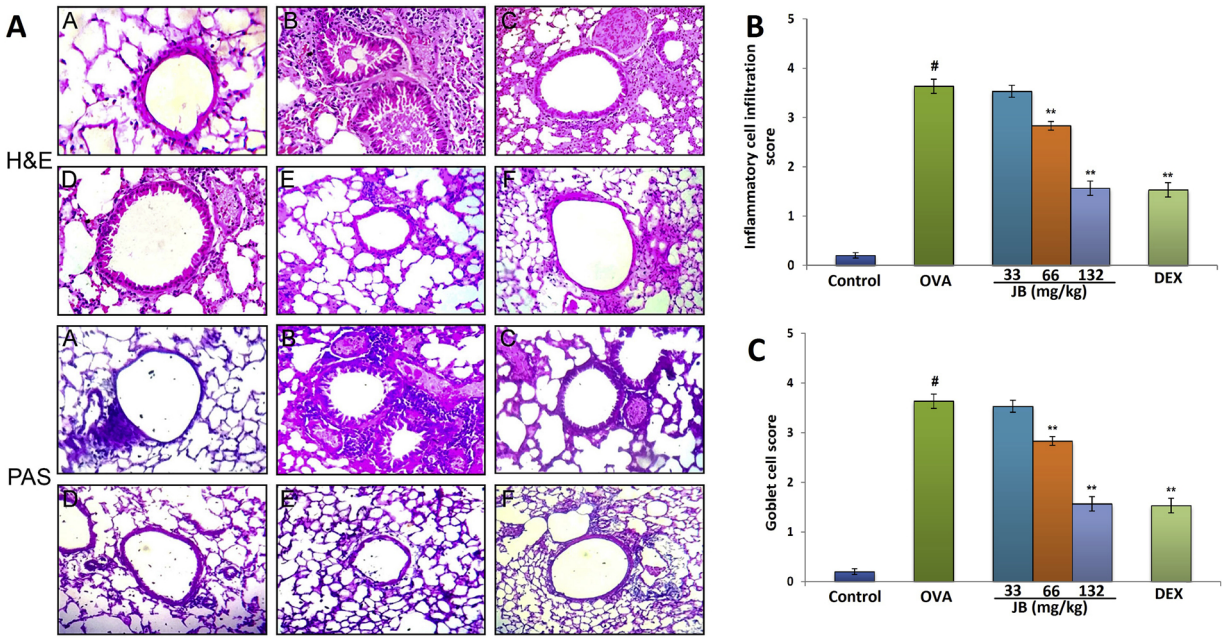

Fig. 6. Effect of JB on histopathological alteration of lung tissue following OVA challenge and treatment with JB. A) Lung tissues were stained with hematoxylin-eosin (H&E) and periodic acid Schiff (PAS) to examine the infiltration of inflammatory cells and hyperplasia of goblet cells respectively (200×). A, Control group; B, OVA group; C, JB (33 mg/kg); D, JB (66 mg/kg); E, JB (132 mg/kg); F, DEX group. B) Lung tissues were analyzed for the infiltration of inflammatory cells. C) The percentage of goblet cells per bronchiole. The data generated were expressed as the mean ± SEM. # $p < 0.001$ vs. control; ** $p < 0.01$ vs. OVA-challenged group.

control group (Fig. 3). Compared to EZJF, the number of leukocytes and eosinophils was greatly reduced by JB administration at all doses against milk-induced leukocytosis and eosinophilia in mice demonstrating greater anti-allergic or adaptogenic activity.

Among the various immunological stimuli, antigen-antibody reactions on the surface of mast cells play a predominant role to cause mast cell degranulation (Kapoor et al., 2011). After degranulation variety of mediators, including histamine, release from mast cells. It is reported that the mobilization of intracellular Ca^{2+} has great significance in the release of histamine from mast cells (Takei et al., 1992). In mast cell degranulation study, the mast cells were found to be intact in EZJF and JB groups exhibiting a protective effect against degranulation induced by clonidine (Table 2). Interestingly, JB offered the protection higher than EZJF against clonidine-induced mast cell degranulation in rats.

Considering occurrence of complex events in the degranulation of mast cells, the inhibitory effect of EZJF and JB against clonidine-induced mast cell degranulation may be due to stabilization of mast cell membrane interrupting mobilization of intracellular Ca^{2+}. However, this needs further confirmation.

In the passive paw anaphylaxis model, administration of egg albumin (antigen) increases antibodies against egg albumin. Injecting these antibodies into the paw passively sensitize the rats. Thereafter, the challenge with egg albumin causes serious antigen-antibody reaction into the paw resulting in paw inflammation (edema) due to the discharge of various inflammatory mediators. Immunomodulators have great significance in the management of allergic ailments as they possess the property to inhibit the antigen-antibody reaction interrupting the release of these inflammatory mediators (Dai et al., 2002). Pretreatment with EZJF and JB protected the rats from antigen-antibody reaction mediated paw inflammation (Table 3). The results obtained in this model suggest that the favorable effect of EZJF and JB may be due to the prevention of antigen-antibody reaction exhibiting potent anti-inflammatory, anti-allergic, anti-anaphylactic, and immunomodulatory activities.

It is now evident that TH2 cytokines play an important role in coordinating, continuing, and intensifying the inflammatory response in asthma (Wang et al., 2017). It has been demonstrated that IL4 takes part in IgE production, pathogenesis of airway remodeling, and eosinophilic inflammation. IL5 involved in the chemoattraction, maturation, as well as activation of eosinophils and augments bronchial hyperresponsiveness in asthma. Increased production of IL13 promotes overproduction of mucus in the airways and goblet cell hyperplasia (Mehta and Mahajan, 2006). In the present study, we examined the number of inflammatory cells in the BAL fluid of allergic mice and the levels of IL4, IL5, and IL13 not only in the serum but also in BAL fluid and lung homogenates of these mice. We found that the levels of studied interleukins in serum, BAL fluid, and lung homogenates were greatly enhanced in the mice challenged with OVA. Treatment with JB dose-dependently lowered the levels of these interleukins in the mice (Fig. 5) probably by stimulating NO generation. In this investigation, dexamethasone was employed as a standard. JB produced parallel effects to those of standard on the cytokine levels in serum, BAL fluid, and lung homogenates.

An eosinophil is the key effector cell for the pathogenesis of allergic inflammation (Duan et al., 2004). As IL5 directly involved in proliferation, activation, and migration of eosinophils, the recruitment of eosinophils into the lungs is directly associated with the production of IL5 (Lee et al., 2010). Our findings demonstrate that JB prevented the recruitment of eosinophils into the lungs, as obvious by a striking fall in the total and differential cell numbers in BAL fluid (Fig. 4). Particularly, we observed a decrease in the number of eosinophils and other cells in BAL fluid and decreased recruitment of inflammatory cells into the airways of the JB treated mice. Thus JB showed a protective effect against airway inflammation possibly due to diminishing TH2 cytokine levels.

Furthermore, our histopathological examination of OVA-challenged lung tissue showed us the loss of normal parenchymal structure, thickening of the airway mucosa, bronchoconstriction, and overproduction of mucus, which are the characteristics seen in asthma. Treatment with JB protected the lung tissue from the deleterious effects of OVA, as was evident from the histological observation and analysis of inflammatory cell infiltration and goblet cell scores (Fig. 6).

The antiasthmatic activity of EZJF may be due to the presence of JB in the ethanolic extract. The protective effect of JB, against various asthmatic triggers, may be due to the inhibition of cytokines.

We tried to explore the identity of the isolated compound by various methods such as melting point determination, FT-IR, UV, 1H-NMR, and HPTLC. But we have not performed mass spectroscopy of the said compound to determine the exact molecular weight. This is the limitation of our study. Despite this limitation, we assume that our results are of great significance in the field of respiratory research.

5. Conclusion

EZJF and JB exhibit significant antihistaminic, anti-allergic, mast cell stabilizing, anti-inflammatory, anti-anaphylactic, and immunomodulatory activities in various experimental animal models and effectively suppressed asthmatic exacerbations. Our data demonstrate that administration of JB controls the exaggerated inflammatory response exhibiting significant anti-inflammatory activity might be by preventing the secretion of selected TH2 cytokines and inflammatory cells infiltration, as observed in this study. Thus our findings validated the traditional claim of EZJF and support the possible use of EZJF and JB as therapeutic drugs in the prevention and treatment of asthma.

Conflict of interest

All authors declare that they have no conflict of interest.

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