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Amelioration of allergic asthma by *Ziziphora clinopodioides* via upregulation of aquaporins and downregulation of IL4 and IL5

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ABSTRACT

Ziziphora clinopodioides has been frequently used as an anti asthmatic plant in traditional medication. Recent work explores the anti-asthmatic activity of *Z. clinopodioides* in allergen-induced asthmatic mice. Intraperitoneal sensitization followed by intranasal challenge were given with ovalbumin (allergen) to develop allergic asthma. Investigational groups of animals were administered with drug methylprednisolone (MP) (15 mg/kg body weight), n-hexane fraction, ethylacetate fraction, and methanolic extract of *Z. clinopodioides* extract (500 mg/kg b.w.) for successive 07 days. Hematoxyline and eosin (H&E) and periodic acid-Schiff (PAS) stains were used to evaluate histopathological parameters on lung tissues. As an index of lungs tissues edema, wet/dry weight ratio of lungs was determined. Evaluation of expression levels of AQP1, AQP5, IL4, and IL5 was conducted by using RT-PCR. The data exhibited that both *Z. clinopodioides* and MP attenuated differential and total leukocyte counts in hematological examination i.e. in BALF and blood. Treatment with *Z. clinopodioides* also caused suppression of inflammatory cell infiltration and expression levels of IL4 and IL5, the later could have caused attenuation of pulmonary inflammation. The study also found decline in lung wet/dry ratio and goblet cellh hyperplasia in treated groups which indicates amelioration of lung edema. Treatment with *Z. clinopodioides* significantly increased the expression levels of aquaporin-1 and -5, which could have led to reduction in lung edema. The treatment with MP showed comparable results to *Z. clinopodioides*. Current investigation revealed that *Z. clinopodioides* possessed anti-asthmatic property which might be accredited to upregulagted AQP1 and AQP5 levels and downregulated IL4 and IL5 levels.

1. Introduction

Asthma comes from the Greek word meaning shortness of breath (Kale et al., 2010). Mortality and morbidity rates of asthma have been increased since last a few years (Masoli et al., 2004). Chronic inflammatory disease asthma is categorized by airway inflammation, edema, and airways obstruction (Abdureyim et al., 2011; Asai et al., 2003). Many evidences showed that cellular elements, such as eosinophils, neutrophils, macrophages, T-lymphocytes, and epithelial cells played a pivotal role in the pathogenesis of allergen-induced asthma (Abdureyim et al., 2011; Barnes, 1992). Role of many cytokines in airway inflammation is established (Levine, 1995). Over production of Th2 mediated cytokines as a result of imbalance between Th1 and Th2 mediated pathways may lead to the development of airway inflammation (Factor, 2003; Finotto et al., 1994; Lampinen et al., 2004; Mueller et al., 1996; Romagnani, 2000). IL5, a Th2 type cytokine, possesses the property to control eosinophils in allergic asthma by regulating the growth and differentiation of eosinophils (Kopf et al., 1993). Previous studies have documented increased IL5 levels in asthmatic individuals with eosinophilic inflammation (Wills-Karp, 1999). IL4 influences the inflammatory process of allergic asthma by driving Th0 differentiation in favor of Th2 cells (Hesselmar et al., 2010; Li et al., 2013). IL4 has the

Abbreviations: BALF, broncho alveolar lavage fluid; OVA, ovalbumin; AQP, aquaporin; IL, interleukin; EA, ethylacetate; n-H, n-hexane; TLC, total leukocyte count; DLC, differential leukocyte count

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ability to induce the expression of vascular cell adhesion on endothelial cells (Dabbagh et al., 1999; Doucet et al., 1998a; Moser et al., 1992). Movement of water is facilitated by a small transmembrane protein, aquaporins, that are abundantly found in lungs (Borok and Verkman, 2002). Four types of aquaporins are found in lungs tissues and the roles of AQP1 and AQP5 in pulmonary edema are well described (King et al., 1997; Kozono et al., 2002; Ma et al., 2000; Song et al., 2001).

Inhaled β2 agonists have been used as a first line treatment for the management of asthma. But due to their many local and systemic effects such as, weight gain, and muscle weakness etc., use of these agents have been limited (Wenzel and Covar, 2006). Use of corticosteroids for the treatment of allergic asthma is effective, but their use is also restricted by the emergence of various side effects such as, bulging eyes, weight gain, moon like face, muscle weakness, bone metabolism reduction, reduced growth in children, and adrenal suppression. Due to these effects both physicians and patients seeks for the use of alternative traditional medicine (Abbas et al., 2005; Markham and Wilkinson, 2004).

Ziziphora clinopodioides (Lamiaceae), also called as Blue Mint Bush in English (Baytop et al., 1996), kakuti-e-kuhi in Persian, and locally as Kirnanesi (Baytop et al., 1996), widely grows in Afghanistan, Iraq, Iran, and estern part of Turkey. Leaves, flowers, aerial part, and stem of this plant are mostly used for medicinal purpose (Abbas et al., 2005). *Z. clinopodioides* is endorsed as a good medication for the treatment of cough, asthma, bronchitis, lung abscess, and tracheitis in traditional medication system (Toprak and Takir, 2017; Yousefbeyk et al., 2016). In Iran too, different part of this plant are used in asthma, cold, cough (Li et al., 2013). *Z.clinopodioides* has also been pharmacological evaluated as antifungal (Khosravi et al., 2011), antibacterial (Ozturk and Ercisli, 2007), anti-larva (Lolestani and Shayesteh, 2009; Rivi, 2008), antioxidant (Amiri, 2009a, b), vasorelaxant (Senejoux et al., 2010), and anti-arthritic (Shabbir et al., 2018).

Previously, (Tian et al., 2011) evaluated the total polyphenolic contents and total flavonoid contents in *Z. clinopodioides* and found their highest concentration in ethyl acetate fraction. The ethyl acetate fraction also showed the highest anti-oxidant activity. Others investigated the phytochemical composition of n-hexane extract and found pulegone in the highest concentration (Yousefbeyk et al., 2016). Pulegone is known to suppress allergic and inflammatory response (Choi et al., 2018). These findings logically linked the folkloric use of *Z. clinopodioides* in inflammatory disorders. The results gave credibility to the traditional medicinal relevance of Z. clinopodioides as remedy of inflammatory disorders of respiratory system, and warranted further scientific investigation of anti-inflammatory activity of n-hexane and ethyl acetate extracts using model of airway inflammation. This study investigates the anti-asthmatic potential of *Z. clinopodioides* in allergen-induced asthmatic mice.

2. Materials and methods

2.1. Collection of plant

Z. clinopodioides Lam was collected from province Balochistan (District Ziarat). Plant was identified by Professor Rasool Baksh Tareeen Department of Botany, University of Quetta, Balochistan. The specimen was also deposited in the herbarium of said department (ZC-RBT-08).

2.2. Preparation of crude methanolic extract and fractionation by using ethyl acetate and n-hexane solvents

The plant was dried and fine powder was prepared by grinding. The obtained powder (500 mg) was immersed in 2 L methanol and incubated for 7 days. All the material was primarily filtered through muslin cloth and afterwards through filter paper. Semi solid thick extract was obtained by concentrating the filtrate using rotary evaporator. About 3% estimated yield was calculated. A solution of crude extract was prepared by dissolving in 200 ml distilled water. Liquid-liquid extraction with n-hexane was done using the separating funnel. Upper layer of n-hexane was collected and evaporated by same procedure earlier mentioned. The aqueous layer was again fractioned with ethyl acetate. Alike procedure was used as designated in preparation of n-hexane fraction (Uroos et al., 2017).

2.3. Housing of animal

36 healthy female mice of 28–33 g weight and of 6–8 weeks age were divided into six group equally. Before start of experiment, all the mice were kept in animal house facility of The University of Lahore for 7 days in order to adapt them to enviorment. Animals were given stanadrd diet and tap water with mainatence of temperature (24–26 °C) and humidity (40–60%) (Kim et al., 2016). The Institutional Research Ethics Committee of The University Of Lahore approved the study (IREC -2017 -30).

2.4. Ovalalbumin immunization and challenge

All groups, except normal control group, were immunized at 0 day and 14 day by administring intraperitoneal injection (I.P) of ovalbumin (20 μg), which was dissolved in an adjuvant. The adjuvant was prepared by mixing aluminum sulphate (2 mg) in phosphate buffer saline (0.1 ml). Next day after second sensitization, mice were challenged with ovalbumin again (1%; intranasal) once daily for one week (i.e. day 15–21). Negative control group was sensitized and challenged with PBS only (Park et al., 2011; Yang et al., 2010).

2.5. Experimental design

36 healthy mice were used which were divided into 6 groups. Each group contained 6 mice. The anti-inflammatory dose of the plant was selected from our previous publication (Shabbir et al., 2018) which demonstrated that *Z. clinopodioides* ameliorated inflammation in different animal models of acute and chronic inflammation.

Disease control group (D. control): Disease control group was treated with vehicle that was (Tween 80 and DMSO 5% each) only.

Negative control group (N. control): Mice were treated with PBS only for consecutive 7 days from day 15 to 21.

Methanolic extract treated group (C. extract): Mice was treated with methanolic extract (500 mg/kg b.w.) orally for consecutive 7 days from day 15–21.

Ethyl acetate treated group (EA fraction): Mice was treated with ethyl acetate (500 mg/kg b.w.) orally for consecutive 7 days from day 15–21.

n-Hexane treated group (n-H fraction): Mice was treated (500 mg/kg b.w.) with n-hexane orally for continuous one week from days 15–21.

Reference drug Treated Group (R. control): Duration of treatment was similar as of extract treated groups. Referemce drug treated animals were intraperitoneally administered (15 mg/kg b.w) with methylprednisolone (Inam et al., 2017).

2.6. Inflammatory cell counts in BALF and blood

BALF is collected by dissecting out trachea with intact lungs. The Lungs were lavaged through trachea with 0.5 ml ice cold PBS using method of gradual instillation and withdrawl with blunt needle (Yun et al., 2014). Total leukocyte count (TLC) were evaluated in BALF. The blood was withdrawn using cardiac puncture technique. Granulocytes and agranulocyte counts were evaluated in blood using automatic hematology analyzer (Inam et al., 2017; Shabbir et al., 2014).

2.7. Determination of lung wet/dry weight ratio

Lungs wet/dry ratio was detemined by taking a fresh lobe of lung and weiging immediately. Then, the lobe was oven dried for 15 min at 56 °C. The weight was measured again after drying (Matsuyama et al., 2008).

2.8. Lungs histopathology

A lobe of dissected lung was fixed in 10% buffered formalin, and subsequently dipped in increasing concentrations of ethanol for dehydration. Tissues was embedded in paraffin wax and 5 µm thick section were cut by using microtome. Infiltration of inflammatory cells was determined by staining the tissues with eosin and hematoxylin, while goblet cell hyperplasia was evaluated by using PAS stain (Inam et al., 2017; Pavuluri et al., 2013). Results were semi-quantified by using histopathological scoring method where 0, 1, 2, 3, and 4 were given to none, minimal, mild, moderate and severe changes, respectively (Khan et al., 2015).

2.9. Determination of mRNA expression levels of IL4, IL5, AQP1 and AQP5

2.9.1. RNA extraction and reverse transcription

mRNA expression levels of pro-inflammatory IL4 and IL5, and aquaporins in lung tissue were measured by reverse transcription polymerase chain reaction (Shahzad et al., 2009). Extraction of total RNA from lungs was conducted by following standard procedure of TriZol method. Nanodrop spectrophotometerical analysis was used to determine the quantity of total RNA. cDNA was synthesized by using total RNA (1000 ng/reaction) as a template. Protocol provided by kit manufacturer was followed to conduct reverse transcription (Enzynomics). Primer (80 µM; Oligo dT_{18}) and nuclease free water were mixed with template. Heating (65 °C for 5 min) and subsequent chilling (on ice) was used to loosen the bonding in GC rich template. Then, MMuLV (200 U/µl; 1 µl), dNTP (2 mM; 2 µl), RNase inhibitors (40 U/µL; 1 µL), and reaction buffer (10X; 2 µl) were mixed in prior prepared mixture. One hour heating of mixture at 42 °C synthesized complementary DNA.

2.9.2. PCR and gel electrophoresis

cDNA (template; 2 µl), nuclease free water, forward and reverse primers (10 µM; 0.5 µl each), and master mix (2X; 10 µl) were added in a PCR tube. PCR protocol was as follows: Denaturation (95 °C for 10 s), annealing (58 °C for 20 s), extension (72 °C for 30 s). Gel (2% with agarose) was made and electrophoresis was conducted for 40 min at 110 V. The results were subjected to densitometry using ImageJ software (Khan et al., 2015). Primers of IL4, IL5, AQP1, and AQP5 were selected from the study of Rana et al. (Rana et al., 2016).

2.10. Statistical evaluation

To determine the statistical significance i.e. P value ≤ 0.05; One way ANOVA and Post hoc Tukey's test were applied. Mean ± standard deviation (SD) was given to present the data. Graphpad prism v.6 was used for all the statistical evaluations.

3. Results

3.1. Z. clinopodioides significantly attenuated TLC in blood

The data showed increase in TLC in D. control (4.25 ± 0.3082; $P < 0.001$) group as compared to N. control (2.65 ± 0.1871) group. Treatment with EA. Fraction (3.083 ± 0.1472), n-H fraction (2.550 ± 0.1871), Cr. extract (1.85 ± 0.1871) and methylprednisolone (1.817 ± 0.1472) attenuated ($P < 0.001$) TLC in blood (Fig. 1A).

3.2. Z. clinopodioides significantly decreased the TLC in BALF

TLC was found elevated in D. control (0.7033 ± 0.0367; $P < 0.001$) as compared with N. control group (0.205 ± 0.0345). EA. fraction (0.07333 ± 0.01633), n-H fraction (0.1 ± 0.03162), Cr. extract (0.1383 ± 0.03869), and R. Control (0.09167 ± 0.02787) decreased ($P < 0.001$) the TLC as compared with D. control (Fig. 1B).

3.3. Z. clinopodioides significantly attenuated the DLC in blood

Granulocytes (8.217 ± 0.3312; $P < 0.001$), monocytes (11.7 ± 1.349; $P < 0.05$) and lymphocytes (85.8 ± 7.988; $P < 0.05$), counts were found increased in D. control as compared to N. control groups (5.933 ± 0.2582; 7.417 ± 1.484; 75.63 ± 6.195, respectively). The data showed that counts of granulocytes, monocytes, and lymphocytes were reduced after treatment with n-H fraction (2.583 ± 0.3189, $P < 0.001$; 3.313 ± 0.7139, $P < 0.001$; and 70 ± 4.935, $P < 0.001$, respectively), EA. fraction (5.333 ± 0.8311, $P < 0.01$; 7.2 ± 1.198, $P < 0.05$; and 71.25 ± 5.018, $P < 0.001$, respectively), and Cr. extract (2.833 ± 0.4546, $P < 0.001$; 5.583 ± 0.9847, $P < 0.001$; and 74.73 ± 2.654, $P < 0.05$, respectivley) of the plant. R. Control (2.35 ± 0.7423, $P < 0.001$; 4.033 ± 1.196, $P < 0.001$; 75.95 ± 2.583; $P < 0.05$) group also showed reduction in DLC (Fig. 1C-E).

3.4. Z. clinopodioides ameliorated inflammatory cell infiltration

An elevation in inflammatory cells infiltration was determined in D. control (2.667 ± 0.5164; $P < 0.01$) as compared with N. control (0.333 ± 0.5164). EA. fraction (0.6667 ± 1.033; $P < 0.01$), n-H fraction (1.0 ± 0.8944; $P < 0.01$), Cr. extract (0.6667 ± 0.8165; $P < 0.05$), and R. control (0.6667 ± 0.8165; $P < 0.01$) reduced inflammatory cells infiltration when compared to D. control (Figs. 2A and 3).

3.5. Z. clinopodioides significantly attenuated goblet cell hyperplasia

Hyperplasia of goblet cells was found enhanced in D.control (2.5 ± 0.5477) as compared with N. control (0.5 ± 0.5477). Treatment with EA. fraction (1.133 ± 0.8165; $P < 0.05$), n-H fraction (1.133 ± 0.5164; $P < 0.05$), Cr. extract (1.167 ± 0.7528; $P < 0.01$), and R. control (1.167 ± 0.4082; $P < 0.01$) resulted in reduced hyperplasia of goblet cells as compared to D. control (Figs. 2B and 4).

3.6. Treatment with Z. clinopodioides downregulated IL4 levels

IL4 expression levels were found upregulated in D. control (1.434 ± 0.02142; $P < 0.001$) as compared with N. control (1.032 ± 0.09753). Treatment with EA. fraction (1.266 ± 0.02996; $P < 0.01$), n-H fraction (1.31 ± 0.06206; $P < 0.05$), Cr. extract (0.8382 ± 0.06002; $P < 0.001$), and R. Control (1.148 ± 0.1059; $P < 0.001$) caused attenuation of IL4 expression levels (Fig. 5A).

3.7. Treatment with Z. clinopodioides downregulated IL5 levels

IL5 levels were found ($P < 0.001$) upregulated in D. control (1.117 ± 0.2639) as compared with N. control (4.417 ± 0.2317). Treatment with EA. fraction (1.984 ± 0.3139), n-H fraction (1.903 ± 0.3074), Cr. extract (1.737 ± 0.2775), and R. control (2.483 ± 0.343) attenuated ($P < 0.001$) IL5 expression levels (Fig. 5B).

3.8. Z. clinopodioides significantly reduced lungs wet/dry weight ratio

Results showed elevated ($P < 0.001$) lung wet/dry weight ratio in

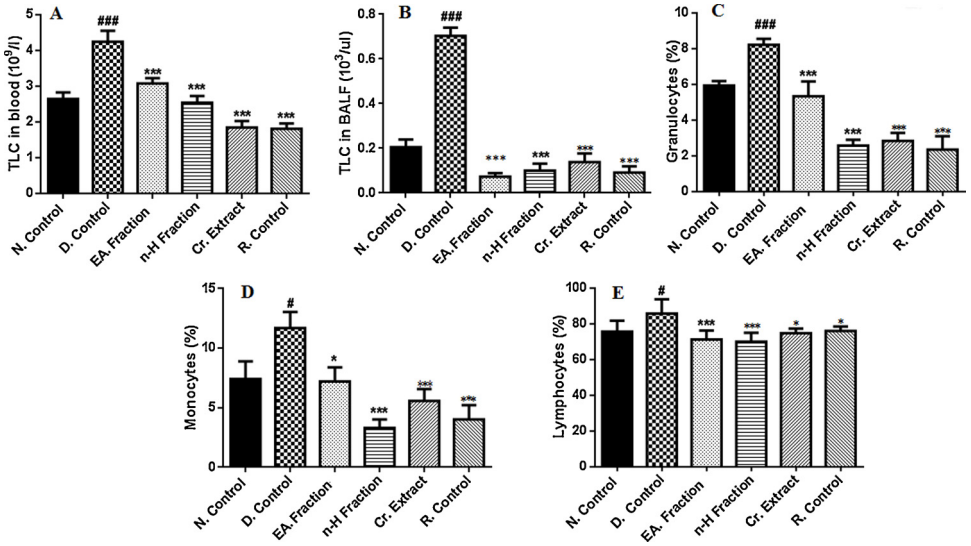

Fig. 1. The data showed significant attenuation of TLC in both blood (A) and BALF (B). Treatment with *Z. clinopodioides* also nearly normalized DLC in blood (C–E). # denotes comparison with normal control while, * denotes comparison with D. control. *P < 0.05, **P < 0.01, ***P < 0.001.

D. control (2.533 ± 0.1547) as compared with N. control (1.458 ± 0.03764). EA. fraction (2.222 ± 0.3658; P < 0.05), n-H fraction (1.778 ± 0.06338; P < 0.001), Cr. extract (2.185 ± 0.03391; P < 0.05), and R. Control (1.828 ± 0.1202; P < 0.001) showed decrease in the lung wet/dry ratio when compared with D. control (Fig. 6A).

3.9. Treatment with Z. clinopodioides enhanced the mRNA expression levels of AQP1

The data showed downregulated AQP1 levels in D. control (1.595 ± 0.409; P < 0.01) as compared with N. control (2.483 ± 0.621). EA. fraction (3.2 ± 0.2966; P < 0.001), n-H. fraction (3.737 ± 0.1498; P < 0.001), Cr. extract (4.082 ± 0.2733; P < 0.001) and R. Control (2.207 ± 0.4139; P < 0.05) enhanced AQP1 expression levels (Fig. 6B).

3.10. Z. clinopodioides significantly elevated the mRNA expression levels of AQP5

The data showed upregulated AQP5 levels in D. control (1.358 ± 0.2217) as compared with N. control (2.146 ± 0.4407). n-H fraction (3.544 ± 0.4463), EA. fraction (3.72 ± 0.194), Cr. extract (2.552 ± 0.3149) and R. control (3.062 ± 0.7844) caused elevation in AQP5 expression levels (Fig. 6C).

4. Discussion

Asthma is the most common and long-term inflammatory disease of lungs which is categorized by symptoms such as, edema, bronchospasm, inflammation, airway obstruction, wheezing, cold, and shortness of breath. Asthma is caused by many environmental factors such as, allergens and air pollutants, while some genetic factors are also responsible for causing asthma. A few medications such as, aspirin and beta blocker are also known to trigger asthma (Asai et al., 2003; Goodwin and Consensus Group of the British Association for Psychopharmacology, 2009; Lemanske and Busse, 2010; Martinez, 2007). Histopathological evaluation in current study showed raised inflammatory cell infiltration in disease group. While hematological evaluation showed increased total leukocytes and differential leukocytes counts. These results are in accordance with the characteristics of allergic asthma. Treatment with *Z. clinopodioides* and methylprednisolone (MP) caused near normalization of all raised hematological and histopathological parameters (Mueller et al., 1996).

Th2 cells are found abundantly in asthma as compared with Th1 cells. Activated form of Th2 cells are involved in the development of allergy by production and release of cytokines (Larché et al., 2003). Th2 cells are responsible for secretion of IL4 and IL5 cytokines (Ashraf et al., 2015). Asthma is caused by imbalance between Th2 and Th1 cells, and shift in favor of Th2 cells mediated by IL4 and IL5 cytokines (Swain, 1995).

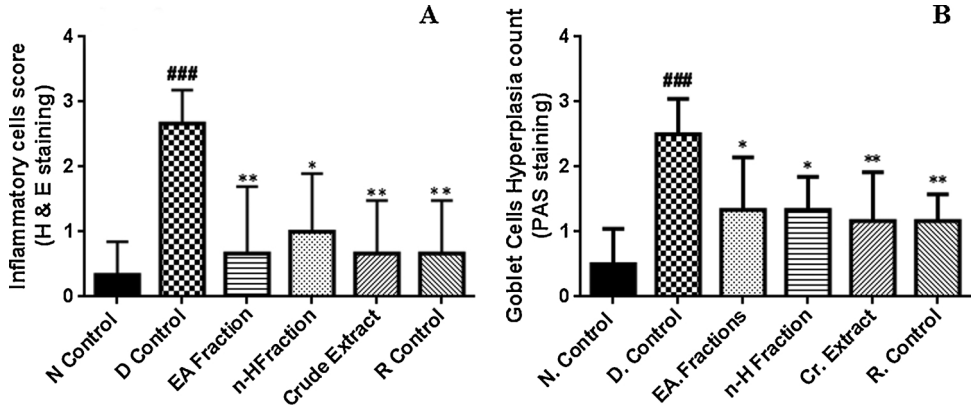

Fig. 2. Treatment with *Z. clinopodioides* significantly attenuated infiltration of inflammatory cells (A) and goblet cell hyperplasia (B) as shown in the histopathological evaluation. # denotes comparison with normal control while, * denotes comparison with D. control. *P < 0.05, **P < 0.01, ***P < 0.001.

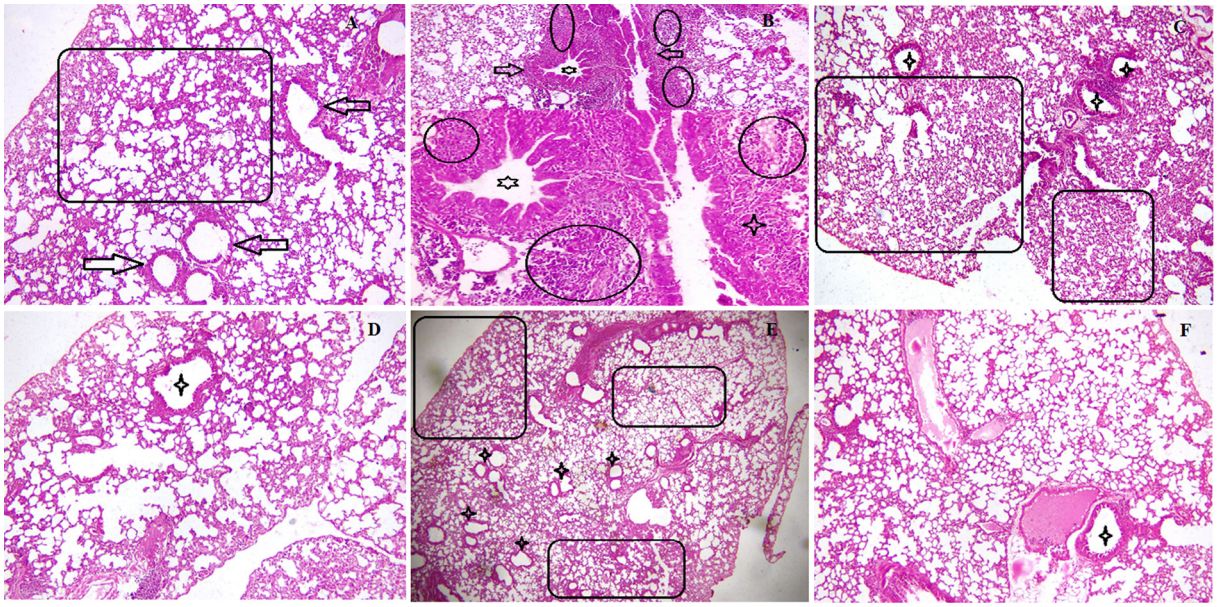

Fig. 3. Histopathological evaluation (H&E staining): The figure A shows absence of the goblet cell hyperplasia in bronchioles (diamond) and lack of inflammation in the alveoli in the intervening areas (rectangle and its adjacent areas). In figure B, The asterisk indicates a bronchiole with goblet cells hyperplasia. The diamond shows the peri-bronchiolar smooth muscle hypertrophy. The oblong circles span the areas of inflammation surrounding the bronchiole. In figures C–F, bronchioles are devoid of goblet cell hyperplasia (diamond) and intervening areas also lack inflammation (rectangle and its adjacent areas).

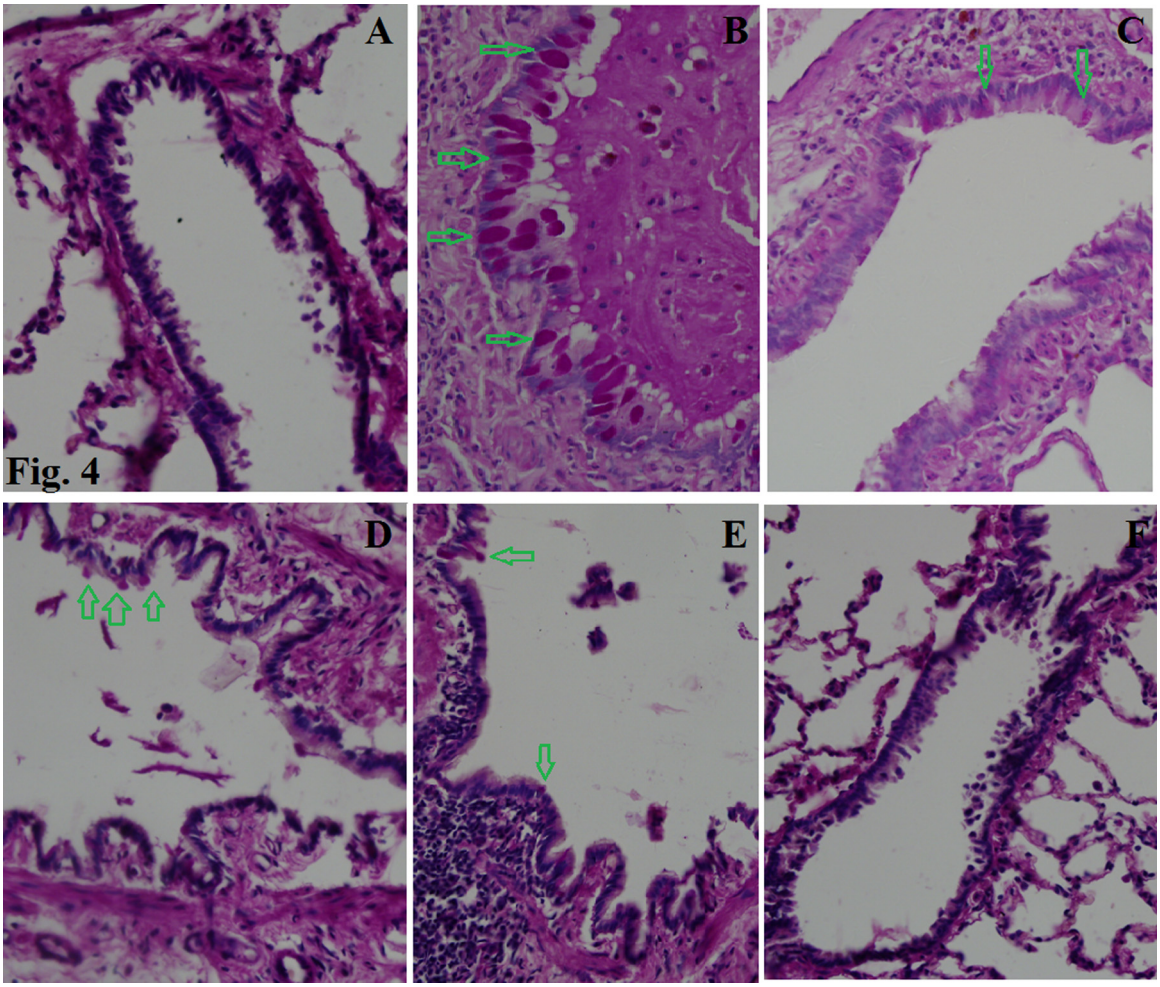

Fig. 4. Histopathological evaluation (PAS staining): No goblet cells are visible in N. control group (A). The arrows indicate a bronchiole with increased number of goblet cells highlighted by PAS stain in D. control group (B). The goblet cell hyperplasia was found decresed in all plant treated groups (C–E), and MP group (F).

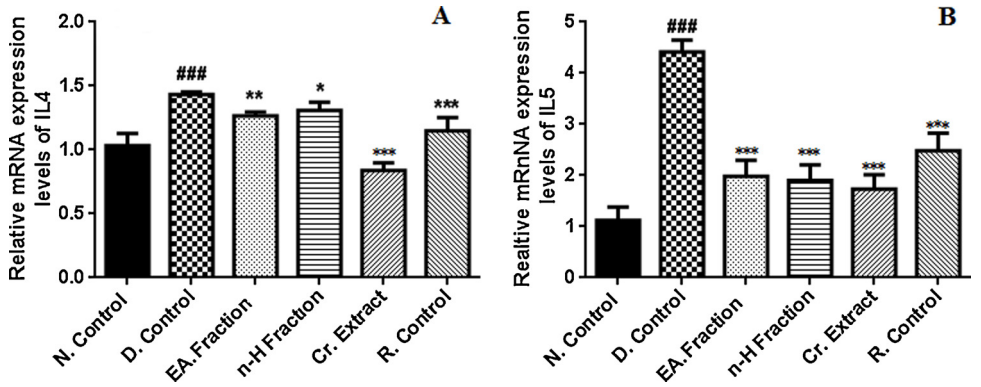

Fig. 5. mRNA expression levels of IL4 (A) and IL5 (B) were found significantly reduced after treatment with *Z. clinopodioides*. # denotes comparison with normal control while, * denotes comparison with D. control. *P < 0.05, **P < 0.01, ***P < 0.001.

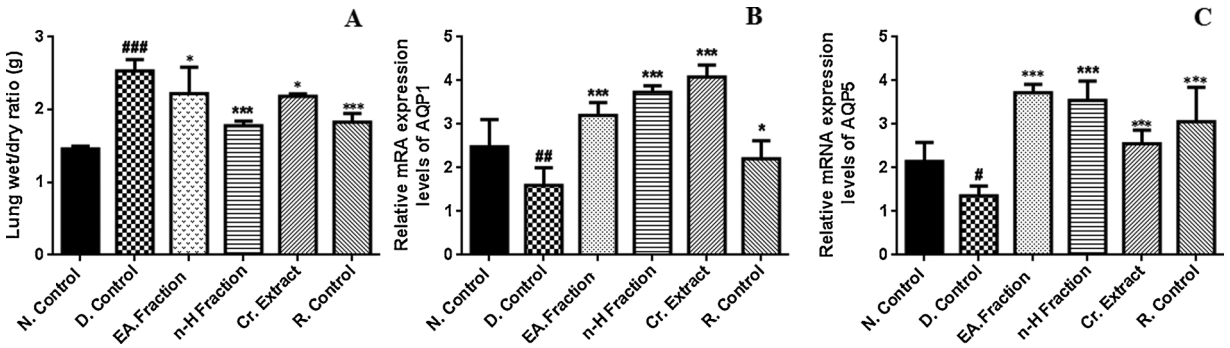

Fig. 6. Pulmonary edema was also found ameliorated after treatment with *Z. clinopodioides* as indicated by significant reduction in lung wet/dry weight ratio (A). The amelioration may be attributed to significant elevation in AQP1 (B) and AQP5 (C) expression levels by the plant. # denotes comparison with normal control while, * denotes comparison with D. control. *P < 0.05, **P < 0.01, ***P < 0.001.

Increased production of IL4 in lungs leads to eosinophils and lymphocytic inflammation (Rankin et al., 1996). IL4 raises the secretions of mucus and excites the mucin gene expression that are involved in airway obstruction. Cytokine IL4 plays a key part in IgE dependent mast cells activation; exotoxin upregulation; adhesion of vascular cells on vascular epithelium; and monocytes, eosinophils, T-lymphocytes, and basophils movement towards the site of inflammation. All these effects lead to allergic inflammation and remodeling of lungs. IL4 is also known to prevent the apoptosis of T-lymphocytes and eosinophils, which leads to acceleration of the proliferation, secretion of cytokines, and eosinophilic inflammation (Dabbagh et al., 1999; Doucet et al., 1998b; Moser et al., 1992; Steinke and Borish, 2001). Increased levels of neutrophils have been found in asthmatic patient. The neutrophils are also involved in the generation, storage, and release of IL4 cytokines (Monteseirin, 2009). Our experimental results revealed that treatment with *Z. clinopodioides* extract significantly decreased IL4 levels when compared with D. control. This inference is in accordance with the reduction of granulocytes and agranulocytes after treatment with *Z. clinopodioides*.

IL5 acts on various types of cells such as, eosinophils and basophils. IL5, another Th2 type cytokine, plays significant part in maturation, survival, activation, and release of eosinophils in the bone marrow. Clinically, upregulation of IL5 in airways is related with severity of asthma (Garcia et al., 2013; Greenfeder et al., 2001; Hirai et al., 1990; Resnick and Weller, 1993). The data showed upregulated levels of IL5 in D. control, which were found downregulated in treated groups. Current study showed co-relation in the reduced levels of eosinophils with the suppression of IL4 and IL5 expression levels.

The anti edematous effects of the plant were evaluated by using wet/dry ratio of the lungs, which is commonly used marker for tissue edema (Shahzad et al., 2009). Results revealed that *Z. clinopodioides* reduced wet/dry ratio of the lungs thus decreasing pulmonary edema (Dong et al., 2012).

Downregulated levels of AQP1 and AQP5 are related with pulmonary edema (Dong et al., 2012). AQP5 has been found to be involved in the lowering of fluid secreted by the nasopharynx and upper airway (Funaki et al., 1998; Ishida et al., 1997; Song and Verkman, 2001). It also plays a part in regulation of pulmonary hyperresponsiveness (Krane et al., 2001) and airway inflammation (Towne et al., 2001). Previous studies showed that elevated mucus production in patient having respiratory disease correlated with downregulation of AQP5 levels (Krane et al., 2009; Wang et al., 2007). Various research studies also revealed that mice deficient in AQP5 levels showed increased lungs resistance (Krane et al., 2001). This study displayed that *Z. clinopodioides* and MP significantly upregulated AQP5 levels as compared with D. control. These inferences are in accordance with the results of Ben et al. (Ben et al., 2008), who displayed that AQP5 levels were increased after Dexamethasone treatment. We also found increased levels of AQP1 expression with the treatment of *Z. clinopodioides*. AQP1 are found in visceral pleura, micro vascular endothelium, and in mesothelial cells (Ben et al., 2008). Formerly, Towne et al. (Towne et al., 2000) confirmed AQP1 role in fluxes of fluid in inflammation. AQP1 also plays a pivotal role in eosinophils movement (Lei et al., 2008). Expression levels of AQP5 and AQP1 level have been found increased in pulmonary edema by anti-asthmatic agents (Dong et al., 2012). The results of Dong et al. (Dong et al., 2012) are in accordance with the current study which displayed that upregulated levels of AQP5 and AQP1 might result in amelioration of pulmonary edema.

5. Conclusion

This study validated the folkric use of *Z. clinopodioides* in airway disorders. The study showed that *Z. clinopodioides* possessed anti edematous property in airway disorders. The inference was based on

alleviatoin of hyperplasia of goblet cells and wet/dry ratio of the lungs. Reduction in pulmonary edema by *Z. clinopodioides* may be attributed to the upregulated levels of AQP5 and AQP1. *Z. clinopodioides* also alleviated pulmonary inflammation. This was validated by reduced infiltration of inflammatory cells found in lungs, and attenuated hematological parameters found in blood and BALF. Reduction in inflammation may be attributed to downregulated IL-4 and IL-5 levels.

There is a need for identification and separation of active phytochemical ingredents that possess possible anti-asthmatic properties and requires further studies in future.

Conflict of interest

The authors declare no conflict of interests.

References

Abbas, A.T., Abdel-Aziz, M.M., Zalata, K., Abd, A.-G.T.-D., 2005. Effect of dexamethasone and Nigella sativa on peripheral blood eosinophil count, IgG1 and IgG2a, cytokine profiles and lung inflammation in murine model of allergic asthma. Egypt. J. Immunol. 12, 95.

Abdureyim, S., Amat, N., Umar, A., Upur, H., Berke, B., Moore, N., 2011. Anti-inflammatory, immunomodulatory, and heme oxygenase-1 inhibitory activities of ravan napas, a formulation of uighur traditional medicine, in a rat model of allergic asthma. Evid. Based Complement. Altern. Med. 2011.

Amiri, H., 2009a. Composition and antioxidant activity of the essential oil and methanolic extract of Ziziphora clinopodioides Lam in preflowering stage. J. Kerman Univ. Med. Sci. 16, 79–86.

Amiri, H., 2009b. Influence of growth phase on the essential oil composition of Ziziphora clinopodioides Lam. Nat. Prod. Res. 23, 601–606.

Asai, K., Kanazawa, H., Kamoi, H., Shiraishi, S., Hirata, K., Yoshikawa, J., 2003. Increased levels of vascular endothelial growth factor in induced sputum in asthmatic patients. Clin. Exp. Allergy 33, 595–599.

Ashraf, M.I., Shahzad, M., Shabbir, A., 2015. Oxyresveratrol ameliorates allergic airway inflammation via attenuation of IL-4, IL-5, and IL-13 expression levels. Cytokine 76, 375–381.

Barnes, P., 1992. Frontiers in medicine: new aspects of asthma. J. Intern. Med. 231, 453–461.

Baytop, T., Turkiyede, B., Tedavi, I., 1996. Yayinlari. Eczacilik Fak 40. pp. 444.

Ben, Y., Chen, J., Zhu, R., Gao, L., Bai, C., 2008. Upregulation of AQP3 and AQP5 induced by dexamethasone and ambroxol in A549 cells. Respir. Physiol. Neurobiol. 161, 111–118.

Borok, Z., Verkman, A., 2002. Lung edema clearance: 20 years of progress invited review: role of aquaporin water channels in fluid transport in lung and airways. J. Appl. Physiol. 93, 2199–2206.

Choi, Y.Y., Kim, M.H., Lee, H., Jo, S.Y., Yang, W.M., 2018. (R)-(+)-pulegone suppresses allergic and inflammation responses on 2,4-dinitrochlorobenzene-induced atopic dermatitis in mice model. J. Dermatol. Sci. 91, 292–300.

Dabbagh, K., Takeyama, K., Lee, H.-M., Ueki, I.F., Lausier, J.A., Nadel, J.A., 1999. IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. J. Immunol. 162, 6233–6237.

Dong, C., Wang, G., Li, B., Xiao, K., Ma, Z., Huang, H., Wang, X., Bai, C., 2012. Anti-asthmatic agents alleviate pulmonary edema by upregulating AQP1 and AQP5 expression in the lungs of mice with OVA-induced asthma. Respir. Physiol. Neurobiol. 181, 21–28.

Doucet, C., Brouty-Boyé, D., Pottin-Clémenceau, C., Canonica, G.W., Jasmin, C., Azzarone, B., 1998a. Interleukin (IL) 4 and IL-13 act on human lung fibroblasts. Implication in asthma. J. Clin. Invest. 101, 2129.

Doucet, C., Brouty-Boyé, D., Pottin-Clemenceau, C., Jasmin, C., Canonica, G.W., Azzarone, B., 1998b. IL-4 and IL-13 specifically increase adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. Int. Immunol. 10, 1421–1433.

Factor, P., 2003. Gene therapy for asthma. Mol. Ther. 7, 148–152.

Finotto, S., Ohno, I., Marshall, J.S., Gauldie, J., Denburg, J.A., Dolovich, J., Clark, D.A., Jordana, M., 1994. TNF-alpha production by eosinophils in upper airways inflammation (nasal polyposis). J. Immunol. 153, 2278–2289.

Funaki, H., Yamamoto, T., Koyama, Y., Kondo, D., Yaoita, E., Kawasaki, K., Kobayashi, H., Sawaguchi, S., Abe, H., Kihara, I., 1998. Localization and expression of AQP5 in cornea, serous salivary glands, and pulmonary epithelial cells. Am. J. Physiol. Cell Physiol. 275, C1151–C1157.

Garcia, G., Taillé, C., Laveneziana, P., Bourdin, A., Chanez, P., Humbert, M., 2013. Anti-interleukin-5 therapy in severe asthma. Eur. Respir. Rev. 22, 251–257.

Goodwin, G.M., Consensus Group of the British Association for Psychopharmacology, 2009. Evidence-based guidelines for treating bipolar disorder: revised second edition—recommendations from the British Association for Psychopharmacology. J. Psychopharmacol. 23, 346–388.

Greenfeder, S., Umland, S.P., Cuss, F.M., Chapman, R.W., Egan, R.W., 2001. Th2 cytokines and asthma the role of interleukin-5 in allergic eosinophilic disease. Respir. Res. 2, 71.

Hesselmar, B., Bergin, A.M., Park, H., Hahn-Zoric, M., Eriksson, B., Hanson, L.Å., Padyukov, L., 2010. Interleukin-4 receptor polymorphisms in asthma and allergy: relation to different disease phenotypes. Acta Paediatr. 99, 399–403.

Hirai, K., Yamaguchi, M., Misaki, Y., Takaishi, T., Ohta, K., Morita, Y., Ito, K., Miyamoto, T., 1990. Enhancement of human basophil histamine release by interleukin 5. J. Exp. Med. 172, 1525–1528.

Inam, A., Shahzad, M., Shabbir, A., Shahid, H., Shahid, K., Javeed, A., 2017. Carica papaya ameliorates allergic asthma via down regulation of IL-4, IL-5, eotaxin, TNF-α, NF-κB, and iNOS levels. Phytomedicine 32, 1–7.

Ishida, N., Hirai, S.-I., Mita, S., 1997. Immunolocalization of aquaporin homologs in mouse lacrimal glands. Biochem. Biophys. Res. Commun. 238, 891–895.

Kale, R., Patil, R., Patil, R., 2010. Asthma and herbal drugs. Pathophysiology 20, 4.

Khan, A.M., Shahzad, M., Raza Asim, M., Imran, M., Shabbir, A., 2015. Zingiber officinale ameliorates allergic asthma via suppression of Th2-mediated immune response. Pharm. Biol. 53, 359–367.

Khosravi, A., Minooeianhaghighi, M., Shokri, H., Emami, S., Alavi, S., Asili, J., 2011. The potential inhibitory effect of Cuminum cyminum, Ziziphora clinopodioides and Nigella sativa essential oils on the growth of Aspergillus fumigatus and Aspergillus flavus. Braz. J. Microbiol. 42, 216–224.

Kim, W., Park, S., Choi, C., Kim, Y.R., Park, I., Seo, C., Youn, D., Shin, W., Lee, Y., Choi, D., 2016. Evaluation of anti-inflammatory potential of the new ganghwaljetongyeum on adjuvant-induced inflammatory arthritis in rats. Evid. Based Complement. Altern. Med. 2016.

King, L.S., Nielsen, S., Agre, P., 1997. Aquaporins in complex tissues. I. Developmental patterns in respiratory and glandular tissues of rat. Am. J. Physiol. Cell Physiol. 273, C1541–C1548.

Kopf, M., Le Gros, G., Bachmann, M., Lamers, M.C., Bluethmann, H., Köhler, G., 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature 362, 245–248.

Kozono, D., Yasui, M., King, L.S., Agre, P., 2002. Aquaporin water channels: atomic structure molecular dynamics meet clinical medicine. J. Clin. Invest. 109, 1395–1399.

Krane, C.M., Fortner, C.N., Hand, A.R., McGraw, D.W., Lorenz, J.N., Wert, S.E., Towne, J.E., Paul, R.J., Whitsett, J.A., Menon, A.G., 2001. Aquaporin 5-deficient mouse lungs are hyperresponsive to cholinergic stimulation. Proc. Natl. Acad. Sci. 98, 14114–14119.

Krane, C.M., Deng, B., Mutyam, V., McDonald, C.A., Pazdziorko, S., Mason, L., Goldman, S., Kasaian, M., Chaudhary, D., Williams, C., 2009. Altered regulation of aquaporin gene expression in allergen and IL-13-induced mouse models of asthma. Cytokine 46, 111–118.

Lampinen, M., Carlson, M., Håkansson, L., Venge, P., 2004. Cytokine-regulated accumulation of eosinophils in inflammatory disease. Allergy 59, 793–805.

Larché, M., Robinson, D.S., Kay, A.B., 2003. The role of T lymphocytes in the pathogenesis of asthma. J. Allergy Clin. Immunol. 111, 450–463.

Lei, F., Zhu, D., Wang, X., Guan, G., Jiang, Y., Dong, Z., 2008. Role of aquaporin 1 in the migration of eosinophils from asthmatic guinea pigs. Zhonghua er bi yan hou tou jing wai ke za zhi= Chin. J. Otorhinolaryngol. Head Neck Surg. 43, 130–133.

Lemanske, R.F., Busse, W.W., 2010. Asthma: clinical expression and molecular mechanisms. J. Allergy Clin. Immunol. 125, S95–S102.

Levine, S.J., 1995. Bronchial epithelial cell-cytokine interactions in airway inflammation. J. Invest. Med. 43, 241–249.

Li, Z., Ju, Z., Frieri, M., 2013. The T-cell Immunoglobulin and Mucin Domain (Tim) Gene Family in Asthma, Allergy, and Autoimmunity, Allergy and Asthma Proceedings. OceanSide Publications, Inc, pp. e21–e26.

Lolestani, F., Shayesteh, N., 2009. Fumigant toxicity of Ziziphora clinopodioides (Boiss.) (Lamiaceae) against adults and eggs of Callosobruchus maculatus (Fab.)(Coleoptera: bruchidae). J. Biol. Sci. 9, 92–95.

Ma, T., Fukuda, N., Song, Y., Matthay, M.A., Verkman, A., 2000. Lung fluid transport in aquaporin-5 knockout mice. J. Clin. Invest. 105, 93–100.

Markham, A.W., Wilkinson, J.M., 2004. Complementary and alternative medicines (CAM) in the management of asthma: an examination of the evidence. J. Asthma 41, 131–139.

Martinez, F.D., 2007. Genes, environments, development and asthma: a reappraisal. Eur. Respir. J. 29, 179–184.

Masoli, M., Fabian, D., Holt, S., Beasley, R., 2004. The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy 59, 469–478.

Matsuyama, H., Amaya, F., Hashimoto, S., Ueno, H., Beppu, S., Mizuta, M., Shime, N., Ishizaka, A., Hashimoto, S., 2008. Acute lung inflammation and ventilator-induced lung injury caused by ATP via the P2Y receptors: an experimental study. Respir. Res. 9, 79.

Monteseirin, J., 2009. Neutrophils and asthma. J. Investig. Allergol. Clin. Immunol. 19, 340–354.

Moser, R., Fehr, J., Bruijnzeel, P., 1992. IL-4 controls the selective endothelium-driven transmigration of eosinophils from allergic individuals. J. Immunol. 149, 1432–1438.

Mueller, R., Chanez, P., Campbell, A., Bousquet, J., Heusser, C., Bullock, G., 1996. Different cytokine patterns in bronchial biopsies in asthma and chronic bronchitis. Respir. Med. 90, 79–85.

Ozturk, S., Ercisli, S., 2007. Antibacterial activity and chemical constitutions of Ziziphora clinopodioides. Food Control 18, 535–540.

Park, Y.N., Lee, Y.J., Choi, J.H., Jin, M., Yang, J.H., Li, Y., Lee, J., Li, X., Kim, K.-J., Son, J.K., 2011. Alleviation of OVA-induced airway inflammation by flowers of Inula japonica in a murine model of asthma. Biosci. Biotechnol. Biochem. 75, 871–876.

Pavuluri, S., Hanus, V., Bergren, D.R., 2013. Interaction of tobacco smoke exposure and ovalbumin-sensitization promotes goblet cell and submucosal gland metaplasia in guinea pigs. Respir. Physiol. Neurobiol. 189, 639–645.

Rana, S., Shahzad, M., Shabbir, A., 2016. Pistacia integerrima ameliorates airway inflammation by attenuation of TNF-α, IL-4, and IL-5 expression levels, and pulmonary

edema by elevation of AQP1 and AQP5 expression levels in mouse model of ovalbumin-induced allergic asthma. Phytomedicine 23, 838–845.
Rankin, J.A., Picarella, D.E., Geba, G.P., Temann, U.-A., Prasad, B., DiCosmo, B., Tarallo, A., Stripp, B., Whitsett, J., Flavell, R.A., 1996. Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: lymphocytic and eosinophilic inflammation without airway hyperreactivity. Proc. Natl. Acad. Sci. 93, 7821–7825.
Resnick, M.B., Weller, P.F., 1993. Mechanisms of eosinophil recruitment. Am. J. Respir. Cell Mol. Biol. 8 349-349.
Rivi, M.V., 2008. Effect of the essential oil composition and biological activity of Ziziphora clinopodiodes Lam. On the against Anopheles stephensi and Culex pipiens Parva from Iran. Saudi J. Biol. Sci. 15, 185–188.
Romagnani, S., 2000. The role of lymphocytes in allergic disease. J. Allergy Clin. Immunol. 105, 399–408.
Senejoux, F., Girard, C., Kerram, P., Aisa, H.A., Berthelot, A., Bévalot, F., Demougeot, C., 2010. Mechanisms of vasorelaxation induced by Ziziphora clinopodioides Lam. (Lamiaceae) extract in rat thoracic aorta. J. Ethnopharmacol. 132, 268–273.
Shabbir, A., Shahzad, M., Ali, A., Zia-ur-Rehman, M., 2014. Anti-arthritic activity of N′-[(2, 4-dihydroxyphenyl) methylidene]-2-(3, 4-dimethyl-5, 5-dioxidopyrazolo [4, 3-c] [1, 2] benzothiazin-1 (4H)-yl) acetohydrazide. Eur. J. Pharmacol. 738, 263–272.
Shabbir, A., Batool, S.A., Basheer, M.I., Shahzad, M., Sultana, K., Tareen, R.B., Iqbal, J., Saeed-ul-Hassan, 2018. Ziziphora clinopodioides ameliorated rheumatoid arthritis and inflammatory paw edema in different models of acute and chronic inflammation. Biomed. Pharmacother. 97, 1710–1721.
Shahzad, M., Yang, X., Asim, M.R., Sun, Q., Han, Y., Zhang, F., Cao, Y., Lu, S., 2009. Black seed oil ameliorates allergic airway inflammation by inhibiting T-cell proliferation in rats. Pulm. Pharmacol. Ther. 22, 37–43.
Song, Y., Verkman, A., 2001. Aquaporin-5 dependent fluid secretion in airway submucosal glands. J. Biol. Chem. 276, 41288–41292.
Song, Y., Jayaraman, S., Yang, B., Matthay, M.A., Verkman, A., 2001. Role of aquaporin water channels in airway fluid transport, humidification, and surface liquid hydration. J. Gen. Physiol. 117, 573–582.
Steinke, J.W., Borish, L., 2001. Th2 cytokines and asthma—Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. Respir. Res. 2, 66.
Swain, S.L., 1995. T-Cell Subsets: Who does the polarizing? Curr. Biol. 5, 849–851.
Tian, S., Shi, Y., Zhou, X., Ge, L., Upur, H., 2011. Total polyphenolic (flavonoids) content and antioxidant capacity of different Ziziphora clinopodioides Lam. Extracts. Pharmacogn. Mag. 7, 65–68.
Toprak, A., Takir, S., 2017. Comparable effects of the different extracts of the same plants on blood pressure and vascular tone. Pharm. Chem. J. 4, 57–65.
Towne, J.E., Harrod, K.S., Krane, C.M., Menon, A.G., 2000. Decreased expression of aquaporin (AQP) 1 and AQP5 in mouse lung after acute viral infection. Am. J. Respir. Cell Mol. Biol. 22, 34–44.
Towne, J.E., Krane, C.M., Bachurski, C.J., Menon, A.G., 2001. Tumor necrosis factor-α inhibits aquaporin 5 expression in mouse lung epithelial cells. J. Biol. Chem. 276, 18657–18664.
Uroos, M., Abbas, Z., Sattar, S., Umer, N., Shabbir, A., Sharif, A., 2017. Nyctanthes arbortristis ameliorated FCA-Induced experimental arthritis: a comparative study among different extracts. Evid. Based Complement. Altern. Med. 2017.
Wang, K., XU, D., YANG, J., 2007. Decreased expression of human aquaporin-5 correlated with mucus overproduction in airways of chronic obstructive pulmonary disease. Acta Pharmacol. Sin. 28, 1166–1174.
Wenzel, S.E., Covar, R., 2006. Update in asthma 2005. Am. J. Respir. Crit. Care Med. 173, 698–706.
Wills-Karp, M., 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. Annu. Rev. Immunol. 17, 255–281.
Yang, X., Sun, Q., Raza Asim, M., Jiang, X., Zhong, B., Shahzad, M., Zhang, F., Han, Y., Lu, S., 2010. Nitric oxide in both bronchoalveolar lavage fluid and serum is associated with pathogenesis and severity of antigen-induced pulmonary inflammation in rats. J. Asthma 47, 135–144.
Yousefbeyk, L.F., Tabaside, J., Ostad, S.N., Sourmaghi, M.H.S., Amin, G.R., 2016. Investigation of chemical composition and cytotoxic activity of aerial parts of Ziziphora clinopodioides. Res. J. Pharmacogn. Phytochem. 3, 47–51.
Yun, L., Xin-sheng, F., Jing-hua, Y., Li, X., Shan-shan, W., 2014. CD4+ CD25+ FOXP3+ T cells, Foxp3 gene and protein expression contribute to antiasthmatic effects of San'ao decoction in mice model of asthma. Phytomedicine 21, 656–662.