ORIGINAL ARTICLE

EXPLORING BETULINIC ACID: A PROMISING THERAPY FOR ASTHMA IN MALE MICE

Sher Afghan Khan, Qadeer Nawaz, Muhammad Tahir*, Sameer Ahmed, Muhammad Junaid Khan***, Saqib Zaman†**

Department of Pharmacology, Gajju Khan Medical College, Swabi, *Department of Pharmacology, Mekran Medical College, Turbat, **Rawal Institute of Health Sciences Islamabad, ***Final Year Student, Gajju Khan Medical College, Swabi, †Pharmacist, Baja, Swabi, Pakistan

Background: Betulinic acid (BA) has been proven to contain diverse functions such as anti-cancer effects, anti-HIV, anti-bacterial, anti-inflammatory, anti-malarial, anti-helminthic, and other pharmaceutical properties. The objective of this study was to assess the immunomodulatory effects of BA on ovalbumin induced airway inflammation in male mice. **Methods:** Forty healthy BALB/c mice were randomly divided into 4 groups. Group I served as normal control (NC) group. Group II as asthmatic control (AC) group and was sensitized intraperitoneally (IP) with ovalbumin (OVA) at day 0 and challenged at day 14 respectively and from day 15 to 21 was challenged intranasally (I/N) for one week. Group III were treated with OVA (I/N) and concomitantly BA at 5 mg/Kg body weight was given orally, and Group IV were treated with OVA (I/N) and concomitantly dexamethasone (DEX) 2 mg/Kg body weight was given orally for 7 days. Terminal sampling at day 22 was performed for blood TLC and DLC and lung tissue sampling for interleukin 4 by *m*RNA expression. **Results:** Betulinic acid significantly lowered both the differential cell count of blood and the *m*RNA expression of IL-4 in asthmatic model of mice. **Conclusion:** Betulinic acid manifested similar results when compared to DEX in lowering the pro-inflammatory markers.

Keywords: Betulinic acid, Dexamethasone, Ovalbumin, Interleukin, Immunomodulatory, Asthma

Pak J Physiol 2024;20(4):40–3, **DOI:** https://doi.org/10.69656/pjp.v20i4.1725

INTRODUCTION

It has been assessed that more than 300 million individuals of any age, and every single ethnic foundation, experience the ill effects of asthma and the burden of this infection to governments, human services frameworks, families, and patients is expanding around the world with each passing day.[1,2] Epidemiological studies from different parts of the world have additionally affirmed that asthma incidence is expanding on the planet, particularly in the under developed nations. As indicated by the Global Initiative for Asthma (GINA), pervasiveness of asthma in Pakistan is 4–5%.[3] At present there is little awareness about asthma in a large proportion of developing nations.[4] Recent economic shift in Pakistan has led to modernization of its cities resulting in rise of asthma cases.[5] In spite of the fact that passing from asthma has diminished with the general utilization of inhaled glucocorticoids, the worldwide effect of asthma stays high, and the prevalence of asthma is by all accounts expanding. There is an unmistakable requirement for new medications for asthma that beat inadequacies of those accessible presently.[4]

Treatment for asthma centres on anticipation as well as treatment of airway route irritation and alleviation of side effects. Studies have discovered inhaled corticosteroids to alleviate bronchial responsiveness and improved lung function.[6,7] Non-steroid mitigating prescriptions have additionally been discovered compelling in a significant extent of patients. Bronchodilators, (for example, inhaled β-agonists, breathed in anti-cholinergic operators, and oral theophylline) work rapidly to diminish indications by unwinding the smooth muscles in the dividers of the airway routes.[8] Asthma scenes that do not react adequately to bronchodilators might be treated with short courses (a few days) of oral steroids. Concerns remain, notwithstanding, about the long term utilization of steroids in youngsters, as they may abatement or postpone physical development and sexual and bone development. Prolonged utilization of oral steroids to control intense intensifications conveys contradictory symptoms, for example, adrenal concealment, osteoporosis, bruising, thinning of skin and cataracts.[9]

The best comprehended immunological pathway has been characterized in unfavourably susceptible asthma, demonstrating that T helper 2 (Th2) cells assume an essential part in allergen-initiated irritation in such patients.[10] Unfavourably susceptible asthma is related with a higher level of bronchial hyper responsiveness, higher serum IgE levels and expanded quantities of eosinophils in blood or sputum. Enacted Th2 cells deliver certain marker cytokines, e.g., interleukin (IL) -4, -5, -9, and -13. IL-4 animates B-cells to create immunoglobulin compose E (IgE) against that particular allergen, and it fortifies Th2 cell multiplication and cytokine generation.[11,12]

Objectives of this study were to compare the effects of betulinic acid (BA) and dexamethasone (DEX) in ovalbumin induced asthma in mice and

measure the *m*RNA expression levels of IL-4 using PCR.

METHODOLOGY

It was an experimental randomized control study. The study was conducted in 1 year (April 2021 to March 2022), after endorsement from Ethics Review Committee of Islamic International Medical College (No. RIPHAH/ERC/16/0200), and was executed within the set regulations by the National Institute for animal experiments with the National Institute for Health (NIH) animal house, Islamabad.

Eight-week-old forty male BALB/c mice were acquired from the animal house of NIH, Islamabad. The animals were housed in a sterile environment with 12-12 hour light and dark cycle and supplied with an OVA-free animal diet and water *ad libitum*. All mice were subjected to euthanasia and treated by the Oswaldo Cruz Foundation rules for laboratory animals.[13] Experiments were performed in accordance with guidelines of Institutional Animal Ethics and Experimentation Committee.[14]

Forty BALB/c mice weighing 30–50 grams were procured from animal house of NIH. The mice were housed in mesh wire confines under standard lab conditions[13] with standard feeds and had free access to tap water. They were permitted to adjust for 2 weeks before the beginning of experiment. Intraperitoneal (IP) injection composed of 10 mg chicken egg OVA (Sigma, St Louis, MO, USA) dissolved in 50 mL of alum (Alu-gel-S, Serva, Heidelberg, Germany) was given to all mice on day 0.[15,16]

The allergic airway inflammation was induced in mice by IP sensitization and airway challenge with OVA through nasal inhalation. Groups B, C and D were sensitized on day 0 and 14 by IP injection of 10 µg of OVA in 50 mg $Al(OH)_3$ (adjuvant) in a volume of 0.1 mL Phosphate Buffer Saline (PBS). Mice of all groups were sensitized with PBS on day 0 and 14 by IP injection and challenged intranasally with PBS once daily for 7 days (Day 14–21).[16]

Betulinic acid was purchased from Sigma (St. Louis, MO, USA) and was administered in the dose of 5 mg/Kg body weight in BA treated groups. DEX was administered of 0.5–2.0 mg/Kg PO. Ovalbumin was purchased from sealed container of Sigma Aldrich from local distributor and administered in dose of 10 µg of OVA in mice.[17]

Forty healthy BALB/c mice were randomly assigned to four groups, each containing 10 animals. The first group served as the Negative Control and received no treatment, remaining under standard conditions for 21 days. The second group, the Positive Control, was sensitized with 10 µg of OVA intraperitoneally (IP) on day 0 and subsequently challenged with OVA for the following 7 days after day 14. Group III (OVA+BA) received OVA 10 µg/Kg IP on days 0 and 14, followed by 5 mg/Kg body weight for 7 days. Group IV (OVA+DEX) was treated with OVA 10 µg/Kg IP on day 0 and day 14, followed by 2 mg/Kg body weight for the next 7 days. Biochemical analyses were conducted using commercially available kits to measure total leukocyte count, blood eosinophil count, and IL-4 PCR levels. Blood cell differentiation was performed using a computerized analyzer equipped with flow cytometry, which counts and classifies blood cells as they pass through a sensing tube. Peripheral blood samples were collected in EDTA tubes and analysed using an automated haemocytometer (Sysmex XT-2000iV; Sysmex, Kobe, Japan).[16,17]

For the Polymerase Chain Reaction (PCR) primer sequence for mice as shown in Table-1 was brought from the Thermo Scientific Company from their regional distributors in Pakistan (South East-Asia region).

1. Thermo Scientific GeneJET RNA Purification Catalog number: K0731
2. RevertAid First Strand cDNA Synthesis Kit. Catalog number: K1621
3. Maxima SYBR Green/ROX qPCR Master Mix. Catalog number: K0221

Table-1: Primer sequence for IL-4

Name	Primer sequence	
IL-4	5′-TATACAGAGCTCCGTAGGAC-3′	Forward
	5′-AGTTGTCTGCAGCCACGAAC-3′	Reverse

Thermo Scientific GeneJET RNA Purification Kit is an easy to use and efficient system for purification of total RNA from mammalian tissue. The kit acts on the principle of silica-based membrane technology in the form of a convenient spin column, excluding the necessity for tedious cesium chloride difference, alcohol precipitation, or toxic phenol-chloroform extractions.

RNA molecules longer than 200 nucleotides can be separated with the GeneJET RNA Purification Kit in 15 minutes after the lysis step. The high-yielded isolated RNA can be used in a wide range of uses, including RT-PCR, RT-qPCR, Northern blotting, and other RNA-based analyses.

RESULTS

There was a considerable (p<0.001) rise in TLC in AC in comparison to NC. Treatment with BA and DEX considerably (p<0.01) reduced TLC as compared to AC group respectively (Figure-1). Our result showed a major (p<0.001) rise in blood eosinophils in AC as compared to NC group. Treatment with BA and DEX considerably reduced blood eosinophils as compared to AC group respectively (p<0.001) (Figure-2). There was a drastic raise (p<0.001) in the expression levels of IL-4 in AC in relation to NC. Treatment with BA and DEX considerably attenuated (p<0.001) the IL4 expression levels in comparison to AC (Figure-3).

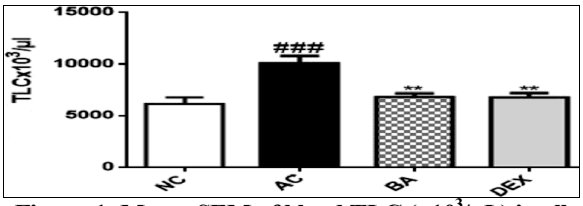

Figure-1: Mean±SEM of blood TLC (×10³/µL) in all groups

BA and DEX significantly decreased levels of total leukocyte count compared to AC. Mean±SEM is shown to symbolize the data. ###p<0.001 when AC compared with NC, **p<0.01 when BA and DEX compared with AC

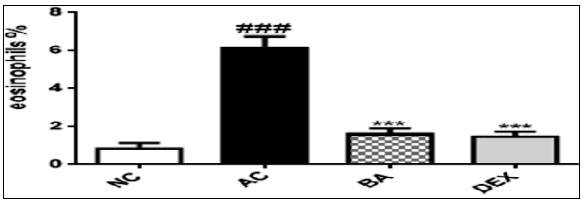

Figure-2: Mean±SEM of eosinophils (%) in all groups

BA and DEX significantly decreased levels of blood eosinophils count in comparison to AC. Mean±SEM is shown to signify the data. ###p<0.001 when AC compared with NC, ***p<0.001 when BA and DEX compared with AC

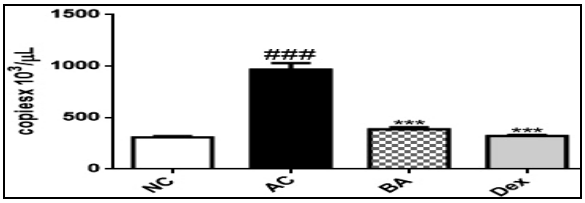

Figure-3: IL-4 copies in all four groups

BA and DEX significantly decreased levels of *m*RNA expression of IL-4 when compared with AC. Mean±SEM is shown to represent the data. ###p<0.001 when AC compared with NC, ***p<0.001 when BA and DEX compared with AC

DISCUSSION

Previous research has described the significance of stability between Th1 and Th2 pathways. Both these pathways work interestingly with each other and have a tendency to hinder each other's reaction. Allergen introduction in aviation routes expanded grouping of Th2 type cytokines which brought about acceptance of a Th2 predominant resistant reaction. IL-4 are considered as Th2 derived cytokines.[18]

Our study showed the immunomodulatory role of BA by decreasing the articulation levels of Th2 composed cytokines. It was demonstrated that BA improved hypersensitive asthma by diminishing the inflammatory cell invasion in airway routes, by decreasing the TLC and DLC in blood which is mainly ascribed to the reduction in expression of both IL-4 levels.

The findings from the present work underscore the crucial role of BA in modulating the balance between Th1 and Th2 immune responses. The observed decrease in Th2-derived cytokines, such as IL-4, aligns with the notion that BA may counteract the Th2-dominant immune response commonly associated with allergic asthma.[19] By reducing the infiltration of inflammatory cells in the airways and lowering total leukocyte count (TLC) and differential leukocyte count (DLC) in the blood, BA appears to mitigate the inflammatory processes characteristic of asthma.[20] This immunomodulatory effect suggests that BA not only reduces the overall inflammatory burden but also helps restore a more balanced immune response, potentially offering a novel therapeutic strategy for managing asthma. These results highlight the promising potential of BA as an adjunctive treatment in asthma management, warranting further investigation to elucidate its mechanism and therapeutic efficacy.[21]

Effects of BA on the expression of IL-4 was determined in this study which showed significant decreased expression levels of IL-4 in AC group after therapy with BA and DEX treatment. Our study is in alignment with the previous studies performed by C Faustino *et al*[22]. The finding of Jung DH *et al*[23], also demonstrated that IL-4 expression was decreased when BA and DEX was used as compared to AC group.

These outcomes further supported our results which suggested that DEX and BA improved allergic asthma in part via attenuation of IL-4 expression levels.

It has been well established that the progression of asthma has a direct role with the rise of differential count due to chemical and cellular effects of asthma. The cellular composition of inflammatory infiltrate in lung is characterized by increased numbers of activated neutrophils, mast cells, eosinophils, and monocytes. Eosinophils and lymphocytes have an important role in patho-physiology of asthma. These mediators release certain chemicals like leukotrienes and prostaglandins that worsen the asthmatic conditions. In these asthmatic models drastic increase in neutrophils have also been found which leads to cascade of asthmatic complications.

We recognized a raise in inflammatory cells like total leukocyte count, eosinophils, lymphocytes and neutrophils in AC group whereas treatment with BA and DEX significantly decreased all the values of the above stated indices. Our results demonstrated a considerable rise in eosinophils count in blood whereas treatment with BA and DEX normalized their values considerably. Findings of Kaushik *et al*[24] are in concordance with our research. BA and DEX reduce the total leukocyte count, blood eosinophils, neutrophils and lymphocytes as compared to the AC is also depicted by Shaban *et al*[25], and Pimentel *et al*[26].

There was a significant increase in inflammatory cell counts including TLC, eosinophils, lymphocytes, and neutrophils in AC group, highlighting the heightened inflammatory state characteristic of asthma. This is consistent with previous findings that associated elevated eosinophil counts with asthma

exacerbations.[19] Treatment with BA and DEX led to a marked reduction in these inflammatory indices, indicating a potent anti-inflammatory effect of these treatments. The normalization of eosinophil counts in particular underscores the potential of BA and DEX in counteracting the excessive inflammatory response often seen in asthma. These results suggest that both BA and DEX are effective in mitigating the inflammatory process, thereby supporting their potential as therapeutic agents in asthma management. The significant reduction in inflammatory cell counts observed with these treatments provides compelling evidence for their efficacy in restoring immune balance and reducing asthma-related inflammation.[20]

CONCLUSION

Betulinic acid has significant immunomodulatory effect on ovalbumin induced airway inflammation in male mice model. It is also noteworthy that immuno-modulatory effects of BA are comparable to that of DEX. Future studies are recommended for the combined effect of BA and DEX.

REFERENCES

1. Yakubu A. Conceptualizing the maturity of citizens for consumer health informatics in low and middle-income countries empirical analysis of Chile, Ghana, Iraq, Kosovo, Turkey, and Ukraine (Doctoral dissertation). Heidelberg: 2023.
2. Samans R. Human-centred national economic policy: Institutionalizing inclusion, sustainability and resilience in domestic economic governance. In: Human-centred economics: The living standards of nations. Cham: Springer International Publishing; 2023.p. 131–234).
3. Soomro RN, Rohi S, Tabasum A. Prevalence of asthma and allergen in Bolan Medical Complex and Sendeman provincial tertiary care hospitals Quetta Balochistan. Mader-e-Milat Int J Nurs Allied Sci 2024;2(1):63–79.
4. Nguyen GT, Gauvreau C, Mansuri N, Wight L, Wong B, Neposlan J, *et al.* Implementation factors of non-communicable disease policies and programmes for children and youth in low-income and middle-income countries: a systematic review. BMJ Paediatrics Open 2024;8(1):e002556.
5. Muralitharan M. The Impact of Race on the Health of South Asians: A Systematic Review. (Doctoral dissertation). McMaster University; 2023. Available from: http://hdl.handle.net/11375/29126.
6. Urs RC, Evans DJ, Bradshaw TK, Gibbons JTD, Smith EF, Foong RE, *et al.* Inhaled corticosteroids to improve lung function in children (aged 6–12 years) who were born very preterm (PICSI): a randomised, double-blind, placebo-controlled trial. Lancet Child Adolesc Health 2023;7(8):567–76.
7. Matsumura Y. Inadequate therapeutic responses to glucocorticoid treatment in bronchial asthma. J Int Med Res 2023;51(6):03000605231175746.
8. Rai JP, Sahi MA, Prajapati M, Verma S, Parthiban MS. Text book of Pharmacology I: Bilaspur: Shashwat Publication; 2024.
9. Dejaco C, Kerschbaumer A, Aletaha D, Bond M, Hysa E, Camellino D, *et al.* Treat-to-target recommendations in giant cell arteritis and polymyalgia rheumatica. Ann Rheum Dis 2024;83(1):48–57.
10. Harker JA, Lloyd CM. T helper 2 cells in asthma. J Exp Med 2023;220(6):e20221094.
11. Sen HN. Elements of the immune system and concepts of intraocular inflammatory disease pathogenesis. In: Scott M. Whitcup, H. Nida Sen. (Eds). Whitcup and Nussenblatt's Uveitis: Fundamentals and Clinical Practice. Elsevier; 2021.p.1–28.
12. Kaboodkhani R, Mehrabani D, Karimi-Busheri F. Achievements and challenges in transplant ation of mesenchymal stem cells in otorhinolaryngology. J Clin Med 2021;10(13):2940.
13. Pereira LCC, Coelho JWR, Elidio HDSM, Silva RCPF, Sobral JGR, Soledade BAB, *et al.* Clinical/behavioral monitoring of rodents and rabbits undergoing scientific experiments. Open J Vet Med 2024;14(5):91–109.
14. Pimenta BL, Menezes TP, Louise V, Dias FCR, Machado BAA, Ribeiro L, *et al.* Protective effects of Theracurmin treatment during experimental infection of the Colombian strain of Trypanosoma cruzi at the testicular site. Front Cell Infect Microbiol 2023;13:1143360.
15. Rizzo GP, Sanches RC, Chavero C, Bianchi DS, Apuzzo E, Herrera SE, *et al.* Poly(allylamine)/tripolyphosphate nanocomplex coacervate as a NLRP3-dependent systemic adjuvant for vaccine development. bioRxiv [Preprint] 2024:2024.07.01.601578.
16. Hussein NA, Gawad HS, Maklad HM, El-Fakharany EM, Aly RG, Samy DM. Empagliflozin inhibits autophagy and mitigates airway inflammation and remodelling in mice with ovalbumin-induced allergic asthma. Eur J Pharmacol 2023;950:175701.
17. Lodi S, Waheed A, Jehangir A, Naeem U, Khan SA, Iftikhar J. Immunomodulatory effect of betulinic acid in ovalbumin induced airway inflammation in male mice. J Islamic Int Med Coll 2019:14(2):86–90.
18. León B. Understanding the development of Th2 cell-driven allergic airway disease in early life. Front Allergy 2023;3:1080153.
19. Kamaraj Y, Dhayalan S, Chinnaiyan U, Kumaresan V, Subramaniyan S, Kumar D, *et al.* Triterpenoid compound betulin attenuates allergic airway inflammation by modulating antioxidants, inflammatory cytokines and tissue transglutaminase in ovalbumin-induced asthma mice model. J Pharm Pharmacol 2021;73(7):968–78.
20. Zaidi A, Srivastava AK, Parveen K. Role of nelumbo nucifera (LOTUS) stem as immunity booster and anti-inflammatory food. Era J Med Res 2021;8(2):1–5.
21. Nagori K, Nakhate KT, Yadav K, Ajazuddin, Pradhan M. Unlocking the Therapeutic Potential of Medicinal Plants for Alzheimer's Disease: Preclinical to Clinical Trial Insights. Future Pharmacol 2023;3(4):877–907.
22. Faustino C, Pinheiro L, Duarte N. Triterpenes as potential drug candidates for rheumatoid arthritis treatment. Life (Basle) 2023;13(7):1514.
23. Jung DH, Lee A, Hwang YH, Jung MA, Pyun BJ, Lee JY, *et al.* Therapeutic effects of Pulsatilla koreana Nakai extract on ovalbumin-induced allergic rhinitis by inhibition of Th2 cell activation and differentiation via the IL-4/STAT6/GATA3 pathway. Biomed Pharmacother 2023;162:114730.
24. Kaushik A, Singh DP, Sahu M, Kumar A, Pratibha, Pandey P, *et al.* Protective effect of Achyranthes aspera against compound 48/80, histamine and ovalbumin-induced allergic disorders in murine model. Mol Biol Rep 2024;51(1):202.
25. Shaban P, Honari N, Erfanian N, Hosseini M, Safarpour H, Nasseri S. Anti-inflammatory Effects of Ziziphus Jujube Mill on LPS-induced Acute Lung Injury in Mice. Iran J Allergy Asthma Immunol 2023;22(3):281–9.
26. Pimentel VD, Acha BT, Gomes GF, de Sousa Cardoso JL, da Costa CL, Batista NJ, *et al.* Anti-inflammatory effect of *Anadenanthera colubrina var. cebil* (Griseb.) Altschul in experimental elastase-induced pulmonary emphysema in rats. J Ethnopharmacol 2024;332:118216.

Address for Correspondence:

Dr Sher Afghan Khan, Assistant Professor, Department of Pharmacology, Gajju Khan Medical College, Swabi, Pakistan. **Cell:** +92-322-5363231

Email: sher_ak@hotmail.com

Received: 15 Jul 2024 **Reviewed:** 30 Oct 2024 **Accepted:** 4 Nov 2024

Contribution of Authors:

SAK: Concept, data collection, write-up **QN:** Concept, data collection, write-up **MT:** Concept and proofreading

SA: Data collection, statistical work **MJK:** Statistical work and write-up **SZ:** Data analysis

Conflict of Interest: None, **Funding:** None