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***Myxopyrum serratulum* ameliorates the airway inflammation in LPS stimulated
RAW 264.7 murine macrophages and OVA induced murine model of allergic
asthma**

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Abstract

Ethnobotanical relevance: *Myxopyrum serratum* (*M.serratum*) is an important medicinal plant traditionally used for the treatment of asthma and cough, and many inflammatory diseases. In this study, the protective effects of *M.serratum* was investigated through airway inflammation in an ovalbumin (OVA)-induced asthma murine model and lipopolysaccharide (LPS)-stimulated inflammation in RAW 264.7 murine macrophages and possible mechanism were studied.

Method

Phytochemicals in the methanolic extract of *M.serratum* (MEMS) were identified using RP-HPLC analysis. For evaluating *in vitro* anti inflammatory activity, LPS stimulated RAW 264.7 murine macrophage method was adopted and to estimate the levels of nitric oxide (NO), reactive oxygen species (ROS) and group of proinflammatory cytokines by ELISA. Allergic asthma was induced in Female BALB/c mice by intraperitoneal injection of OVA on days 0 and 14 and then challenged with OVA from days (21 to 23). The MEMS at 200 & 400 mg/kg was administered by oral gavage 1h before the OVA challenge. The airway hyperresponsiveness (AHR) was measured after 24h OVA challenge, bronchoalveolar lavage fluid (BALF) & lung tissues were collected after 48h last OVA challenge. Total cell count, differential cell eosinophil peroxidase assay, NO, Prostaglandin (PGE₂), ROS assay and the cytokine release (IL-4, IL-5 & IL-13), were performed in BALF. Serum total IgE level and histopathological changes of lung tissues and cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were detected by Western blot.

Results

From the chromatographic identification, the gallic acid, protocatechuic acid, catechin, ellagic acid, rutin, *p*-coumaric acid, quercetin, naringenin, apigenin were found in MEMS. In LPS stimulated RAW 264.7 cells, MEMS (125,250 µg/mL) treatment significantly reduced the levels of NO, ROS and proinflammatory cytokines including IL1 α , IL1 β , IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, IFN- γ , TNF- α , G-CSF and GM-CSF). MEMS (200 & 400 mg/kg) significantly reduced the

number of inflammatory cells, NO, PGE₂, ROS and IL-4, IL-5, IL-13 in BAL fluid, IgE in serum of OVA-sensitized/challenged mice and also inhibit the COX-2 & iNOS protein expression in lung tissues of OVA-sensitized/challenged mice.

Conclusion

From this finding it was suggested that the MEMS exhibited significant effects on airway inflammation by reducing the level of oxidative stress, pro-inflammatory cytokines and inhibiting the COX-2, iNOS protein expression.

Keywords: *Myxopyrum serratum*; anti-inflammatory; anti-asthma; HPLC; COX-2.

1. Background

Bronchial asthma is a chronic inflammatory disease of the airway (bronchial tubes in the lungs) that involves multiple components and is orchestrated by several cell types, particularly eosinophils, goblet cells, mast cells and CD4⁺ lymphocytes. Activation of these cells due to allergen exposure, releases the inflammatory and pro-inflammatory mediators. The pathophysiology of asthma involves activation and maturation of many inflammatory cells like mast cells, macrophages/monocytes, eosinophils, dendritic cells, T-helper type-2 lymphocytes (Th2), basophil and neutrophils (Barnes et al., 2008). Macrophages plays a central role during inflammation, upon activation it elicit a variety of inflammatory reactions including the release of inflammatory & proinflammatory mediators and cytokines such as NO, PGE₂, ROS and IL1 α , IL1 β , IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, IFN- γ , TNF- α , G-CSF and GM-CSF. The proinflammatory mediators such as NO and PGE₂ are fashioned by iNOS and COX-2 respectively. A huge amount of NO, produces oxidative damage to the cells and induce nitrogen stress. ROS plays a pivotal role in the pathogenesis of number of airway diseases, including cystic fibrosis, chronic obstructive pulmonary disorder (COPD) and asthma (Gillissen A et al., 1998; Repine et al., 1997). Airway cells and smooth muscles are exposed to oxidative stress, produces increased levels of ROS, which damage the bio molecules and may directly activate the inflammatory pathways. In addition, the elevated level of ROS leads to upregulation of iNOS and COX-2 expression along with the

induction of proinflammatory cytokines. It is well known that cytokines play a crucial role in orchestrating, propagating and amplifying the inflammatory response in asthma. (Adcock et al., 2008). Cytokines regularly possess to overlapping many biological activities, exhibit different effects at different concentrations, can either synergize or provoke the effects of other cytokines and controlled in a complex manner and function (Papadakis & Targan., 2000). The analysis of the function of the complete set of cytokines expressed at the site of inflammation is often of more value than the analysis of a single isolated cytokine.

Phytoconstituents such as phenolic compounds, flavonoids and terpenoids are reported to possess antioxidant, anti-proliferative, anti-inflammatory, anti-cancer, and neuroprotective activities (Park et al., 2011). In this view many scientific communities showed interest in the protection of oxidative stress induced disease by natural antioxidants.

The plant *M. serratum* is an important medicinal plant belonging to the family *Oleaceae*. It is traditionally useful for cough, rheumatism, nerve complaints, asthma, fever, and neuropathy, the leaves paste taken with ghee as a good remedy for asthma (The Wealth of India., 2011). In addition, the plant has been reported to possess antioxidant, anti inflammatory, antimicrobial and wound healing activity. The important phytochemicals like ursolic acid and iridoid glycosides only were reported from the leaves of the plant. So far the antiasthmatic activity of this plant was not yet been reported by *in vitro* and *in vivo* model. Therefore, evaluate the ability of MEMS on antiasthmatic efficacy via *in vitro* and *in vivo* model using RAW 264.7 murine macrophages and a mouse model of allergic lung inflammation.

2. Materials and Methods

2.1. Plant material and extract preparation

The leaves of *M. serratum* A. W. Hill was collected from Western ghats, Kerala in the month of May-2013 and taxonomic identification of the plant material was confirmed by Dr. V. Chelladurai, Central Council of Research in Ayurveda and Siddha, Government Siddha Medical

College, Palayamkottai, Tamilnadu, India. The voucher specimen (MS-0713/BIT) was deposited in the Department of Pharmaceutical Technology, Anna University, Tiruchirappalli. The leaves were freshly collected, shade dried and powdered, defatted using petroleum ether (60-80°C) and then extracted with methanol using hot extraction technique. The solvents were removed under vacuum yielding the concentrated green colour sticky mass, it was dried and stored in a vial at low temperature (4°C) prior to analysis, the percentage yield of the extract was 20.5%.

2.2. Quantification of bioactive compounds by RP-HPLC analysis

2.2.1. Sample preparation

For preparation of sample, 100 mg of methanolic extract of *M.serratulum* was dissolved in a volumetric flask in methanol/water/TFA (50:50:0.02) and the volume was adjusted to 10 mL accurately. The mixture was sonicated in a bath sonicator (Sonics, USA, 40 W, 20 KHZ) for 30 min at room temperature and filtered (0.45 µm PTFE, acrodisc, 25 mm, Pall Gelman, USA) prior to injection into the HPLC system.

2.2.2. Standard preparation

For standard mixture preparation 2.5 mg of each of the nine phenolics viz. naringenin, rutin, quercetin, flavone, catechin, Ferulic acid, protocatechic acid, *p*-coumaric acid and Gallic acid were weighed accurately and dissolved in 1:1 (methanol/water) into a 50 mL volumetric flask to obtain stock solutions (50 µg/mL). The standard solution was filtered (0.45 µm PTFE, acrodisc, 25-mm, Pall Gelman, USA) prior to HPLC analysis.

2.2.3. HPLC conditions

The separation of bioactive compounds was accomplished in a SHIMADZU (Shimadzu Corp., Japan) LC2010 CHT series equipped with on-line degasser, quaternary pump (LC2010 AHT), Autosampler (LC2010 CHT), column oven and UV-VIS detector. Data was acquired three-dimensionally (absorbance time-wavelength) using LC solutions software. A reversed-phase C-18 column Phenomenox Luna (4.6x250 mm, 5 µM particle size, Phenomenox, USA) was used. Mobile phase A was water containing 0.02% TFA and B was methanol containing 0.02% TFA. The

gradient conditions were as mentioned below: 0-5 min, 25% B; 5-10 min, 25-30% B; 10-16 min, 30-45% B, 16-18 min, 45% B; 18-25 min, 45-80% B, 25-30 min 80% B; 30-40 min, 80-25% B. The column temperature was maintained at 25°C. Elution was performed at a flow rate of 0.5 mL/min and detected at a wavelength of 280 nm. Injection volume of 20 µL was used. Phenolic compounds present in the extract were identified by comparing chromatography peaks with the retention time (RT) of reference standards. The peak area of the chromatogram was used for calculating the concentration of the compounds in the extract (Wen et al., 2005).

2.3. Determination of Free radical scavenging activity

2.3.1. DPPH radical scavenging assay

MEMS of different concentrations (10, 20, 30, 40 and 50 µg/mL) was mixed with 2 mL of 0.2 mM DPPH ethanolic solution and vortexed, then shaking vigorously and allowed to stand for 30 min at room temperature. The absorbance was measured at 517 nm, the decrease in absorbance indicating the free radical scavenging ability of the extract. The synthetic antioxidant ascorbic acid was used as standard and purified water was used as a control (Liyana-pathiyana and Shahidi, 2005).

2.3.2. ABTS scavenging assay

One ml of the ABTS⁺ (2, 2'-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) solution was added into different concentrations of MEMS (5-25 µg/mL) and kept for 6 min at room temperature and the absorbance was directly measured at 734 nm. The percentage inhibition of scavenging activity of the extract was calculated and the results were compared with reference standard ascorbic acid (Re et al., 1999).

2.4. *in vitro* investigation of anti-inflammatory activity of MEMS using LPS-stimulated RAW

264.7 murine macrophages

2.4.1. Cell viability assay

The effect of MEMS on cell viability was studied in RAW 264.7 murine macrophages. MEMS was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mg/mL. The final concentration of DMSO was less than 0.02% for every treatment which served as negative control.

The cells were seeded in 96-well microtiter plates at a density of 10^4 cells/well in DMEM, supplemented with 10% heat-inactivated FBS and grown at 37°C in a humidified atmosphere with 5% CO_2 . After 24h of incubation, the medium was discarded and the cells were incubated separately with different concentrations of MEMS (62.5,125,250 & 500 $\mu\text{g/mL}$) for 24h. Untreated cells were used as normal control and diclofenac sodium (5 $\mu\text{g/mL}$), a standard anti-inflammatory drug was used as positive control. The morphology of cells was observed with an inverted microscope (TiE, Nikon, Japan) and the phase-contrast images were captured at 20X magnification (Park et al., 2015).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the cell viability. The assay depends on the reduction of MTT by mitochondrial dehydrogenase, an enzyme present in the mitochondria of viable cells to a purple coloured formazan product. After 24h treatment, 10 μL of MTT (5 mg/mL) was added and incubated for 4h at 37°C , 5% CO_2 . The medium was discarded and add 100 μL of DMSO to dissolve the formazan crystals. The absorbance was read at 570 nm in a multimode microplate reader (EnSpire, Perkin Elmer, Singapore) and the cell viability were expressed as a percentage of surviving cells over control cells.

2.4.2. Measurement of NO Production

The concentration of NO in the culture supernatant was determined based on the accumulation of nitrite, a major stable product of NO, using a Griess reagent-based colorimetric assay. Equal volumes of the culture supernatants from the respective treatment groups and 1 $\times$ Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diamine dihydrochloride in water) were incubated at room temperature for 15 min. The absorbance of the mixture was measured at 540 nm using a Multimode microplate reader (EnSpire, PerkinElmer, Singapore). A standard curve with known concentrations of sodium nitrite was plotted to extrapolate the nitrite concentration of the unknown samples (Lou et al., 2009).

2.4.3. Measurement of intracellular ROS Production

The production of intracellular ROS was detected by DCFH₂-DA assay. Briefly, RAW 264.7 murine macrophages at a density of 10⁵ cells per well in a 24-well plate were incubated with MEMS (125 & 250 µg/mL) with or without LPS (1 µg/mL) at 37°C, 5% CO₂ for 24h. After 24h, the cells were washed with PBS and 10 µM of DCFH₂-DA was added and incubated at 37°C for further 30 min. After incubation, the cells were washed thrice with PBS and the fluorescence images were captured at 20X magnification using an inverted fluorescence microscope (Nikon, Ti-E, Japan). The levels of ROS was measured by the intensities in fluorescence due to the intracellular production of DCF caused by the oxidation of DCFH₂. The fold increase of intracellular ROS was compared with the positive & negative controls and was quantified by measuring the intensity of DCF fluorescence in the microscopic field (Yang et al., 2015).

2.4.4. Measurement of pro-inflammatory cytokines

Multi-analyte ELISArray kit (Qiagen, USA) was used for the simultaneous detection of mouse pro-inflammatory cytokines (IL1 α , IL1 β , IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, IFN- γ , TNF- α , G-CSF and GM-CSF). Briefly, RAW 264.7 murine macrophages at a density of 10⁶ cells per well in a 6-well plate were incubated with MEMS (125 & 250 µg/mL) with or without LPS (1 µg/mL) at 37°C, 5% CO₂ for 24h. After 24h, the concentrations of cytokines in the culture supernatants were quantified as per manufacturer's protocol.

2.5. Evaluation of *in vivo* antiasthmatic activity

2.5.1. Experimental Animal

Pathogen free 7-8 weeks old female BALB/c mice, weighing between 25-30g were obtained from Sri Venkateshwara agencies, Bangalore. The mice were maintained under standard laboratory conditions of humidity (50-60%) and temperature 25 $\pm$ 2°C. Animals were given an adequate pellet diet and water *ad libitum*. The animal study was performed according to the CPCSEA guidelines and approved by an institutional animal ethical committee of Bharathidasan Institute of Technology, Anna University, Tiruchirappalli.

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2.5.2. Animal sensitization and treatment

Animals were randomly divided into five groups (6mice/group). Asthma is induced by ovalbumin (OVA) (Sigma Aldrich, USA). Group 1 received only saline, served as a normal control (untreated), group-2 OVA sensitized and challenged, Group 3&4 received different concentration of text extract (200,400 mg/kg of MS extract). Group-5 was served as a positive control received 3mg/kg of dexamethasone. Animals of Group 2-5 were sensitized on day 0 and 14 by intra peritoneal injection of 20 μ g/200 μ L OVA adsorbed on 1.00 mg/50 μ L alum adjuvant. The mice were then exposed with 1% OVA in PBS for 20 min from day 21 to 23 using ultrasonic nebulizer. The test extracts were made suspension with CMC sodium and administered orally from day 18 once daily for 6 days. (King et al., 2004).

2.5.3. Measurement of Airway Hyper responsiveness (AHR)

After twenty-four hours of the final OVA challenge, the AHR was assessed in conscious and unrestrained mice through whole-body plethysmography (Emka Technologies, France). Each mouse was placed in a plastic chamber and exposed to aerosolized PBS followed by aerosol containing methacholine at increasing concentrations (3.125-25 mg/mL in PBS) for 3 min (Kim *et al.*, 2008). After each methacholine challenge, the Penh values were recorded for 3 min, the Penh value was expressed as a proportion of the Penh value obtained in response to PBS challenge.

2.5.4. Measurement of inflammatory cell counts in BALF

The mice were anesthetized through an intraperitoneal injection of Pentobarbital (Sigma Aldrich, USA) 48h after the last challenge, and tracheostomies were performed. To obtain the bronchoalveolar lavage fluid (BALF), ice-cold PBS (0.4 mL) was infused into the lung and withdrawn via tracheal catheter three times (total volume of 1.2 mL). The obtained BALF was centrifuged; the supernatant was collected and stored at -80°C for further biochemical analysis. Total inflammatory cell counts were assessed by counting the cells in at least five squares of a hemocytometer after the exclusion of dead cells through trypan blue staining. To determine the

differential cell counts, 100 μ L of BALF was centrifuged (200g, 4°C, 10 min) and fixed the cells onto slides. The slides were dried and stained with the Giemsa staining reagent according to the manufacturer's instructions. (Li et al., 2014; Khan et al., 2015).

2.5.5. Eosinophil Peroxidase activity

EPO activity was determined by adding 100 μ L of BALF into 100 μ L of reaction mixture containing 0.1mM-O-Phenylene-diamine-dihydrachloride, 0.1 % Triton X-100 and 1mM hydrogen peroxide in Tris-Hcl in microtitre plate. The plate was incubated in dark for 30 min and the reaction was blocked by adding 50 μ L of 4M sulfuric acid per well. The absorbance was measured at 490 nm using Perkin Elmer, Multimode Plate Reader, Singapore. (Kobayashi et al., 2006).

2.5.6. Determination of Prostaglandin (PGE₂)

Commercially available enzyme-linked immunosorbent assay antibodies were used to determine the PGE₂ concentration in BALF, according to the manufacturer's instruction.

2.5.7. NO release in BALF

NO was measured in BALF according to the method described by Feelisch and Noack (1987). 100 μ L of BALF was added to 10 μ L Griess reagent (1% sulfanilamide 0.1% naphthylene diamine dihydrochloride and 2.5% phosphoric acid). The plate was incubated in dark for 30 min at 37°C and the absorbance was read at 542 nm in a PerkinElmer, Multimode Plate Reader, Singapore. A standard curve of sodium nitrite was constructed and used for quantification (Tiwari et al., 2014).

2.5.8. 2,7- Dichlorofluorescein (DCF) fluorescence assay

The intracellular ROS using 2,7- dichloro fluorescein diacetate (Sigma Aldrich, Germany) which reacts with cellular peroxides such as hydrogen peroxide and converted into the highly fluorescent DCF. An aliquot of BALF was treated with 25 μ m DCF-DA for 10 min at 37°C. To determine the intracellular ROS activity, fluorescence was read at 488 nm excitation and 525 nm emission using a fluorescence plate reader (Perkin-Elmer, Singapore).

2.5.9. Measurement of Cytokine in BALF and IgE in serum (ELISA)

The Enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instruction (Ray Biotech, Norcross, GA). The concentration of IL-4, IL-5 and IL-13 in BALF and IgE in serum was determined using sandwich ELISA kits. The absorbance was measured at 450 nm using a micro plate reader.

2.5.10. Histopathological evaluation of lungs

After 48h of last OVA challenge, the mice were sacrificed and lungs were taken out and fixed at 10% solution of buffered formalin. After fixation, the 5 μ M thick paraffin sections were cut and stained with Haematoxylin and Eosin (H & E) for the measurement of inflammatory cell infiltration and alveolar thickening. In addition, the lung tissues were stained with Periodic Acid-Schiff (PAS) stain and used for the identification of goblet cell hyperplasia and mucus hyperproduction. (Myou et al., 2003).

2.5.11. Western blot analysis

Lung protein was separated using SDS-PAGE analysis and the protein were transferred to a membrane and incubated at 4°C with suitable primary antibodies such as β -actin, COX-2 and iNOS. The membrane was washed with Tris-buffered saline containing Tween 20 (TBST) and incubated with secondary antibody for 1h at room temperature. Again the membranes were washed thrice with TBST and developed using a chemiluminescence kit. The quantitative band densitometric band value were determined by chemi-Doc analysis (Jeon et al., 2014).

2.6. Statistical analysis

All the experiments were performed in triplicate and the results are represented as Standard Error Mean (SEM) n=3. Statistical significance using analysis of variance (ANOVA), followed by a Dunnett's test for pairwise comparisons was analyzed with SPSS 11.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Quantification of bioactive compounds by HPLC

HPLC is an excellent tool for identification and quantification of phenolic compounds in the plant extract. The phenolic compounds were identified from the MEMS and quantified by RP-HPLC method. The present study is the first report on this plant for the determination of gallic acid, *p*-coumaric acid, ferulic acid, and five flavonoids, catechin, rutin, quercetin, naringenin and flavone using HPLC analysis. The concentration of the compounds was determined by comparing chromatographies peak area obtained in the standards and sample (Table.1) and the chromatogram was represented in (Fig.1). From the results it was observed that the amount of phytochemicals present in the MEMS were found to be naringenin (100.20 ± 0.69 mg/g), rutin (57.66 ± 0.75 mg/g), ellagic acid (47.37 ± 0.54 mg/g), quercetin (35.91 ± 0.42 mg/g), catechin (6.10 ± 0.03 mg/g), *p*-coumaric acid (3.05 ± 0.04 mg/g), apigenin (2.12 ± 0.02 mg/g) and gallic acid (1.34 ± 0.05 mg/g). Among the eight compounds, naringenin, rutin, ferulic acid and quercetin are found in higher concentration when compared to others.

***In vitro* evaluation**

3.2. Free Radical -Scavenging assay of MEMS

DPPH radical scavenging capacity of MEMS was studied and depicted in (Fig.2A). The MEMS was particularly reduced the DPPH free radical in the dose dependant manner, the inhibition ability was in support with a good IC_{50} values. The higher percentage inhibition and lower IC_{50} value indicate the highest antioxidant potential. From the results it was found that the percentage inhibition of MEMS exhibited radical scavenging activity of about 95.51% at 50 μ g/mL with the IC_{50} value was 15.75 μ g/mL which was comparable to that of standard ascorbic acid (5.50 μ g/mL). In similar to DPPH radical scavenging activity, ABTS is also a protonated radical which exhibit the maxima absorbance at 734nm. ABTS (2,2-azino-bis (3- ethylbenzthiazoline- 6- sulfonic acid) assay was based on the principle that the blue green colored (ABTS radical) chromogen reduces and decolorize into pale yellow color by the addition of antioxidant compounds. ABTS radical cation scavenging activity of MEMS extract was depicted in (Fig. 2B). The results showed a high percentage inhibition in the methanolic extract was 88.55%, even at low concentration (10

µg/mL) with respect to the IC_{50} of 3.87 µg/mL in a dose dependent manner which was comparable to that of standard ascorbic acid (94.95 % at 10 µg/mL) with the IC_{50} of 2.96 µg/mL.

3.3. Effects of MEMS on LPS induced inflammation in RAW 264.7 murine macrophages

3.3.1. Effects of MEMS on cell viability

The cell viability upto 250 µg/mL for 24h of incubation MEMS exhibited 95.97% cell viability. As shown in (Fig. 3), the morphology of the RAW 264.7 murine macrophages appeared as small round and smooth surface. MEMS (125 & 250 µg/mL) treated at increasing concentration does not significantly altered the morphology of the cells compared with normal control and DS treated RAW 264.7 murine macrophages. Based on these data, the non-cytotoxic concentrations (i.e., IC_{50} values) were used to evaluate their anti-inflammatory effects in RAW 264.7 murine macrophages. Accordingly, the concentrations of MEMS 125 & 250 µg/mL and DS 5 µg/mL used for the subsequent experiments.

3.3.2. Effects of MEMS on the morphology of LPS-stimulated RAW 264.7 murine macrophages

To examine the effects of MEMS on the morphological changes related with LPS-stimulated RAW 264.7 murine macrophages. The unstimulated (normal control) RAW 264.7 murine macrophages were observed as small, round and no vacuoles are present (Fig. 4). The LPS-stimulated RAW 264.7 murine macrophages shown bigger and their surface became crude and rough; some projection was presented in outer to the cell wall. In contrast, the MEMS (125 & 250 µg/mL) reduced the LPS-induced morphological changes in RAW 264.7 murine macrophages, somewhat similar to control. This study demonstrated that the MEMS exhibited a little effect on LPS-induced cell morphological changes as compared to standard diclofenac sodium (5 µg/mL).

3.3.3. Effects of MEMS on NO and ROS generation

In order to examine the possibility of MEMS to inhibit LPS-induced NO production, found that LPS 1 $\mu\text{g/mL}$ treatment increased the NO level to $49.50 \pm 1.02 \mu\text{M}$. Pre-treatment with MEMS at 125 & 250 $\mu\text{g/mL}$ decreased the NO level to $38.24 \pm 1.26 \mu\text{M}$, $31.25 \pm 1.00 \mu\text{M}$ respectively (Fig.5). LPS-induced intracellular ROS accumulation in RAW 264.7 murine macrophages was examined using the oxidant-sensing fluorescent probe 2',7-dichlorodihydrofluorescein diacetate (DCFH₂-DA) fluorescence assay. This method is extensively used to measure oxidative stress in cells attributable to the high sensitivity of fluorescence-based assays. The non-fluorescent molecule DCFH₂-DA diffuses into cells and converts into a fluorescent molecule dichlorofluorescein (DCF) in the presence of free radicals, signifying the intracellular production of oxidative molecule having an excitation wavelength of 485 nm and an emission band between 500 and 600 nm (Marchesi et al. 1999). The elevated level of ROS was observed in LPS-stimulated RAW 264.7 murine macrophages. Treatment with MEMS at different concentrations (125&250 $\mu\text{g/mL}$) were significantly inhibits the elevated ROS level (Fig. 6).

3.3.4. Effects of MEMS on IL-1 α , IL-1 β , IL-2, TNF- α , IFN- γ , IL-6, IL-12, IL-17A & GM-CSF

The effect of MEMS at different concentration (125 & 250 $\mu\text{g/mL}$) on pro-inflammatory cytokine IL-1 α , IL-1 β , IL-2, TNF- α , IFN- γ , IL-6, IL-12, IL-17A and GM-CSF production in LPS-stimulated RAW 264.7 murine macrophages was studied using ELISA analysis and presented (Fig.7,8 & 9). The MEMS significantly suppressed the LPS-stimulated IL-1 α , IL-1 β and IL-2 production on RAW 264.7 murine macrophages in a dose dependent manner. The concentration of IL-1 α , IL-1 β and IL-2, TNF- α , IFN- γ , IL-6, IL-12, IL-17A and GM-CSF in LPS alone treated cells was observed as $345.10 \pm 4.02 \text{ pg/mL}$, $397.03 \pm 2.95 \text{ pg/mL}$, 678.56 ± 4.53 , 3237.43 ± 6.52 , $987.00 \pm 5.82 \text{ pg/mL}$, 417.83 ± 5.64 , 201.68 ± 4.26 , 409.46 ± 6.05 and $289.49 \pm 4.63 \text{ pg/mL}$ respectively. Pre-treatment with 125 $\mu\text{g/mL}$ of MEMS significantly decreased the production of IL-1 α , IL-1 β and IL-2, TNF- α , IFN- γ , IL-6, IL-12, IL-17A and GM-CSF to $310.52 \pm 5.89 \text{ pg/mL}$, $362.57 \pm 3.54 \text{ pg/mL}$, $478.36 \pm 5.20 \text{ pg/mL}$, $3098.46 \pm 6.02 \text{ pg/mL}$, 849.57 ± 6.00 , 391.26 ± 5.31 , 189.32 ± 4.51 , 387.90 ± 2.98

and 271.27 ± 3.19 pg/mL respectively ($**p < 0.01$). The MEMS at $250 \mu\text{g/mL}$ exhibited a significant reduction in the production of IL- 1α , IL- 1β and IL-2, TNF- α , IFN- γ , IL-6, IL-12, IL-17A and GM-CSF as 278.62 ± 5.64 pg/mL, 314.62 ± 4.08 pg/mL and 388.21 ± 4.91 pg/mL, 2856.00 ± 5.34 pg/mL, 724.35 ± 6.25 pg/mL, 350.67 ± 4.97 , 168.92 ± 3.05 , 337.52 ± 3.15 and 235.69 ± 3.02 pg/mL respectively. The increased production of IL- 1α , IL- 1β , IL-2, TNF- α , IFN- γ , IL-6, IL-12, IL-17A and GM-CSF was observed in LPS-stimulated RAW 264.7 murine macrophages. In contrast, MEMS at 125 & $250 \mu\text{g/mL}$ significantly ($*p < 0.05$) reduced the production of these cytokines in a dose dependent manner.

3.3.5 Effects of MEMS on anti-inflammatory cytokines (IL-4, IL-10 and G-CSF)

The anti-inflammatory effect of MEMS of different concentrations (125 & $250 \mu\text{g/mL}$) on the production of anti inflammatory cytokines including IL-4, IL-10 and G-CSF in LPS-stimulated RAW 264.7 murine macrophages were presented (Fig.10). The decreased concentration of IL-4, IL-10 and G-CSF was observed in LPS-treated RAW 264.7 murine macrophages. These cytokine levels were significantly increased in MEMS and DS treated RAW 264.7 murine macrophages when compared with LPS-stimulated cells. The concentration of IL-4, IL-10 and G-CSF in the LPS-treated RAW 264.7 murine macrophages were observed as 249.77 ± 6.52 , 223.58 ± 5.82 and 167.98 ± 6.71 pg/mL respectively at low concentration ($125 \mu\text{g/mL}$). The MEMS at high concentration ($250 \mu\text{g/mL}$) exhibited that the increased concentration of IL-4, IL-10 and G-CSF were 306.88 ± 4.62 , 324.57 ± 6.13 and 232.78 ± 5.62 respectively when compared with LPS-treated cells. These results clearly demonstrated that the MEMS exhibited the concentration dependent anti-inflammatory activity in LPS-induced inflammation in RAW 264.7 murine macrophage.

3.4 In vivo evaluation

3.4.1 The effect of MEMS on Airway hyper responsiveness (AHR)

The Airway hyper responsiveness is one of the important features of asthma. The effect of extract on AHR in response to increasing doses of methacholine (3.125 to 25 mg/mL) (Fig. 11).

The data exhibited the AHR was significantly increased ($^{***}p<0.001$) in the OVA induced asthmatic mice when compared to normal PBS treated mice. The MEMS (400 mg/kg) treated group have significantly reduced ($^{***}p<0.001$) the AHR at all doses of methacholine as compared to OVA treated group. The effect of extract on AHR also compared with positive control dexamethasone, it was found that the similar effect was observed. These results indicate that MEMS treatment significantly reduced the OVA-induced AHR in a dose dependent manner.

3.4.2 The effect of MEMS on inflammatory cell count

The elevated level of total cell count in BALF of OVA sensitized and challenged mice were ($53.66\pm2.55\times10^4$ cells), MEMS at 200 & 400 mg/kg was significantly reduced these elevated to 22.40 ± 1.96 and $10.41\pm1.72\times10^4$ cells respectively. The results clearly demonstrate that the elevated level of the total leukocyte cell count was observed in BALF of OVA sensitized and challenged group when compared with the saline treated group. The MEMS treated group exhibited significant reduction ($^{**}p<0.01$) of elevated total leukocyte cell count in a dose dependent manner compared to diseased group. Similarly, the data exhibited the elevated level of differential cell counts, including eosinophils macrophages in BALF of OVA treated group (diseased group) when compared with normal PBS treated group. The MEMS (400 mg/kg) significantly ($^{**}p<0.01$) reduced the macrophage and eosinophils count from 11.50 ± 0.64 to $4.45\pm0.56\times10^4$ cells and $20.52\pm4.56\times10^4$ cells respectively. (Fig. 12).

3.4.3 The effect of MEMS on EPO activity, PGE₂, NO and ROS production in BALF of OVA sensitized and

Significantly higher EPO activity was observed in BALF of OVA sensitized and challenged mice when compared to the normal PBS, receiving group, it clearly indicates that the eosinophil infiltrate in the BALF of diseased group was increased during inflammation (Fig.13A). The data showed that the significant reduction ($^{**}p<0.01$) of EPO activity in MEMS treated group was observed as $73.37\pm0.004\%$ and $83.70\pm0.011\%$ at the respective concentration of 200 mg/kg, 400 mg/kg. The similar activity was observed in dexamethasone treated group ($77.72\pm0.001\%$).

The secretion of PGE₂ was significantly increased in BALF of OVA sensitized and challenged mice (increase from 180.79±5.58 pg/mL to 478.50±9.88 pg/mL) (^{##}*p*<0.01). MEMS (200 & 400 mg/kg) treatment was significantly reduced the level of PGE₂ in BALF was about 380.59±8.38 & 270.59±7.29 pg/mL respectively in a dose dependent manner, this reduction might alleviate the asthmatic airway inflammation (Fig. 13B).

Activity of MEMS against NO level in OVA-induced mouse model was shown in Fig. 13C. The data showed the elevated level of NO released in BALF of OVA-treated mice (18.17±0.96 μM) as compared to normal control PBS group (4.83±0.43μM) (^{###}*p*<0.001). The MEMS (200 & 400 mg/kg) treated groups exhibited significant reduction of NO released in BALF was 6.94±0.75μM, and 4.04±0.68μM respectively compared to OVA treated mice, the standard drug dexamethasone treated mice was observed as 4.01±0.54μM.

The effect of MEMS on ROS generation in BALF of OVA-induced asthmatic mice was shown in Fig.13D. The ROS level was significantly higher in BALF of asthmatic mice (3.99±0.31 folds of control) compared to normal PBS treated mice (1.00±0.15) (^{##}*p*<0.01). Whereas the BALF of MEMS (200 & 400 mg/kg) treated mice significantly reduced the ROS level to 2.49±0.22, 1.04±0.22 folds of control (^{**}*p*<0.01) in a dose dependent manner when compared to diseased group

3.4.4 Effect of MEMS on Th2 cytokines (IL-4,IL-5&IL-13) and serum IgE level of OVA sensitized and challenged mice

To establish the other possible mechanisms underlying the inhibitory effect of MEMS in airway inflammation, the Th2 cytokines (IL-4, IL-5 & IL-13) levels were examined in BALF and depicted in Fig. 14 A,B & C respectively, and IgE level in serum was also examined and shown in Fig. 14D. The concentration of IL- 4, IL-5, and IL-13 in BALF were markedly increased in OVA - sensitised/challenged mice, compared with those in PBS-treated mice (^{###}*p*<0.001). The results

clearly indicated that the MEMS exhibited a significant and dose dependent reduction of IL-4 concentration in BALF. The MEMS was reduced the OVA-induced IL-4 production in BALF was about 57.03 ± 0.04 and $95.06\pm0.05\%$ at the respective concentration of 200 & 400 mg/kg. The reduction ability of MEMS at 400 mg/kg showed similar effect as that of normal control PBS received group ($95.26\pm0.03\%$). This is due to MEMS exhibiting allergen specific immune response in asthma. The concentration of IL-5 in BALF was markedly increased in OVA-induced mice (84.41 ± 0.52 pg/mL) compared with those in PBS-treated mice (26.66 ± 0.40 pg/mL) ($^{###}p<0.001$). The MEMS (200 & 400 mg/kg) treated mice significantly reduced the concentration of IL-5 in BALF to 60.44 ± 0.69 and 27.14 ± 0.40 pg/mL respectively in a dose dependent manner ($^{***}p<0.001$). The concentration of IL-13 in BALF was markedly increased in OVA-induced mice (118.32 ± 2.18 pg/mL) as compared with those in PBS-treated mice (51.82 ± 1.42 pg/mL). The MEMS (200 & 400 mg/kg) treated mice significantly reduced the concentration of IL-13 in BALF of asthmatic mice to 67.26 ± 1.65 pg/mL and 51.66 pg/mL respectively in a dose dependent manner. The effect of MEMS at higher concentration is similar that of positive control ($^{***}p<0.001$). The concentrations of total IgE level in serum were drastically increased in OVA-treated mice (19.58 ± 0.82 ng/mL) compared with PBS-treated mice (3.34 ± 0.45 ng/mL) ($^{###}p<0.001$). Whereas, the MEMS (200 & 400 mg/kg) treated mice exhibited significant reduction in total IgE concentration into 9.16 ± 0.40 ng/mL and 3.20 ± 0.20 ng/mL respectively when compared with OVA-treated mice. The positive control dexamethasone (3 mg/kg) also exhibited similar inhibitory effect as MEMS (5.52 ± 0.32 ng/mL).

3.4.6. Effect of MEMS on histological changes of OVA induced asthma model

The protective effect of the MEMS on inflammation in lung tissue was further established by histological examination. The lung tissues were examined for inflammation due to infiltration of leukocytes, mast cells and eosinophils using H&E staining. Micrographs are shown in Fig.15 lung tissues of normal control mice (A) showed normal histology with no significant occurrence of inflammatory cells in peribronchial space confirming the lower eosinophils count in

BALF. The lung tissues of OVA-sensitized and challenged mice (B) showed an increase eosinophils infiltration in peribronchiolar and perivascular space and the erosion in peribronchiolar region. The lung tissues of MEMS (200 & 400 mg/kg) treated mice Fig. (C) & (D) reduced the indication of inflammation and infiltration of cells representing the protective effect of the extract. The positive control group, i.e. dexamethasone treated mice (E) also exhibited a protective effect against inflammatory symptoms. The effect of MEMS extracts on mucus hyper-secretion caused by goblet cell hyperplasia was measured by PAS staining of the lung tissue (Fig. 16). The lung tissues of OVA-challenged mice (B) showed an increase in the levels of goblet cell hyperplasia as increased mucus production. The MEMS (200 & 400 mg/kg) treated mice (C&D) and dexamethasone treated group (D) also showed reduced PAS stained airways as marked by slighter violet colour stain in the airways. Hence, the treatment of MEMS exhibited a protective effect on goblet cell hyperplasia and mucus over-production.

3.4.7 Effects of MEMS on expression of iNOS and COX-2 in the lung tissues of OVA induced asthma model

The western blot analysis of present study clearly showed that the iNOS expression in the lung tissue was drastically up regulated in OVA-sensitized/challenged mice compared with the normal control group. Whereas, the MEMS treatment dramatically decreased the iNOS protein expression which is consistent with the result of NO production observed in BALF of asthmatic mice in this study. In addition, the level of COX-2 was also evaluated in this study, it was markedly increased in OVA-sensitized/challenged group compared to normal group and the significant reduction of COX-2 protein expression was observed by MEMS treated group compared with the OVA sensitized/challenged mice (Fig. 17).

4. Discussion

In this study, *in vitro* evaluation of anti-inflammatory activity of MEMS on LPS-stimulated inflammation in mouse RAW 264.7 murine macrophages was explored. Our results demonstrated

that MEMS significantly inhibited the increased production of IL-1 α , IL-1 β , IL-6, IL-12, IL-2, IL-17A, TNF- α , IFN- γ and GM-CSF in LPS-stimulated RAW 264.7 murine macrophages in a dose dependent manner. The another findings, the MEMS exhibited potent antiasthmatic property through significantly reduced the AHR, inhibition of inflammatory cells infiltration in the BALF of asthmatic mice, decreased EPO activity, PGE₂, NO & ROS generation in BALF and also reduced goblet cell hyperplasia, mucus hyperproduction in lung tissues. This is the first report to evaluate the antiasthmatic ability of the MEMS in OVA induced asthmatic mouse model and in RAW murine macrophages.

Macrophages play a crucial role in inflammation and are main targets for the action of lipopolysaccharides (LPS). LPS-treated RAW 264.7 murine macrophages have been widely used as a model for studying the inflammatory responses (Wynn & Barron., 2010). When RAW 264.7 murine macrophages are activated by LPS, the inflammatory mediators such as NO as well as pro-inflammatory mediators such as iNOS were markedly up-regulated (Blatteis et al. 2005). NO may played a governing role at every stage of the development of inflammation. As a result, its embarrassment may be an important target in the treatment for chronic inflammation (Guzik et al., 2003). In this study, we found that the MEMS exhibited a significant inhibitory activity upon LPS-induced NO production in RAW 264.7 murine macrophages.

IL-1 α , IL-1 β and TNF- α are prototypic pro-inflammatory cytokines exerting pleiotropic effects on an assortment of cells and play significant roles in acute and chronic inflammatory disorders (Dinarello., 2010). Therapeutic blockade of TNF- α is highly beneficial in the treatment of chronic inflammatory conditions (Tracey et al. 2008). Interferon (IFN)- γ is another pleiotropic nature of cytokines and is considered a pro-inflammatory cytokine because it augments TNF- α activity and induces nitric oxide (NO) which produces tissue damage at the site of inflammation. In addition to that the IL-1 and TNF- α acted synergistically in this process and initiate the cascade of inflammatory mediators by targeting the endothelium. Contrarily, the anti-inflammatory cytokines,

including IL-4, IL-10 and IL-13 blocks this process and consequently, suppress the production of IL-1, TNF- α and vascular adhesion molecules. IL-1 and TNF- α are being readily produced at the site of local inflammation and stimulate the synthesis of prostaglandin (PGE₂) produces the pain. The important biological function of IL-1 is the induction of phospholipid A2 (PLA₂) and COX-2 gene expression (Charles & Dinarello., 2000). IL-6 augments the differentiation of Th17, mainly produces IL-17 and is responsible for the development of various autoimmune disorders in mice (Nishihara et al., 2007). IL-6 antibody is under clinical development for the treatment of many autoimmune diseases. So, the blockade of IL-6 and IL-17 signalling is an important therapeutic target for developing new anti-inflammatory drugs (Jones et al., 2011, Miossec & Kolls., 2012). IL-12 is a heterodimeric cytokine mainly involved in phagocytic mechanism and it promotes cytokine production, particularly IFN- γ , in T and NK cells, and many of biological effects of IL-12 are, at least in part, mediated through IFN- γ (Manetti et al., 1993). Our data showed that the MEMS exhibited dose dependent reduction of IL-6, IL-12, IL-17A and GM-CSF production in LPS-induced RAW 264.7 murine macrophages. IL-4 is a pleiotropic cytokine that contributes in the regulation of the immune system and it is a growth and survival factor for lymphocytes. The significant biological function of IL-4 is promoting Th2 cell differentiation whereas inhibiting Th1 cell differentiation. In contrast to allergic diseases, autoimmune diseases are mainly due to Th1 and monocytic cytokines. IL-4 can therefore act as an anti-inflammatory cytokine as evidenced by its protective effect in murine models of rheumatoid arthritis and Diabetes. Therefore, IL-4 could be considered a target for the treatment of allergic diseases like asthma but is a potential tool for the treatment of autoimmune diseases (Benedetti et al., 2000).

IL-10 is an anti-inflammatory cytokines which potentially inhibits itself the production of IL-1 α , IL-1 β , IL-6, IL-10, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF- α by activated monocytes/macrophages. The inhibitory effects of IL-10 on IL-1 and TNF- α production are crucial to its anti-inflammatory activities, because these cytokines often have synergistic activities on inflammatory pathways and processes and amplify these responses by inducing secondary mediator

such as chemokine, prostaglandins and PAF. In this study, in contrast to pro-inflammatory cytokine, this cytokines level was decreased in LPS-activated RAW 264.7 murine macrophages when compared with normal control. These present findings is direct evident that the anti-inflammatory property of these cytokines (IL-4, IL-10 and G-CSF).

In the present study, MEMS inhibited the LPS-induced production of NO, ROS and several proinflammatory cytokines. These cytokines have profound effects on the regulation of immune reactions, hematopoiesis, inflammation. In addition to the in vitro results, in vivo results suggest that MEMS suppressed the OVA-induced airway inflammatory responses by inhibiting the proinflammatory cytokine production and the expression of COX- 2 and iNOS in the lung tissues.

The protective effect of several plant extracts and plant derived compounds on airway inflammation have been previously reported (Tiwari et al., 2014; Kim et al., 2008). Traditionally, the leaves of *Myxopyrum serratum* taken with ghee are the best medicine to reduce the signs of asthma. Based on the traditional claim, the antiasthmatic effect of MEMS was evaluated using the OVA induced mouse model of allergic asthma. Lee et al., 2011, demonstrated that 80% ethanolic extract of *Petasites japonicus* (50, 250,500 mg/kg) significantly inhibited eosinophil infiltration and mucus hyper production, reduced ROS production and IL-5 release in lung tissues in a dose dependent manner which were support of our study.

Reactive oxygen species (ROS) play a major role in the asthma pathogenesis through direct contraction of airway smooth muscle produces airway hyper responsiveness (Hulsmann et al., 1994). The inflammatory cells, primarily eosinophils activated by external stimuli and produce increased amounts of ROS and RNS. (Vachier et al., 1992). An additional source of ROS for the period of inflammation is the activation of eosinophil resulting the release of eosinophil peroxidase (EPO) and oxidative damage to proteins via the bromination of tyrosine (Mitra et al., 2000). Consequently the EPO release causes increased production of peroxynitrite by the interaction of HOBr with NO leads to depletion of intracellular NO storage can also induce hyper responsiveness

571 (Foster et al., 2003). Similarly, reactive nitrogen species (RNS) can also produce cellular injury
572 through nitrosative stress. Inducible nitric oxide synthase (iNOS) is responsible for nitrosative
573 stress, as it generates huge amounts of nitric oxide (NO) in response to environmental stimuli. NO
574 is a gaseous signalling molecule involved in a range of biological processes. In response to
575 proinflammatory cytokines and lipopolysaccharides, epithelial cells and T lymphocytes produce NO
576 through iNOS. NO reacts with superoxides to produce the highly reactive peroxynitrites that have
577 been shown to produce airway inflammation (Ricciardolo et al., 2006).

578 In this study, the OVA sensitized/challenged mice showed the marked increasing number of
579 inflammatory cells, particularly eosinophils, in the BALF. However, the MEMS significantly
580 reduced the number of inflammatory cells compared with the OVA-sensitized/challenged mice.
581 These findings indicate that the treatment of MEMS suppresses the eosinophilia induced by OVA
582 challenge. In addition, the antiasthmatic effect of MEMS strongly consistent with the results of
583 histological analysis of the lung tissues. Previous studies evidenced that the asthmatic mice
584 exhibited the inflammation due to infiltration of inflammatory cells into the airway and mucus
585 overproduction with goblet cell hyperplasia (Lee et al., 2011). These important features of asthma
586 closely associated with Th2 cytokines, which play significant roles in expressing B-cell
587 development, differentiation and secretion of IgE, which activates mucus hypersecretion and goblet
588 cell hyperplasia (Oh et al., 2006). Furthermore the increased level of serum OVA-specific IgE is
589 provided as a characteristic of the Th2-driven immune response (Bao et al., 2009). The present
590 study, the OVA-sensitized/challenged mice exhibited markedly increased inflammatory cell
591 infiltration in peribronchiolar and perivascular lesions and mucus overproduction with goblet cell
592 hyperplasia in epithelial lesions. In contrast, the MEMS treated mice exhibited a reduced
593 inflammatory response and mucus production compared to the OVA-sensitized/challenged mice in
594 a dose dependent manner.

595 The airway inflammation is mainly related with increased production of T-helper-2 (Th2)
596 cells, they secrete interleukins 4, 5, and 13 (IL-4, IL-5 and IL-13) which ultimately increased the

production of IgE and eosinophilic inflammation. The significance of Th2 cells in the asthma pathogenesis was direct evidence by murine models of AHR (Lee et al., 2011). After allergen exposure into the airway, IL-4 is immediately produced from T cells and activation of B cells was happen, ultimately leads to increased secretion of IgE, this is a central pathway of allergen specific immune response in asthma. IL-5 is significantly involved in the proliferation, differentiation, recruitment and prolonged survival of eosinophils. The eosinophil has been recommended that it is a key effector cell plays a vital role in chronic inflammation of asthmatic airway. (Sakuishi et al., 2007). Nowadays, IL-13 is an important therapeutic target for the treatment of allergic asthma, because it induces airway hyper responsiveness (AHR) and several structural alterations in chronic asthma, including goblet cell hyperplasia, airway smooth muscle proliferation, and subepithelial fibrosis (Wills-Karp, 2004). The concentration of these cytokines (IL-4, 5 and IL-13) was observed as the elevated level in the OVA induced asthmatic mice, whereas the MEMS treated mice significantly reduced the concentration of IL-4, IL-5, IL-13 and IgE in the BALF.

The antioxidant (sheelarani et al., 2013) and anti-inflammatory (sheelarani et al., 2014) efficiency of the extract of *Myxopyrum serratum* have also been reported previously. The medicinally important pentacyclic triterpenoid ursolic acid was identified and reported from the methanolic extract of *Myxopyrum serratum* previously by (Sudharmini & Nair., 2008). It exhibited potent antiasthmatic activity through the reduction of Th2 cytokines and eosinophil infiltration in BALF (Kim et al., 2013). These are consistent with our study, additionally, the determination of more phenolic compounds were also examined from MEMS by RP-HPLC method.

The experimental data showed Naringenin, a predominant phenolic compound quantified in MEMS, which is known to possess excellent antiallergic property through the inhibition of iNOS and Th2 cytokine production in lung and thus, reduces airway hyper responsiveness against an OVA induced allergen model (Shi et al., 2009). Moreover, the plant is also found to possess higher concentration of quercetin, rutin, catechin that are known to exhibit potent antiasthmatic via

inhibition of the recruitment of eosinophils and neutrophils into the lung, the production of histamine, Phospholipase A₂ and Eosinophil peroxidase (Jung et al., 2007; Prochazkov et al., 2011). Similarly, the higher concentration of ferulic acid and *p*- coumaric acid, gallic acid was identified and reported in MEMS possessing potent antioxidant properties, it exhibited an excellent protection against oxidative damage caused by ROS produced in biological system (Zheng et al., 2001). Moreover, a iridoid glycoside were isolated and reported from the plant *M.serratulum* (Franzyk et al., 2001), the several author reported that the iridoid glycoside exhibited wide pharmacological activities especially anti-inflammatory, neuroprotective, antioxidant and antiallergi (Tundis et al., 2008). The presence of these bioactive compounds in the MEMS attributed the potent antioxidant and antiasthmatic property of the plant *M. serratulum*.

iNOS plays an central role in airway disease, including allergic asthma. Numerous studies have confirmed, iNOS expression was increased in the airway epithelium of asthmatic patients and releases toxic radical NO, which can cause tissue and cell damage. The increased production of NO might recruit eosinophils into the asthmatic airway following in the augmentation of airway inflammation. The amplified iNOS expression may play an important role in inflammation of the upper and lower airways, and may contribute to asthma. In addition, iNOS expression is closely associated with Th2 cytokines release in aggravating the asthmatic response, including inflammatory cell infiltration, airway constriction, and remodeling processes (Ricciardolo et al., 2006). Therefore, suppression of NO production via inhibition of iNOS expression might be an attractive therapeutic target for the treatment of inflammation. During asthma, COX-2 expression has also been increased by a number of stimuli including cytokines and is associated with inflammation. Accumulating evidence suggests that the stimulation and regulation of COX-2 may be key elements in the pathophysiological process of a number of inflammatory disorders and may play an important role in the pathogenesis of asthma (Redington et al., 2001). COX-2 is markedly induced in airway smooth muscle cells in vitro by proinflammatory cytokines and other mediators that exist in asthmatic airways, it is reasonable to speculate that COX-2 expression in airway

smooth muscle is also up regulated in asthma. PGE₂, the foremost product of COX-2 stimulation, is a key anti-inflammatory mediator which has considerable bronchoprotective effects in the airways. It is possible that PGE₂ production as a result of COX-2 generation may reduce the inflammatory process in asthmatic airways. However, PGE₂ at higher concentrations also causes contraction of airway smooth muscle via thromboxane receptors. Amplified COX-2 expression brings increased expression of PGE₂ which exacerbate inflammatory responses. In this study, MEMS significantly inhibited expression of iNOS and COX-2 in the lung tissue compared with the OVA-sensitized/challenged mice. These results were seemed consistent with the histological examination. MEMS also reduced the inflammatory cell infiltration into the airway against OVA challenge. In addition, MEMS produced a significant reduction in NO, and cytokine production in LPS-stimulated RAW 264.7 murine macrophages. Therefore, these results recommend that the reduction in iNOS and COX-2 expression is one possible mechanism by which MEMS attenuates the airway inflammation in experimentally induced allergic asthma.

5. Conclusion

This study has provided the first experimental evidence of the effect of MEMS on OVA induced airway inflammation in a mouse model of allergic asthma and the identification and quantification of phenolic compounds using RP-HPLC analysis. The data indicating that the MEMS containing a large amount of phenolic compounds could significantly reduced the characteristic features of asthmatic inflammation through suppression of EPO, NO, PGE₂ & ROS production and IL-4, IL-5 & IL-13 in BALF and IgE level in serum. These effects are deliberated to be concomitant with the down-regulation of iNOS and COX-2 expression. Therefore, MEMS plays numerous roles as an anti-inflammatory and anti-asthmatic activity via the regulation of several inflammatory & proinflammatory mediators. Therefore, *Myxopyrum serratum* as a therapeutic alternative for treating inflammatory diseases, including asthma. Further studies may be desirable to describe the inhibitory mechanism of MEMS.

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Tables and figures

Table. 1: Quantification of phenolic compounds in the methanolic extract of *M.serratum* by RP- HPLC analysis

Peak no	R _t of std (min)	R _t of MEMS (min)	Analyte	Mol formula	Concentration (mg/mL)	Detection (λ)
1	11.33	11.31	Gallic acid	C ₇ H ₆ O ₅	1.34±0.05	280
3	17.67	17.59	Catechin	C ₁₄ H ₁₄ O ₆	6.10±0.03	280
4	27.63	27.86	Ferulic acid	C ₁₀ H ₁₀ O ₄	40.54±0.54	280
5	28.66	28.66	Rutin	C ₂₇ H ₃₀ O ₁₆	57.66±0.75	280
6	30.02	30.02	<i>p</i> -coumaric acid	C ₉ H ₈ O ₃	3.054±0.04	280
7	31.77	31.59	Quercetin	C ₁₅ H ₁₀ O ₇	35.91±0.42	280
8	32.07	31.92	Naringenin	C ₁₅ H ₁₂ O ₅	100.20±0.69	280
9	37.17	37.26	Apigenin	C ₁₅ H ₁₀ O ₂	2.12±0.02	280

Each value represents the mean ±SD (n=3)

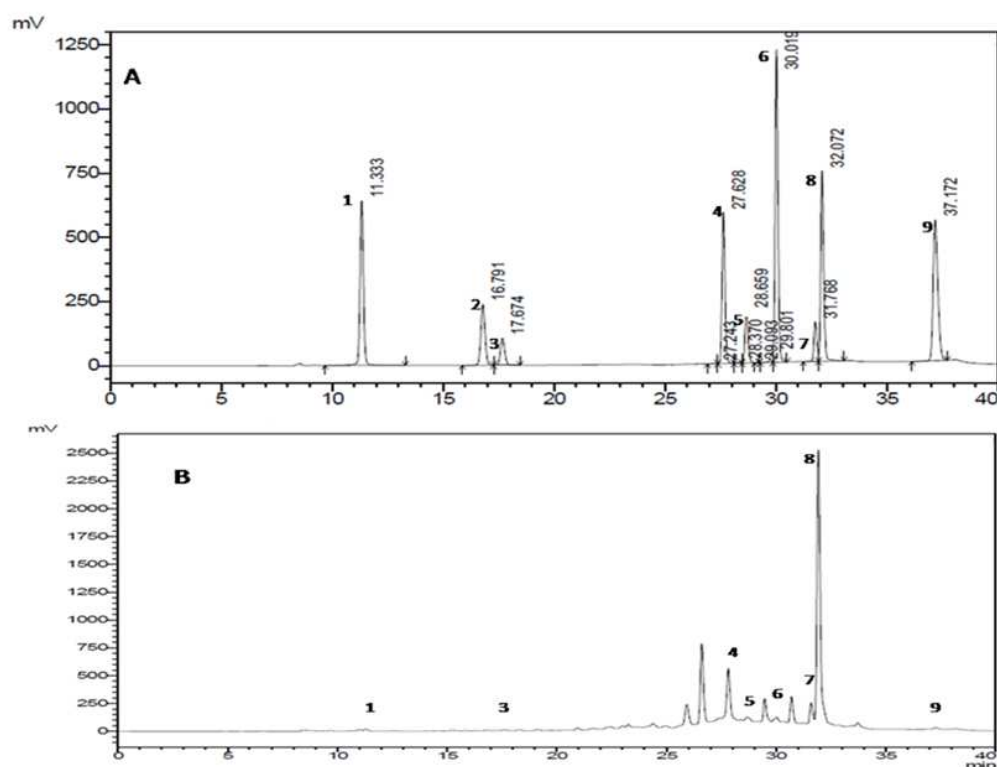


Figure. 1 (A); HPLC chromatogram of standard phenolic compounds. The peak number is indicated as follows: 1- gallic acid, 2- protocatechuic acid, 3- catechin, 4- ellagic acid, 5- rutin, 6-*p*-coumaric acid, 7-quercetin,

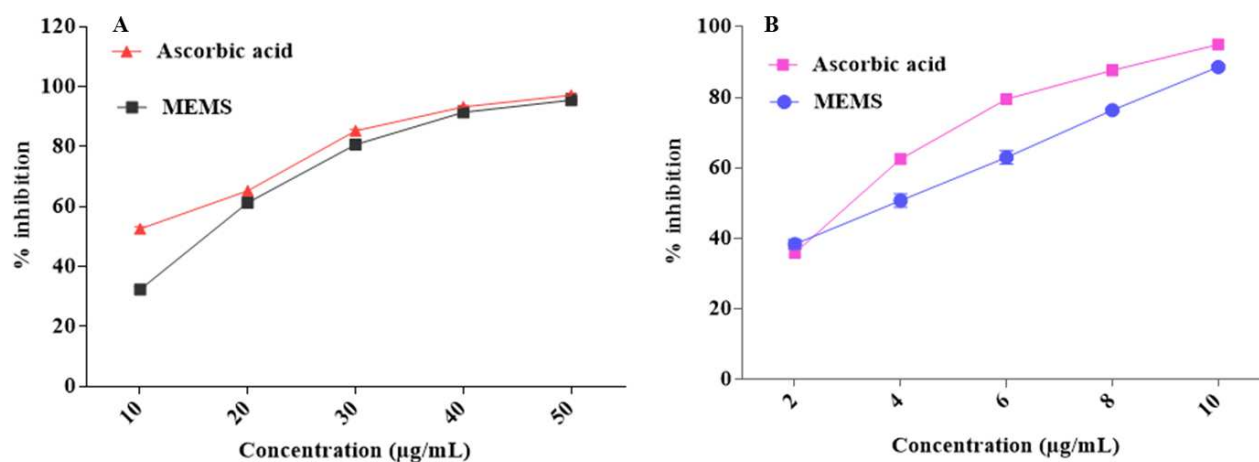


Figure. 2 (A); Percentage inhibition of DPPH radical scavenging activity of methanolic extract of *M.serratum*. (B); Percentage inhibition of ABTS radical scavenging activity of methanolic extract of *M.serratum*. Each value represent the mean $\pm$ SEM, n=3.

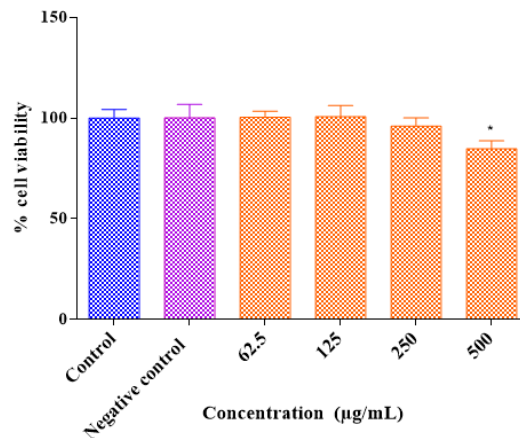


Figure. 3 Effect of MEMS (62.5-500 µg/mL) on the viability of RAW 264.7 murine macrophages was determined by MTT assay after 24h incubation. Control-untreated cells, negative control-DMSO (0.02%) treated cells. Each value represents mean $\pm$ SEM (n=3).

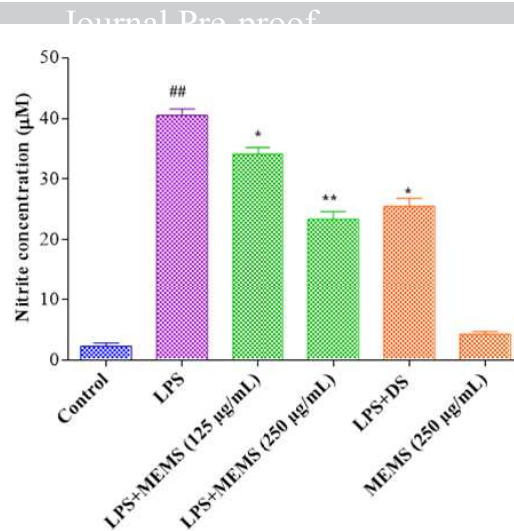


Figure. 4 Effect of MEMS on the production of nitric oxide on LPS-induced RAW 264.7 murine macrophages

The control group was treated with media only. The LPS group was treated with Lipopolysaccharide 1µg/mL alone. DS (5 µg/mL). Values are mean ± SEM of the three independent Experiments. ^{##}*p*<0.01 was considered statistically significant for LPS-stimulated cells when compared to control cells; *p*<0.05 for MEMS (125 µg/mL) & DS vs LPS group; *p*<0.01 for LPS treated vs MEMS (250 µg/mL).

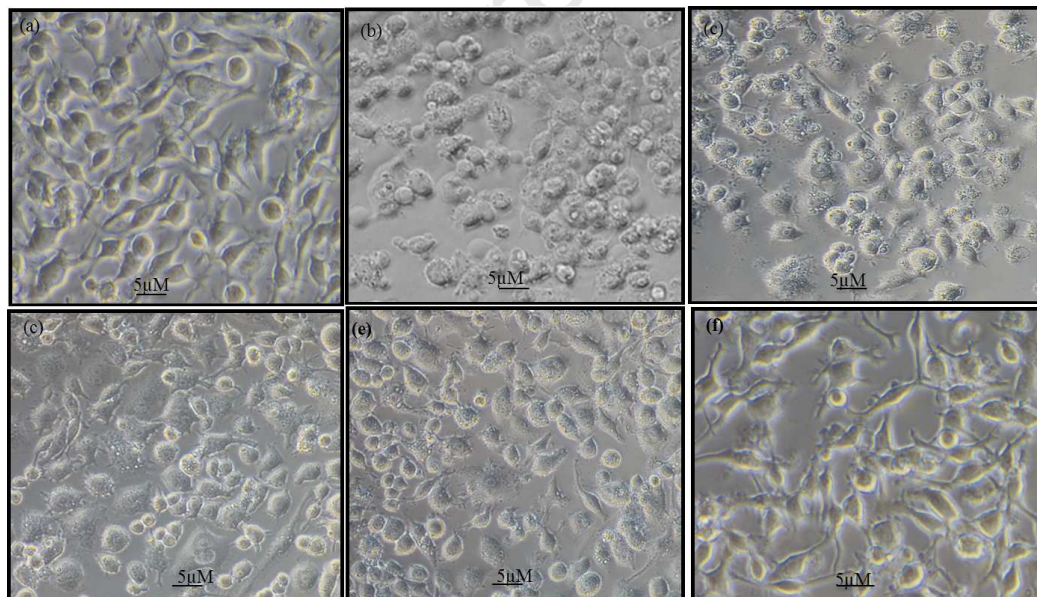


Figure. 5 Effects of MEMS on morphological changes of RAW 264.7 murine macrophages after LPS induction

Morphological observation of MEMS on treated RAW 264.7 murine macrophages in the presence of LPS (1 µg/mL) after 24h of incubation. (a) untreated RAW 264.7 murine macrophages as control; (b) LPS (1 µg/mL); (c) LPS+ DS (5 µg/mL); (d) LPS+MEMS (125 µg/mL); (e) LPS+MEMS (250 µg/mL) (f). MEMS (250 µg/mL). All the images were acquired at 40X magnification (Scale bar = 5µM).

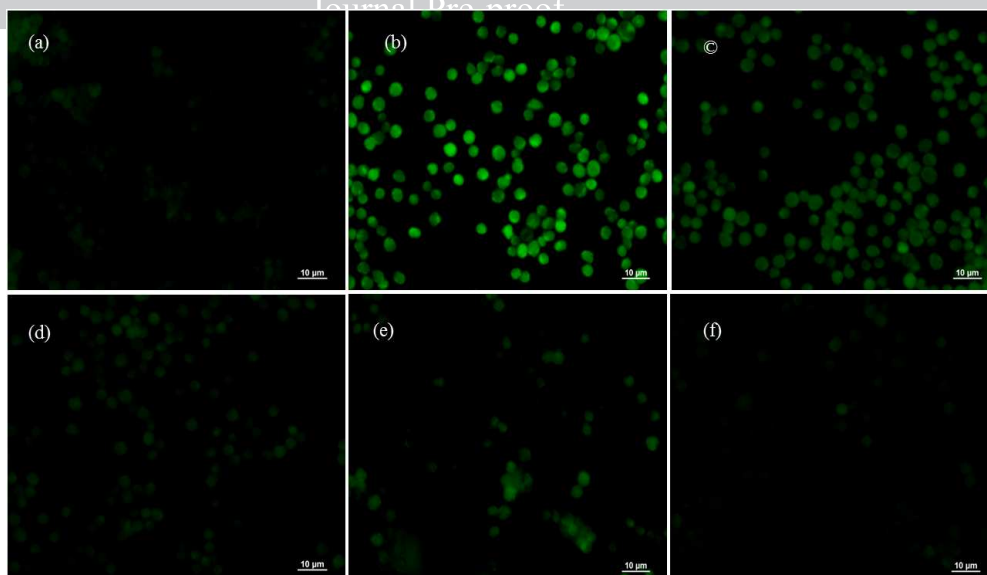


Figure. 6 Effects of MEMS on ROS accumulation in RAW 264.7 murine macrophages examined by DCFH₂-DA fluorescence assay

The green DCF fluorescence as a result of ROS production. (a) DCFH treated LPS unstimulated RAW 264.7 murine macrophages as control; (b). LPS (1 µg/mL) treated cells; (c) LPS+ DS (5 µg/mL); (d) LPS+MEMS (125 µg/mL); (e) LPS+MEMS (250 µg/mL) (f). MEMS (250 µg/mL). Magnification 40X, (Scale bar =5µM).

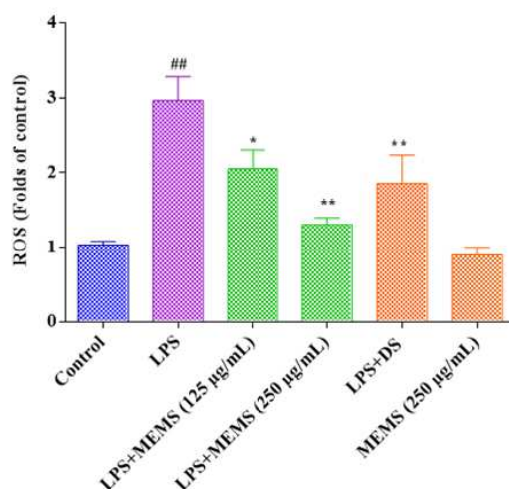


Figure. 7 Quantification of intracellular ROS in fold increase compared to control by measuring DCF fluorescence intensity assay. DS, diclofenac sodium (5 µg/mL); LPS, Lipopolysaccharide (1µg/mL). Values are the mean ± SEM of the three independent experiments. ##*p*<0.01 was considered statistically significant for LPS- stimulated cells when compared to control cells; **p*<0.05 for LPS-stimulated DS and LPS-stimulated MEMS when compared to LPS alone.

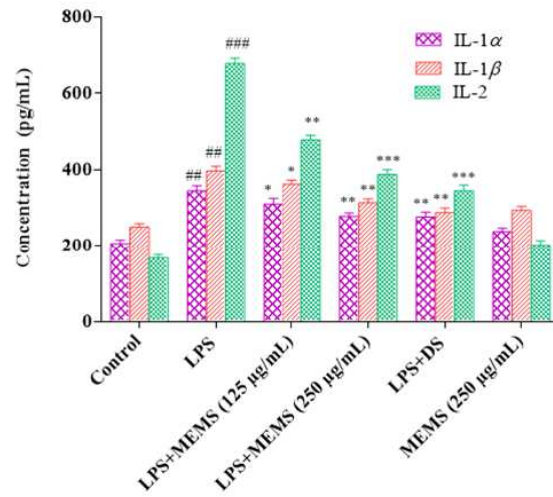


Figure. 8

Effects of MEMS on pro-inflammatory cytokines (IL-1 α , IL-1 β and IL-2). The Data presented are mean $\pm$ SD of three independent experiments. Significant differences between i) non-stimulated control vs LPS group (^{##} $p < 0.01$); ii) significant difference between LPS vs MEMS treated group ($p < 0.05$) and iii) significant differences between DS vs LPS-treated group ($p < 0.01$) were observed.

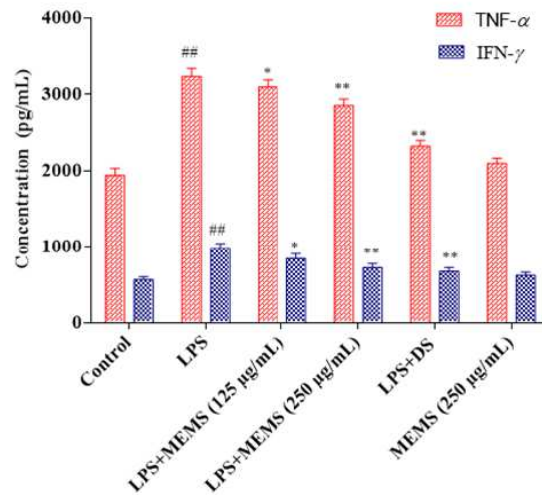


Figure. 9

Effects of MEMS on pro-inflammatory cytokines (TNF- α and IFN- γ). The Data presented are mean $\pm$ SEM of three independent experiments. Significant differences between non-stimulated control vs LPS group (^{##} $P < 0.01$); LPS vs MEMS (125 μ g/mL) treated group ($p < 0.05$) and MEMS (250 μ g/mL) treated group, DS vs LPS-treated group ($p < 0.01$) was observed.

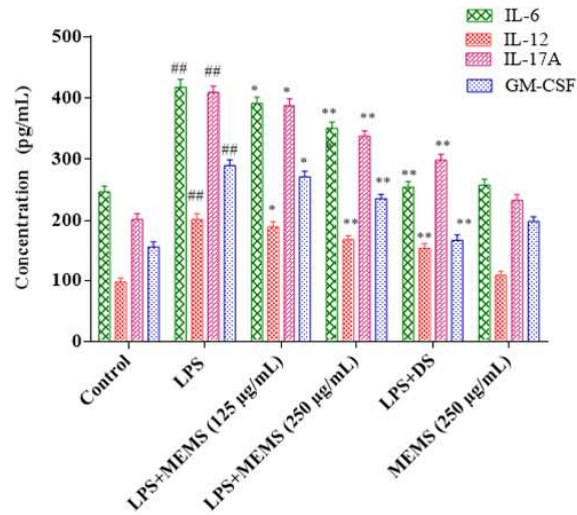


Figure. 10

Effects of MEMS on pro-inflammatory cytokines (IL-6, IL-12, IL-17A and GM-CSF). The Data presented are mean $\pm$ SEM of three independent experiments. Significant differences between non-stimulated control vs LPS ($^{##}p < 0.01$) and LPS vs MEMS treated group ($^{*}p < 0.05$, $^{**}p < 0.01$) and DS vs LPS-treated group ($^{**}p < 0.01$) was observed.

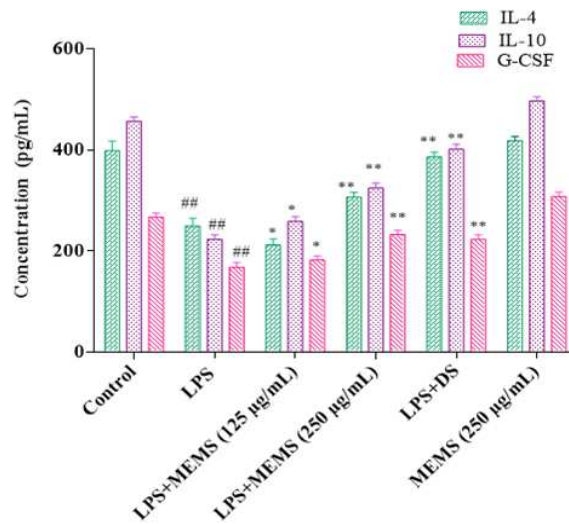


Figure. 11

Effects of MEMS on anti-inflammatory cytokines (IL-4, IL-10 and G-CSF)

RAW 264.7 murine macrophages were treated with indicated concentrations of MEMS for 24h and then stimulated with 1µg/mL of LPS for 12h. IL-4, IL-10 and G-CSF levels in supernatants were analysed by ELISA. The Data presented are mean $\pm$ SEM of three independent experiments. Significant differences between non-stimulated Control vs LPS ($^{##}p < 0.01$) and LPS vs MEMS treated group ($^{*}p < 0.05$) and DS vs LPS-treated group ($^{**}p < 0.01$) was observed.

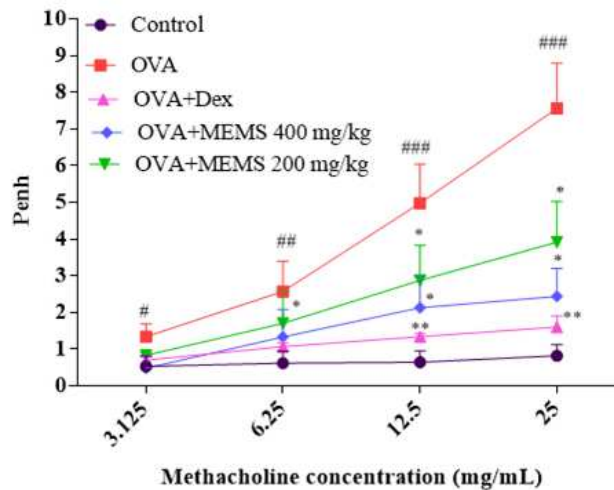


Figure. 12

Effect of MEMS on airway hyperresponsiveness (AHR) to methacholine in OVA-induced mice (n=6). Mice were given aerosolized saline and various doses of methacholine to evaluate the enhanced pause (Penh). Control (PBS only); OVA, OVA-sensitized/challenged mice; Dex, dexamethasone (3 mg/kg)+OVA sensitized/challenged mice; MEMS+OVA sensitized/challenged mice. All data were expressed as mean±SEM. Significant difference from PBS group vs OVA (### $p<0.001$); OVA vs MEMS (400 mg/kg) & Dex treated group (* $p<0.01$) and MEMS (200 mg/kg) vs OVA-treated group (* $p<0.05$) was observed.

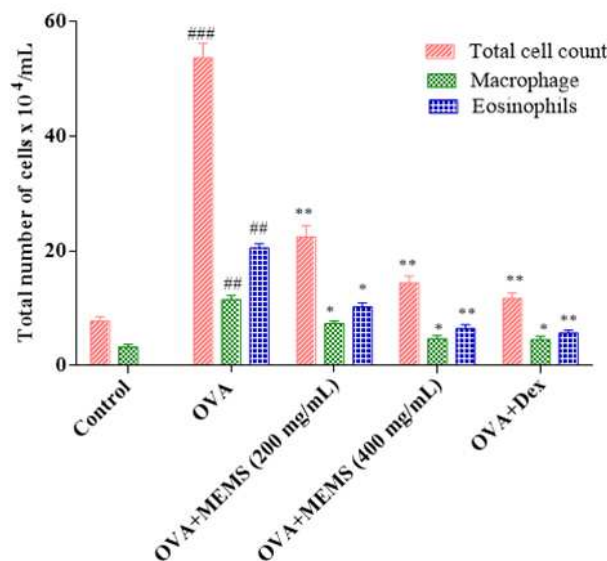


Figure. 13

Effect of MEMS on the recruitment of inflammatory cells in BALF obtained from the mice 48h after the final OVA challenge (n=6). After staining with trypan blue, the total cell numbers were assessed by counting with a haemocytometer. Control (PBS only); OVA, OVA sensitized/challenged mice; Dex, dexamethasone (3 mg/kg). Significant difference from PBS group vs OVA (### $p<0.001$); OVA vs MEMS (400 mg/kg) & Dex treated group (*** $p<0.001$) and MEMS (200 mg/kg) vs OVA-treated group (** $p<0.01$) was observed.

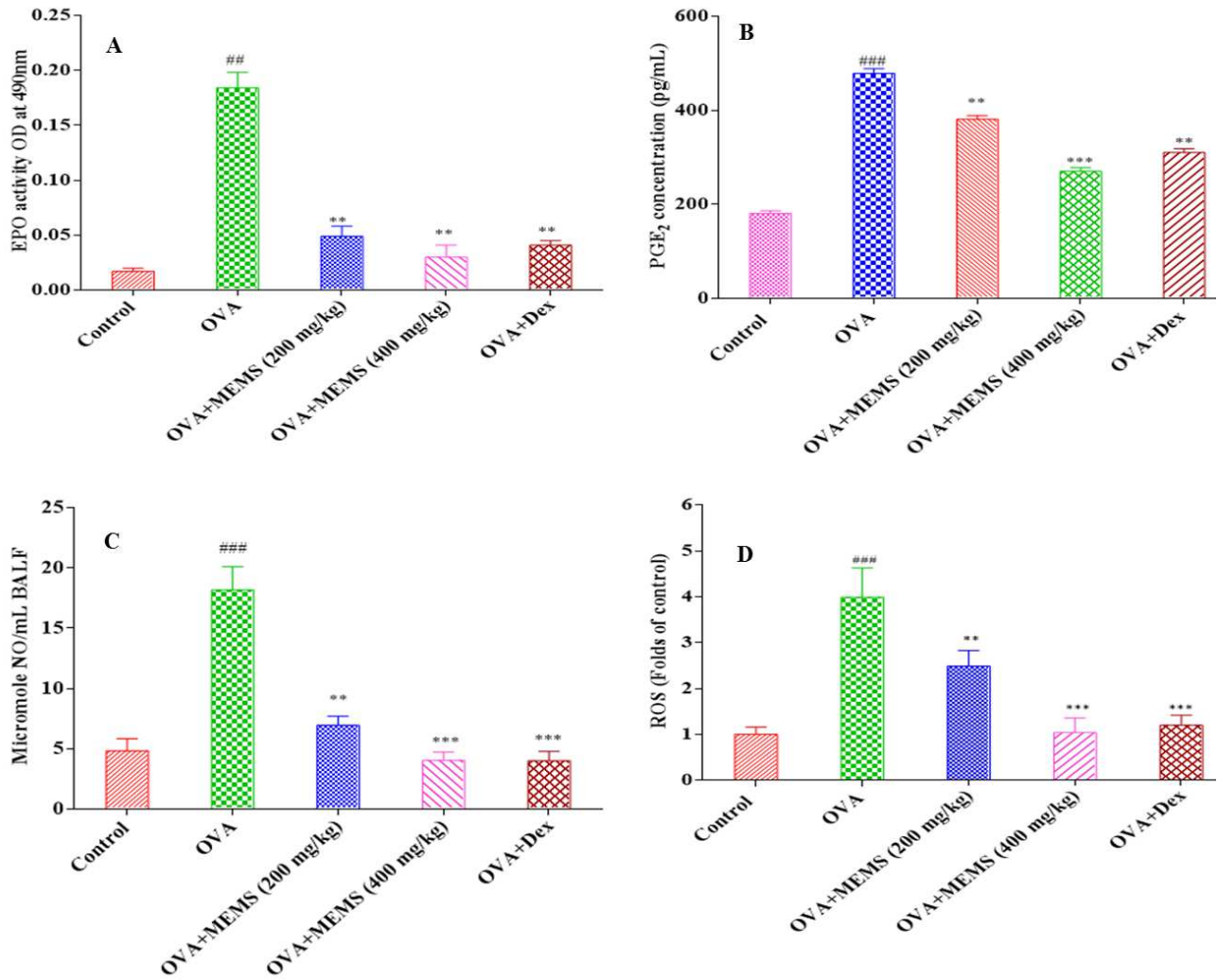


Figure. 14 (A): Inhibitory effect of MEMS (200 & 400 mg/kg) on Eosinophil peroxidase (EPO) activity in BALF, (B): The effect of MEMS (200 & 400 mg/kg) on inhibition PGE₂ production in BALF. (C): The effect of MEMS (200 & 400 mg/kg) on inhibition NO production in BALF. *** $p < 0.001$. (D): The intracellular ROS in the BALF were determined based on DCF fluorescence assay. Control (PBS only); OVA, OVA sensitized/challenged mice; OVA+Dex, Dexamethazone 3mg/kg treated mice. Each value represented as mean $\pm$ SEM, significant difference were observed from PBS group vs Dex treated (** $p < 0.01$) and PBS vs OVA treated group (### $p < 0.001$). (** $p < 0.01$); MEMS 200 mg/kg vs OVA & (** $p < 0.001$); MEMS 400 mg/kg vs OVA treated group.

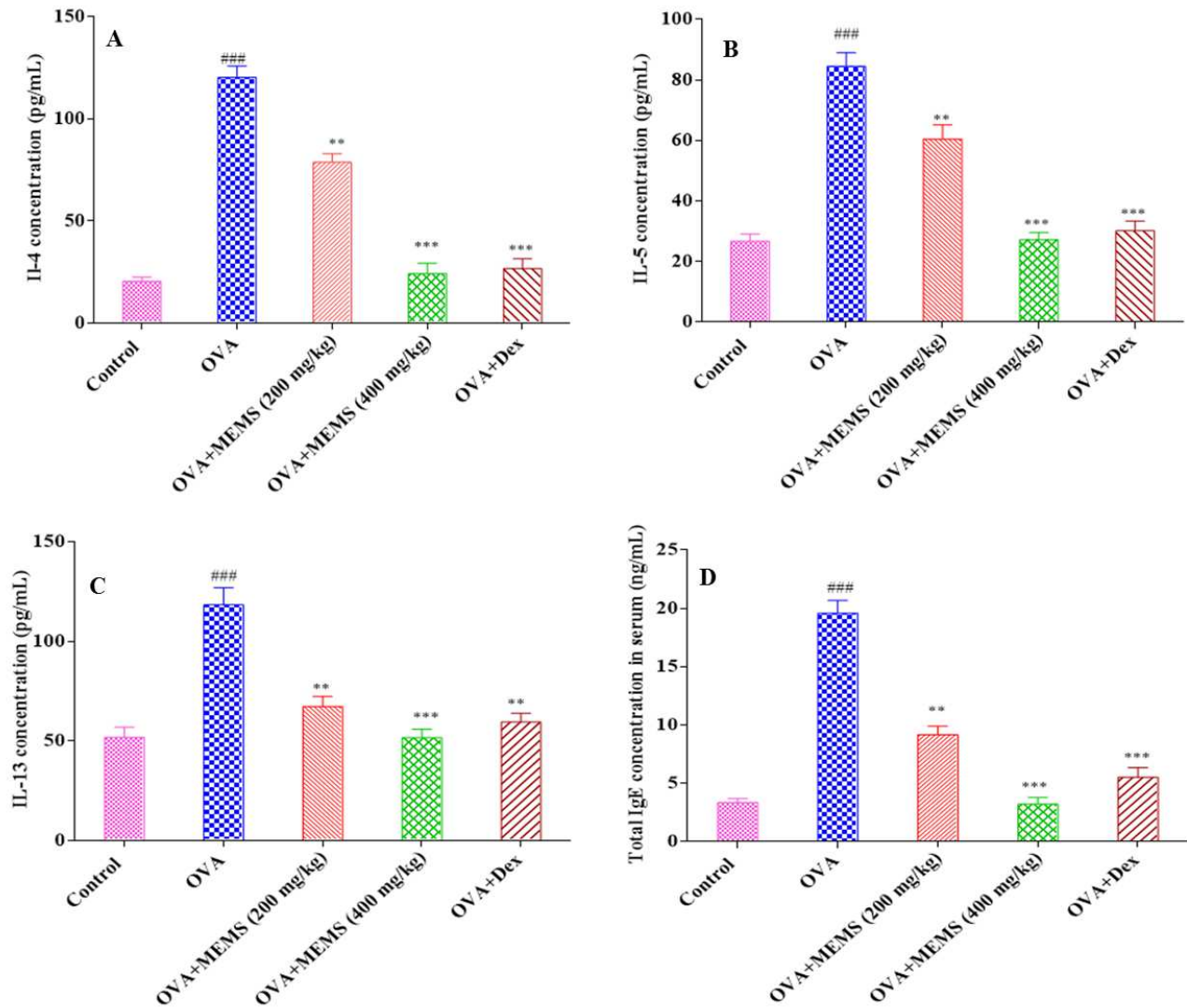
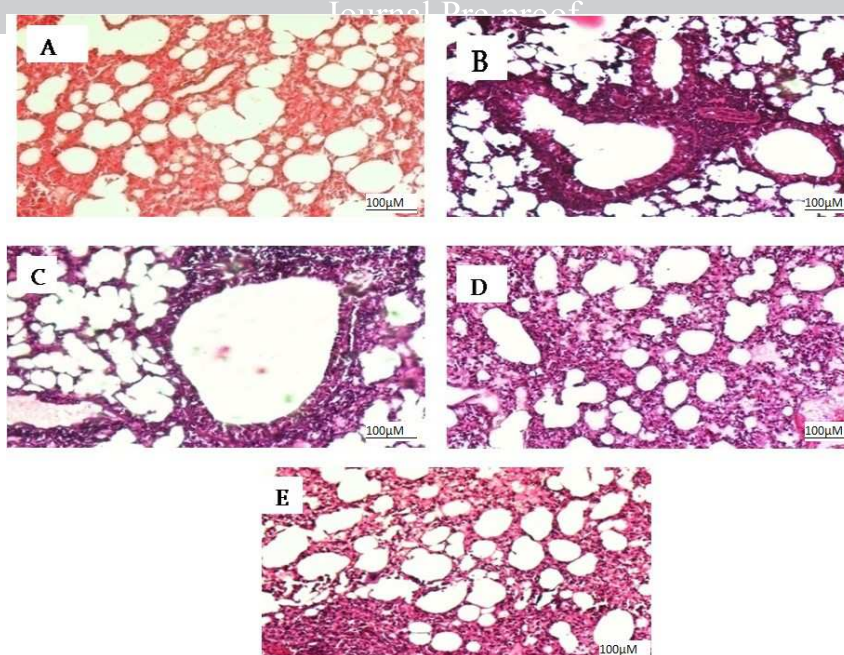


Figure. 15 BALF was collected 48h after the last OVA challenge. The effects of MEMS on (A) IL-4; (B) IL-5; (C) IL-13 levels in BALF and (D); IgE levels in serum samples were analyzed using ELISA kits. Control (PBS only); OVA, OVA-sensitized/challenged mice; Dex, Dexamethasone (3 mg/kg); MEMS 200 & 400 mg/kg. Each value represents as mean $\pm$ SEM with $n=6$ in each group, (^{###} $p<0.001$), a significant differences between OVA-treated and PBS-treated group. (^{**} $p<0.01$) & (^{***} $p<0.001$), significant differences between OVA vs drug treated group.



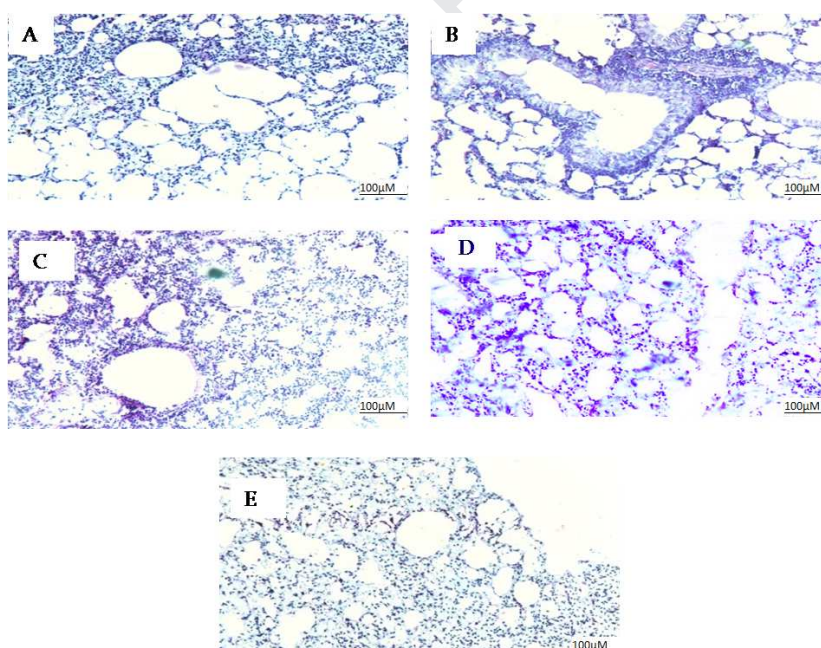
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1039 **Figure. 16**

Effect of MEMS on histopathologic changes in the lung tissues (H&E Staining)

The effect of MEMS on histopathological changes in lung tissues of OVA-challenged allergic mice was observed. Inflammation was evaluated in the lung tissues by analyzing inflammatory cell infiltration using H&E staining; A, PBS-challenged mice; B, OVA-challenged mice; C, 200 mg/kg of MEMS treated mice; D, 400 mg/kg of MEMS treated mice and E, Dexamethasone 3 mg/kg treated mice. Magnification 20X, (scale bar = 100µM).

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The effect of MEMS on histopathological changes in lung tissues of OVA-challenged allergic mice using PAS staining indicating the mucus production; A, PBS-challenged mice; B, OVA-challenged mice; C, 200 mg/kg of MEMS treated mice; D, 400 mg/kg of MEMS treated mice and E, Dexamethasone (3 mg/kg) treated mice. Magnification 20X, (scale bar = 100 µM).

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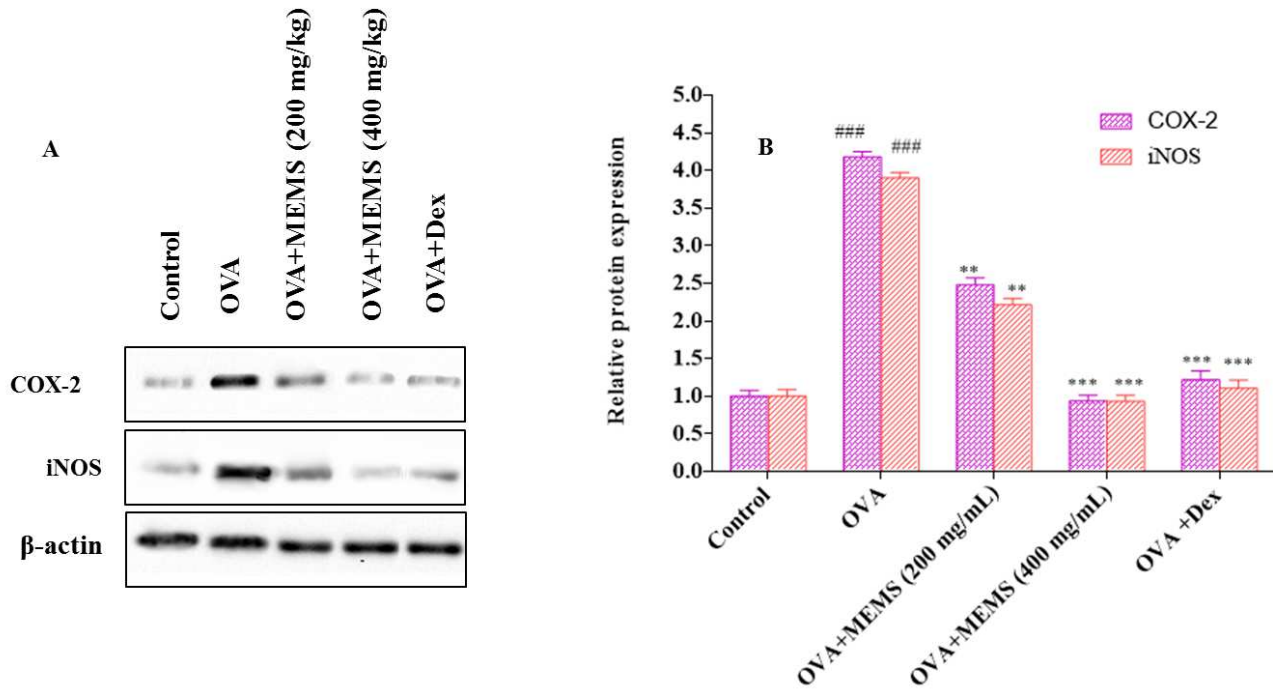
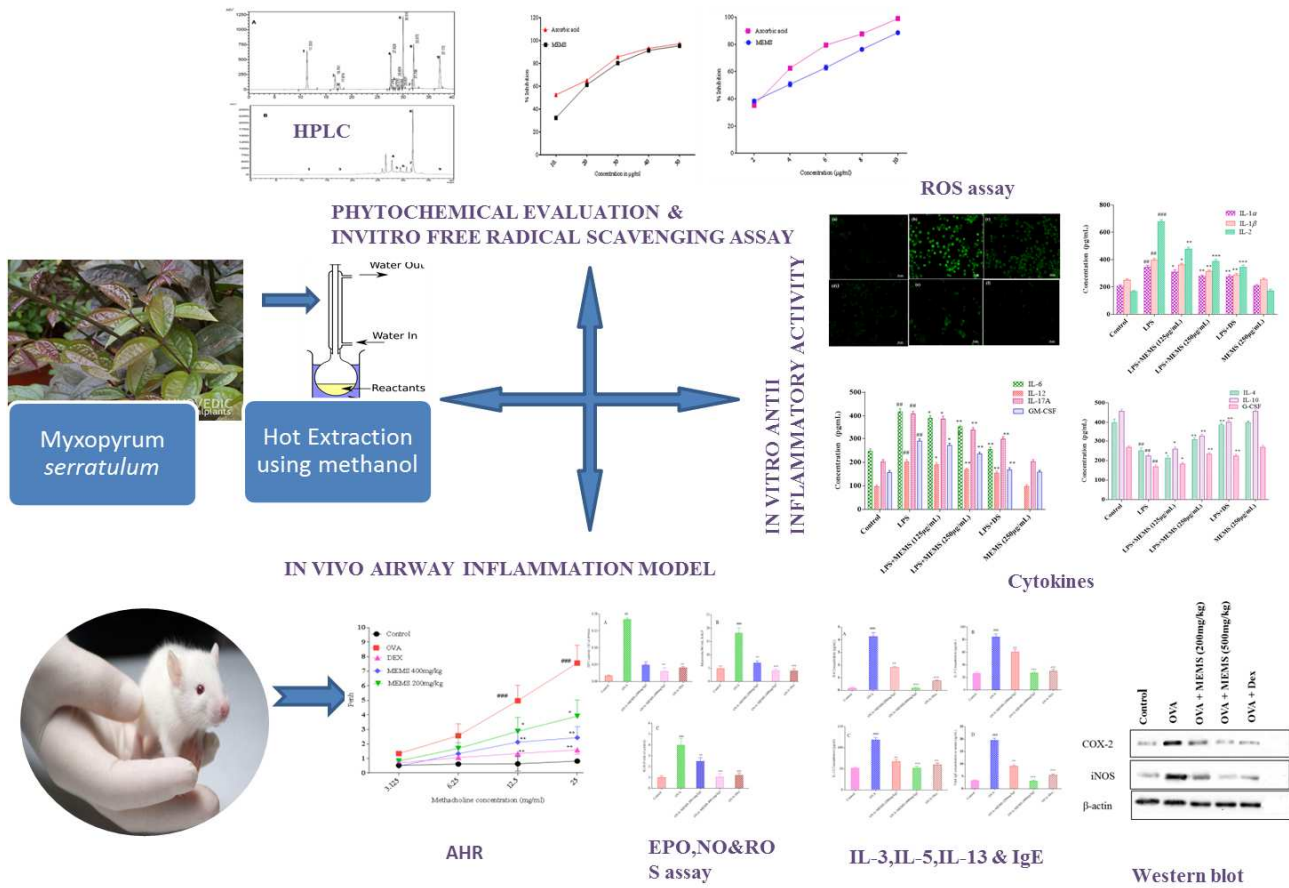


Figure. 18

MEMS inhibits the expression of proinflammatory proteins in the lung tissue that induced by OVA challenge. (A), Expression of iNOS and COX-2 in the lung tissue. (B), Relative protein expressed as the ratios of the densities of the proinflammatory mediators to β -actin. ###Significantly different from control vs OVA-sensitized/challenged mice, ($**P \leq 0.01$); significantly different from the OVA vs MEMS 250 μ g/mL. ($***P \leq 0.001$); significantly different from the OVA vs MEMS 500 μ g/mL.

Graphical abstract



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