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Research Article

Anti Asthmatic Effect of Finger Millet Bran

Phenolic Rich Extract Of Finger Millet Bran Attenuates Lung Inflammation And Fibrosis In A Mouse Model Of Ovalbumin Induced Asthma

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Abstract: Asthma is a serious global health risk characterized by chronic airway inflammation of lungs with infiltration of inflammatory cells, enhanced mucus secretion and narrowing of airways that leads to poor respiratory functions. The clinical symptoms of asthma includes recurrent episodes of cough, shortness of breath, wheezing and chest tightness. One of the pathophysiology of asthma was mediated by overproduction of pro-inflammatory asthmatic leukotrienes through arachidonic acid pathway catalyzed phospholipase A_2 (PLA_2) and 5-lipoxygenase (5-LOX) enzymes. The available conventional therapies to treat asthma may induce severe side effects on health and high cost. To overcome the disadvantages to treat asthma and to improve the current therapeutic strategy, we used natural products as an alternative therapy. Finger millet (*Eleusine coracana*) has been used by folk and traditional medicinal practitioners to treat various inflammatory conditions. Previously, we have found the promising anti-inflammatory phytochemicals in finger millet bran (Fmb). Hence, aim of the present study was to identify and characterize anti-asthmatic phytochemicals from Fmb and the objective was to test its anti-inflammatory activity in ovalbumin induced asthma mouse model. Phytochemical analysis of Fmb revealed the presence of phenolics, saponins and anthraquinone. Among all the extracted fractions, finger millet bran methanol extract (*FbMe*) possessed strong *in vitro* anti-inflammatory activity by inhibiting pro-inflammatory PLA_2 and 5-LOX enzymes activity. Further, *in vivo* anti-inflammatory activity of *FbMe* was evaluated in an ovalbumin induced asthma mouse model. *FbMe* (50 mg/kg) significantly reduced the infiltration of inflammatory cells, lung fibrosis by reducing the deposition of collagen in the tissue. It also inhibited the PLA_2 as well as 5-LOX enzymes activity in collected BAL fluid. This finding concludes the presence of strong anti-inflammatory and anti-fibrotic phenolic compounds in the *FbMe*, which attenuates the lung inflammation and fibrosis probably via inhibition of PLA_2 and 5-LOX enzymes activity.

Keywords: Asthma, anti-inflammation, fibrosis, finger millet, phospholipase A_2 and 5-lipoxygenase.

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1. INTRODUCTION

Asthma is a non-communicable chronic inflammatory disease of the lungs characterized by reversible airway obstruction, infiltration of cells, increased mucus production, airway hyperresponsiveness and narrowing of airways.[1] Hyperresponsiveness of airways that leads to intermittent episodes of wheezing, chest tightness, breathlessness, and coughing.[2,3] Asthma affects people of different ethnic group, age and gender. WHO was estimated around 262 million people affected and caused 4,61,000 deaths by asthma worldwide in 2019.[4] The incidences of asthma increases with repeated exposure to pollutants, allergens, tobacco smoking and other factors.[5] Though, the pathogenesis and progression of asthma is mediated via various pathways, the eicosanoid pathway synchronized by phospholipases A_2 (PLA_2) and 5-lipoxygenases (5-LOX) plays a pivotal role.[6,7] Arachidonic acid released from the membrane phospholipids by the action of PLA_2 is further metabolized by 5-LOX to produce pro-inflammatory leukotrienes.[8] Of note, liberated leukotrienes were the major candidate in the progression of asthma.[9] Pathogenesis of asthma can be reduced with decreased release of pro-inflammatory leukotrienes by blocking the eicosanoid pathway through inhibition of PLA_2 and 5-LOX enzymes activity.[9] It is known that, nutraceuticals are safe and reasonable when compared to synthetic drugs in the treatment of various diseases.[10] Finger millet (*Eleusine coracana*) is extensively used as one of the main food crop in the region of Karnataka and Tamil Nadu due its health benefits.[11] Apart from nutritional importance, finger millet also considered as therapeutically important crop due to various biological activities such as antiulcer,[12] antioxidants,[13] anti-inflammatiory,[14,17] anti-diabetic,[15] and anti-cancer properties.[16] A number of therapeutically important compounds such as γ-oryzanol,[18] arabinoxylan,[19] xylan[20] and feruloyl arabinose[21] have been isolated from various part of the finger millet plant. Our attention was to isolate the therapeutically important compounds that existed within the bran. The outer cover of the seeds or cereals are considered as the bran, which was thrown away as it was not suitable for human consumption and used as fodder for animals. In recent past, researchers have identified, isolated and characterized various therapeutically important compounds from the bran of different cereals that grabbed the considerable attention of the scientific communities.[22] However, none of the recent studies have established the mechanism related to anti-inflammatory activity of finger millet bran. Considering this, the aim of this study was to test the hypothesis that bran extracts for the inhibition of PLA_2 and 5-LOX, and ovalbumin-induced airway inflammation and fibrosis in mouse model. Our results suggested the presence of anti-inflammatory molecules in finger millet bran extract. Further, complete purification of these molecules may lead to a novel potent anti-inflammatory compound which can be used to treat asthma-like inflammatory conditions.

2. MATERIALS AND METHODS

2.1 Materials

Arachidonic acid and 4-nitro-3-octanoyloxy-benzoic acid were purchased from Cayman Chemicals (Michigan, USA) and *abcam* Cambridge, UK, respectively. Ovalbumin (grade IV), bacterial lipopolysaccharide, adenosine triphosphate and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, USA). Finger millet bran was procured from a cereal processing unit. All the other chemicals and reagents used in this study were of analytical reagent grade.

2.2 Animals

Six weeks old male Swiss albino mice were obtained from Central Animal House Facility, Department of Studies in Zoology, University of Mysore, Mysuru, India. The animal care and handling were conducted in compliance with National Regulations for Animal Research. The animal experiments were carried out after reviewing the protocols by the Animal Ethical Committee of the University of Mysore, Mysuru, India (Order No: UOM/IAEC/13/2018).

2.3 Extraction and analysis of phytochemicals

Finger millet bran (Fmb) was filtered to remove the seeds and other contaminants. About 20 g of Fmb was hot extracted using soxhlet apparatus according to Yadav et al. (2011) with slight modifications.[23] The phytochemicals were extracted in different solvent such as n-hexane, ethyl acetate, methanol and distilled water. The obtained extracts were filtered and dried in a flash evaporator and the dry weight and percent yield was calculated. Extracts were re-dissolved in respective solvents and diluted serially in phosphate buffer for further use. From obtained extracts the phytochemicals were qualitatively analysed as describe by Verma et al., (2020).[24]

2.4 Ovalbumin induced asthma and treatment regimen

Asthma was induced as described by Hoffman et al., (2013) with slight modifications.[25] Six weeks old male Swiss albino mice were taken for the ovalbumin induced asthma and they were divided into 5 groups (n=5, each group). Group 1: control, group 2: ovalbumin challenged, group 3: ovalbumin challenged plus 5 mg/kg finger millet bran methanol extract (*FbM*e), group 4: ovalbumin challenged plus 50 mg/kg *FbM*e and group 5: vehicle control (0.5 % DMSO). The framed asthma induction procedure was 23 days long and it was divided into two stages; sensitization and challenge phase. Mice were sensitized with 150 µg of ovalbumin and 100 ng of LPS suspended in 40 µl of sterile PBS on *day 0* and boosted again on *day 7*. Mice were then challenged using four to five doses of aerosolized 1% ovalbumin in sterile PBS consecutively on *days 14-18*. Treatment groups of mice were administered with *FbM*e (5 and 50 mg/kg body weight) via intraperitoneally on *days 19-22*. Control group received sterile PBS (vehicle) all the time. Mice were sacrificed 24 h following the final *FbM*e treatment.

2.5 Isolation of bronchoalveolar lavage (BAL) fluid

BAL fluid was isolated as described by Hoffman et al. with slight modifications.[25] The mice were euthanized after treatment regimen by administration of ketamine and xylocaine and brought to a biosafety cabinet. Mice were placed front side up on a Styrofoam panel and the arms and legs were fixed with needles, scissors were used to make incision in the skin from abdomen to neck and skin was torn apart with forceps to expose thoracic cage and neck. The muscle around the neck was gently removed to expose trachea and with help of a forceps ~10 cm long surgical nylon string under the trachea was inserted carefully, then a 22-gauge Exel Safelet Catheter was inserted into the trachea and

the stylet hub was removed, the catheter and the trachea were tied together firmly with a nylon string. BAL was performed by installation of 0.8 ml of chilled sterile PBS through the trachea into the lungs, perfused 2 to 3 times and draws the BAL fluid. The differential cell count was determined by visualizing the cells with Giemsa stain and counted manually using a hemocytometer.

2.6 Source of phospholipase A_2 and 5-lipoxygenase

Human synovial fluid phospholipase A_2 (HSF-PLA_2) was partially purified according to the method described by Vishwanath et. al. (1988)[26] and 5-lipoxygenase (5-LOX) was isolated from human polymorphonuclear leukocytes (PMNL) according to Mohamed et. al., (2014).[27]

2.7 Determination of phospholipase A_2 activity

The PLA_2 enzyme activity was determined by using HSF-PLA_2 and BAL fluid collected from different experimental mice groups. The activity was measured using 4-nitro-3-octanoyloxy-benzoic acid (NOB) as a chromogenic substrate in 96 well plate as described previously by W H Lee et al., (1999) with slight modifications.[28] A standard reaction mixture composed of 200 µl of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 7.8) 60 mM of NOB and 30 µg of HSF-PLA_2 enzyme or 30 µg of protein from BAL fluid was added to a final volume of 260 µl and the content was incubated for 40 min at 37 °C. The absorbance of the product formed was recorded at 425 nm (Varioskan multimode plate reader, Thermo Scientifics, USA) for 0, 10, 20, 30 and 40 min intervals. Enzyme activity was calculated based on the increase in absorbance after 20 min. The resultant PLA_2 activity in HSF-PLA_2 was considered as 100 % activity, whereas the PLA_2 activity in BAL from mice was compared in different experimental animal groups.

2.8 Determination of 5-lipoxygenase activity

The enzyme activity was determined by using 5-LOX enzyme from human polymorphonuclear leukocytes and BAL fluid collected from different experimental mice groups. The reaction was carried out by pre-incubating 5 µg of human 5-LOX enzyme or 5 µg of protein from BAL fluid with 100 mM phosphate buffer (pH 7.4) containing 0.05 mM dithiothreitol (DTT), 0.3 mM $CaCl_2$ and 0.2 mM ATP along with 0.15 mM of arachidonic acid. The product formed was measured by taking the absorbance at 234 nm.[27] The 5-LOX enzyme activity obtained in human 5-LOX was considered as 100 %, whereas the 5-LOX activity in BAL from mice was compared in different experimental animal groups.

2.9 Lung tissue histopathology

Following BAL procedure, the upper lobe right lung tissue was fixed in 4 % paraformaldehyde for 24 hours. The fixed lung tissues were embedded in paraffin, 5 µM sections were made using microtome and stained with hematoxylin and eosin (H&E).[29] The stained sections were observed in bright field microscope.

2.10 *Determination of collagen content*

Collagen content in the lung was measured indirectly by determining the hydroxyproline content as described by Yariswamy (2013).[30] Briefly, 10 mg of de-fatted lung tissue was hydrolysed with 2 ml of 6 N hydrochloric acid at 110 °C overnight. 1 ml of 0.01 M copper sulphate, 2.5 M sodium hydroxide and 6 % hydrogen peroxide was added to equal amounts of hydrolyzed samples. The contents were thoroughly mixed and heated in a boiling water bath at 70 °C for 5 min. Samples were cooled immediately on ice and 4 ml of 3 N H_2SO_4 and 2.5 ml of 5% p-dimethyl aminobenzaldehyde was added and mixed thoroughly. Then the tubes were again heated at 70 °C and the pink color developed was measured at 530 nm. The hydroxyproline standard was used to calculate the concentration of hydroxyproline. The total collagen content was calculated using the formula,

$$Collagen\ content = \frac{Amount\ of\ hydroxyproline\ in\ the\ sample \times 7.45}{Amount\ of\ sample}$$

2.11 Statistical analysis

The results were expressed as mean ± SD. One-way ANOVA with Bonferroni's multiple comparison post hoc tests was performed to assess statistical significance using Graph Pad Prism 5.0 software. The comparison between the groups was considered significant if $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Extraction of phytochemicals from finger millet bran

Plant extracts are comprised of various therapeutically important bioactive constituents such as phenolics, alkaloids, flavonoids, saponins, steroids, anthraquinone and terepenoids.[31-36] Especially phenolic compounds which are the largest category of phytochemicals found ubiquitously in the plant kingdom[37] gained the considerable attention by scientific communities due its wide therapeutic importance.[38] Phenolic compounds were reported to play beneficial roles towards various disease conditions and exerts anti-oxidant, anti-cancer, anti-diabetic, anti-inflammation, anti-malarial and anti-viral properties.[39] Recently, researchers have identified anti-inflammatory phenolic molecules from various bran of cereals grain. For instance, in 1954 Kaneko et al., was first to isolate therapeutically important γ-oryzanol from rice bran oil, which attracted the researcher to isolate more therapeutically important compounds from bran. In view of this, the aim of our present study is to isolate therapeutically important phytochemicals from finger millet bran (Fmb). The Fmb extraction was carried out by using soxhlet apparatus with solvents of different polarity to extract all possible phytochemicals. Phytochemical analysis of Fmb revealed the presence of glycosides, phenolics and saponins (Table 01 A). It was observed that the highest amount of phenolics were present in finger millet bran methanol extract (*Fbm*e). The yield of phytochemicals was determined by taking dry weight of phytochemicals of respective solvents (Table 01 B).

A

Phytochemicals	Finger millet bran			
	Hexane	Ethyl acetate	Methanol	Water
Alkaloids	-	-	-	-
Anthraquinone	-	-	-	-
Flavonoids	-	-	-	-
Glycosides	-	+	-	+
Phenolics	-	+	+ + +	+ +
Saponins	-	-	+ +	-
Steroids	-	-	-	-
Tannins	-	-	-	-
Terepenoids	-	-	-	-

B

Solvents	Fmb (% yield)
Hexane	0.10
Ethyl acetate	0.15
Methanol	0.30
Water	1.20

Table 01: (A) Phytochemical analysis of Fmb hot extract. Note: + (Low concentration), ++ (Moderate concentration) and +++ (High concentration) and – (absent). (B) Percent yield. (Adopted from our previous publication).[17]

3.2 Effect of FbMe on inflammatory enzymes

Phospholipases A_2 (PLA_2s) are the hydrolytic enzymes specifically cleaves the phospholipid at *sn-2* position to release arachidonic acid.[8] The released arachidonic acid enters into eicosanoid pathway and was further metabolized by different enzymes. In lungs by the action of 5-lipoxygenase (5-LOX), pro-inflammatory leukotrienes were released which are known to be involved in many inflammatory reactions including the pathogenesis of asthma.[40] Generally, non-steroid anti-inflammatory drugs (NSAIDs), corticosteroids and antagonists of cysteinyl leukotriene receptor are still being used to relieve asthma even they exert serious side effects on health.[41-43] Hence, a better approach is to use a naturally occurring anti-inflammatory compounds with minimal side effects. In this study we have extracted phytochemicals from different extracts of Fmb to determine their effect on *in vitro* PLA_2 and 5-LOX enzyme activities. Our results show that, among all the extracts tested, *FbMe* significantly inhibited the *in vitro* activity of PLA_2 and 5-LOX enzymes, whereas phytochemical constituents of other extracts have failed to inhibit these enzymes. The extent of inhibition by *FbMe* is up to 89 ± 3.0 % and 91 ± 0.9 % for PLA_2 and 5-LOX enzymes activity respectively (Fig 01 A and B).This data indicated the presence of anti-inflammatory phytochemicals in *FbMe*. Hence, the further anti-inflammatory studies were carried out by using *in vivo* mouse asthma model.

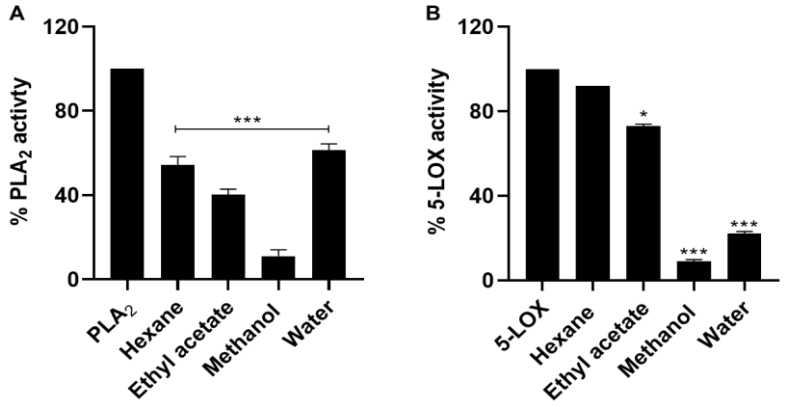
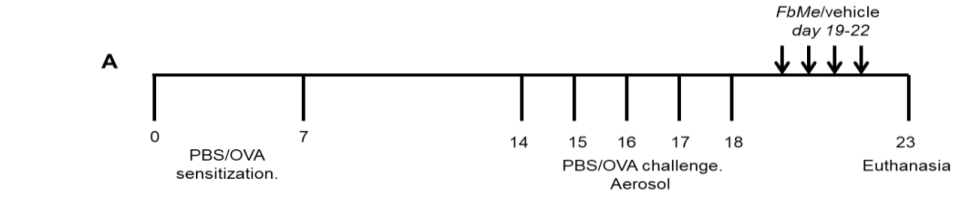


Fig 01: Effect of *FbMe* on *in vitro* inflammatory enzymes (A) Inhibition of PLA_2 enzyme activity. Data represents mean ± S.D (n=3). ***P < 0.001 as compared to PLA_2 alone and (B) Inhibition of 5-LOX enzyme activity. Data represents mean ± S.D (n=3). *P < 0.05 and ***P < 0.001 as compared to 5-LOX alone.

3.3 Effect of FbMe on OVA-induced inflammatory cells and enzymes

Asthma is a well-known inflammatory condition characterized by impaired airflow due to inflamed airways of lungs.[44] Asthma can be triggered by various allergens such as pollen, dust mite, cigarette smoking, rodents, air pollution, respiratory infections and chemicals.[45] Previously, Takayuki Yamamoto et al. reported elevated levels of leukotrienes as one of the main factor to cause asthma.[46] The initiation of leukotrienes biosynthesis was due to the liberated arachidonic acid from membrane phospholipids by the enzymatic action of PLA_2. Later, the released arachidonic acid was metabolized by 5-LOX enzyme as a result leukotrienes were produced.[40] Therefore, the dual inhibitor of PLA_2 and 5-LOX enzymes that can block the eicosanoid pathway thereby release of leukotrienes is the crucial step in the management of asthma. In this study, we induced asthma to mice by sensitization with OVA and LPS as an allergen (Fig 02 A). The mice trachea was exposed and inserted with 22-guage needle to isolate the BAL fluid from lungs as described in the methods (Fig 02 B). Different groups of mice were sensitized, challenged and treated as mentioned (Fig 02 C). Metzger et al., reported that sudden and significant raise in the inflammatory cells population taken place in the lungs of asthma patients.[47] Another study by Bisgaard et al., highlighted the pivotal role of PLA_2 and 5-LOX enzymes in mounting asthma.[48] Therefore, decrease of cell counts and pro-inflammatory enzymes activity were considered as the best strategy in management of asthma. Promising *in vitro* inhibition of these enzyme activities possessed by *FbMe* lead us to test on the OVA-induced asthma to the mice. Group of mice sensitized with OVA shows increased in number of total cells 36.85 x 10^3/ml (neutrophils, eosinophils, lymphocytes and macrophages), where PBS (control) treated mice counts for 2.12x 10^3/ml of cells. Treatment with *FbMe* (50 mg/kg) significantly reduces the total cell count to 17.37 x 10^3/ml of BAL fluid (Fig 03 A to D). It has been observed that drastically increased activity of PLA_2 and 5-LOX in the BAL fluid of OVA treated mice. Notably, 50 mg/kg of *FbMe* significantly reduced the *in vivo* enzyme activity of PLA_2 and 5-LOX in BAL fluid of the mice up to 49.66 ± 5.02 % and 48.33 ± 3.05 % respectively (Fig 03 E and F).



B

C

Group	Sensitization *day 0 and 7* (*i.n*)	Challenge *day 14-19* 5 to 6 dose (Aerosol)	Treatment *day 19-22* (*i.p*)
Control	PBS	PBS	PBS
OVA	150 µg OVA + 100 ng LPS	1 % OVA	PBS
OVA + 5 mg/kg	150 µg OVA + 100 ng LPS	1 % OVA	5 mg/kg *FbMe*
OVA + 50 mg/kg	150 µg OVA + 100 ng LPS	1 % OVA	50 mg/kg *FbMe*
Vehicle	150 µg OVA + 100 ng LPS	1 % OVA	0.5 % DMSO

Fig 02: (A) Experimental regimen for the induction and treatment of asthma to the mice. (B) Tracheal insertion of needle to collect the BAL fluid from the lungs. (C) Treatment protocol for the different groups of mice.

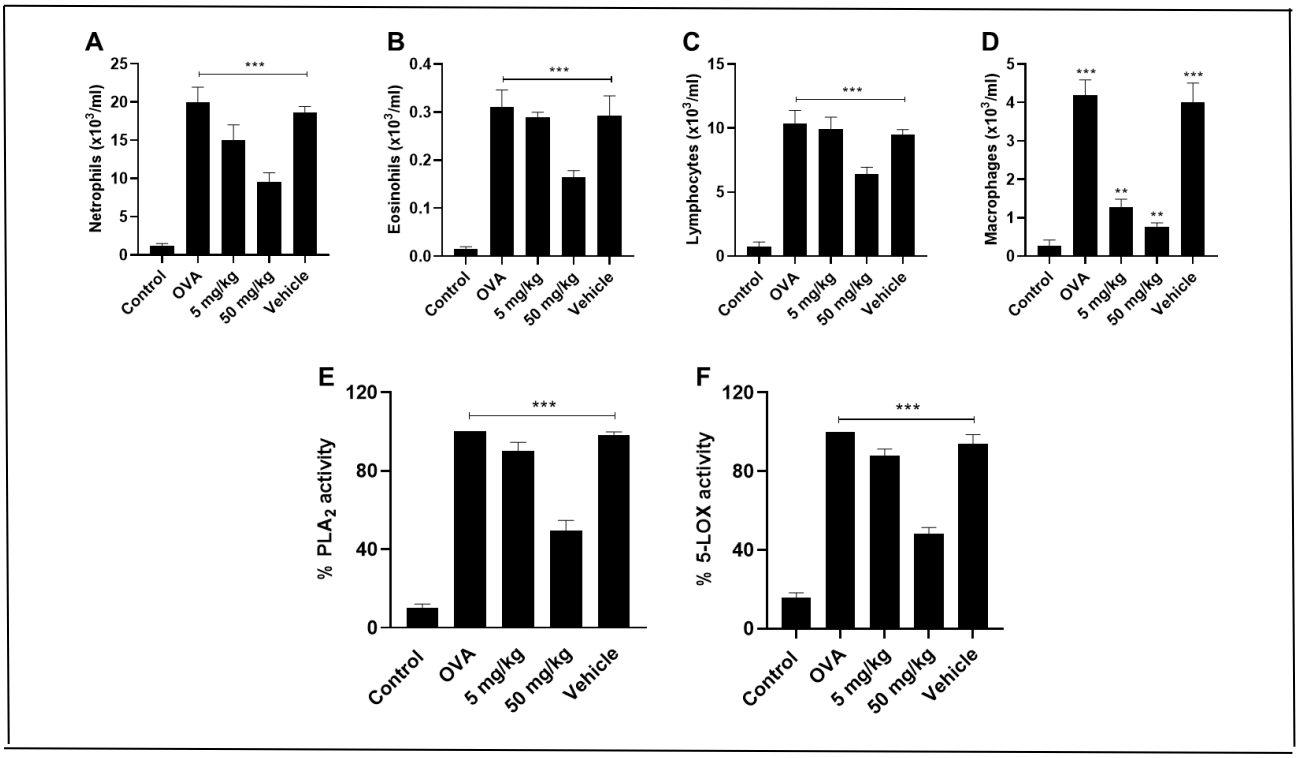


Fig 03: Effect of *FbMe* on (A to D) inflammatory cell count. Data represents mean ± S.D (n=5). **P < 0.01 and ***P < 0.001 as compared to the control group. (E) PLA_2 and (F) 5-LOX enzyme activity in collected BAL fluid. Data represents mean ± S.D (n=5). ***P < 0.001 as compared to the control group.

3.4 Effect of FbMe on histopathological change and collagen content of lungs

The hallmark characteristic of asthma is the infiltration of inflammatory cells such as neutrophils, eosinophils, lymphocytes and macrophages in and around the airways of lungs due to inflammation.[49] Asthma is also characterized by constriction of airways that eventually develops the persistent wheeze, night cough, and shorten the breath.[50] Hematoxylin and eosin staining was performed on the lung tissues to analyze the effect of *FbMe* on pathological changes in the lungs. In lung tissue obtained from OVA sensitized group showed increased cell infiltration with narrowed airways compared to control mice. Treatment with 50 mg/kg *FbMe* reduced the cells infiltration and widens the airways (Fig 04 A).

Lung fibrosis also takes place in persistent asthma conditions due to accumulation of collagen. Therefore, measurement of collagen content is an important marker to check the lung fibrosis. Hydroxyproline is a non-standard amino acid, an important constituent of collagen and other extracellular proteins. The levels of hydroxyproline is directly correlated with the levels of collagen in the tissue.[51] To measure the collagen content in the lungs were determined by hydroxyproline assay. As a result, of asthma in the OVA sensitized group of mice was characterized by highest collagen content in the lung which was corroborated with hydroxyproline concentration. Group of mice treated with 50 mg/kg of *FbMe* showed decreased collagen content (5.10 ± 0.36 %) compared to the OVA treated group (8.00 ± 0.43 %) or vehicle treated mice (Fig 04 B). This data suggests that, *FbMe* reduces lung fibrosis which is a characteristic feature of asthma.

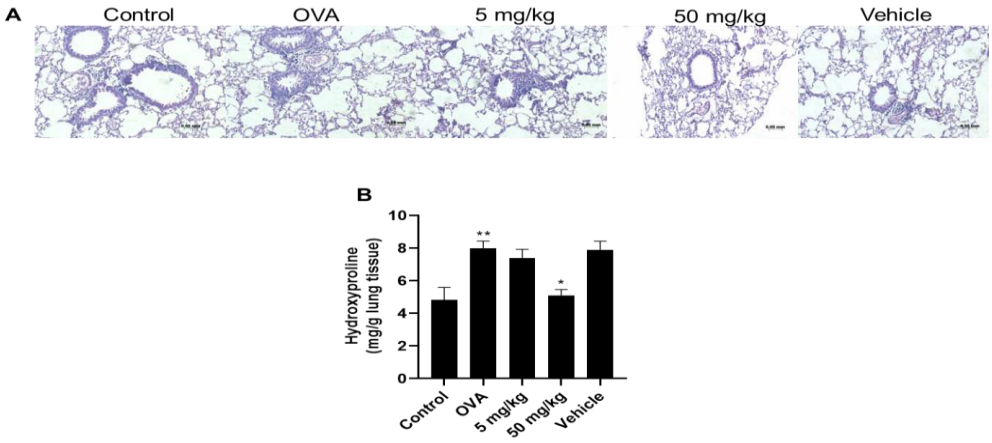


Fig 04: Effect of *FbMe* on (A) H&E staining of lung tissue and (B) hydroxyproline content of mice lung tissue. Data represents mean ± S.D (n=5). *P < 0.05 and **P < 0.001 as compared to the control group.

In these experiments, finger millet bran was subjected to soxhlet extraction by using different solvent such as hexane, ethyl acetate, methanol and water. The extracted phytochemicals were tested for inhibition of pro-inflammatory PLA_2 and 5-LOX *in vitro* enzymes activity. Among all the extracts, phytochemicals isolated by finger millet bran methanol extract (*Fb*Me) significantly inhibited the PLA_2 and 5-LOX enzymes activity and inhibition of was more than 80%. So all other experiments were carried out using *Fb*Me. *In vivo* anti-inflammatory activity was carried out by using OVA induced asthma in mice. Similar to previous reports,[52-54] treatment of OVA induced asthmatic mice with *Fb*Me alleviates the asthma by decreasing the cell infiltration in BAL fluid, accumulation of collagen content in lungs by inhibiting pro-inflammatory enzymes activity.

4. CONCLUSION

Based on the results of the present study we can conclude the definitive presence of anti-inflammatory, anti-fibrotic and anti-asthma molecules in *Fb*Me. This study also highlighted the beneficial effect of phenolic compounds present in finger millet bran; this can also justify the anti-inflammatory activity of phenolic molecules. However, future study should investigate their role in detail to identify the mechanism and confirm these studies by purification and characterization of the active compounds present in *Fb*Me.

5. AUTHOR CONTRIBUTION STATEMENT

Bannikuppe S. Vishwanath and Noor Mohamed Jameel conceived the original concept, designed the experiments and critically evaluated the manuscript. Milan Gowda M D performed the major experiments, collected, and analyzed the data and wrote the manuscript. Jayachandra K performed some experiments. Siddesha J M designed the experiments and critically evaluated the manuscript.

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7. CONFLICT OF INTEREST

The authors involved in this study declare no conflict of interest.

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