A Thesis

For The Degree of Master of Interdisciplinary Graduate Program in Advanced Convergence Technology And Science

Oleuropein Rich Olive Leaf Curtails PM-Induced Allergic Airway Inflammation in Mouse

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Department of Interdisciplinary Graduate Program in Advanced Convergence Technology & Science

GRADUATE SCHOOL

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A thesis in partial fulfilment of the requirement for the degree of Master of Interdisciplinary Graduate Program in Advanced Convergence Technology & Science

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ABC	Avidin-Biotin Complex
BALF	Broncho Alveolar Lavage fluid
CD4	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
COPD	Chronic Obstructive Pulmonary Disease
CRM	Certified Reference Material
DAB	3,3'-Diaminobenzidine
DNA	Deoxyribonucleic Acid
EDTA	Ethylene-diamine-tetra acetic acid
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
H&E	Haematoxylin and Eosin
HRP	Horseradish Peroxidase
IFN-γ	Interferon Gamma
IgE	Immunoglobulin E
IL	Interleukin
MUC2	Mucin 2
MUC5AC	Mucin 5AC
MUC5B	Mucin 5B
NIES	National Institute for Environmental Studies
NIH	National Institute of Health

NO	Nitric Oxide
OCP	Organochlorine Pesticides
OVA	Ovalbumin
PAHs	Polycyclic Aromatic Hydrocarbons
PAS	Periodic Acid of Schiff
PBS	Phosphate-buffered saline
PCBs	Bisphenols and Polychlorinated Biphenyls
PCR	Polymerase Chain Reaction
PM	Particulate Matter
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
Th2	T helper type 2
Th17	T helper 17
TLR	Toll Like Receptor
TNF-α	Tumour Necrosis Factor alpha

Abstract

PM is well-known as an asthma aggravating crucial factor frequently existing in air pollutants. Oleuropein rich olive leaf (*Olea europea*) extract (OLE) has been recognized as an effective therapeutic compound to suppress multiple health issues including inflammatory conditions in respiratory tract. This study was mainly focused on exploring beneficial suppressive abilities of olive leaf for asthma inflammations. During this study, we developed a mouse model, by intraperitoneally injecting BALB/c mice with Ovalbumin (OVA) followed by challenge with PM (30mins/day) for 7 days consecutively in a nebulizer. Afterwards, the oral gavage of OLE and the positive control, prednisone was done. As results, we observed that the OLE significantly decreased eosinophil and basophil infiltration in blood and BALF, further inflammatory cell infiltration and more specifically eosinophil infiltration in both lung and trachea which were aberrantly increased by PM. OLE abated PM-induced TLR4 expression. This proceeds to reduced mRNA expression of pro-inflammatory cytokines by Th1 response (IL-1β, TNF-α and IFN-γ) and inflammatory cytokines by Th2 and Th17 response (IL-4, IL-10, IL-13, IL-17, and IL-22) resulting reduced mucus secretion in airway epithelia and reduced inflammatory conditions. In conclusion, the Olive leaf extract is proficient in alleviating inflammatory conditions by suppressing Th2 and Th17 cell activation, mast cell degranulation and mucus secretion in PM-induced allergic asthma by rectification of TLR4 induced signalling as a substituting conventional drug.

Key words: Particulate matter, Olive leaf extract, Oleuropein, allergic asthma, Th2 and Th17 response

Introduction

Asthma is a heterogeneous disease characterized by hostility in airflow altered by varied inflammatory mediators and immune cells (Canonica et al., 2016, Alberto et al., 2022, Posberg et al., 2023). Concerning the statement of with Hammad and Lambrecht (2021) the main hallmark of asthma is bronchoconstriction along with alterable airflow obstruction. They have further noted the pathophysiology of this condition as the inflammation of airway wall characterized by infiltration and activation of immune cells. This condition leads to symptoms of wheeze, shortness of breath, chest tightness and cough which are triggered by mucus over production. Airway remodeling and bronchial hyperresponsiveness (Aaron et al., 2018; Hammad and Lambrecht, 2021; Brusselle and Koppelman, 2022). In accordance with Hammad and Lambrecht (2021) many allergens and air pollutants are related with asthma generation and exacerbation by production of cytokines and chemokines via the activation of toll-like receptors (TLRs) and protease-activated receptors (PARs) leading to activation of immune cells and ultimately generating airway inflammation by producing many responsible mediators.

Environs particulate matter (PM) has become a crucial index of air pollution (Zhao and Cai, 2021). Currently, the part of PM for engendering and aggravating human respiratory diseases has become prominent, by negatively impacting on human health, even increasing the mortality rate playing a role as a main orchestrator in allergic asthma. As stated by Xing et al (2023) exposure to PM is an affidavit for activating the lung oxidative stress and interact with different components of the immune system and ultimately aggravating allergic inflammatory responses. In accordance with Yang et al (2021) as the consequences of the physiochemical characteristics of PM, it has a

great potential to absorb harmful substances of the surface and exist in the air for a long time. Particulate matter has a great propensity for penetrating into the deeper part of the respiratory tract and deposited in different parts of the respiratory tract including bronchioles due to its small particle size and that process is supported via inhalation (Kyung and Jeong, 2020; Yang et al, 2021). Paplinska-Goryca et al (2021) has further mentioned that the inhaled large PM are deposited in conducting airways and the smallest PM are deposited in peripheral airways and in the terminal bronchioles and alveoli also. Altogether, increment of ambient particulate matter concentration prominently has become one of the major causes for inducing asthma as well as for exacerbating pre-existing asthmatic conditions (Kim et al., 2017). As evidenced by Herath et al (2020) in their studies by investigating higher eosinophil infiltration in PM induced mice, there is a strong relationship between PM and asthma. PM inhalation induces secretion of inflammatory cytokines from immune cells like macrophages and structural airway cells (Leikauf et al., 2020). With the introduction of PM, Th2 cells induce IgE switching resulting enhancement of allergic inflammation and bronchial hypersensitivity (Xu et al., 2020). Besides, Liu et al (2022) has mentioned that TNF-α, IL-1β can be up-regulated and some other cytokines and chemokines levels of the airway epithelial also can be increased by introducing PM2.5. According to Herath et al (2020) PM has aggravated IgE level in serum, mucus secretion and goblet cell metaplasia and MUC5AC expression in goblet cells which are among main physiological changes leading to asthma. Moreover, Sun et al (2020) has found in his experiments that PM2.5 created immunological imbalance by promoting Th17 imbalance. As Yang et al (2021) noted in his study, airway inflammatory responses increased by PM activate eosinophil associated with allergic inflammation. On the top of all, Liu et al (2020) has found

through their studies that PM is known to be responsible for activating NF-kB signalling pathway which may lead to secret several anti-inflammatory and pro-inflammatory cytokines. Interestingly, according to several studies IL-17 seems to be highly increased in patients with moderate to severe asthma (Luo et al., 2022). It is further noted that IL-13 induces excessive mucus secretion by impeding respiration. Moreover, Liu et al (2022) has mentioned that the expression level of MUC5AC is increased in asthmatic conditions. Therefore, seeking for effective treatments for asthma has become a necessitate.

However, prevailing medications for asthma are composed of corticosteroids combined with bronchodilator which have the possibility to prevent asthma with low doses (Reddel et al., 2017). These drugs do not respond well in many asthmatic patients and have reported some side effects (Herath et al., 2020) implying that it is insecure to be taken as medications. Therefore, experiments on alternative medicines for asthma have become a necessity. Thus, as a substitute, Olive (*Olea europea* L.) leaf has been selected by our research group for further analyzing its anti-inflammatory effects against PM induced allergic asthma due to its historical medicinal values. Olive is an evergreen (Cavalheiro et al., 2015) small annual tree native to Mediterranean countries belongs to family Oleacea (Medina et al., 2019) which is subjected to many researches as a medicinal herb. In several studies it has been mentioned that olive leaves are used as a folk prophylactic treatment in traditional medicine especially in Mediterranean countries (Medina et al., 2019). Through several studies it has been investigated that olive leaf extract has a great potential as a treatment for inflammatory diseases (Silvan et al, 2021).

Olive leaves are considered to have outstanding chemical composition due to

their polyphenolic content (Persuric et al., 2019; Silvan et al., 2021) composed with hydroxytyrosol, some secoiridoids (elenolic and demethyl elenolic acids and their glycosidic forms); flavones (luteolin, luteolin-7-glucoside, apigenin-7-glucoside, diosmetin, and diosmetin-7-glucoside); flavonols (rutin and kaempferol); flavan-3-ols (catechin), and phenolic acids (tyrosol, caffeic acid, chlorogenic acid, cinnamic acid, and vanillic acid) (Silvan et al., 2021). Olive leave extract (OLE) is supplemented with active chemicals belonging to secoiridoids, hydroxytyrosol, polyphenols, triterphenes and flavonoids. Oleuropein is considered as the main phenolic compound, which is accounted about 6-9% of dry matter content of the olive leave and Medina et al (2020) has reported in their study that oleuropein content is 88-94% of total phenolic content along with a characteristic bitter taste. Oleuropein is a secoiridoid, a component of many parts of the olive tree notably in olive leaves and it is the main component responsible for many characteristics of OLE. Due to this composition, olive leaves are known to give several health benefits like antioxidant, hypoglycemic, anti-hypertensive, hypercholesterolemia, and anti-inflammatory properties (Persuric et al., 2019). Furthermore, phenolic compounds of OLE possess antimicrobial and neuroprotective activity and the ability to modulate blood pressure, plasma lipid profiles and insulin sensitivity in overweight people and beneficial effects for cardiovascular diseases also (Persuric et al., 2019). OLE is known to be potent in inhibiting the inflammatory conditions as evidenced by several research conducted in current studies.

As per the results of atopic dermatitis mouse model of Huang et al (2022) oleuropein is an auspicious suppressor of mast cell and eosinophil infiltration. Geyukoglu et al (2017) have found out in their rat model in stomach and lung using high dose oleuropein that inflammatory cell infiltration in lung has been attenuated by

oleuropein. Interestingly, Xu et al (2022) have explored through their studies conducted via a lung I/R (Ischemia-Reperfusion) mouse model that oleuropein is successful in obstructing TLR4 signalling cascade in alveolar macrophage. The mRNA expressions exhibited by the q-PCR results of oleuropein treated groups in lung I/R mouse model have shown a decreased TLR4 levels in lung tissues and alveolar macrophages. Silvestrini et al (2023) has discovered the inhibition of pro-inflammatory cytokine production in their study. These results evidence that OLE is competent for regulating inflammatory conditions by inhibiting TLR4 signalling. According to Xu et al (2022), the q-PCR results gained in I/R mouse model has shown a decreased level of IL-1β and TNF-α pro-inflammatory cytokines in lung tissues and BALF of groups treated with oleuropein confirming the impact of oleuropein for anti-inflammation.

In the present study, we investigated the anti-inflammatory effect of OLE for suppression of responsible cytokines transcription and impedementation of mucus hypersecretion of PM induced asthmatic mice by restraining TLR4 signalling.

Research Hypothesis

Interestingly, OLE has been proved to have anti-inflammatory effects by attenuating pro-inflammatory cytokine secretion like IL-1β, TNF-α (Silvan et al., 2021). Therefore, through our study we investigated mitigating PM-induced allergic asthma through regulating TNF-α, IFN-ϒ, IL-13 and Th17 cytokines IL-17, IL-22 using OLE. When PM triggers the epithelial tissue of airway the antigen presenting dendritic cells activate CD4 cells and make them differentiate into T helper cells. IL-4, IL-10 and IL-13 cytokines are produced by Th2 cells. According to our data, IL-13 was significantly reduced by olive leaf extract. IL-17 and IL-22 are produced by Th17 cells and our data shows that OLE significantly suppresses Th17 cytokine levels. The active ingredient responsible for suppressing inflammatory condition is oleuropein. Abdelgawad et al (2022) has mentioned in their study that oleuropein has the potential to diminish the influx of eosinophil and lymphocytes in the respiratory system and to reduce the secretion of IL-4 suppressing the infiltration of macrophages. Moreover, Qabaha et al (2017) has mentioned in their experiment that the most significant component in olive leaf extract for the reduction of pro-inflammatory cytokine TNF-α was Oleuropein. We hypothesize that oleuropein is the responsible component in olive leaf extract for suppressing Pro-inflammatory and inflammatory cytokines as well as attenuating mucus over-production from goblet cells. Other than these, OLE suppress production of B lymphocytes. Thereby OLE attenuates mast cell degranulation. Thus, we hypothesize that OLE has the potential to curtail inflammatory effects of asthmatic mice.

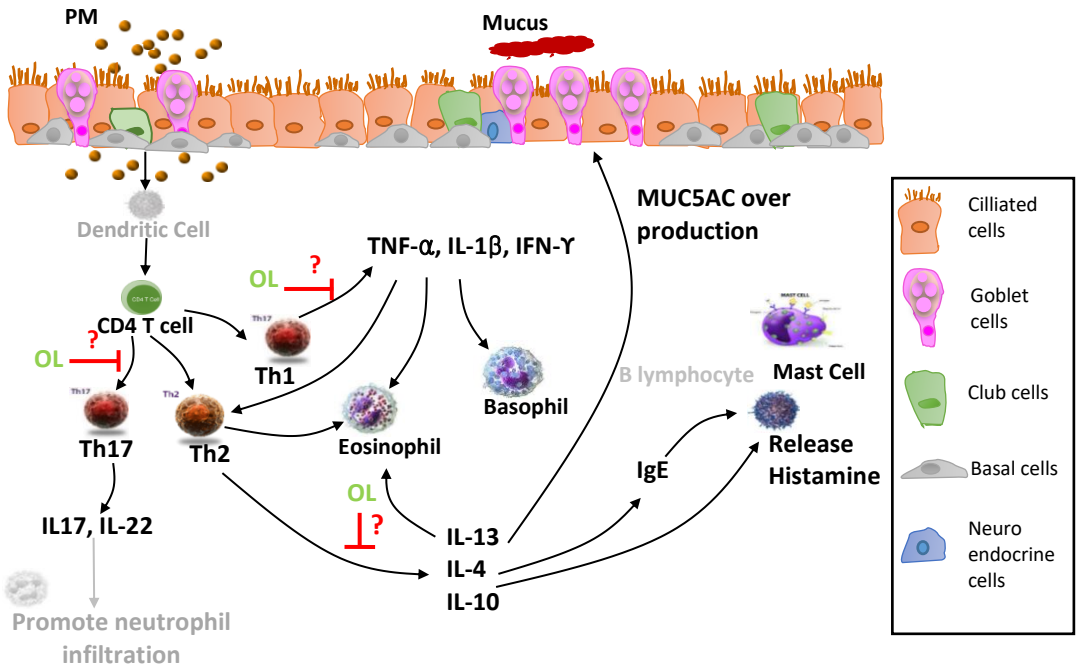

Figure 1. Hypothesis of the ramification of OLE on allergic asthma-induced mice exposed to PM

Aim

Oleuropein in Olive leaves possesses anti-inflammatory qualities. The main aim of this research is to investigate anti-inflammatory effects against asthma condition by oleuropein in olive leaf extract (OLE).

Specific aims are:

1. To investigate the role of OLE on Th17 mediated cell signalling for secretion of IL-17 and IL-22 and reduction of IL-13 from Th2 cells.
2. To investigate the role of OLE on TNF-α, IL-1β, IFN-ϒ pro-inflammatory cytokine production from Th1 cells.
3. To investigate the role of OLE on mucus over production in goblet cells and mast cell degranulation

Materials and Methods

Mice

For this study BALB/C mice were purchased from Orientbio, Inc. (Sungnam, Korea) and used for experiments at the age of 4-5 weeks. From the arrival up until conduct experiments, mice were housed in well-ventilated cages arranged in an animal room facilitated with a constant temperature (23±1°C) and humidity (50±10%) and exposed to 12h/12h light-dark cycle, along with supplementation of NIH-07 approved diet and water *ad libitum*. All experiments were conducted according to the approved ethics of Institutional Ethical Committee of Jeju National University.

Particulate matter

Certified Reference Material of PM (CRM No. 28) collected over 10 years in Beijing, China was purchased from the National Institute for Environmental Studies (NIES), Ibaraki, Japan.

Table 1. Mass fraction of elements in CRM No.28

Element	Unit	Content
Al	%	5.04
Ca	%	0.24
Mg	%	1.40
Ti	%	0.292
Zn	%	0.114
Cd	mg/kg	5.60
Ld	mg/kg	403
Ni	mg/kg	63.8
Cu	mg/kg	104
V	mg/kg	73.2

National Institute for Environmental Studies (NIES), Ibaraki, Japan

Table 2. Mass fraction of PAH in CRM No. 28

Component name	Unit	Content
Fluoranthene	mg/kg	7
Pyrene	mg/kg	0.145
Benz(a) anthracene	mg/kg	14.9
Benzo(b) fluoranthene	mg/kg	3.91
Benzo(k) fluoranthene	mg/kg	20.1
Benzo(a) pyrene	mg/kg	5.09
Benzo(ghi) perylene	mg/kg	65.6
Indeno(1,2,3-cd) pyrene	mg/kg	22.0

PAH: Polycyclic Aromatic Hydrocarbon
(National Institute for Environmental Studies (NIES), Ibaraki, Japan, 2008)

Plant extract of Olive leaf

Comvita Fresh-Picked Olive Leaf Extract was purchased from Comvita, Queensland, Australia. Olive leaves were grown, harvested, and extracted on olive leaf plantations in Queensland, Australia.

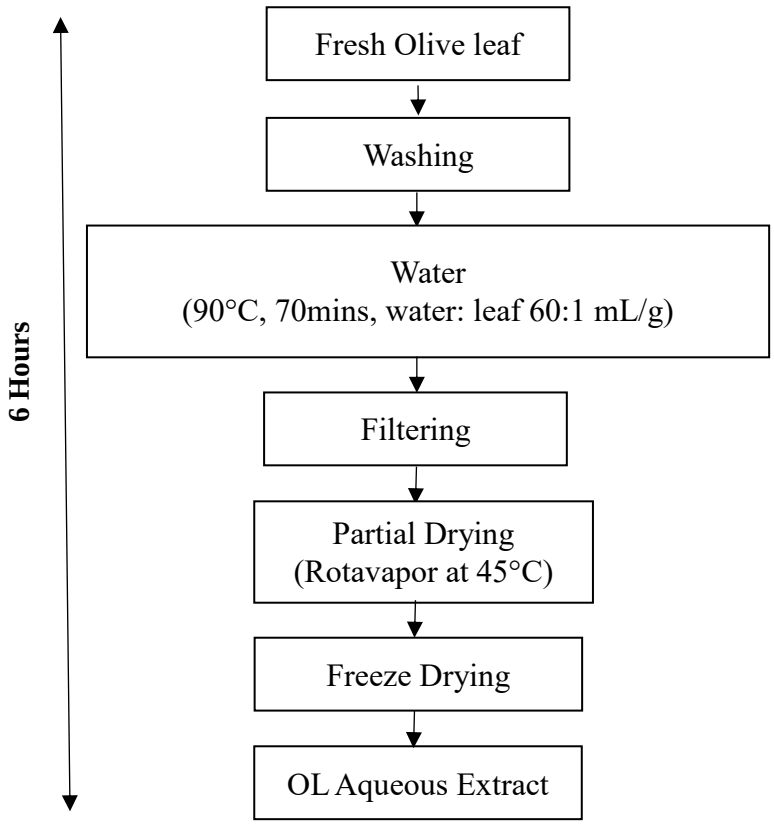

Figure 2. Schematic diagram of extraction

Animal model

4-5 weeks old female and male BALB/C mice were randomly divided into seven groups (n=4~8 each): 1. Healthy control, 2. PM only, 3. OVA only, 4. OVA+PM, 5. OVA+PM+OLE 50mg/kg (OVA+PM+OLE50), 6. OVA+PM+OLE 200mg/kg (OVA+PM+OLE200), 7. OVA+PM+Prednisone 5mg/kg (OVA+PM+Prednisone). In this experiment, Prednisone is used as the positive control as it is abundantly used as a drug to control inflammation in asthma patients. On day 1, all mice except healthy control were sensitized by intraperitoneally injecting 10µg OVA in 2 mg Al $(OH)_3$ along with 200µl saline. From day 15 to day 21, for 7 consecutive days all mice except healthy control and OVA only were challenged with sonicated PM (5 mg/m^3) in a nebulizer for 30 minutes per day. Alongside each other, oral administration of OLE 50

mg/kg, OLE 200 mg/kg and Prednisone 5 mg/kg were done to each respective group prior to PM inhalation. The rest of the groups were orally administered by saline. On 21st day mice were sacrificed and extracted tissues and BALF. The experimental procedure is illustrated in Fig. 3.

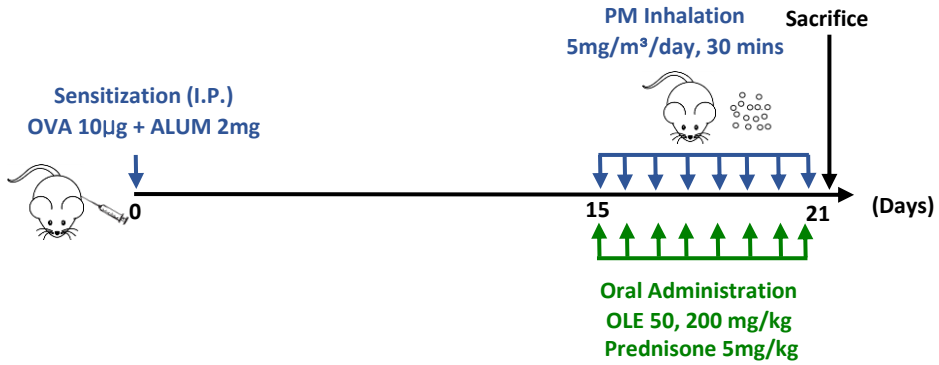

Figure 3. Schematic diagram for animal model of allergic asthma-induced mice by exposed to PM

Contrast of Differential cell quantity of blood in asthma induced mice exposed to PM

Mice were taken into an unconsciousness status and extracted the blood via cardiac puncture using a heparinized syringe and put into an ethylene diamine tetra acetic acid (EDTA) tube. Smears were made and stained using Diff Quick to take the count of each inflammatory cell; neutrophils, lymphocytes, monocytes, eosinophils, and basophils using Olympus DP-72 microscope camera (Olympus, Tokyo, Japan) system.

Contrast of Differential cell quantity of Bronchoalveolar Lavage Fluid (BALF) in asthma induced mice exposed to PM

Sacrificed mice were intratracheally cannulated and BALF were extracted by installation of 0.5 ml DPBS into the lungs gently to maximize the BALF recovery. The BALF

taken from each mouse was centrifuged separately at 300g for 5min at 4℃. The cells were methanol fixed and smears were made and stained using Diff Quick to take the count of each inflammatory cell; neutrophils, lymphocytes, monocytes, eosinophils, and basophils using Olympus DP-72 microscope camera (Olympus, Tokyo, Japan) system.

Histopathological analysis of inflammatory cell infiltration in trachea and lung in asthma induced mice exposed to PM

The extracted lung and trachea were sectioned and fixed in 10% formalin, following embedded in paraffin, and sectioned to 3μm sections and thereby performed histological staining to conduct histological analysis. Microscope slides made with sectioned tissues were stained with Hematoxylene and Eosin (H&E) for appraising the histopathological changes in lung and trachea by analysing inflammatory cell infiltration. Tissue slides were stained with congo red to analyse eosinophil infiltration in lung and trachea. Toludine blue staining was performed to appraise the mast cell production and the degranulated mast cells in trachea. To detect mucus secreting cells in both lung and trachea, periodic acid of Schiff (PAS) staining was performed with Basic Fuchsin Dye. Each stained tissue slide was dehydrated in ethanol series and cleared in xylene. Ultimately stained tissues observed and analysed using Olympus DP-72 microscope camera system (Olympus, Tokyo, Japan).

Immunohistochemistry analysis of lung and trachea in asthma induced mice exposed to PM

Paraffin embedded tissue sections were subjected for de-waxing and re-hydration in

ethanol. Tissues were immersed in 0.3% hydrogen peroxide for 40 minutes and thereafter incubation in horse serum to block non-specific binding. Then tissues were incubated with primary anti-mucin 5AC (MUC5AC) antibody (1:500, Abcam, Cambridge, MA, USA), MUC5B antibody (1:1000, Abcam, Cambridge, MA, USA), MUC2 (1:1000, Abcam, Cambridge, MA, USA) overnight at 4°C. After washing, tissues were incubated with biotinylated anti mouse serum for 45 minutes. Tissues were then washed with PBS and avidin-biotin peroxidase complex binding reaction was performed using horseradish peroxidase (HRP)-labeled Vectastain Elite ABC kit (Vector). HRP binding sides were detected using 3-3'- diaminbenzidine (DAB, Vector). Counterstain was performed using hematoxylene. Thereafter positive cells were analyzed using Olympus DP-72 (Olympus, Tokyo, Japan) microscope.

RNA extraction, cDNA synthesis and real-time PCR analysis in asthma induced mice exposed to PM

Sacrificed mouse lung tissues were used to isolate total RNA using Trizol reagent (Life technologies) and the RNA yield was measured using NanoVue Plus spectrophotometer (GE Healthcare Life Sciences, UK). Single strand cDNA was synthesized using promega A3500 cDNA synthesis kit (St Louis, Cam, USA) according to manufacturer's protocol and qPCR was carried out with StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) using the Power SYBER Green PCR Master Mix (Applied Biosystems, USA). The fold changes in expression were calculated using 2-ΔΔCT method and endogenous control GAPDH was used for normalization. The primers used are indicated in Supplementary Table 3.

Table 3. Mouse primer sequences for quantitative PCR in lung

Gene	Sequence	
	Forward (5'-3')	Reverse (5'-3')
IL - 4	ACG GAG ATG GAT GTG CCA AAC GTC	CGA GTC ATC CAT TTG CAT GAT GC
IL-13	CAA TTG CAA TGC CAT CTA CAG GAC	CGA AAC AGT TGC TTT GTG TAG CTGA
IL-17a	TCA ACC GTT CCA CGT CAC CCT GGA C	TCA GCA TTC AAC TTG AGC TCT CAT GC
IFN-γ	AGG TCA ACA ACC CAC AGG TCC A	CCA GAT ACA ACC CCG CAA TCA C
IL-10	GCT ATG CTG CCT GGT CTT ACT G	TCC AGC TGG TCC TTT GTT TG
IL-22	ACC TTT CCT GAC CAA ACT CA	AGC TTC TTC TCG CTC AGA CG
TNF-α	GGC AGC TTC TGT CCC TTT CAC TC	CAC TTG GTG GTT TGC TAC GAC G
IL-1β	GCT ACC TGT GTC TTT CCC GTC G	TTG TCG TTG CTT GGT TCT CCT TG
MUC5AC	AAA GAC ACC AGT AGT CAC TCA GCA A	CTG GGA AGT CAG TGT CAA ACC
MUC5B	AAA GAC ACC AGT AGT CAC TCA GCA A	CTG GGA AGT CAG TGT CAA ACC
MUC2	GCT GAC GAG TGG TTG GTG AAT G	GAT GAG GTG GCA GAC AGG AGA C

IL: Interleukin, IFN-γ: Interferon- γ, TNF-α: Tumor necrosis factor-α, MUC: Mucin

Statistical analysis

Numerical data are presented as the means ± S.E.M. for each group. Statistical analysis was performed by using Student's *t*-test. $P < 0.05$ was considered significant in this study.

Results

OLE diminishes Differential cell quantity of blood in asthma induced mice exposed to PM

During the first part of this study, the number of inflammatory cells in blood tissue were analysed to appraise the potential of OLE to restrain the inflammatory responses of PM induced mice. The results of the differential counting of blood have shown a significant increase in OVA+PM group in eosinophils and basophils compared to healthy control. Inflammatory cells of blood tissue in OVA+PM group has shown a significant increase in eosinophil content (by 3.6 folds, $p<0.05$) while inflammatory cells in the group treated with OVA+PM+OLE 200 group (by 2.3 folds, $p<0.005$) had indicated a significant decrease in the eosinophil content (Fig. 4D). As well, the basophil content was significantly increased in each group treated with OVA only (by 10 folds, $p<0.005$), OVA+PM group (by 11.2 folds, $p<0.005$), PM only group (by 14.6, $p<0.005$) compared to healthy control respectively. The high concentration of OLE was discovered as most effective as prednisone to deplete basophil count in the blood (by 9.3 folds, $p<0.01$) (Fig. 4E). As per the inflammatory cell number in blood, elevated eosinophil and basophil counts due to PM in blood was significantly decreased by OLE. The OLE were recognized as competent to modulate the typical inflammatory cell numbers (Fig. 4).

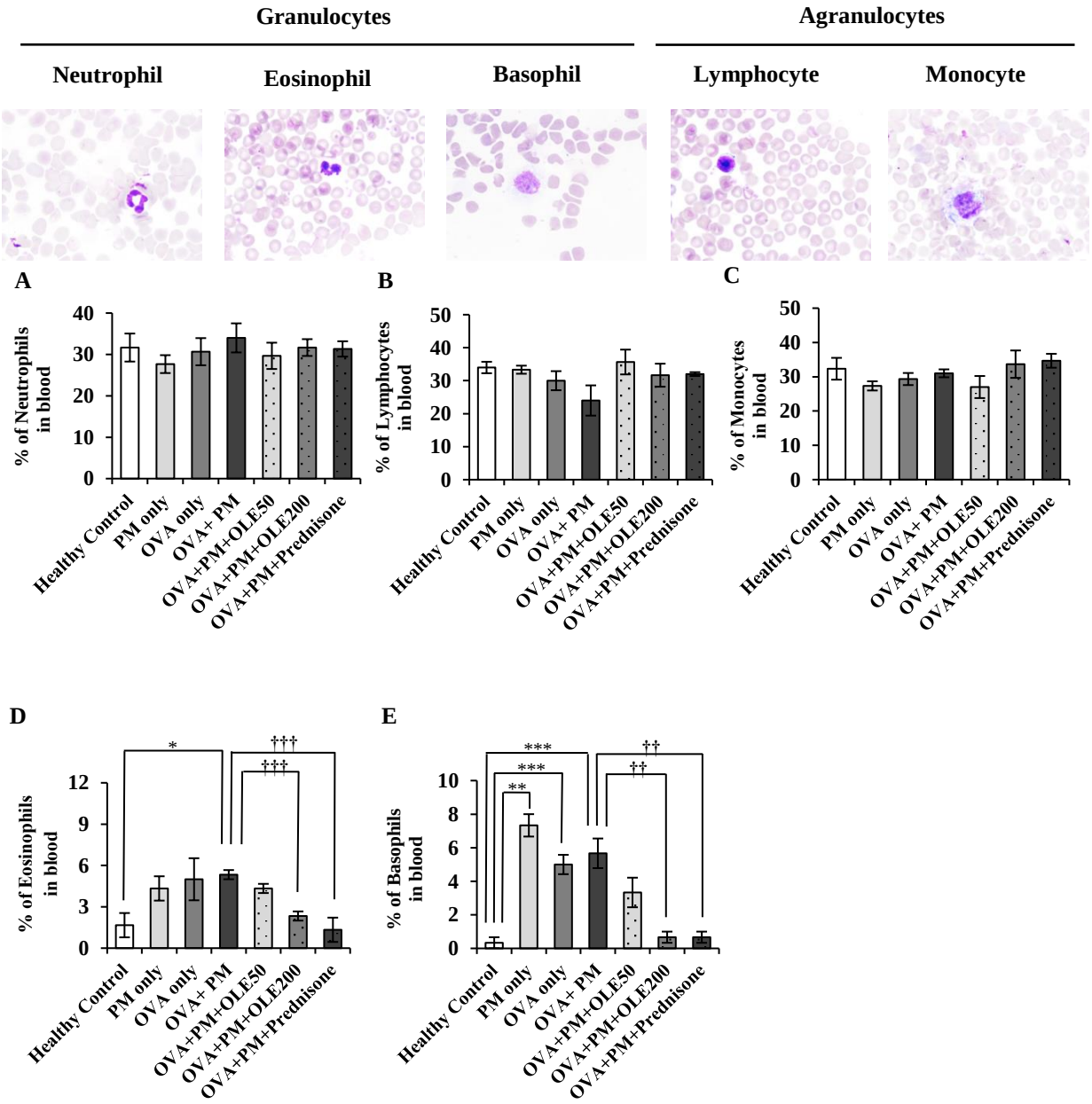

Figure 4. Contrast of Differential cell quantity of blood in asthma induced mice exposed to PM. Percentages of (A) neutrophils, (B) lymphocytes, (C) monocytes, (D) eosinophils and E basophils in blood. All data are represented as the mean ± S.E. (n=3). *($p<0.05$), ** ($p < 0.01$), *** ($p < 0.005$) represent significant increase compared to untreated control. † ($p<0.05$), †† ($p<0.01$), and ††† ($p<0.005$) represent significant decrease compared to OVA+PM group.

OLE diminishes Differential cell quantity of BALF in asthma induced mice exposed to PM

The number of inflammatory cells in BALF tissue were also analysed to ascertain the potential of OLE to suppress the inflammatory responses of PM induced mice. The results of the differential counting of BALF have shown a significant increase in OVA+PM group in eosinophils and basophils compared to healthy control. Inflammatory cells of BALF in PM group, OVA and OVA+PM groups have shown a significant increase in eosinophil content (by 2.0 folds, $p<0.0005$; 1.6 folds, $p<0.01$; by 1.8 folds, $p<0.0005$ respectively) similar with prednisone group while inflammatory cells in the group treated with OVA+PM+OLE 50 group (by 0.7 folds, $p<0.01$) and OVA+PM+OLE 200 group (by 0.6 folds, $p<0.01$) had indicated a significant decrease in the eosinophil content (Fig. 5D). As well, the basophil content was significantly increased in each group treated with OVA only group (by 2.5 folds, $p<0.05$) followed by OVA+PM group (by 2.7 folds, $p<0.05$) compared to healthy control respectively. Both concentrations of OLE, OVA+PM+OLE 50 and OVA+PM+OLE 200 were discovered as significantly effective to deplete basophil count in the BALF (by 0.4 folds, $p<0.05$) and (by 0.3 folds, $p<0.05$) respectively (Fig. 5E). In accordance with the data, PM elevated eosinophil and basophils counts in BALF was significantly decreased by OLE. The OLE was recognized as competent to regulate the typical inflammatory cell numbers (Fig. 5).

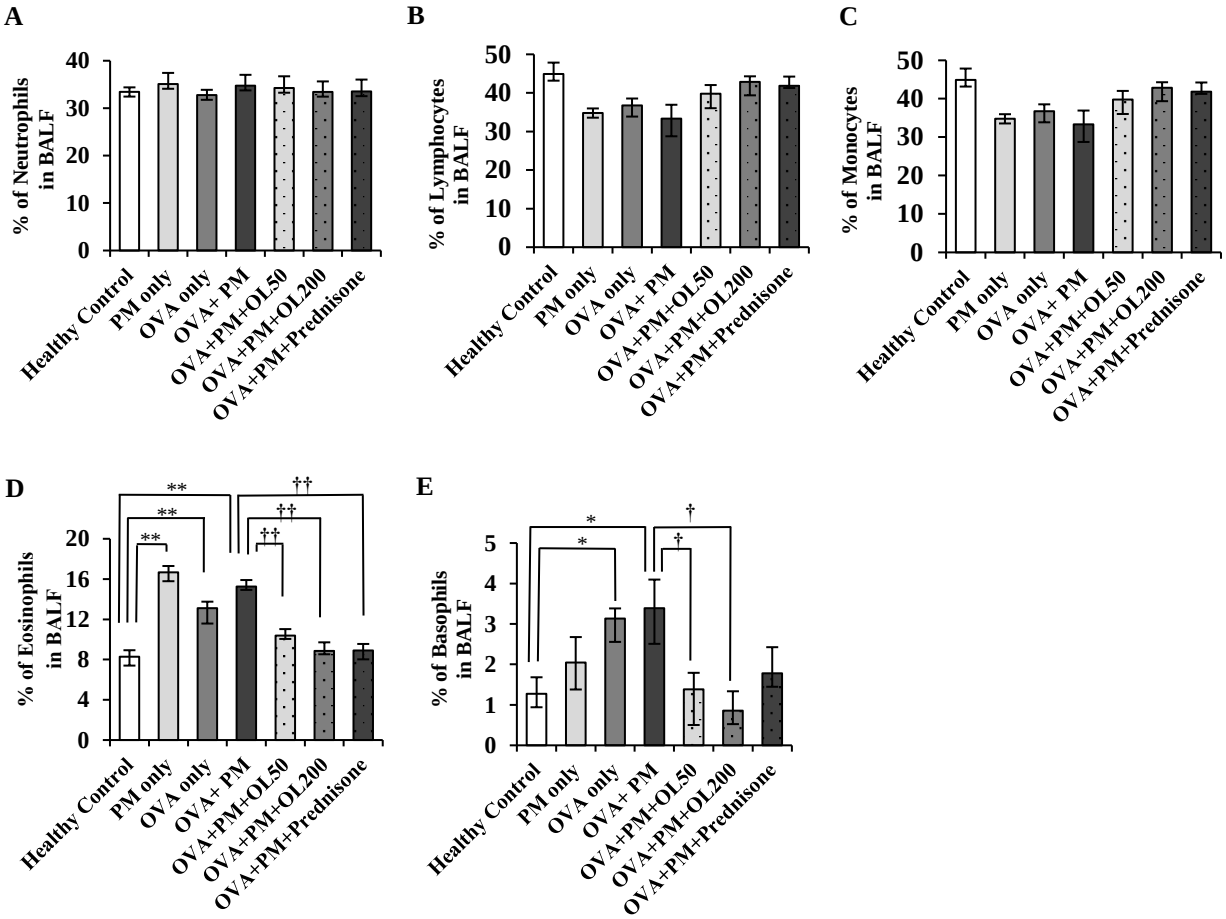

Figure 5. Contrast of Differential cell quantity of BALF in asthma induced mice exposed to PM. Percentages of (A) neutrophils, (B) lymphocytes, (C) monocytes, (D) eosinophils and E basophils in BALF. All data are represented as the mean ± S.E. (n=3). *($p<0.05$) and ** ($p < 0.01$) represent significant increase compared to untreated control. † ($p<0.05$) and †† ($p<0.01$) represent significant decrease compared to OVA+PM group.

OLE curtails histopathological inflammatory cell infiltration in asthma induced mice exposed to PM

The H&E staining of lung and trachea sections of each group were analysed to assess the degree of peri-bronchial and peri-vascular inflammatory cell infiltration for further corroboration of histopathological changes of respiratory system. In trachea, OVA+PM group has shown a significant increase in inflammatory cell infiltration in compared to healthy control and relative inflammatory score in OVA+PM group was even higher than that in PM only or OVA only groups. The relative inflammatory score in the group treated with OVA+PM+OLE 50 was lower in tracheal mucosa. Compared with OVA+PM+OLE 50 group, it has shown a lesser severity in infiltration of inflammatory cells in group treated with OVA+PM+OLE 200 and interestingly it was similarly effective as Prednisone. In lung tissues, severe inflammatory cell infiltration was recognized in OVA only and OVA+PM groups which were similar to each other compared with healthy control. Relative inflammatory score was lower in PM only group than that in OVA+PM group. The group treated with OVA+PM+OLE 50 also has shown a moderately severe infiltration of inflammatory cells, and compared with trachea it was more severe in lung tissues. Comparatively, OVA+PM+OLE 200 group has indicated a lower inflammatory cell infiltration, and it was similarly effective as Prednisone. According to these results, it was explicit that PM exacerbated the pulmonary histopathological changes in OVA sensitized asthmatic mice. Decisively, augment of inflammatory cell infiltration caused by PM was efficaciously reversed by OLE (Fig. 6).

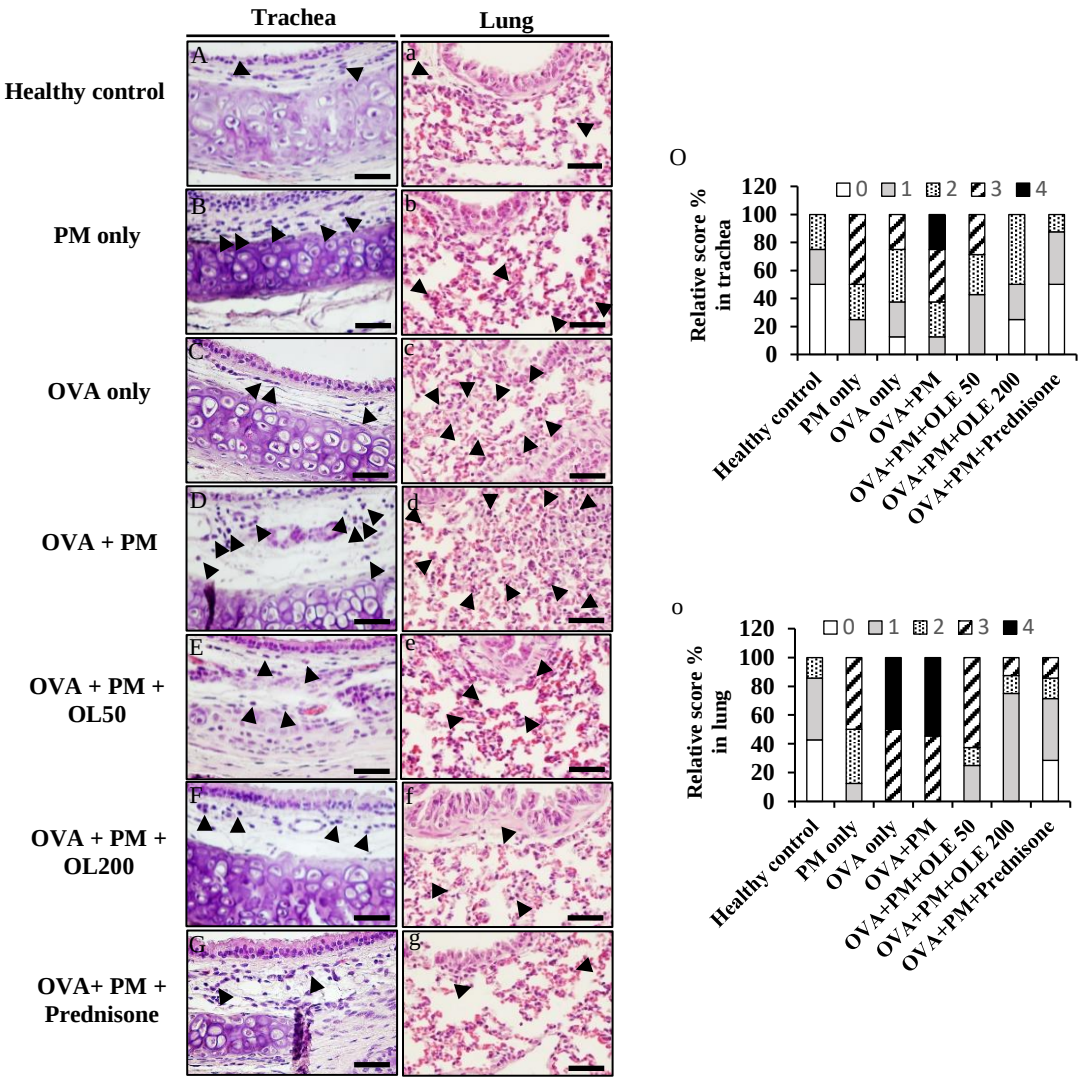

Figure 6. Impact of OLE on histopathological changes of inflammatory cell infiltration in asthma induced mice exposed to PM.

Representative images of trachea and lung sections stained with H&E. Inflammatory cell infiltration is represented by arrow heads. The scoring for inflammatory cell infiltration of trachea and lung is represented in (O) and (P) respectively at least by n=2. The degree of inflammatory cell infiltration in trachea and lung of at least two mice per each group were separately analysed according to a score as follows: 0-normal; 1-few cells infiltrated; 2-a ring of inflammatory cells one cell layer deep; 3-a ring of inflammatory cells 2-4 cell layers deep; 4-a ring of inflammatory cells >4 cell layers

deep.

OLE drastically reduces eosinophil infiltration in asthma induced mice exposed to PM

The influx of eosinophil among the infiltrated inflammatory cells in lung and trachea was estimated through Congo red analysis. In trachea, OVA+PM group (by 4.0 folds, $p<0.0005$) has shown a significant increase in eosinophil infiltration compared with healthy control group. OVA only (by 1.9 folds, $p<0.05$) and PM only (by 3.1 folds, $p<0.0005$) groups also alone shows significant increase in eosinophil infiltration but lower than the OVA+PM group. OVA+PM+OLE 200 group (by 2.8 folds, $p<0.0005$), OVA+PM+OLE 50 group (by 3.1 folds, $p<0.0005$) and OVA+PM+Prednisone group indicates significantly reduced eosinophil infiltration respectively in trachea sections. In lung tissues, OVA+PM group (by 7.3 folds, $p<0.005$) has shown a significant increase in infiltration of eosinophils compared with healthy control group. OVA only (by 4.8 folds, $p<0.0005$) and PM only (by 4.1 folds, $p<0.0005$) groups also alone shows significant increase in eosinophil infiltration but lower than the OVA+PM group which has the similar pattern with trachea. But in lung tissues OVA+PM+OLE 50 (by 2.2 folds, $p<0.0005$), OVA+PM+OLE 200 (by 4.0 folds, $p<0.0005$) and OVA+PM+Prednisone groups indicated significantly lesser infiltration of eosinophils respectively. OLE had effectively brought up the PM enhanced eosinophil count particularly into the typical levels of healthy control (Fig. 7).

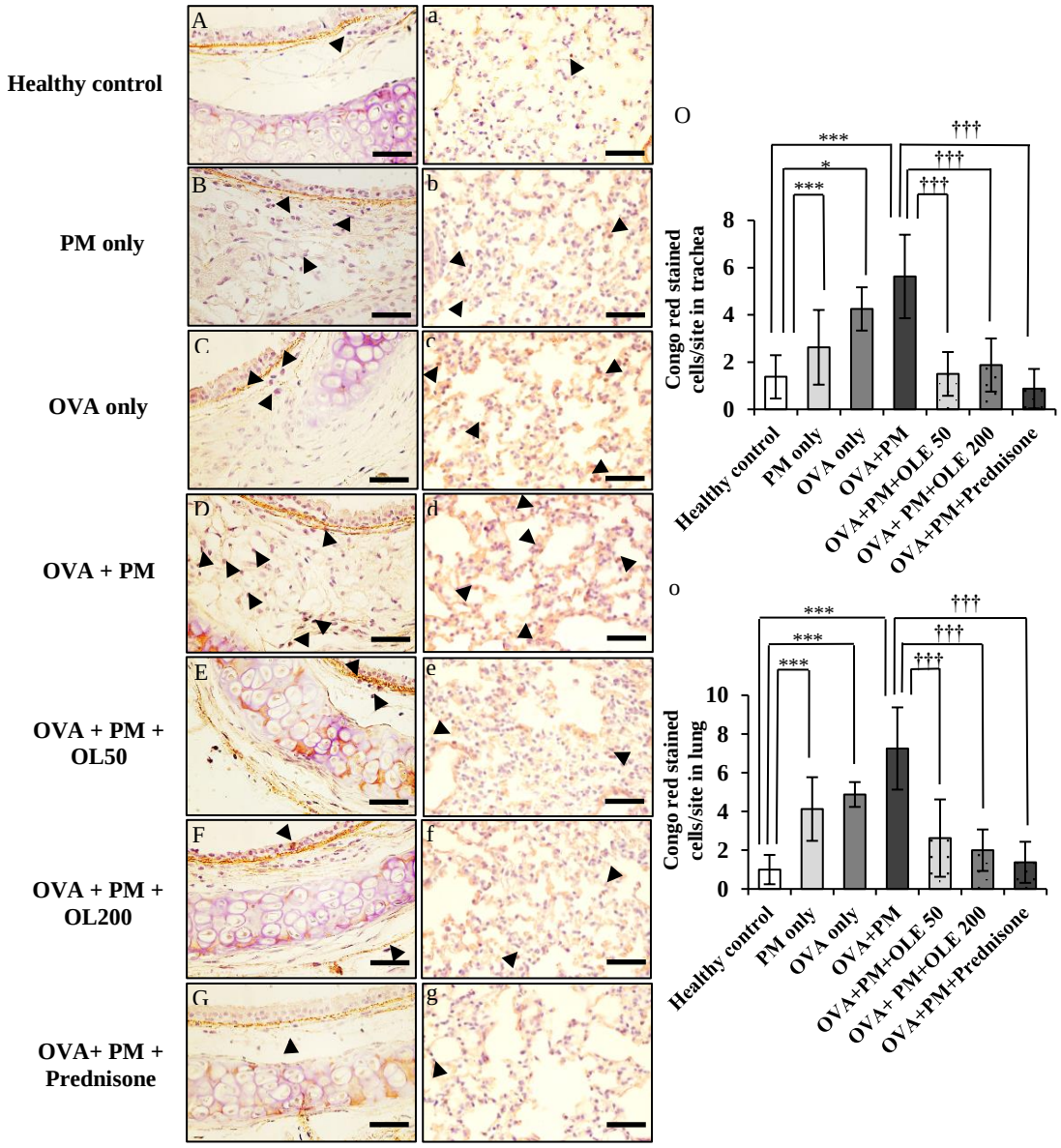

Figure 7. Congo red staining of trachea and lung in asthma induced mice exposed to PM. (A, a) Healthy control group, (B, b) PM Only group, (C, c) OVA Only group, (D, d) OVA+PM group, (E, e) OVA+PM+OLE 50 group, (F, f) OVA+PM+OLE 200 group, (G, g) OVA+PM+Prednisone group. (O) Congo red stained cells/site in trachea, (o) Congo red stained cells/site in lung. * ($p < 0.05$), ***($p < 0.0005$) represent significant increase compared to healthy control and ††† ($p<0.005$) indicate significant decrease compared to OVA+PM.

OLE attenuates mast cell degranulation in asthma induced mice exposed to PM

Toluidine blue analysis in PM exposed trachea was conducted to estimate the effect of OLE on mast cell infiltration and degranulation. A significant increase in infiltration of mast cells could be observed in OVA+PM group (by 3.3 folds, $p<0.005$) and followed by OVA only (by 2.4 folds, $p<0.05$) and PM only (by 2.0 folds, $p<0.05$) groups compared to the healthy control respectively. Mast cell activation was significantly reduced in OVA+PM+OLE 50 (by 1.6 folds, $p<0.01$), OVA+PM+OLE 200 (by 2.1 folds, $p<0.05$) and OVA+PM+Prednisone groups respectively. A significant increase in activated mast cells could be observed in OVA+PM group (by 5.3 folds, $p<0.005$) and followed by OVA only (by 4.2 folds, $p<0.05$) and PM only (by 4.0 folds, $p<0.05$) groups compared to the healthy control respectively. Mast cell activation was significantly reduced in OVA+PM+OLE 50 (by 1.6 folds, $p<0.05$), OVA+PM+OLE 200 (by 1.8 folds, $p<0.05$) and OVA+PM+Prednisone groups respectively. As demonstrated by the data, OLE showed a potential to restraint the mast cell infiltration and activation which were enhanced by exposing to PM (Fig. 8).

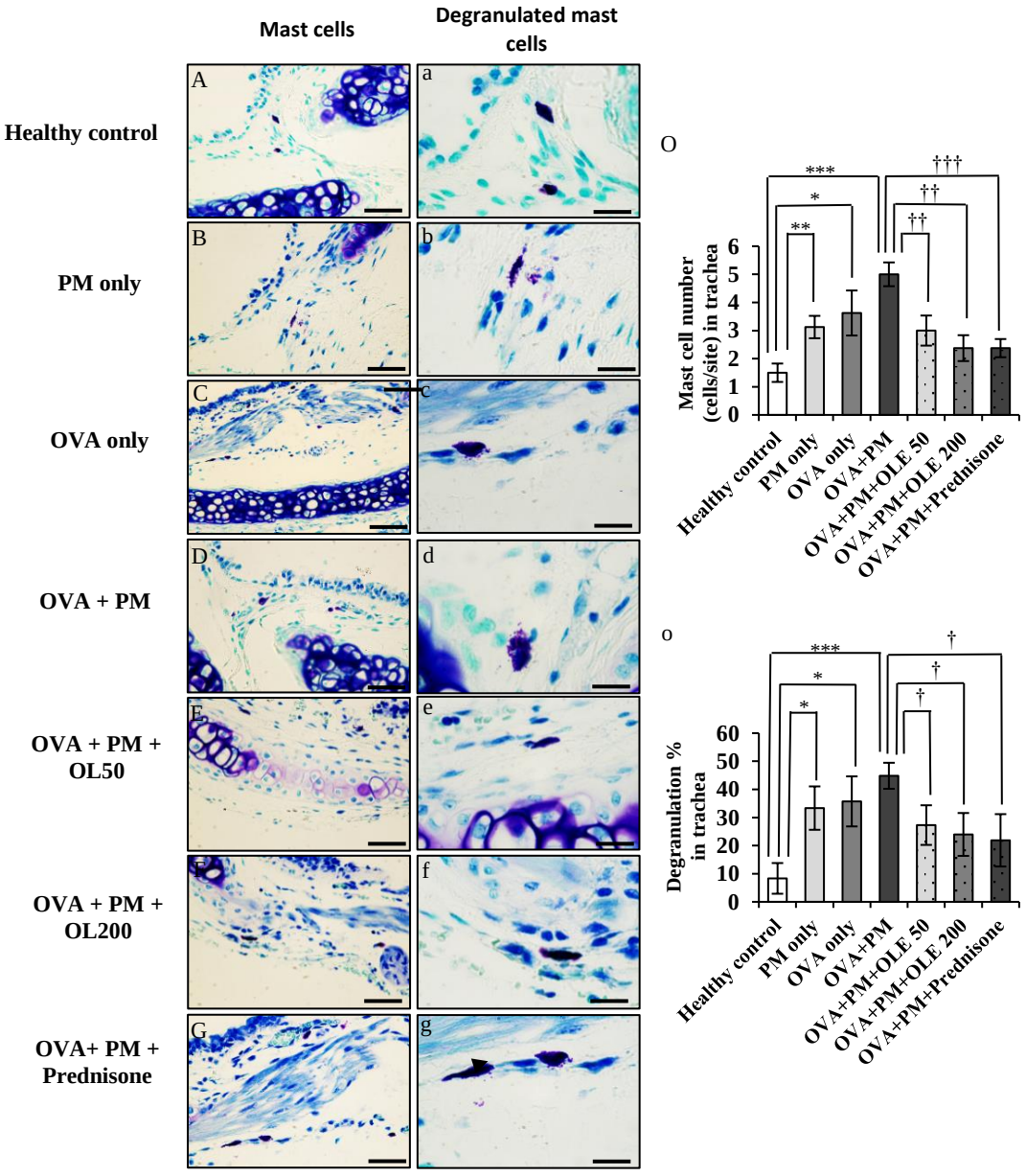

Figure 8. Toludine blue staining of trachea in asthma induced mice exposed to PM. (A, a) Healthy control group, (B, b) PM Only group, (C, c) OVA Only group, (D, d) OVA+PM group, (E, e) OVA+PM+OLE 50 group, (F, f) OVA+PM+OLE 200 group, (G, g) OVA+PM+Prednisone group. (O) mast cell number/site in trachea, (o) mast cell degranulation percentage in trachea. * ($p < 0.05$), **($p < 0.01$), ***($p < 0.0005$) represent significant increase compared to healthy control and † ($p<0.05$), †† ($p<0.01$), ††† ($p<0.005$) indicate significant decrease compared to PM+OVA.

OLE involves in masking the expression of Toll-Like Receptors (TLRs) in asthma induced mice exposed to PM

The results we gained through RT-PCR for mRNA expression of TLR1-TLR9 with a view of scrutinize the TLRs for being activated by PM, verifies that PM is a crucial factor in up-regulating the expression of TLRs by denoting significant increase of TLR2 and TLR 4 in OVA+PM group compared to untreated group by 12.8-fold ($p<0.005$) and 4.1 folds ($p<0.5$) respectively. In consonance with RT-PCR data, the groups OVA+PM+OLE 50 and OVA+PM+OLE 200 unveil a significant decrease in TLR4 mRNA expressions by 95.5 folds($p<0.05$) and 66.2 folds($p<0.05$) respectively. Interestingly, TLR2 expressions by 188.6 folds ($p<0.005$) and 172.5 folds ($p<0.005$) respectively. Moreover, mRNA levels of TLR1 expression showed a significant decrease in the groups OVA+PM+OLE 50 and OVA+PM+OLE 200 accordingly by 7.9 folds ($p<0.005$) and 6.1 folds($p<0.005$) while TLR7 shows significant reduction in the group OVA+PM+OLE 200 by 0.5 folds ($p<0.005$) and TLR8 shows a significant reduction in OVA+PM+OLE 50 group by 20.0 folds ($p<0.005$). Nevertheless, it hypothecates the fact that OLE is influential in masking TLR2, TLR4, TLR1, TLR7 and TLR8 expression in PM exposed asthmatic mice according to the vigour of the significance, while there can be a combine effects on down regulating TLR activities by OLE in asthma (Fig.9).

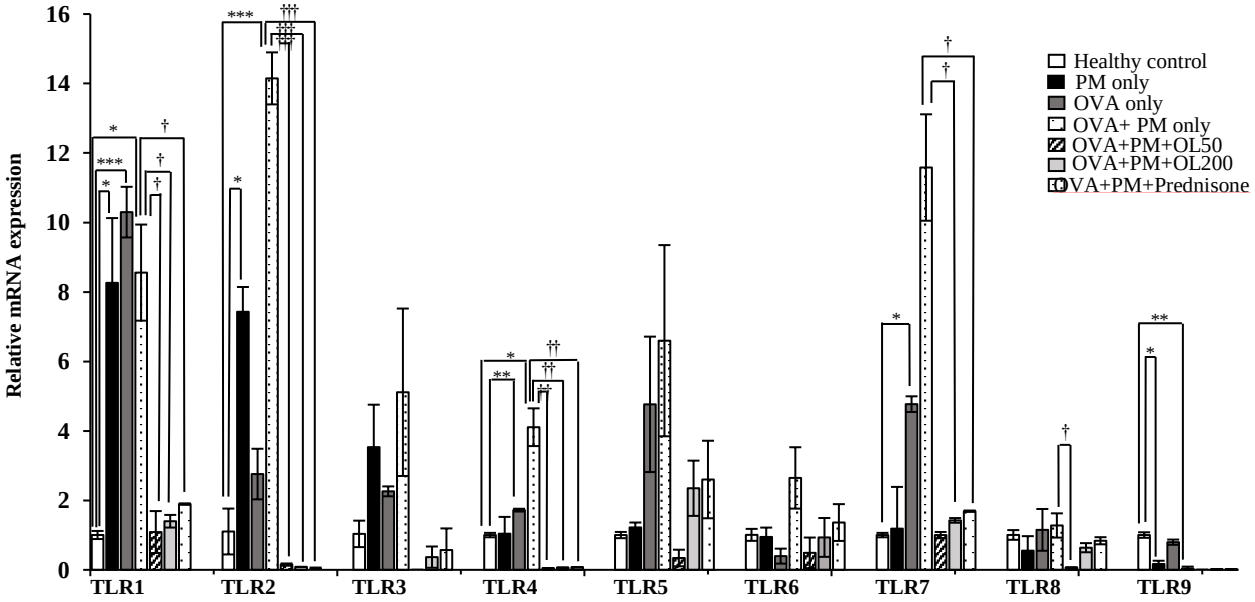

Figure 9. Quantitative real time PCR analysis of mRNA expression of TLR1, 2, 3, 4, 5, 6, 7, 8 and 9 in asthma induced mice exposed to PM. * ($p < 0.05$), **($p < 0.01$), ***($p < 0.0005$) represent significant increase compared to healthy control and † (p<0.05), †† (p<0.01), ††† (p<0.005) indicate significant decrease compared to OVA+PM group.

OLE mitigates anti-inflammatory cytokine production from Th2, Th17 cells in asthma induced mice exposed to PM

To analyse the effect of OLE against PM induced cytokine secretion, cytokine levels produced by Th1, Th2 and Th17 cells were measured. IL-4 expression was significantly increased in OVA+PM group (by 1.1 folds, $p<0.05$) compared to the healthy control. It showed significant decrease in OVA+PM+OLE 50 group (by 1.0 folds, $p<0.05$), OVA+PM+OLE 200 group (by 1.0 folds, $p<0.05$). IL-10 expression was significantly increased in OVA+PM group (by 1.2 folds, $p<0.05$) compared to the healthy control. It showed significant decrease in OVA+PM+OLE 50 group (by 1.2 folds, $p<0.05$), OVA+PM+OLE 200 group (by 1.1 folds, $p<0.05$). IL-13 expression was significantly increased in OVA+PM group (by 0.9 folds, $p<0.05$) compared to the healthy control. It showed significant decrease in OVA+PM+OLE 50 group (by 1.0 folds, $p<0.05$), OVA+PM+OLE 200 group (by 1.1 folds, $p<0.05$). IL-17 expression was significantly increased in OVA+PM group (by 1.4 folds, $p<0.05$) and in PM only group (by 1.1 folds, $p<0.005$) compared to the healthy control. It showed significant decrease in OVA+PM+OLE 50 group (by 1.1 folds, $p<0.05$), OVA+PM+OLE 200 group (by 1.1 folds, $p<0.05$). IL-22 expression was significantly increased in OVA+PM group (by 1.2 folds, $p<0.01$) compared to the healthy control. It showed significant decrease in OVA+PM+OLE 50 group (by 1.2 folds, $p<0.01$), OVA+PM+OLE 200 group (by 1.1 folds, $p<0.01$). OLE significantly reduced anti-inflammatory cytokines production from Th2, Th17 cells and pro-inflammatory cytokine production from Th1 cells allowing to illustrate the potential of restraining the inflammatory conditions caused by PM inhalation. (Figs. 10).

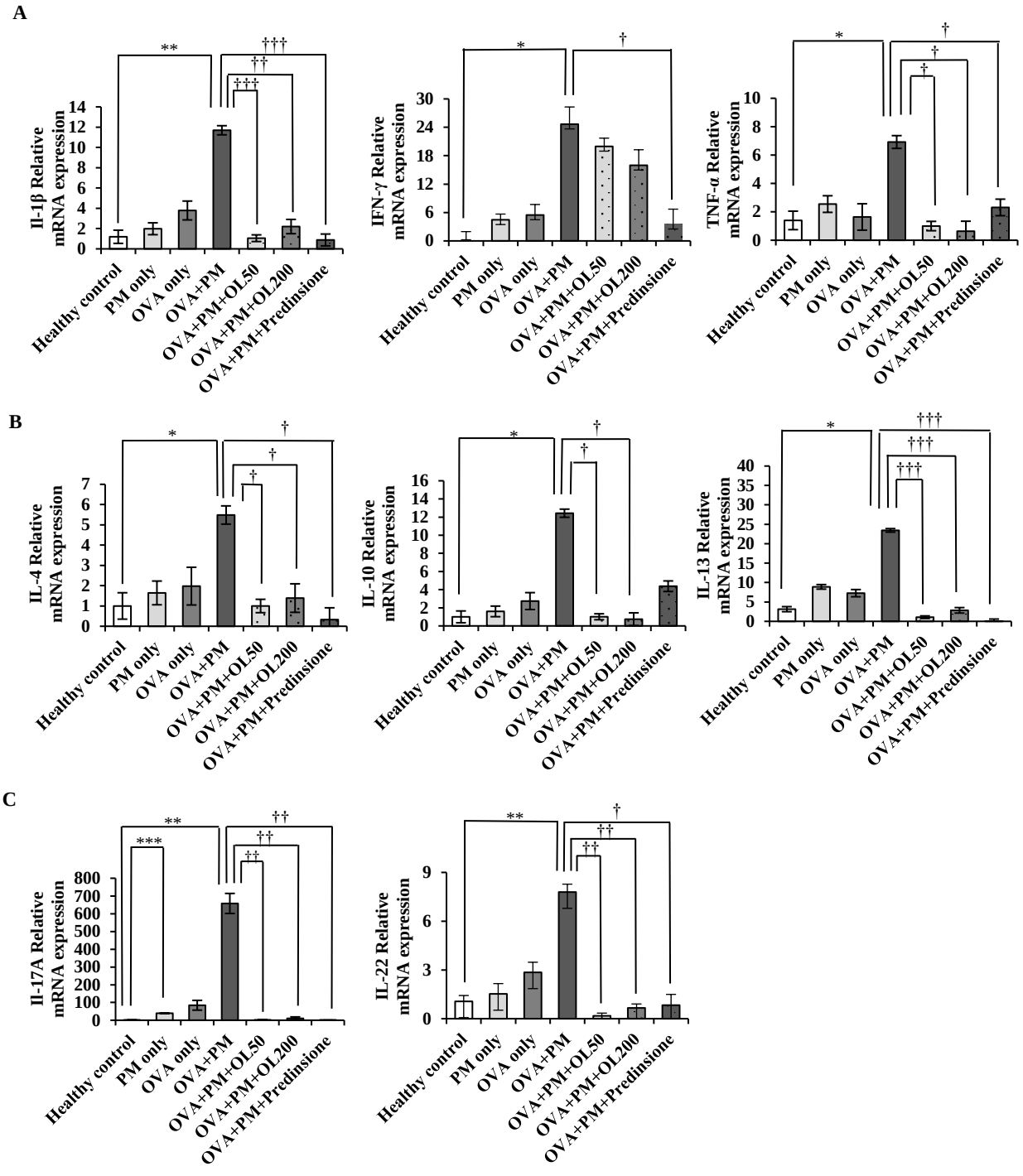

Figure 10. Impact of OLE on pro-inflammatory cytokine expression in asthma induced mice exposed to PM. (A) Th1 cytokine (TNF-α, IFN-γ and IL-1β); (B) Th2 cytokines (IL-4, IL-10, IL-13); (C) Th17 cytokines (IL-17, IL-22). * ($p < 0.05$), **($p < 0.01$), ***($p < 0.0005$) represent significant increase compared to healthy control

and † ($p<0.05$), †† ($p<0.01$), ††† ($p<0.005$) indicate significant decrease compared to PM+OVA.

OLE suppress the mucus hypersecretion from goblet cells in asthma induced mice exposed to PM

PAS analysis was carried out in lung and trachea of PM exposed mice for particularizing the consequences of OLE against mucus hyper secretion from the goblet cells. PAS staining in trachea has shown a significant over-production of mucus and goblet cell hyperplasia of the OVA+PM group (by 7.8 folds, $p<0.005$) and in OVA only group (by 4.2 folds, $p<0.005$) respectively compared to the healthy control. Mucus and goblet cell hyperplasia was higher in OVA+PM group than OVA only and PM only groups separately. It shows a significant decrease of over-production of mucus and goblet cell hyperplasia in both OVA+PM+OLE 50 group (by 1.8 folds, $p<0.005$) and OVA+PM+OLE 200 group (by 1.8 folds, $p<0.005$) which are similar to each other. In lung tissues, it has shown a significant over-production of mucus and goblet cell hyperplasia of the OVA+PM group (by 6.4 folds, $p<0.005$), in PM only group (by 5.3 folds, $p<0.005$) and in OVA only group (by 4.8 folds, $p<0.005$) respectively compared to the healthy control. The mucus and goblet cell hyperplasia have been significantly decreased in OVA+PM+OLE 50 (by 2.2 folds, $p<0.005$) and OVA+PM+OLE 200 (by 6.6 folds, $p<0.005$) groups respectively according to the dose. The capacity of OLE to suppress the PM-induced goblet cell in lung and trachea giving results similar with prednisone was effectively demonstrated. (Fig. 11).

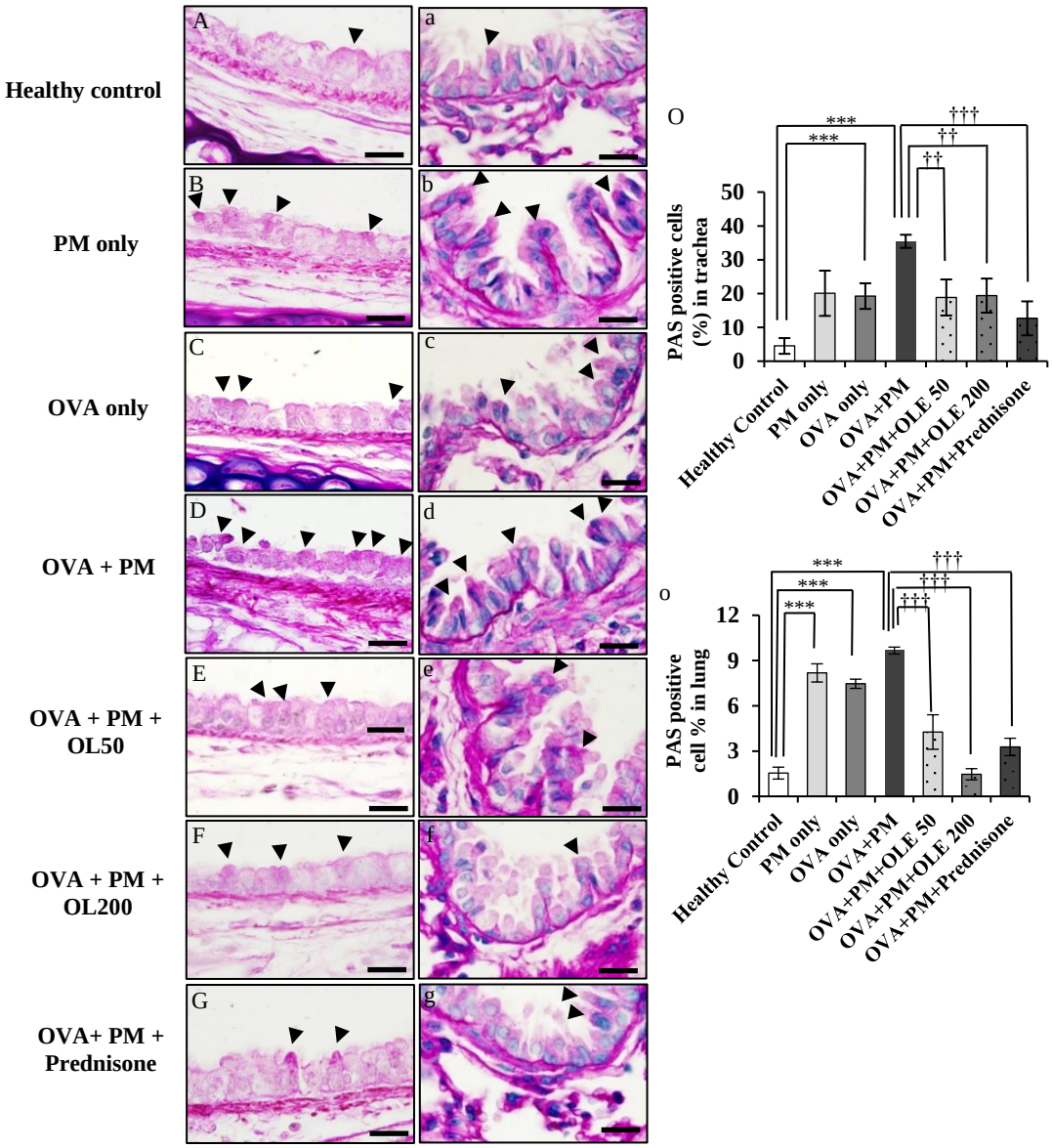

Figure 11. PAS staining for mucus secretion of goblet cells in asthma induced mice exposed to PM. (A, a) Healthy control group, (B, b) PM Only group, (C, c) OVA Only group, (D, d) OVA+PM group, (E, e) OVA+PM+OLE 50 group, (F, f) OVA+PM+OLE 200 group, (G, g) OVA+PM+Prednisone group. (O) PAS positive cell percentage in trachea, (o) PAS positive cell percentage in lung. ***($p < 0.0005$) represent significant increase compared to healthy control and †† ($p<0.01$), ††† ($p<0.005$) indicate significant decrease compared to PM+OVA.

OLE downgrade MUC5AC over expression lung in asthma induced mice exposed to PM

As the intensity of mucin expression is imitated by the expression levels of the mucin genes, expression levels were measured in MUC5AC, MUC2 and MUC5B genes to find out the responsible gene for exacerbating asthma. Moreover, to investigate how efficient the OLE is to suppress this mucus over production, we conducted PAS analysis by counting mucus secreted cells. According to the results taken by Real-time PCR to investigate the mRNA expression of mucin genes in the lung, it was recognized that MUC5AC expression level was higher than MUC2 and MUC5B. MUC5AC expression in trachea was significantly highest in OVA+PM group (by 7.6 folds, $p<0.005$), followed by PM only group (by 4.0 folds, $p<0.05$) and OVA only group (by 2.6-fold, $p<0.005$) compared with healthy control respectively OVA+PM+OLE 50 group (by 3.7 folds, $p<0.005$), OVA+PM+OLE 200 group (by 3.3 folds, $p<0.005$) and OVA+PM+Prednisone group show a similar expression of MUC5AC which are lesser than expression in OVA+PM group. MUC5AC expression in lung tissues was significantly highest in OVA+PM group (by 5.0 folds, $p<0.005$), followed by PM only group (by 2.1 folds, $p<0.05$) and OVA only group (by 2.5-fold, $p<0.05$) compared with healthy control respectively. OVA+PM+OLE 50 group (by 1.7 folds) showed a decreased expression of MUC5AC but was higher than OVA+PM+OLE 200 group (by 2.7 folds, $p<0.05$) and OVA+PM+Prednisone group show a similar expression of MUC5AC which are lesser than expression in OVA+PM group. According to the results of MUC5AC expression analysis of lung and trachea it is obvious that OLE gives a similar effective result with prednisone by reducing mucus secretion which was exacerbated by PM expose (Fig. 12).

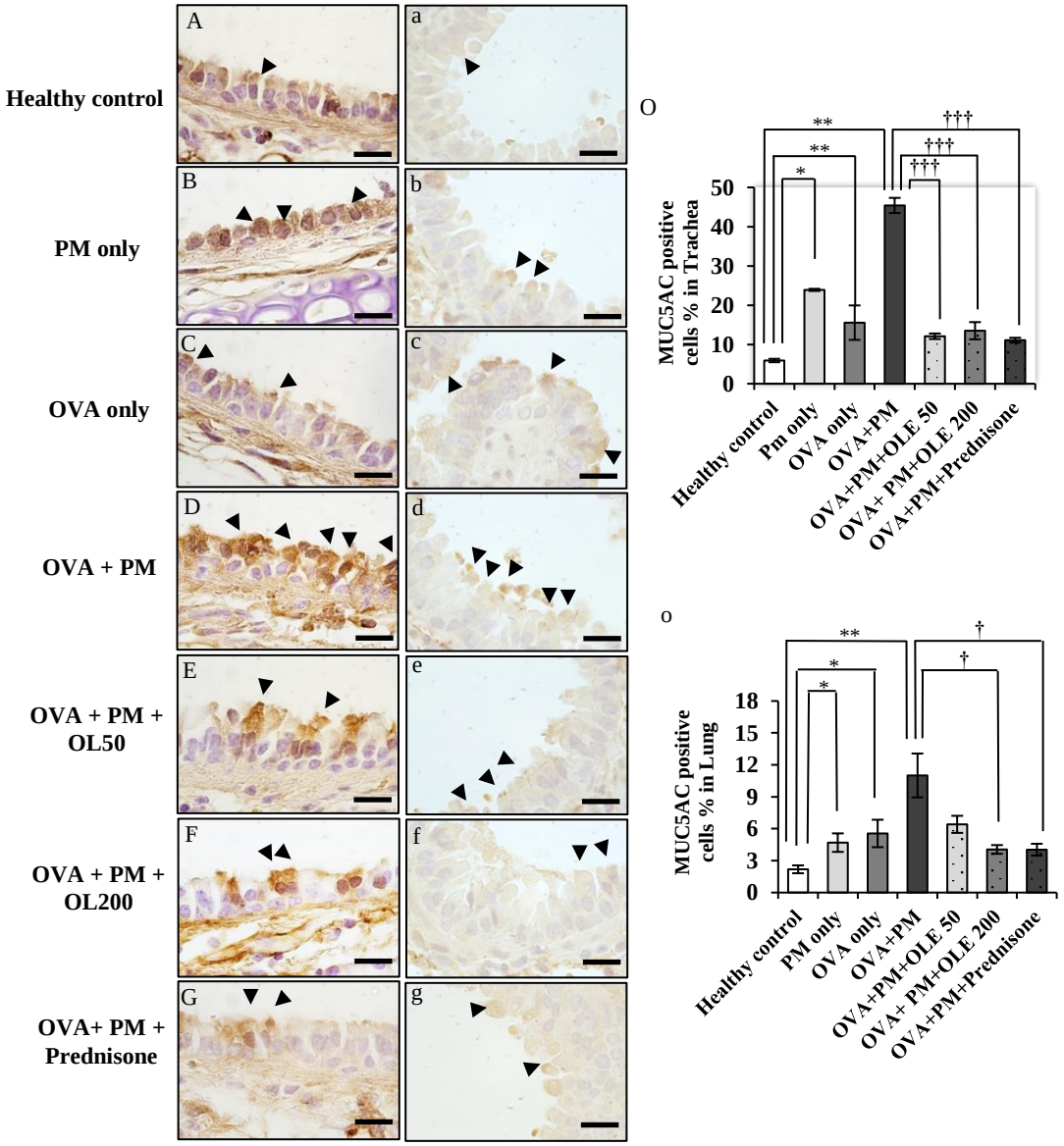

Figure 12. Impact of OLE on MUC5AC-positive cells of trachea and lung in asthma induced mice exposed to PM. (A, a) Healthy control group, (B, b) PM Only group, (C, c) OVA Only group, (D, d) OVA+PM group, (E, e) OVA+PM+OLE 50 group, (F, f) OVA+PM+OLE 200 group, (G, g) OVA+PM+Prednisone group. (O) MUC5AC-positive cell percentage in trachea, (o) MUC5AC-positive cell percentage in lung. * ($p < 0.05$), **($p < 0.01$), ***($p < 0.0005$) represent significant increase compared to healthy control and † (p<0.05), ††† (p<0.005) indicate significant decrease

compared to PM+OVA.

Discussion

This study was effectuated with an intend of appraising the consequences of PM on asthma related inflammatory status, and particularly the profound effects of the extract of olive leave (OLE) to reverse the asthma symptoms of PM-induced mice. Contemplating imitation to human exposure to environmental PM, we unveiled mice to inhale PM via nebulizer with an assumption of generating asthma with PM, which our study was mainly focused on.

As advocated by recent studies, BALF and blood eosinophil count is a crucial factor for prognosticating the aggravation of asthma. In our trial, the magnitude of eosinophil count increment was significantly higher than the baseline in both blood and BALF of OVA+PM grouped PM induced mice. Interestingly, basophil count also showed same increment behaviour in same group of OVA-sensitized PM-inhaled mice. However, OLE seems to be efficacious with regulating inflammatory cells in blood and BALF of asthma-induced mice to an impeccable number. Airway inflammation is a crucial factor for asthma generation and exacerbation. According to McAlinden et al (2019) their results proclaim that asthmatic lungs manifest a greater immune cell influx compared to non-asthmatic lungs. In line with Herath et al (2020) a huge histopathological change could be observed in mice treated with both OVA and PM concomitantly (OVA+PM group) in their asthma model. According to Kennedy-Feitosa et al (2016), these inflammations lead to structural changes in airways which have potent to aggravate asthmatic conditions. It is worth noting that, the OVA+PM group of our study markedly showed a huge difference in histopathological grading in airways. It is noteworthy that the highest concentration of OLE was ascertained rehabilitation of the histopathological changes of the asthmatic airways.

Moreover, to attest the inflammatory status aggravation, the increment of eosinophil infiltration also notably high in mimicable asthma induced group. Eosinophils are known as complex cells possessing surface molecules and receptors that are relied in a wide range as well as cytoplasmic granules too, while having the potency of forming extracellular DNA traps when the eosinophils are activated which expose negative correlation with lung function of asthmatic patients, thus, airway remodelling with inhibiting epithelial cell surface plasmin generation supported by self-released TGF-β (Siddiqui et al., 2023). Confirming the correspondence between eosinophils and asthma impacted airway Herath et al (2020) has noted that there is a significantly enhanced eosinophil infiltration could have been observed in the OVA+PM group in their study of asthma model. Similar to that, in our study also we detected a significant increase of eosinophil count in the group treated with both OVA and PM and this effect was significantly declined by OLE treatments advocating its applicability to use as a remedy for eosinophilic inflammation in asthma.

Mast cell degranulation is known to be an endorsement of allergy conditions and these cells dwell in tissues unveiled to the environment, it is recognized as the immune cell type that firstly defence against infections (Banafea et al., 2022). The most outstanding mediator that is secreted by mast cells in the course of allergy increases the permeability of airway epithelial barrier accompanying with the inducement of smooth muscle contraction (Dale et al., 2018; Steelant et al., 2018). Our results suggest that, OLE competent for alleviating mast cell infiltration and degranulation which was induced by asthma. As a consequence of PM inhalation, TLRs that are acquired in antigen presenting cells and airway epithelial cells provoke inflammatory responses by secreting inflammatory mediators (Castaneda et al.,2017). As indicated

by Herath et al (2020), PM boost the expression of TLR2, TLR4 and TLR7 in asthma induced mice. Attesting the preceding state, our study also showed highest significant increase in TLR2, TLR4 and TLR7. Beyond that, we distinguished a significant decline in TLR2, TLR4, TLR1, TLR7 and TLR8 in the groups treated with OLE.

As reported by Herath et al (2019), the over-activation of immune system leads to autoimmune diseases with the progression of inflammatory conditions, and it is benign to use autoimmune suppressants to regulate immune balance. With the exception of that, pro-inflammatory cytokines are known to be upregulating autoimmune conditions while inflammatory cytokines cause the retrogression of acute conditions of the disease (Moudgil and Choubey, 2011). It is known that IL-4 and IL-13 cytokines upregulate (VCAM)-1 (Alpha 4 integrin/ vascular cell adhesion molecule) expression which allow eosinophils to adhere with vascular endothelial cell through to accumulate in inflamed asthmatic airways. Besides, IL-17 is known to be potentiate with IL-8 upregulation which plays a major role in neutrophil accumulation in inflammatory sites (Nakagome and Nagata, 2018). The resulted data of OLE treated groups of our study revealed that it could revert the upregulated inflammatory cytokine levels released by Th2, Th17 cell activation, implying its ability to suppress Th2 and Th17 cell activation.

Mucus hypersecretion is a causal effect for airflow obstruction (Rubin et al., 2014). Ling et al (2015) in their study has noted that, IL-13 is influential in up-regulating mucus hyper-secretion in airways. Further, Yan et al (2014) has mentioned that it is effective to inhibit Th2 cytokine production to overcome airway mucus surfeit. Our results suggest that mucus overproduction from goblet cells can be reversed in a lucrative manner with the treatment of OLE directing towards getting a relief from

airway obstruction. Added to this, MUC2, MUC5AC and MUC5B are found as primary mucin types prevail in human air tract and MUC5AC is the major type which is overproduced in asthma among the aforementioned mucins (Xia et al., 2017). Kim et al (2019) has reported that IL-4 has an influence on aggravating MUC5AC synthesis in airway epithelia. However, as per our results OLE is worthwhile to be prescribed as a remedy to suppress *MUC5AC* gene expression and down-regulate mucus hypersecretion by virtue of being similarly effective as the results of prednisone.

For further evolution of this research conducting LDH assay for assessing its toxicity in in-vitro and in-vivo as well western blotting to verify the signalling pathway also can be suggested.

Conclusion

In conclusion, our study corroborated that OLE restrain Th17 cell activation, hence dampen IL-17 and IL-22 secretion. By the same token, OLE could inhibit IL-13 cytokine secretion from Th2 cells in accordance with our results. Conjointly, we demonstrated that OLE undermine the mast cell infiltration and degranulation up to an effective level as positive control. On top of everything, treatment with OLE diminish TNF-α, IFN-γ and IL-1β secretion from Th1 cells directing to a declined eosinophil and basophil infiltration. In other respects, OLE offers a great reduction in goblet cell number that over-produce mucus as well over-express *MUC5AC* gene.

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