



Investigation of anti-asthmatic potential of Kanakasava in ovalbumin-induced bronchial asthma and airway inflammation in rats

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ABSTRACT

Ethno-pharmacological relevance: Kanakasava is an Indian traditional Ayurvedic formulation containing *Datura (Datura metel)*, *Vasaca (Adhatoda vasica)*, *Dhataki (Woodfordia fruticosa)* and *Grape (Vitis vinifera)* extracts as major constituents and used to treat pulmonary diseases including coughing, breathing difficulty and asthma. The present study was designed to assess the safety and therapeutic efficacy of Kanakasava against ovalbumin-induced bronchial asthma and related airway inflammation in rats due to lack of evidence based therapeutic efficacy data.

Material and methods: Male wistar rats were sensitized with allergen (ovalbumin, 40 mg/rat + aluminum hydroxide, 2.0 mg/rat) and treated orally with standard dexamethasone (2.5 mg/kg, b.w.) or Kanakasava (1.23 and 2.46 ml/kg, b.w.) from day 1 to day 28. Inflammatory markers, including cell counts and cytokines such as interleukins (IL-4, IL-5, IL-1 β), tumor necrosis factor (TNF- α), leukotriene (LTD-4), immunoglobulin (IgE), nitric oxide and nitrite levels in both blood and broncho alveolar lavaged fluid (BALF) were analyzed. Abdominal mesentery was studied histologically for mast cell degranulation, whereas lung functions were investigated by spirometer. Method was also developed to quantify gallic acid and ethyl gallate content in Kanakasava by HPTLC for its quality control.

Results: None of the rats exhibited mortality and Kanakasava was found to be safe at the tested doses. Treatment with Kanakasava significantly ($P < 0.01$) reversed elevated levels of IgE, cytokines, nitrites and influx of eosinophils and neutrophils in blood and BALF. These findings were further supported by the significant improvement in lung functions ($P < 0.01$) and suppression ($P < 0.01$) of degranulation of mast cells. The content of gallic acid and ethyl gallate in Kanakasava was found to be 1.94% and 0.98%, respectively.

Conclusion: These findings demonstrated the preventive effect of Kanakasava in allergen induced model of asthma providing scientific basis for its traditional use in Ayurveda, since long time.

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1. Introduction

Bronchial asthma is a chronic inflammatory disease characterized by airway obstruction, airway inflammation, and airway hyper responsiveness (Shifren et al., 2012). The prevalence of asthma has

increased in recent decades affecting approximately 334 million people worldwide and ranks third most cause for hospitalization (Behera and Sehgal, 2015). The bronchial asthma can be provoked by factors such as allergens, respiratory infection, dust, cold air and chemicals leading to stimulation of inflammatory cells and infiltration of cell derived mediators such as lymphocytes, eosinophils, neutrophils, histamine, interleukins and immunoglobulin-E (IgE) into the bronchial lumen as well as elevation of reactive nitrogen species and oxygen species (Brightling et al., 2002; Roh et al., 2008).

Although conventional anti-asthmatic drugs have been widely used for the management of bronchial asthma but the relief offered by them is symptomatic and these drugs possess more systemic and local side effects (Fukushima et al., 2003; Roland et al., 2004; Noonan et al., 2009). On the contrary, a number of drugs from indigenous plant sources have been explored for the anti-asthmatic activities during the last few decades with minimal side effects (Abdureyim et al., 2011).

Abbreviations: API, Ayurvedic Pharmacopoeia of India; AFI, Ayurvedic Formulary of India; HPTLC, high performance thin layer chromatography; TLC, thin layer chromatography; BALF, bronchoalveolar lavaged fluid; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin-E; IL-4, interleukin-4; IL-1 β , interleukin-1 β ; IL-5, interleukin-5; LTD-4, leukotriene-D4; NO, nitric oxide; TNF- α , tumor necrosis factor- α ; 5-HT, 5-hydroxytryptamine; Th2, T-helper 2 cell; COX-2, cyclooxygenase -2; NF- κ B, necrosis factor- κ B; PGE₂, prostaglandins-E₂; iNOS, eNOS and nNOS, isoforms of nitric oxide synthases (NOS); S.E.M., standard error of mean; ANOVA, one-way analysis of variance; b.w., body weight

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In Ayurveda bronchial asthma is described as “*tamak-swasa*” with pathogenesis of *dosha* imbalance caused by simultaneous augmentation of *vata* & *kapha*. According to Ayurveda anti-asthmatic drugs should have properties such as *anti-vata* and *anti-kapha*. The Ayurvedic classical text, Bhesajya Ratnavali, has a long-standing and unique insight into comprehensive approach for bronchial asthma management through proper care of the respiratory tract. Ayurveda comprises of various types of polyherbal formulations for *tamak swasa* (bronchial asthma) including fermented decoctions (*arishtas* such as Khadjarishta, Draksharishta) and fermented infusions (*asavas* such as Kumaryasava, Lohasava), which are regarded as valuable therapeutics due to their efficacy and desirable features (Mishra, 2004).

Kanakasava is one of the polyherbal formulations which has been widely used for treatment of allergic conditions like chronic cough, bronchial asthma and breathing disorders by Ayurvedic physicians and recommended in compendium, Bhaisajyaratnavali (AFI, Part-I, 1: 9) as well as in Hikkāśvādhikāra (Anonymous, 2003). Since, there are no scientific preclinical work reported on Kanakasava to validate its traditional claims therefore, in the present study, an attempt has been made to investigate the anti-asthmatic potential of Kanakasava against ovalbumin-induced bronchial asthma in rats.

2. Material and methods

2.1. Chemicals and reagents

The gallic acid (purity 98%, PubChem CID: 370), ethyl gallate (purity 98%, PubChem CID: 13250), ovalbumin (PubChem CID: 71311993), heparin (PubChem CID: 22833565), dexamethasone (PubChem CID: 5743) were purchased from Sigma Aldrich, (St. Louis, MO, USA). Commercially available standard ELISA kits were used for the determination of interleukin (IL-4, IL-1 β), tumor necrosis factor (TNF- α) (Ray Biotech, Inc., IL, USA). Similarly, immunoglobulin (IgE) (Immunology Consultants Laboratory, Inc., Portland, OR), IL-5 and leukotriene (LT)-D₄ (Cusabio Biotech, Hubei, China) as well as nitric oxide (NO) calorimetric kit was obtained from BioVision Research Products, USA. Silica gel 60F-254 pre-coated high performance thin layer chromatography (HPTLC) aluminum sheets (10 $\times$ 10 cm, 0.2 mm thick) were purchased from E. Merck Ltd., Mumbai. All other chemicals used were of analytical reagent grade.

Table 1
Composition of Kanakasava with their voucher specimen number.

S.No	Common name	Botanical name of ingredients (Family)	Part used	Qty (g)	Voucher specimen
1.	Dhatura	<i>Datura metel</i> Linn. (Solanaceae)	Whole plant	192	NISCAIR/RHMD/consult/-2011-12/1752/52
2.	Vasaka	<i>Adhatoda vasica</i> Nees. (Acanthaceae)	Roots	192	NISCAIR/RHMD/consult/-2011-12/1770/70
3.	Yasti	<i>Glycyrrhiza glabra</i> Linn. (Leguminosae)	Roots	96	NISCAIR/RHMD/consult/-2011-12/1770/70
4.	Pippali	<i>Piper longum</i> Linn. (Piperaceae)	Fruits	96	NISCAIR/RHMD/consult/-2011-12/1784/84
5.	Kantakari	<i>Solanum xanthocarpum</i> Scrad & Wendl. (Solanaceae)	Whole plant	96	NISCAIR/RHMD/consult/-2011-12/1784/84
6.	Nagakesaera	<i>Mesua ferrea</i> Linn. (Guttiferae)	Stamens	96	NISCAIR/RHMD/consult/-2011-12/1784/84
7.	Sunthi	<i>Zingiber officinalis</i> Rosc. (Zingiberaceae)	Rhizomes	96	NISCAIR/RHMD/consult/-2011-12/1821/121
8.	Bharangi	<i>Clerodendrum serratum</i> (Linn.) Moon. (Verbenaceae)	Roots	96	NISCAIR/RHMD/consult/-2011-12/1770/70
9.	Talispatra	<i>Abies webbiana</i> Lindl. (Pinaceae)	Leaves	96	NISCAIR/RHMD/consult/-2011-12/1770/70
10.	Dhatki	<i>Woodfordia fruticosa</i> Kurz. (Lythraceae)	Flowers	768	NISCAIR/RHMD/consult/-2011-12/1770/70
11.	Daraksa	<i>Vitis vinifera</i> Linn. (Vitaceae)	Fruits	960	NISCAIR/RHMD/consult/-2011-12/1821/121
12.	Water	Distilled water	–	24.576 l	–
13.	Sarkara	Sugar	–	4.800 kg	–
14.	Madhu	Kasudra	–	2.400 kg	–

2.2. Collection and authentication of ingredients

All plant materials were collected from authorized dealer and authenticated by Dr. H. B. Singh at National Institute of Science Communication and Information Resources, Delhi, India. The voucher specimen numbers of all the plants are mentioned in Table 1 and same was deposited in the laboratory of Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi for future reference. Sugar and pure honey were purchased from the local grocery shop and checked for quality grade parameters as per the Ayurvedic Pharmacopoeia of India. Water used in the preparation of formulation was distilled water.

2.3. Preparation of Kanakasava

Formulation was prepared by anaerobic fermentation as per the method described in Ayurvedic Pharmacopoeia of India (Anonymous, 2000). Briefly, the crude drugs 1–9 in Table 1) were washed, dried in shade, powdered and sieved (# 44). Dhatki (*Woodfordia fruticosa*) flowers and grape (*Vitis vinifera*) fruits were washed and cleaned, separately. The sugar solution was prepared in distilled water, filtered through muslin cloth and transferred to formulation vessel (a porcelain jar fumigated with pippali (*Piper longum*) powder and smeared with ghee). Then, coarsely powdered crude drugs, honey and grape (*V. vinifera*) fruits were added to aqueous sugar base followed by addition of flowers of dhatki (*W. fruticosa*), as an initiator of fermentation. Then, the jar was sealed with a cloth smeared with clay and stacked in seven consecutive layers. The container was shifted to an isolated chamber filled with paddy, specially prepared for fermentation and maintained at 25 $\pm$ 2 °C temperature and 45 $\pm$ 5% of relative humidity. The fermentation process was checked on 8th, 15th, 22nd, 30th and 35th day for release of CO₂ and to get a stable pH as indicators of end of the fermentation (Katiyar, 2006; Vyas et al., 2010; Mishra et al., 2010). The formulation was completely ready by the end of day 35, which was then analyzed for alcohol content using distillation method as mentioned in Ayurvedic Pharmacopoeia of India (2008).

2.4. Quantification of gallic acid and ethyl gallate by HPTLC in Kanakasava

2.4.1. Preparation of sample and standards

Fifty ml of Kanakasava was successively partitioned with equal volume of *n*-hexane and chloroform, thrice each followed by drying of remaining aqueous ethanolic fraction in vacuum to get 30–35 mg of residue from each 50 ml formulation. The 10 mg/ml

solution of extract was prepared in ethanol. The stock solutions of gallic acid and ethyl gallate (1000 µg/ml) were prepared in ethanol, separately and further diluted to 100 µg/ml. Sample and standard solutions were filtered using a 0.22 µm membrane filter and used for application on HPTLC plate.

2.4.2. Chromatographic conditions

HPTLC analysis was carried out similar to previous method (Praveen et al., 2014) reported for analysis of terpenoids. For quantifying gallic acid and ethyl gallate, toluene: ethyl acetate: formic acid: methanol (3.5:3.5:0.8:0.5, v/v/v/v) mobile phase was used. The standards and sample were spotted in the form of bands (width 8 mm with a CAMAG microliter syringe) on pre-coated Silica gel plate 60F-254 aluminum sheets (20 × 10 cm with 0.2 mm thickness; Merck KGaA, Darmstadt, Germany) using a CAMAG Linomat-V applicator (Muttens, Switzerland) attached to CAMAG HPTLC system and TLC scanner III, controlled by winCATS-IV software. The plates were pre-washed by methanol and activated at 105 °C for 5 min prior to chromatography. The standard and sample loaded plates were kept in the TLC twin trough glass developing chamber (after saturating with solvent vapor for 15 min at 28 ± 2 °C) with mobile phase for plate run up to 80 mm. The developed plates were dried in hot air to evaporate solvents and scanned at 292 nm with CAMAG TLC densitometric scanner using deuterium lamp.

2.4.3. Calibration curves for standards

The working standards of gallic acid and ethyl gallate (100 µg/ml each) were spotted on separate TLC plates in different volumes 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 µl, to get concentrations of 100, 200, 400, 600 and 800 and 1000 ng/spot. The regression equations for gallic acid was $y = 1216 + 6.484x$ (co-relation coefficient, $r^2 = 0.996$) whereas for ethyl gallate, it was $y = 4239 + 5.432x$ (co-relation coefficient, $r^2 = 0.992$), which were used for quantification of gallic acid and ethyl gallate, respectively in sample.

2.5. Experimental design for anti-asthmatic potential

The guidelines of committee for the purpose of control and supervision of experiments on animals, Government of India, were followed and prior permission was sought from the Institutional Animal Ethics Committee for conducting the study (Registration no. 173/CPCSEA/748/20.07.2011). Male wistar rats (180–220 g; 8–10 weeks old) were housed in polypropylene cages under controlled conditions (room temperature 25 ± 2 °C; humidity $50 \pm 15\%$ RH; and photoperiod of 12 h light: dark cycle). All the animals had free access to standard laboratory diet and water ad libitum throughout the study. After acclimatization to the environment for one week, the animals were randomly divided into five groups (n=6 rats in each group) and anti-asthmatic study was carried out as per the Abdureyem et al. (2011).

Group 1 was non-sensitized control (N) and received vehicle only (8% ethanolic water, oral, 1.8 ml/kg b.w., daily); however all other groups received intraperitoneal injection (1.0 ml) of allergen suspension containing ovalbumin 40 mg and aluminum hydroxide 2 mg from day 1 to day 15. Further, after 15 days of sensitization, animals were challenged by exposure to aerosol consisting of 1% ovalbumin in normal saline for 20 min in a closed chamber (dimensions 40 × 32 × 32 cm), once daily for 8 consecutive days i.e., from day 15 to day 22, and thereafter, on day 25th and day 28th.

Group 2 served as sensitized control (S) and received vehicle as above in addition to ovalbumin sensitization whereas Group 3 served as reference standard (S+D) and received Dexamethasone (oral, 2.5 mg/kg, b.w., daily) in addition to ovalbumin sensitization. Group 4 and 5 served as test groups (S+K1 and S+K2) and orally administered Kanakasava (1.23 and 2.46 ml/kg,

b.w., daily, respectively) with ovalbumin sensitization. The human dose of Kanakasava (12–24 ml, oral) has been mentioned in Ayurvedic Pharmacopoeia of India (API) for management of bronchial asthma and airway disorders was used in this study after converting to rat dose as per the method of Reagan-Shaw et al. (2008).

2.5.1. Lung function and bronchoconstriction test

On day 28, five minutes after ovalbumin exposure, the rats were anaesthetized with intra-peritoneal injection of urethane (1200 mg/kg) and tracheostomy was performed using a 12 gauge cannula (2.0 mm internal diameter) (Parasuraman, Raveendran, 2012). Breathing rate (f , breaths/min), tidal volume (V_T , ml/s) and minute ventilation volume (V_E) of the animals were measured with a spirometer (Model no: FE141, AD Instrument, Australia). After tracheostomy, heparinized saline (0.1 ml) was injected intravenously through the femoral vein to avoid coagulation of blood. Excessive bronchial secretions, if any, were discharged using a small polyethylene tube without disturbing the trachea.

2.5.2. Bronchoalveolar lavaged fluid (BALF) and cell counting

Immediately after the measurement of lung function parameters, lungs were lavaged with 5.0 ml aliquots of normal saline solution via cannulated tracheal tube for three times (5.0 ml × 3). The BALF was pooled (approximately 11–12 ml/rat) and centrifuged (1500 rpm, 10 min at 4 °C). The supernatant was separated and stored at -80 °C until analysis for IgE and cytokines, whereas cell pellet was re-suspended in 1.0 ml of physiological saline for determination of total and differential leukocyte count as per the reported method (Jung et al., 2007).

2.5.3. Serum preparation and blood cell count

Following collection of BALF, the blood was collected by cardiac puncture in two different portions. The first aliquot (2.5–3.0 ml) was placed in a non-heparinized tube; serum was separated by centrifugation (3000 rpm, 10 min) and stored at -80 °C for estimation of IgE and cytokines. The second aliquot (0.5 ml) was collected in a heparinized tube and stored at 4 °C for determination of leukocyte count.

Within 30 min of collection of heparinized blood, total leukocyte count and differential leukocyte count in blood was determined using hemocytometer as per the reported method (Jung et al., 2007). Differential cell count was made from cytospin smears stained with Leishman's stain (1.5% in methanol for six min). At least 500 cells were counted under an optical digital microscope and classified into eosinophils, neutrophils, lymphocytes, macrophages or monocytes using standard morphologic criterion.

2.5.4. Cytokines, nitric oxide and nitrite levels in serum and BALF

The levels of IgE, TNF α , IL-4, IL-5, IL-1 β and LT-D4 in serum (500 µl) and BALF (5.0 ml) were measured using enzyme-linked immunosorbent assay (ELISA) kits. The samples were analyzed on an automated plate reader (Model no. ELX-80MS, Biotek, USA). Concentration of total nitric oxide and nitrite levels in serum and BALF were determined by nitric oxide calorimetric kit.

2.5.5. Mast cell degranulation study

After collection of BALF and blood, the anaesthetized rats were exsanguinated and mesentery was dissected away from the small intestine. After cleaned off from lobes of fat and blood vessels well-vascularized mesenteric windows were cut and stained for 15 min in a solution containing 50% ethanol, 10% formaldehyde, 5% acetic acid and 0.2% toluidine blue. Mast cells were readily identifiable by their metachromatic cytoplasmic granules, which stain purple after staining with blue color of aqueous toluidine blue. The

stained mesentery pieces were viewed within each circular $\times 20$ field of view under an optical digital microscope (Motic, B1 Series System Microscope with field area 1.13 mm^2). Mast cell degranulation was assessed by counting the percentage of cells with extruded granules using the criteria of intact and degranulated mast cells (Kwasniewski et al., 1998; Acharya et al., 2003).

3. Statistical analysis

Results were reported as mean $\pm$ S.E.M. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by post hoc Tukey's test. Differences were considered statistically significant at value of $p < 0.05$. All statistical analyses were performed using the Graph Pad software (San Diego, CA, USA).

4. Results

4.1. Determination of end point for fermentation and preliminary phytochemical screening of Kanakasava

During ongoing fermentation process, carbon dioxide is produced as a result of anaerobic reactions taking place inside the reaction mixture. Release of carbon dioxide decreases the pH of the medium and extinguishes the burning match stick when brought near the mouth of the vessel. The pH of reaction mixture was decreased to 3.85 on 35th day from 6.05 on 8th day and keeps on constant, further. On other hand match stick kept burning near the mouth of vessel on 35th day. The pH of formulation was found stable on three consecutive days 35, 36, and 37 which confirmed the end of fermentation process and complete development of Kanakasava. The alcohol content in prepared Kanakasava formulation was found as $7.18 \pm 0.012\%$ v/v. Preliminary phytochemical screening of Kanakasava showed the presence of alkaloids, phenolic compounds, flavonoids, terpenoids, saponins and steroidal glycosides.

4.2. Quantification of gallic acid and ethyl gallate by HPTLC in Kanakasava

The finger print profile with developed solvent system is shown in Fig. 1. The R_f values of standard gallic acid and ethyl gallate were found to be 0.43 and 0.48, respectively, (Fig. 1). The ethanolic fraction of Kanakasava showed 8 peaks where fourth and fifth peaks at R_f values 0.43 and 0.48 with corresponding peak areas of 1237.01 and 4247.31 were coinciding with gallic acid and ethyl gallate, respectively (Fig. 1). The percent content of gallic acid and ethyl gallate in Kanakasava were calculated to be 1.94% and 0.92% w/w, respectively in Kanakasava ethanol fraction.

4.3. Anti-asthmatic potential of Kanakasava

4.3.1. Effect of Kanakasava on lung function parameters in the ovalbumin-sensitized rats

Respiration rate was significantly ($p < 0.001$) increased in ovalbumin-sensitized control group as compared to non-sensitized control group. Treatment with Kanakasava (1.23 and 2.46 ml/kg b.w.) exhibited significant ($p < 0.01$) reversal of respiratory rate as compared to the ovalbumin-sensitized control group. There was a significant decrease in tidal volume ($p < 0.001$) and minute ventilation ($p < 0.05$) volume in ovalbumin-sensitized control group as compared to non-sensitized control group. Treatment with Kanakasava (2.46 ml/kg b.w.) significantly ($p < 0.01$) increased the tidal volume as compared to the ovalbumin-sensitized control group (Fig. 2) and maintained up to normal.

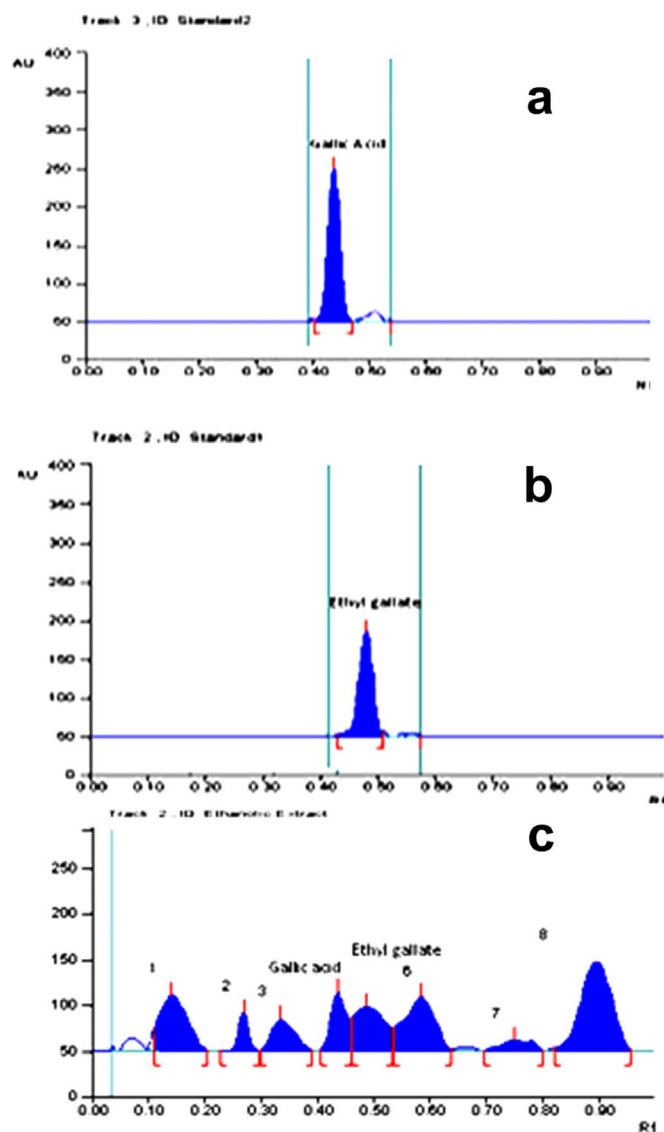


Fig. 1. HPTLC chromatogram of gallic acid (a), ethyl gallate (b) and ethanol extract (c) of Kanakasava.

4.3.2. Effect of Kanakasava on circulating cell count in blood of ovalbumin-sensitized rats

The total number of leukocytes and each differential count in blood samples were significantly ($p < 0.001$) increased whereas lymphocytes were significantly ($p < 0.01$) decreased in ovalbumin-sensitized control animals compared to the non-sensitized control group animals. Treatment with Kanakasava (2.46 ml/kg, b.w.) led to significant reduction in total number of leukocytes ($p < 0.001$), circulating eosinophils ($p < 0.01$), and neutrophils ($p < 0.05$), while significant ($p < 0.05$) increase in lymphocyte count as compared with the ovalbumin-sensitized control group (Table 2).

4.3.3. Effect of Kanakasava on inflammatory cellular counts in BALF of ovalbumin-sensitized rats

The ovalbumin-sensitized control group showed a significant ($p < 0.001$) increase in the total cell count and differential cellular count in BALF compared to the non-sensitized control group. Treatment with Kanakasava (2.46 ml/kg, b.w.) led to significant ($p < 0.001$) reversal of total cells, eosinophils, lymphocytes and macrophages and ($p < 0.05$) neutrophils. However, treatment with Kanakasava (1.23 ml/kg, b.w.) also caused significant ($p < 0.05$) reversal of all these leukocytes except neutrophils (Table 3).

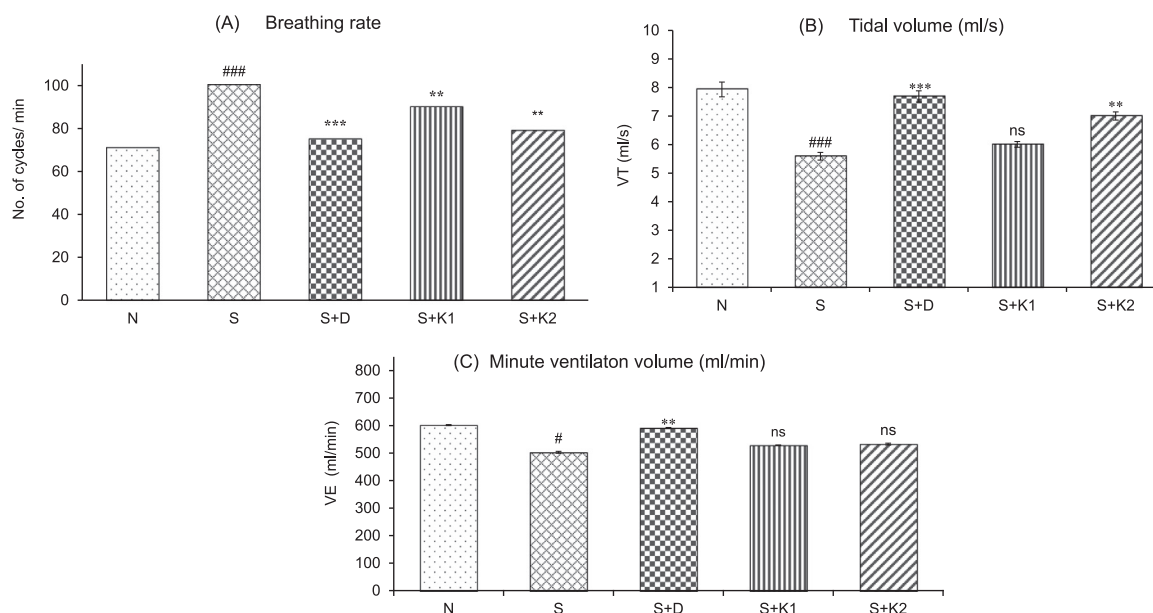


Fig. 2. Effect of Kanakasava on breathing rate (A), tidal volume (B), and mean ventilation volume (C) in ovalbumin-sensitized rats. Values shown are mean $\pm$ S.E.M. (n=6); *p < 0.05 and ***p < 0.001 compared to the non-sensitized control (N); **p < 0.01 and ***p < 0.001, compared to the ovalbumin-sensitized vehicle-treated model control (S). S+D, sensitized and treated with dexamethasone; S+K1, sensitized and treated with Kanakasava, 1.23 ml/kg; S+K2, sensitized and treated with Kanakasava, 2.46 ml/kg.

Table 2

Effect of Kanakasava on total cells and differential leukocyte count in blood ($\times 10^5$ cells/ml) of ovalbumin-sensitized rats.

Groups	Total cells	Eosinophils	Lymphocytes	Macrocytes	Neutrophils
Normal control (N)	9.52 $\pm$ 0.46	0.63 $\pm$ 0.06	10.85 $\pm$ 0.43	6.40 $\pm$ 0.65	0.35 $\pm$ 0.08
Sensitized control (S)	17.33 $\pm$ 1.14 ^{###}	4.87 $\pm$ 0.35 ^{###}	7.15 $\pm$ 0.77 [#]	6.36 $\pm$ 0.68 ^{###}	4.19 $\pm$ 0.46 ^{###}
Sensitized and dexamethasone (standard) treated (S+D)	11.50 $\pm$ 0.43 ^{***}	1.93 $\pm$ 0.27 ^{***}	10.60 $\pm$ 0.36 [*]	6.35 $\pm$ 0.31 ^{***}	1.94 $\pm$ 0.26 ^{***}
Sensitized and Kanakasava (low dose) treated (S+K1)	15.17 $\pm$ 0.60	3.85 $\pm$ 0.47	9.43 $\pm$ 1.27	6.33 $\pm$ 0.35	3.87 $\pm$ 0.30
Sensitized and Kanakasava (high dose) treated (S+K2)	12.83 $\pm$ 0.70 ^{***}	3.03 $\pm$ 0.47 ^{**}	10.30 $\pm$ 0.43 [*]	6.30 $\pm$ 0.40	3.40 $\pm$ 0.29 [*]

Values shown are mean $\pm$ S.E.M. (n=6); [#]p < 0.01 and ^{###}p < 0.001 compared to the non-sensitized control (N); ^{*}p < 0.05, ^{**}p < 0.01, and ^{***}p < 0.001, compared to the ovalbumin-sensitized vehicle-treated model control (S). S+D, sensitized and treated with dexamethasone; S+K1, sensitized and treated with Kanakasava, 1.23 ml/kg; S+K2, sensitized and treated with Kanakasava, 2.46 ml/kg.

Table 3

Effect of Kanakasava on total cells and differential leukocyte counts in BALF ($\times 10^5$ cells/ml) of ovalbumin-sensitized rats.

Groups	Total cells	Eosinophils	Lymphocytes	Macrophages	Neutrophils
Normal control (N)	4.500 $\pm$ 0.52	1.51 $\pm$ 0.56	9.92 $\pm$ 0.44	5.13 $\pm$ 0.52	1.05 $\pm$ 0.05
Sensitized control (S)	11.17 $\pm$ 0.87 ^{###}	8.50 $\pm$ 0.44 ^{###}	18.37 $\pm$ 0.77 ^{###}	12.08 $\pm$ 0.77 ^{###}	5.60 $\pm$ 0.46 ^{###}
Sensitized and dexamethasone (standard) treated (S+D)	4.83 $\pm$ 0.48 ^{***}	3.07 $\pm$ 0.42 ^{***}	10.15 $\pm$ 0.71 ^{***}	6.55 $\pm$ 0.81 ^{***}	1.16 $\pm$ 0.08 ^{***}
Sensitized and Kanakasava (low dose) treated (S+K1)	8.83 $\pm$ 0.60 [*]	6.37 $\pm$ 0.31 [*]	13.25 $\pm$ 0.74 [*]	9.12 $\pm$ 0.60 [*]	4.87 $\pm$ 0.53
Sensitized and Kanakasava (high dose) treated (S+K2)	5.83 $\pm$ 0.48 ^{***}	3.80 $\pm$ 0.43 ^{***}	11.22 $\pm$ 0.58 ^{***}	6.75 $\pm$ 0.09 ^{***}	4.12 $\pm$ 0.39 [*]

Values shown are mean $\pm$ S.E.M. (n=6); ^{###}p < 0.001, compared to the non-sensitized normal control (N), ^{*}p < 0.05 and ^{***}p < 0.001, compared to the ovalbumin-sensitized vehicle-treated model control (S). S+D, sensitized and treated with dexamethasone; S+K1, sensitized and treated with Kanakasava, 1.23 ml/kg; S+K2, sensitized and treated with Kanakasava, 2.46 ml/kg.

4.3.4. Effect of Kanakasava on levels of IgE and cytokine levels in serum of ovalbumin-sensitized rats

The ovalbumin-sensitized control animals showed significant (p < 0.001) increase in serum levels of IgE, IL-4, IL-5, LTD4 and other pro-inflammatory cytokines such as TNF- α and IL-1 β compared to the non-sensitized controls. Treatment with both dexamethasone and Kanakasava (1.23 and 2.46 ml/kg, b.w.) significantly decreased the levels of IgE (p < 0.01 and p < 0.001) and cytokines IL-4 (p < 0.001), IL-5 (p < 0.01 and p < 0.001), TNF- α (p < 0.001), IL-1 β (p < 0.05 and p < 0.01) and LTD-4 (p > 0.05 and p < 0.05) as compared to the ovalbumin-sensitized control group (Fig. 3A and B).

4.3.5. Effect of Kanakasava on levels of IgE and cytokine in BALF of ovalbumin-sensitized rats

The levels of IgE and cytokines were increased (p < 0.001) significantly in BALF of the ovalbumin-sensitized control group compared to non-sensitized animals. Kanakasava (2.46 ml/kg, b.w.) significantly decreased the elevated levels of IgE (p < 0.01) and cytokines [TNF- α (p < 0.001), IL-4 (p < 0.001), IL-5 (p < 0.001), IL-1 β (p < 0.05) and LTD4 (p < 0.05)] whereas treatment with lower dose of the formulation Kanakasava (1.23 ml/kg, b.w.) resulted in significant reduction in TNF- α (p < 0.001), IL-4 (p < 0.001) and IL-5 (p < 0.05) only, (Fig. 4A and B).

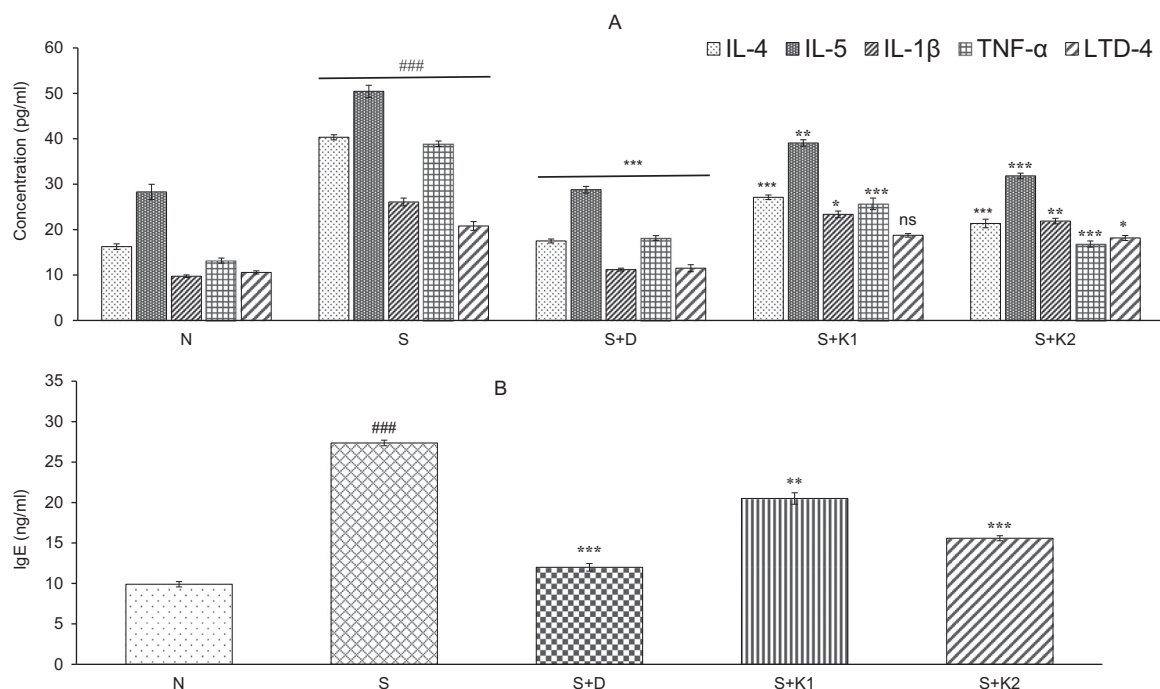


Fig. 3. A. Effect of Kanakasava on serum cytokine levels in ovalbumin-sensitized rats, B. Effect of Kanakasava on serum IgE levels in ovalbumin-sensitized rats. Values shown are mean $\pm$ S.E.M. (n=6); ###p < 0.001 compared to the non-sensitized control (N); *p < 0.05, **p < 0.01 and ***p < 0.001, compared to the ovalbumin-sensitized vehicle-treated model control (S). S+D, sensitized and treated with dexamethasone; S+K1, sensitized and treated with Kanakasava, 1.23 ml/kg; S+K2, sensitized and treated with Kanakasava, 2.46 ml/kg.

4.3.6. Effect of Kanakasava on total nitric oxide and nitrite concentration of ovalbumin-sensitized rats

A significant increase ($p < 0.001$) in total NO and nitrite levels were observed in ovalbumin-sensitized control group as compared to non-sensitized control animals. However, treatment with Kanakasava caused significant (1.23 ml/kg, b.w., $p < 0.05$; 2.46 ml/kg, b.w., $p < 0.001$) reversal in levels of total

nitric oxide and nitrites compared with the ovalbumin-sensitized control group (Fig. 5A).

4.3.7. Effect of Kanakasava on mast cell degranulation of ovalbumin-sensitized rats

The number of degranulated mast cells increased significantly ($p < 0.001$) in the mesentery of ovalbumin-sensitized control

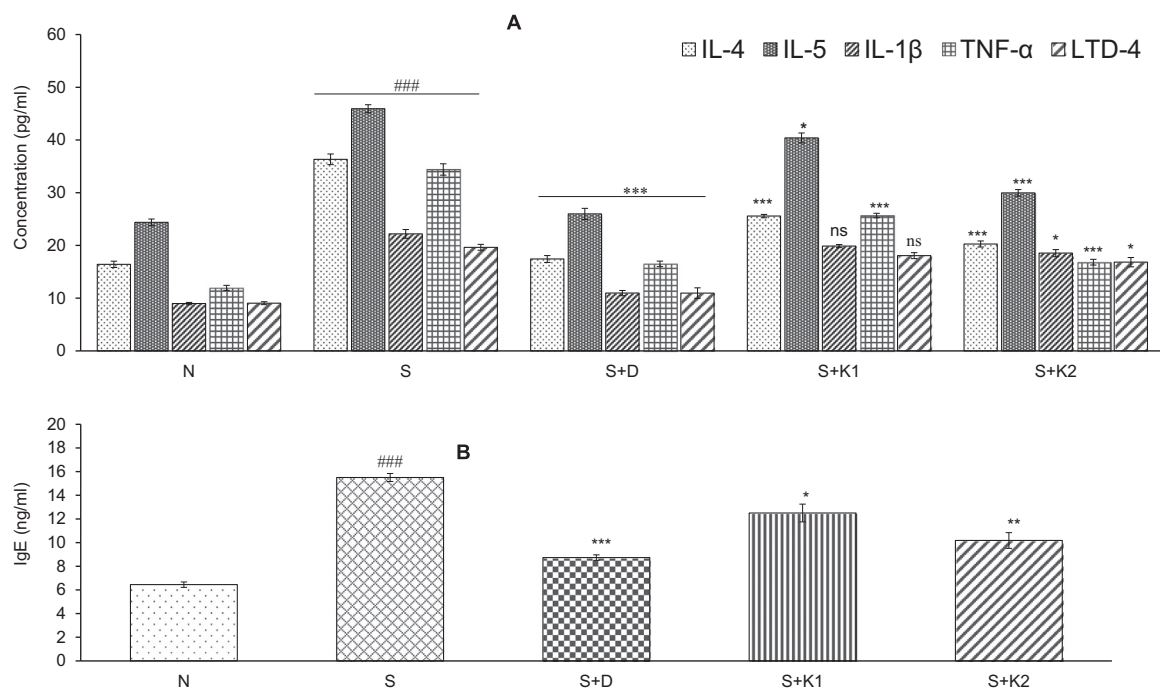


Fig. 4. A. Effect of Kanakasava on cytokine levels in BALF of ovalbumin-sensitized rats, B. Effect of Kanakasava on IgE levels in BALF of ovalbumin-sensitized rats. Values shown are mean $\pm$ S.E.M. (n=6); ###p < 0.001 compared to the non-sensitized control (N); *p < 0.05 and ***p < 0.001, compared to the ovalbumin-sensitized vehicle-treated model control (S). S+D, sensitized and treated with dexamethasone; S+K1, sensitized and treated with Kanakasava, 1.23 ml/kg; S+K2, sensitized and treated with Kanakasava, 2.46 ml/kg.

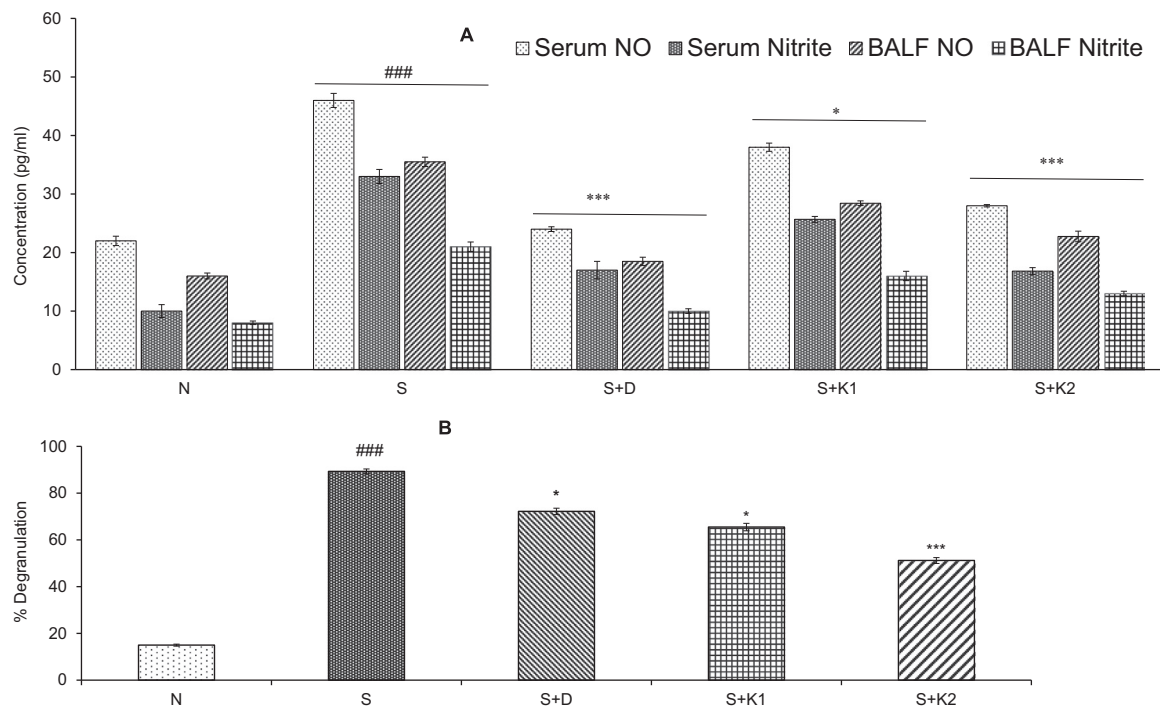


Fig. 5. A. Effect of Kanakasava on nitric oxide (NO) and nitrite level in serum and BALF of ovalbumin-sensitized rats. B. Effect of treatment on mast cell degranulation in abdominal mesentery of ovalbumin-sensitized rats. Values shown are mean $\pm$ S.E.M. (n=6); ^{###}p < 0.001 compared to the non-sensitized control; ^{*}p < 0.05 and ^{***}p < 0.001, compared to the ovalbumin-sensitized vehicle-treated model control (S). S+D, sensitized and treated with dexamethasone; S+K1, sensitized and treated with Kanakasava, 1.23 ml/kg; S+K2, sensitized and treated with Kanakasava, 2.46 ml/kg.

animals compared to non-sensitized control group. Treatment with Kanakasava resulted in significant (1.23 ml/kg, b.w., $p < 0.05$; 2.46 ml/kg, b.w., $p < 0.001$) reduction in the number and extent of degranulation of mast cells as compared to the ovalbumin-sensitized control group (Fig. 5B). However, effects of dexamethasone was similar to the lower dose treatment with Kanakasava.

5. Discussion

Ovalbumin-induced rat model of bronchial asthma is widely used model for investigating anti-asthmatic potential of drugs (Martin and Tamaoka, 2006; Zosky and Sly, 2007). The main characteristic feature of this model is bronchial hyper responsiveness to specific stimuli accompanied with airway inflammation. In our study, rats sensitized with ovalbumin showed bronchial asthma characteristics with increased breathing rate, decreased tidal volume and mean ventilation volume. The other characteristics observed were: 1) increased IgE and cytokines (IL-4, IL-5, IL-1 β , TNF- α , LTD-4) levels in serum and BALF, 2) elevated nitric oxide and nitrite levels in serum and BALF, 3) increased mast cell degranulation in abdominal mesentery, and 4) altered total cells and differential leukocyte count in serum and BALF; demonstrating the features of airway allergy and allergic bronchial asthma such as immediate and late asthmatic responses including IgE production and accumulation of inflammatory cells. These observations are analogous with the results of earlier studies (Abdureyim et al., 2011). Dexamethasone was selected as the standard drug in this study since it has been widely used as a comparator in the previous studies in ovalbumin-induced asthmatic models (Abdureyim et al., 2011).

Allergic reactions through activation of IgE and mast cells leads to the release of inflammatory mediators and cytokines with clinical manifestation of immediate asthmatic response followed by recruitment of inflammatory cells (eosinophils, lymphocytes,

and neutrophils) into the airways resulting in late asthmatic response in asthmatic individuals that can last for 48–72 h or even longer (Arvidsson et al., 2007). Kanakasava is a polyherbal formulation which is standardized for gallic acid and ethyl gallate contents through HPTLC.

In the present study, Kanakasava treatment significantly reversed the ovalbumin-induced elevated serum and BALF levels of IgE and infiltration of inflammatory cells in to the rat airways suggesting that Kanakasava might elicit its anti-inflammatory activity through inhibition of leukocyte subpopulation. In allergic pathways airway hyperresponsiveness is closely related to elevated levels of eosinophils in both lungs and blood (Walford and Doherty, 2014). Inhibition of eosinophil level in serum and BALF with Kanakasava indicates that polyherbal formulation plays an important role in the treatment of allergen induced bronchial hyperresponsiveness. These results were further supported by the observations of lung functioning where reversal of breathing rate and corresponding tidal volume in Kanakasava treated groups showed its bronchodilatory effect. Some common physiological features such as irritability, sneezing, and hyperrhinorrhea were also resolved in all the animals treated with Kanakasava. Mast cells release a number of allergic mediators such as histamine, 5-HT leading to immediate hypersensitivity reaction through an array of released cytokines (IL-4, IL-5, LTD4) and pro-inflammatory mediators (Mahajan and Mehta, 2006). Treatment with Kanakasava exhibited significant protection of mast cells from degranulation suggesting protection from immediate and late bronchial asthmatic reaction phase.

Gallic acid is a common constituent of grape (*V. vinifera*) and dhatki (*W. fruticosa*). During fermentation, gallic acid may forms compounds with ethyl alcohol generated in the process and results in ethyl gallate. The same have also been mentioned as reference standard compound in Ayurvedic Pharmacopoeia for quality control of Kanakasava. Therefore, gallic acid and ethyl gallate were taken as standards for HPTLC analysis. In experimental trials, gallic

acid exhibited inhibitory effects release of TNF- α , and histamine from mast cells (Kim et al., 2006). Whereas ethyl gallate inhibited secretion of Th2 cytokines, IL-4 and IL-5 which are important cytokines to be investigated in bronchial asthma (Kato et al., 2001). Amelioration of allergic consequences associated with inflammatory processes in asthmatic rats treated with Kanakasava may be attributed due to the presence of bioactive constituents (including gallic acid and ethyl gallate) in the formulation justifying that Kanakasava is a potential candidate for the management of airway disorders. Expression of iNOS, COX-2, TNF- α , IL-1 β , IL-6 is primarily regulated by NF- κ B, which plays a central role in regulating functional activity of immune system and pathogenesis of inflammation. Phenolic compounds present in natural drugs have been reported to possess potent anti-oxidant and anti-inflammatory properties mediated through suppression of IL-1 β , TNF- α , COX-2 and production of NO₂ and PGE₂ (Na et al., 2006; Kang et al., 2009; Kuppann et al., 2010).

Reactive oxygen species secreted by inflammatory mediators can modulate airway constriction. NO is a major product and its production is mediated through enzyme nitric oxide synthases (NOS), which is expressed in three isoforms iNOS, eNOS, and nNOS. High concentration of iNOS cause mucus secretion, cellular infiltration epithelial cell damage and contribute in inflammatory process of airways. In the present study, inhibition of NO and nitrites by Kanakasava supports its protective role in bronchial asthma through reduction of lung inflammation.

6. Conclusion

In conclusion, significant ($p < 0.001$) reversal of increased breathing rate, leukocytes, IgE and cytokines (IL-4, IL-5, IL-1 β , TNF- α , LTD4, Nitric oxide and Nitrite level in blood/serum and in BALF as well as significant ($p < 0.01$) reversal of decreased tidal volume and increased mast cell degradation as compared to ovalbumin sensitized control on treatment with 2.46 ml/kg b.w. of Kanakasava. Finally, we may conclude that the results support the traditional use of Kanakasava in airway disorders such as bronchial asthma and confirm the presence of active chemical compounds.

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References

- Abdureyim, S., Amat, N., Umar, A., 2011. Anti-inflammatory, immunomodulatory, and hemeoxygenase-1 inhibitory activities of Ravan Napas, a formulation of uighur traditional medicine, in a rat model of allergic asthma. *Evid. Based Complement. Altern. Med.* 2011, 725926.
- Acharya, S.B., Yanpallewar, S.U., Singh, R.K., 2003. A preliminary study on the effect of *Azadirachta indica* on bronchial smooth muscles and mast cells. *J. Nat. Remedies* 3, 78–82.
- Anonymous, 2000. The Ayurvedic Pharmacopoeia of India: Part II, 1st English edition. Ministry of Health and Family Welfare, Government of India. The Controller of Publications, Civil Lines, Delhi, India.

- Anonymous, 2003. The Ayurvedic Pharmacopoeia of India: Part I, 2nd English edition. Ministry of Health and Family Welfare, Government of India, The Controller of Publications, Civil lines, Delhi, India, pp. 53–54.
- Anonymous, 2008. The Ayurvedic Pharmacopoeia of India: Part II, 1st English edition. Ministry of Health and Family Welfare, Government of India. The Controller of Publications, Civil Lines, Delhi, India, pp. 215–217.
- Arvidsson, M.B., Lqwhagen, O., Rak, S., 2007. Early and late phase asthmatic response in lower airways of catallergic asthmatic patients – a comparison between experimental and environmental allergen challenge. *Allergy* 62, 488–494.
- Behra, D., Sehgal, I.S., 2015. Bronchial asthma – issues for the developing world. *Indian J. Med. Res.* 141, 380–382.
- Brightling, C.E., Bradding, P., Symon, F.A., 2002. Mast-cell infiltration of airway smooth muscle in asthma. *N. Engl. J. Med.* 346, 1699–1705.
- Fukushima, C., Matsuse, H., Tomari, S., 2003. Oral candidiasis associated with inhaled corticosteroid use: comparison of fluticasone and beclomethasone. *Ann. Allergy Asthma Immunol.* 90, 646–651.
- Jung, C.H., Lee, J.Y., Cho, C.H., Kim, C.J., 2007. Anti-asthmatic action of Quercetin and Rutin in conscious guinea-pigs challenged with aerosolized ovalbumin. *Arch. Pharm. Res.* 30, 1599–1607.
- Kang, M.S., Jang, H.S., Oh, J.S., Yang, K.H., 2009. Effects of methyl gallate and gallic acid on the production of inflammatory mediators interleukin-6, interleukin-8 by oral epithelial cells stimulated with *Fusobacterium nucleatum*. *J. Microbiol.* 47, 760–767.
- Katiyar, C.K., 2006. Aqueous alcoholic extraction of MAPs by fermentation process. South-East Asian (SEA) Regional Workshop on "Extraction Technologies for Medicinal and Aromatic Plants". Central Institute of Medicinal and Aromatic Plants (CIMAP) Lucknow, India.
- Kato, K., Yamashita, S., Kitanaka, S., Toyoshima, S., 2001. Effect of gallic acid derivatives on secretion of Th1 cytokines and Th2 cytokines from anti CD3-stimulated spleen cells. *Yakugaku Zasshi* 121, 451–457.
- Kim, S.H., Jun, C.D., Suk, K., 2006. Gallic acid inhibits histamine release and pro-inflammatory cytokine production in mast cells. *Toxicol. Sci.* 91, 123–131.
- Kuppann, G., Balasubramanyam, J., Monickaraj, F., 2010. Transcriptional regulation of cytokines and oxidative stress by gallic acid in human THP-1 monocytes. *Cytokines* 49, 229–234.
- Kwasniewski, F.H., Lima, T.D., Bakhle, Y.S., Jancar, S., 1998. Impairment in connective tissue mast cells degranulation in spontaneously hypertensive rats: stimulus dependent resistance. *Br. J. Pharmacol.* 124, 772–778.
- Mahajan, S.G., Mehta, A.A., 2006. Role of cytokines in pathophysiology of asthma. *Iran. J. Pharmacol. Ther.* 5, 1–14.
- Martin, J.G., Tamaoka, M., 2006. Rat models of asthma and chronic obstructive lung disease. *Pulm. Pharmacol. Ther.* 19, 377–385.
- Mishra, A.K., Gupta, A., Gupta, V., Sannd, R., Bansal, P., 2010. Asava and arishta: an ayurvedic medicine – an overview. *Int. J. Pharm. Biol. Arch.* 1, 24–30.
- Mishra, L.C., 2004. Scientific Basis for Ayurvedic Therapies. CRC Press, New York, London, pp. 209–229.
- Na, H.J., Lee, G., Oh, H.Y., 2006. 4-O-Methyl gallic acid suppresses inflammation-associated gene expression by inhibition of redox-based NF-kappa B activation. *Int. Immunopharmacol.* 6, 1597–1608.
- Noonan, M., Leflein, J., Corren, J., Staudinger, H., 2009. Long-term safety of mometasone furoate administered via a dry powder inhaler in children: results of an open-label study comparing mometasone furoate with beclomethasone dipropionate in children with persistent asthma. *BMC Pediatr.* 9, 43.
- Parasuraman, S., Raveendran, R., 2012. Measurement of invasive blood pressure in rats. *J. Pharmacol. Pharmacother.* 3, 172–178.
- Praveen, R., Ahmad, F.J., Iqbal, Z., Singh, M., Kamal, Y.T., Ahmad, S., 2014. Simultaneous estimation of anti-cancer terpenoids in pharmaceutical nano-formulation by RP-HPLC and HPTLC. *Acta Chromatogr.* 26, 391–400.
- Reagan-Shaw, S., Nihal, M., Ahmad, N., 2008. Dose translation from animal to human studies revisited. *FASEB J.* 22, 659–661.
- Roh, S.S., Kim, S.H., Lee, Y.C., Seo, Y.B., 2008. Effects of radix adenophorae and cyclosporine A on an OVA-induced murine model of asthma by suppressing T cells activity, eosinophilia, and bronchial hyper-responsiveness. *Mediat. Inflamm.* 781425, 1–11.
- Roland, N.J., Bhalla, R.K., Earis, J., 2004. The local side effects of inhaled corticosteroids: current understanding and review of the literature. *Chest* 126, 213–219.
- Shifren, A., Witt, C., Christie, C., Castro, M., 2012. Mechanisms of remodeling in asthmatic airways. *J. Allergy*, 316049.
- Vyas, M., Shukla, V.J., Patgiri, B.J., Prajapati, P.K., 2010. A Unique Concentrated & Fermented Dosage Form i.e. *Pravahi kwatha*. *Inter. J. Pharm. Biol. Arch.* 1, 287–290.
- Walford, H.H., Doherty, T.A., 2014. Diagnosis and management of eosinophilic asthma: a US perspective. *J. Asthma Allergy* 7, 53–65.
- Zosky, G.R., Sly, P.D., 2007. Animal models of asthma. *Clin. Exp. Allergy* 37, 973–988.