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ORIGINAL ARTICLE

Ameliorative effect of barbaloin in experimentally induced allergic rhinitis In Wistar rats

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ABSTRACT
Allergic rhinitis is an inflammatory immune disorder that occurs due to IgE-mediated hypersensitivity characterized by nasal symptoms such as sneezing, coughing, fatigue, headache, itching, phlegm, or throat irritation. To investigate the anti-allergic potential of Barbaloin in ovalbumin induced allergic rhinitis in experimental rat. Acute oral toxicity study of barbaloin was performed in rats according to OECD (425) guidelines. The experimental study was then conducted with on wistar rats for 22 days. In first phase all rats were sensitized with 50 mg ovalbumin for 14 day (first phase). Rats were divided into Six groups (n = 5). In second phase of the study, 10 µl of (20mg/ml) ovalbumin was intranasally instilled for alternate 8days and simultaneously, Levocetirizine 10mg/kg (group 3), Barbaloin 100mg/kg (group 4), Barbaloin 200mg/kg (group5) and Levocetirizine+ barbaloin(10mg/kg+200mg/kg) (group 6) was given to rats. A control group (group 1) and Negative Control (group 2) was planned. Behavioral changes were observed throughout the study. At the end of study serum IgE, IL-4, Histamine and NO level were estimated. Rats were sacrificed to perform histopathological estimation of nasal tissue and spleen. Barbaloin treatment significantly decreases the nasal symptoms and improved pathological alternation in nasal and a spleen tissue, Barbaloin treatment significantly prevented elevated IgE, IL-4, Histamine and NO in serum and it also significantly decreased MDA level and increased SOD and GSH level. In the investigation barbaloin treatment showed neither sedative nor anxiety like behavior. ***Conclusion:*** *Barbaloin is a promising anti-allergic agent that may be useful in the clinical management of allergic rhinitis with further clinical investigation.*

Keywords: Barbaloin, Allergic rhinitis, OVA albumin, inflammatory mediators, Antioxidant.

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INTRODUCTION

Allergic rhinitis (AR) is an abnormal nasal mucosa inflammation that is caused by the activation of antigen specific IgE. The predominant clinical signs of AR are nasal congestion, rhinorrhoea, sneezing, itching of the nose, and lacrimation. The last phase response includes eyelid oedema, postnasal discharge, and nasal blockage with irregular coughing. Rhinitis is linked to severe morbidity, high treatment expenses, decreased work output, and poor quality of life. The incidence of AR varies by high-income country (more than50%), middle-income country (inversely), and low-income country (very low), with a range of 12.8% in Spain to 65.9% in New Zealand. The demographic shift has caused an enormous increase in the epidemiology of AR morbidity, which places a significant socioeconomic burden on patients. The socioeconomic cost of AR in Asia ranges from $30.7 to $105.4 billion, with the cost of RA accounting for over $272.92 million in direct medical expenses [1]. Immunotherapy, however, is a time-consuming, difficult approach that is not fully devoid of issues, constraints, and failure. Medications include antihistamines, nasal decongestants, and glucocorticoids which can provide symptomatic relief. However, majority of them have negative side effects include sleepiness (H1antagonists) and bone marrow

suppression (corticosteroids). Therefore, it is imperative that we find a reliable, safe, and well-tolerated alternative—especially a natural phytoconstituent [2].

Barbaloin (10-beta-D-glucopyranosyl-1, 8-dihydroxy-3-hydroxymethyl-9(10H)-anthracenone) is one of the key inventive phytoconstituents of the Aloevera plant. This chemical is highly sought after as a therapy for a number of disorders because of its wide range of therapeutic qualities. Due to the diverse pharmacological and therapeutic characteristics of barbaloin, numerous research investigations attest to its effectiveness. The various studies discuss the efficacy of the molecule against numerous diseases due to its diverse features like antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, and anti-microbial. [3] Barbaloin showed affinity for phospholipid membranes whereas barbaloin stabilized lamellar structures of cells [4]. Barbaloin showed antiviral activity and may be used as a potential candidate for an alternative for anti microbial, pharmaceutical or cosmetic applications considering that the pathogenesis of AR resembles that of allergic asthma and that of atopic dermatitis, it is unclear whether barbaloin plays a role in the treatment of AR. We sought to investigate the potential therapeutic effects of barbaloin in an ovalbumin-induced AR model.

MATERIAL AND METHODS

Test chemicals

Barbaloin was obtained from Yucca Enterprises, Mumbai (Yucca/BA/2020/17/11) with > 95% of purity. Ova albumin was obtained from Modern Industries, Nashik and Levocetirizine was obtained from local store. All chemical reagents used were of analytical grade and purchased from standard manufacturer.

Experimental Animals

Wistar rats weighing 150-200 g were obtained from LACSMI Bio Farms, Pune, India. They were housed in polypropylene cages with husk bedding, renewed every 48h under12:12h light dark cycle at around18 to 29°C. They were fed with commercial pellet rat chow and water ad libitum. The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi and the Institutional Animal Ethical Committee (IAEC) approved protocol of this study (IAEC/2021-22/05).

Acute Oral Toxicity Study (OECD425)

The acute toxicity study was performed according to OECD guidelines 425. The female Wistar rats were used for toxicity studies. The animals were fasted overnight prior to the acute experimental procedure. The dose level was selected from the four fixed doses i.e. 2000 mg/kg body weight. Immediately, after dosing, the animals were observed at 0, 0.5, 1, 2, 3, 4, 24 hr and then after each day till 14 days. Special attention observational changes such as hyperactivity, ataxia, convulsion, salivation, tremors, diarrhoea , lethargy, sleep were observed and systematically recorded.

Experimental Design

Animals were randomly divided into six groups containing 5 animals in each group. Group – Normal Control (water for injection), Group II: Negative Control (Ovalbumin), Group III: Standard (Ovalbumin+Levocetirizine 10mg/kg, p.o.), Group IV: Test 1 (Ovalbumin+ Barbaloin 100mg/kg p.o.), Group V: Test 2 (Ovalbumin+ Barbaloin 200mg/kg p.o.), GroupVI: Standard + Test (Ovalbumin+ Levocetirizine 10mg/kg, p.o.+ Barbaloin 200mg/kg p.o.).

Sensitization Protocol

The sensitization solution (SS) consisted of 50 mg of ovalbumin; 1000 mg of aluminium hydroxide dissolved in 500 ml on saline. Out of 22 days of experiment the rats were administered 0.5 ml of SS from Day 1 to Day 14 for all groups by i.p. route, except Group i.e. Between Day 15 to Day 22 Intra-nasal Ovalbumin (20mg/ml -10µl/animal) was given on every alternate day i.e. on 15th, 17th, 19th, 21st day to all groups except Group I. The treatment drug was also administered from Day 15 to Day 22 for each day to all groups except Group I and Group II. On day 22,all animals were sacrificed [5].

Assessment of Nasal Symptoms

Nasal symptom scores were assessed independently by two blinded observers to the experimental groups. After a 10-min of adaptation period, rats were observed over 10 min and nasal symptoms, including sneezing, itching, and rhinorrhoea, were rated on a 0–3 point scale. Allergic rhinitis scores were recorded on day 15,16, 18 , 20, 21, and 22.The allergic rhinitis model was considered to be successful when the total score exceeded five points [6]

Elevated Plus Maze

The animals were brought to the experiment room and acclimated for at least 1 h before starting with the sessions. Procedure of 5 min was held under 5 lux of illumination and movement were recorded with a video camera mounted on top of the maze. The time spent in open arms, total distance travelled and number of entries in open arms was analysed with the use of the Maze Master system from V. J.

Instruments. Data was analysed as a percentage of time spent in the open arms vs time spent in closed arms [7].

Assessment of locomotors Activity

The animal locomotor behaviour was monitored using Actophotometer (Omega Scientific Industries). Each animal was placed in actophotometer for 5 minutes and basal activity score was recorded for all animals. Each animal was treated with respective drug and activity score was recorded after 30 min and 1hr.Decreased activity score was taken as index of sedation [8].

Measurement of serum total IgE level

Retro-orbital blood specimens were collected and centrifuged for 10 min at 1000 gat + 4°C.The separated serum was stored in a deep freeze at − 80 °C until the day of study. IgE is the important inflammatory mediators released to stimulate immune response. The estimation of IgE in the serum was carried out immediately after challenge. Total IgE was measured at H.S. Pathology Private Limited, Thane by ELISA method [9].

Measurement of serum IL-4 level

Retro orbital blood specimens were collected and centrifuged for10 min at 1000 gat+ 4°C.The separated serum was stored in a deepfreeze at −80°C until the day of study. IL-4 was measured at SCITESLA Pvt.Ltd., Navi Mumbai 400710 by ELISA using microplate reader, EPOCH (Bio Tek-Agilent, USA) [10].

Measurement of histamine concentration

Histamine is a biogenic amine, noted for manifestation of certain allergic reactions and implicated as a mediator of hypersensitivity. Thus, histamine concentration in serum by an O-phthaladehyde Spectrofluorometric procedure was estimated. The fluorescence intensity was measured at 460 nm (an excitation at 355 nm) using a Spectrofluorometric method [10].

Estimation of nitric oxide

Nitric oxide is one of the important mediators in regulating patency of airways and its concentration increases in allergies related to the upper respiratory tract. The nitric oxide concentration in serum samples was measured by the Griess reagent method, the absorbance was measured at 548nm using the UV–visible spectrophotometer (V630, Jasco Analytical Instrument).The concentrations of nitrite were determined from standard curves constructed with serial dilutions of sodium nitrite [11].

Estimation of Biochemical and antioxidant parameters

The activity of albumin in nasal tissues were measured using commercially available reagent kits and ultraviolet-visible spectrophotometer (JASCO-V-530). Nasal tissue of individual rats was isolated and washed with ice-cold saline. Tissue homogenates were prepared with 0.1M Tris-HCl buffer (pH7.4). The supernatant obtained was used to estimate superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation (MDA). The level of SOD, GSH, and MDA in nasal tissue homogenate was determined according to reported methods [12].

Histological Examination

On day 22, after blood withdrawal, rats were sacrificed and Nasal tissue and spleen tissues were dissected, weighed and stored for 24 h in 10% formalin for histological examination. The histological examination was carried at Optimus Pathology Laboratory, Nashik [3].

Statistical Analysis

Group means and standard error mean was calculated using Graph pad prism (version5.00). Statistically significant differences between group means were determined using Tukey's test where a P-value<0.001wereconsidered statistically significant.

RESULTS AND DISCUSSION

Acute toxicity study 425

Acute toxicity study was performed for 14 days. There are no any toxic changes seen in animals. Whereas the LD50 was found > 2000mg/kg in on behavioural parameters during acute toxicity study**.**

Table 1. Effect of Barbaloin on behavioural parameters during acute toxicity study

Sr No	Parameters	Barbaloin(2000mg/kg)
1	Alertness	Normal
2	Restlessness	Absent
3	Touch response	Absent
4	Pain response	Normal
5	Food intake	Normal
6	Skin and fur	Normal
7	Eye	Normal

8	Water intake	Normal
9	Convulsion	Absent
10	Tremor	Absent
11	Diarrhea	Absent
12	Morbidity	Absent
13	Mortality	Absent

Effect of Barbaloin, Levocetirizine on Nasal Symptoms

The number of rubbing and sneezing increased significantly ($p < 0.05$) after the OVA challenge on day 22 in the AR control mice as compared with normal mice. Treatment with barbaloin (100 mg/kg) and Levocetirizine (10mg/kg) significantly ($p<0.05$) protected the mice from all nasal symptoms induced by OVA challenge as compared with AR control group. Treatment with barbaloin (200 mg/kg) and combination of Levocetirizine + barbaloin shows significant ($p>0.05$) protection against OVA-induced alterations in nasal symptoms as compared with AR control group.

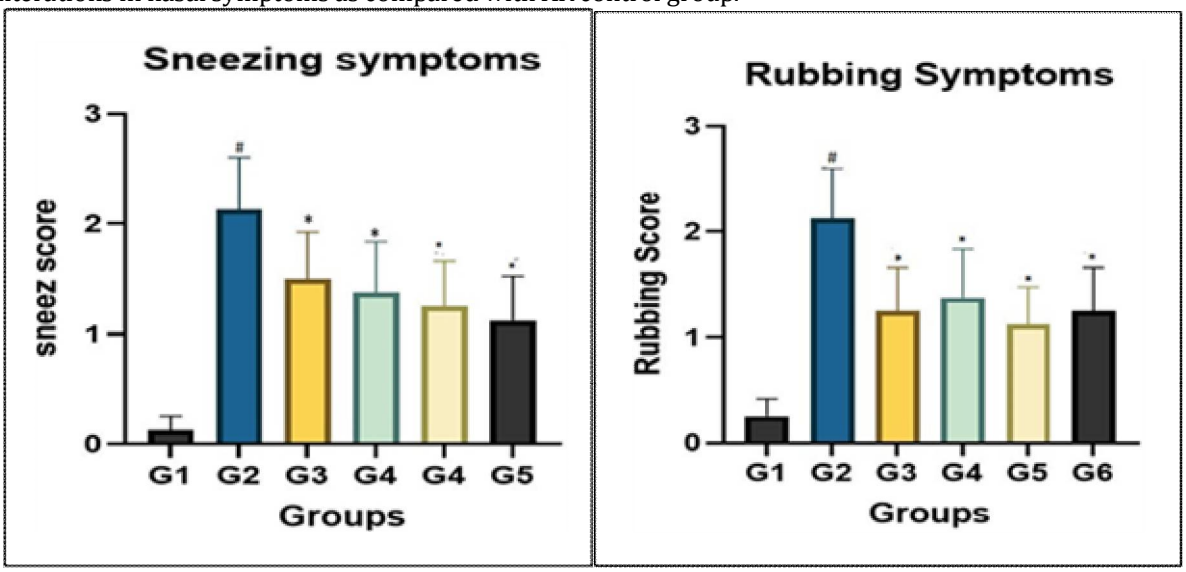

Fig 1. Effect of Barbaloin, levocetirizine and combination of levocetirizine + Barbaloin on ovalbumin induced (A) Sneezing and (B) Rubbing frequencies: G1-Control, G2-ARcontrol,G3-Levocetrizine(10mg/kg), G4-Barbaloin (100mg/kg) G5-Barbaloin (200mg/kg), G6- Levocetrizine + Barbaloin.

Assessment of anti-anxiety activity

AR control group significantly ($p<0.0001$) spent less time in open arm as compared to control.

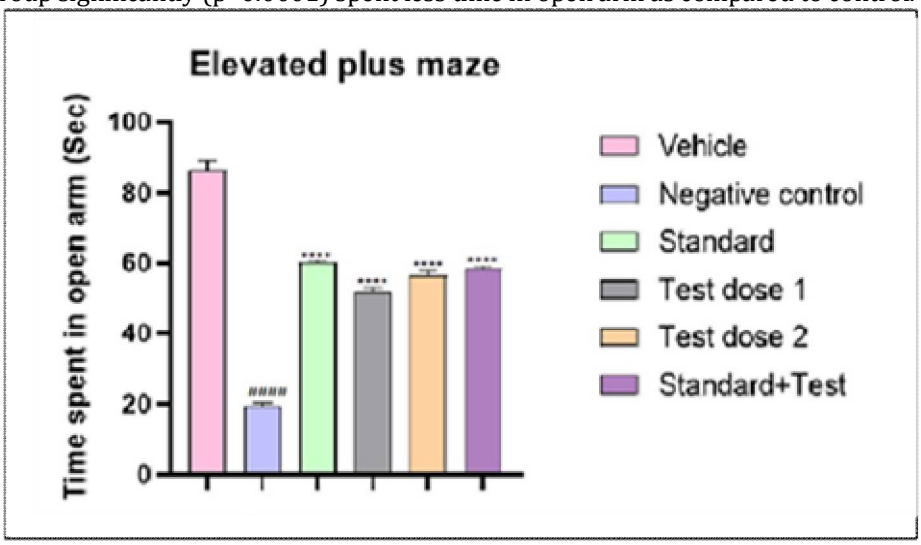

Fig 2. Effect of Barbaloin against allergic rhinitis induced anxiety like behavior Barbaloin (100mg/kg), Levocetirizine, Barbaloin (200mg/kg), Levocetirizine Barbaloin groups animals significantly ($p<0.0001$)spent more time in open arm as compared to AR control

Effect of barbaloin and Levocetirizine on locomotors Activity

Significantly ($p<0.01$) decrease in locomotor activity in AR control as compared to control. Levocetirizine ($p>0.05$) shows the decrease in locomotor activity as compared to AR control. While Barbaloin (100mg/kg), Barbaloin (200mg/kg) and Levocetirizine + Barbaloin shows significantly ($p<0.0001$) improve the locomotor activity.

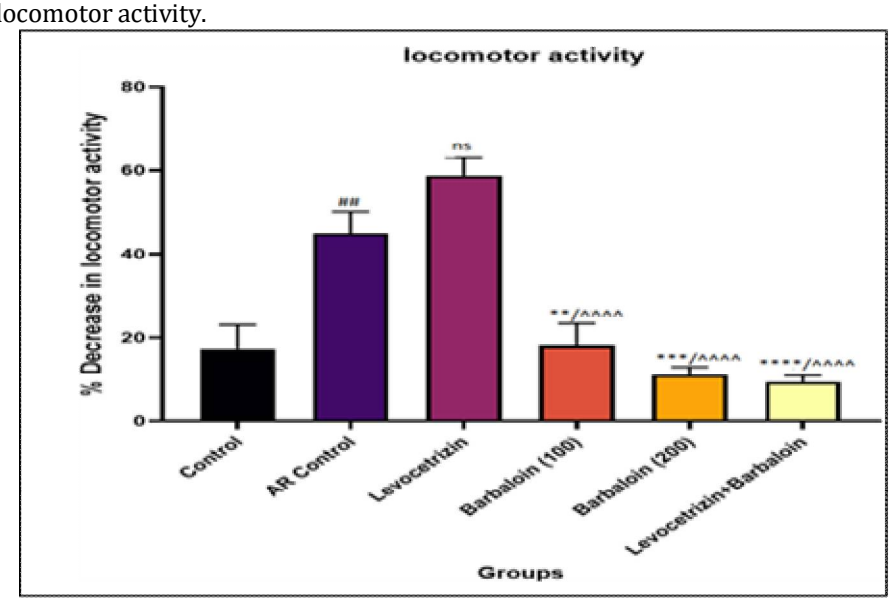

Fig. 3. Effect of Barbaloin (100mg/kg and 200mg/kg) on sedation caused by levocetirizine by performing locomotor activity using actophotometer

Effect of Barbaloin, Levocetirizine on spleen weight

The spleen weight significantly ($p<0.0001$) increases in AR control as compared to control. Barbaloin (100mg/kg), Barbaloin (200mg/kg), Levocetirizine (10mg/kg) and Levocetirizine + Barbaloin shows significantly ($p<0.0001$) decreases in spleen weight as compared to AR control.

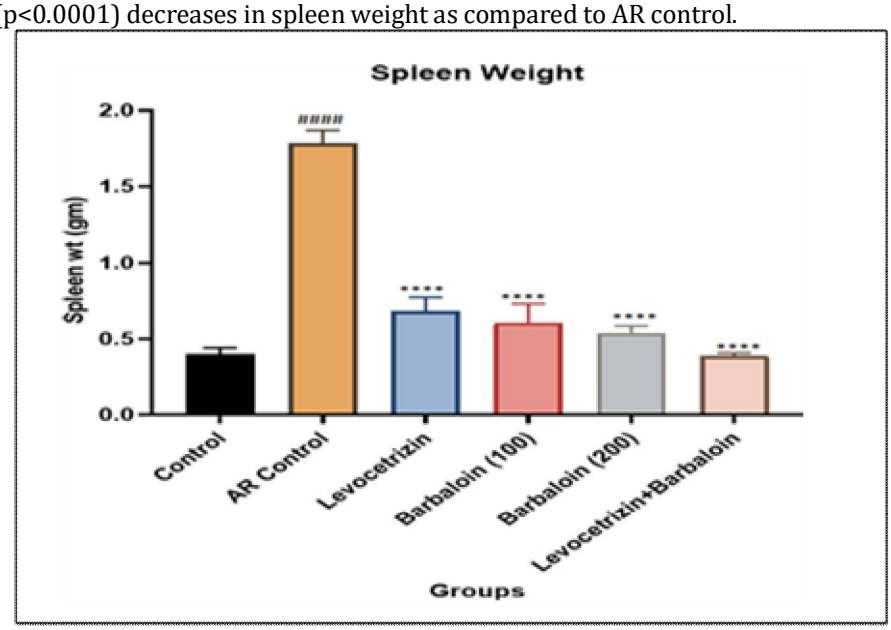

Fig. 4. Effect of Barbaloin and Levocetirizine on allergic rhinitis affected spleen weight

Effect of barbaloin and Levocetirizine on total serum Ig E level

IgE level significantly ($p<0.0001$) increases in AR control as compared to control. Barbaloin (100mg/kg), Barbaloin (200mg/kg), Levocetirizine (10mg/kg) and Levocetirizine + Barbaloin shows significantly ($p<0.0001$) decrease IgE level as compared to AR control.

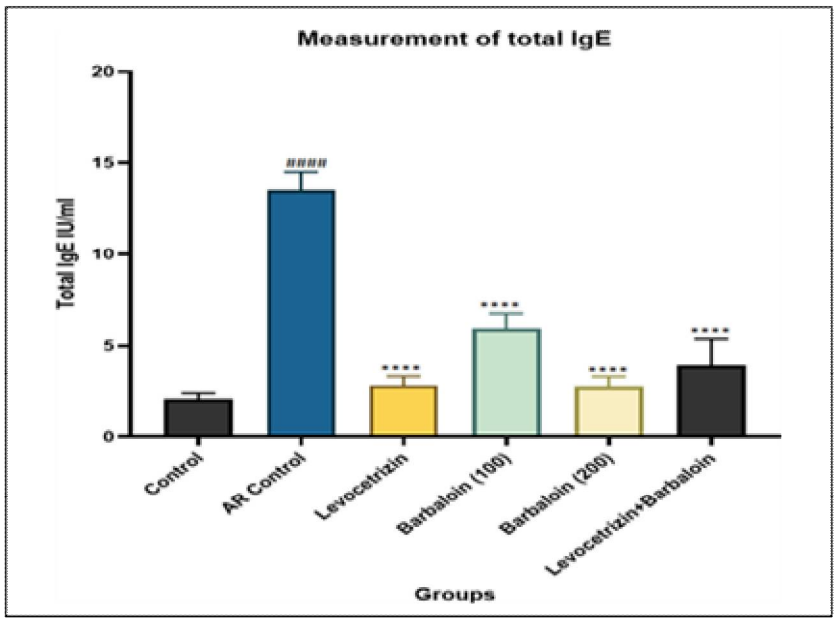

Fig 5. Effect of barbaloin, levocetirizine on total serum IgE level

Effect of barbaloin and Levocetirizine on total serum IL-4 level

IL-4 level significantly ($p<0.0001$) increases in AR control as compared to control. Barbaloin (100mg/kg) ($p<0.01$), Barbaloin (200mg/kg) ($p<0.001$), Levocetirizine (10mg/kg) ($p<0.05$) and Levocetirizine Barbaloin (0.0001) shows significantly decrease IL-4 level as compared to AR control.

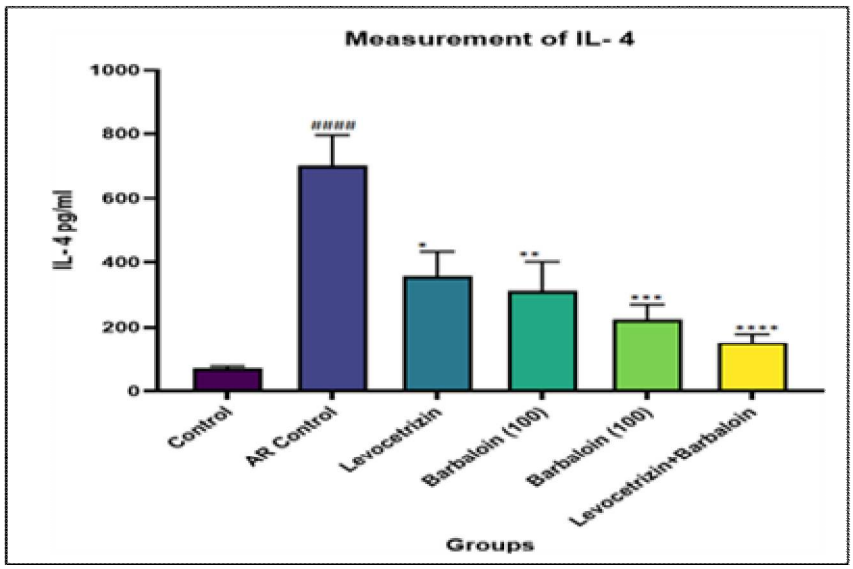

Fig 6. Effect of barbaloin, levocetirizine on serum IL-4level

Effect of barbaloin and Levocetirizine on total serum histamine level

Serum histamine level significantly ($p<0.0001$) increases in AR control as compared to control. Barbaloin (100mg/kg) ($p<0.05$), Barbaloin (200mg/kg)($p<0.0001$), Levocetirizine (10mg/kg)($p<0.0001$) and Levocetirizine Barbaloin (0.0001) shows significantly decrease serum histamine level as compared to AR control.

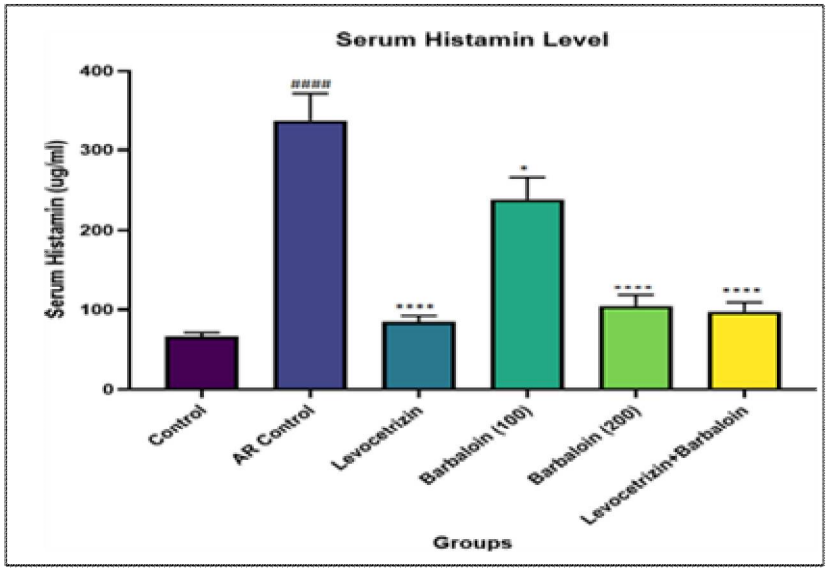

Fig 7. Effect of barbaloin, levocetirizine on serum histamine level

Effect of barbaloin and Levocetirizine on total serum NO level

Serum NO level significantly ($p<0.0001$) increases in AR control as compared to control. Barbaloin (100mg/kg) ($p<0.01$), Barbaloin (200mg/kg) ($p<0.001$), Levocetirizine + Barbaloin ($p<0.01$) shows significantly and Levocetirizine (10mg/kg) non-significantly decrease serum histamine level as compared to AR control.

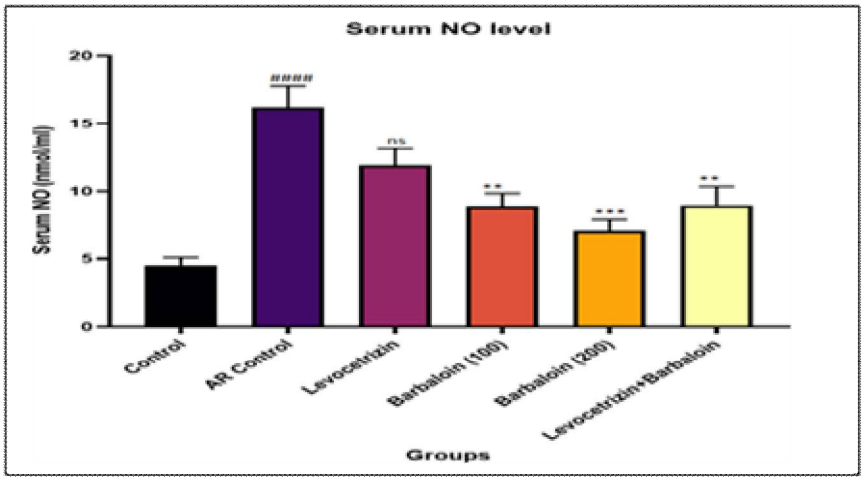

Fig 8. Effect of barbaloin, levocetirizine on serum NO level

Effect of barbaloin and Levocetirizine on MDA, GSH and SOD

MDA level ($p<0.0001$) significantly increases, GSH and SOD level ($p<0.0001$) significantly decreases in AR control as compared to control. While, MDA level significantly decreases, GSH and SOD level significantly increases in Barbaloin (100mg/kg), Barbaloin (200mg/kg), Levocetirizine (10mg/kg) and Levocetirizine +Barbaloin.

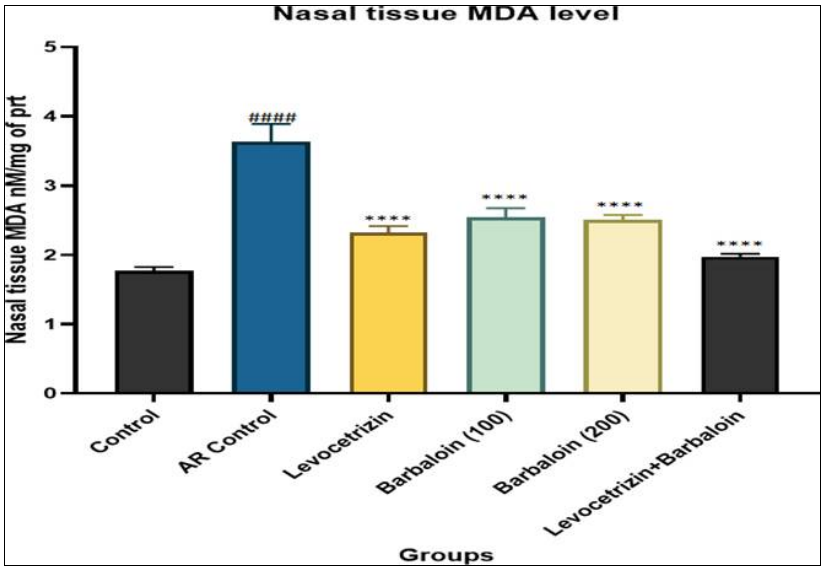

Fig 9. Effect of barbaloin, levocetirizine on MDA level

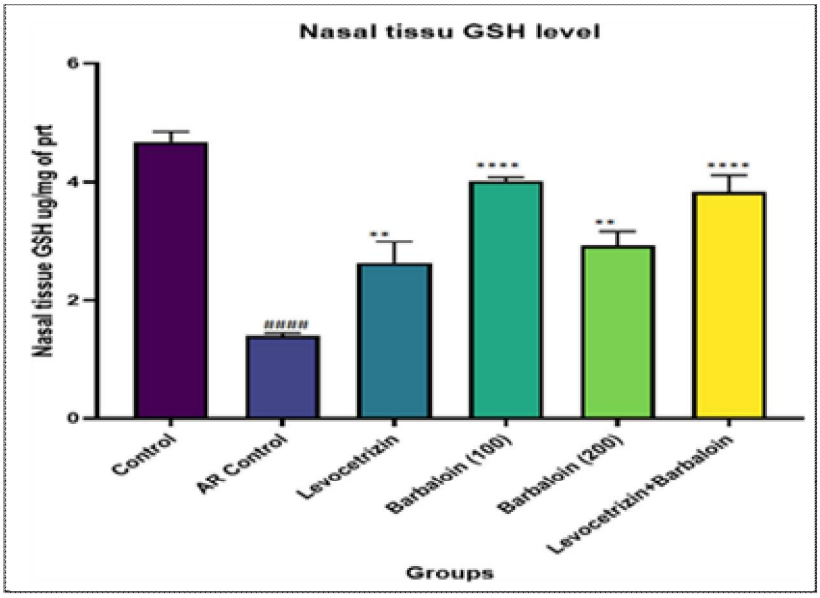

Fig 10. Effect of barbaloin, levocetirizine on GSH level

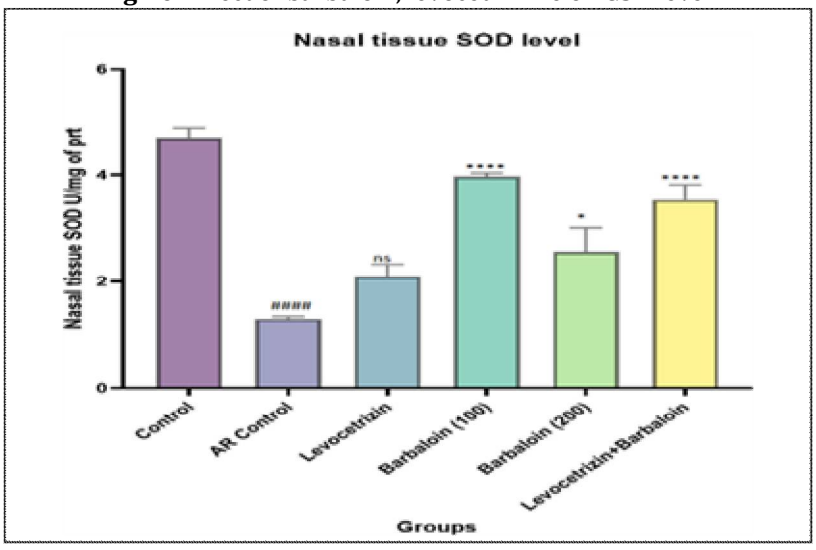

Fig 11. Effect of barbaloin, levocetirizine on SOD level

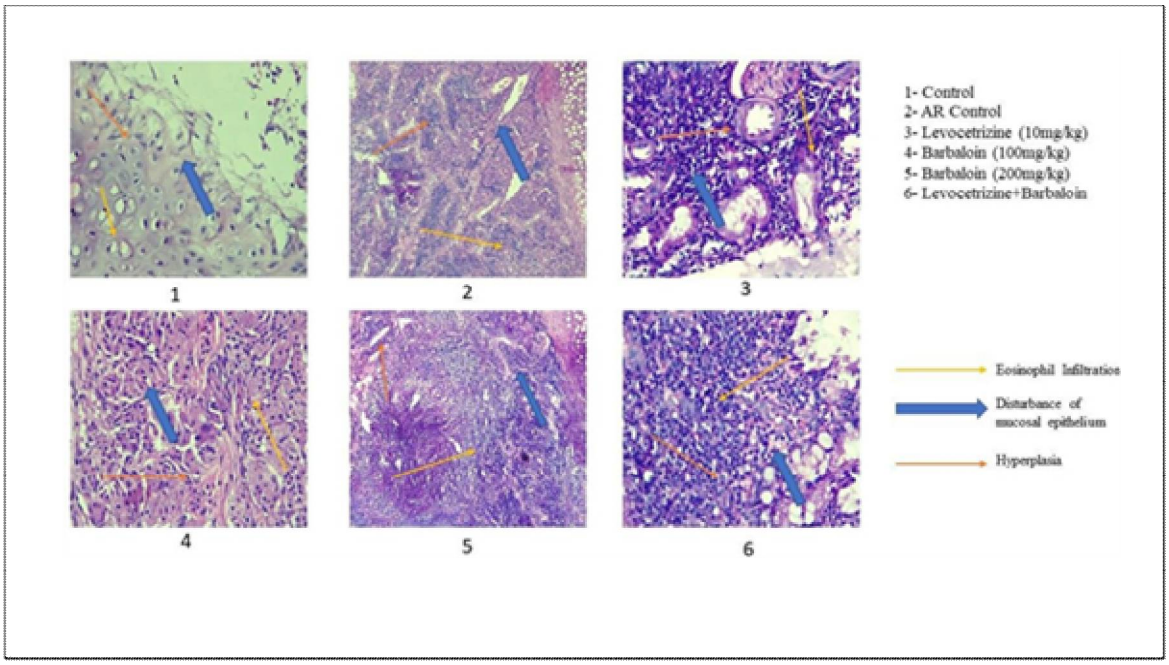

Fig 12. Effect of barbaloin, Levocetirizine on histopathology of spleen and nasal tissues

AR control group shows the nasal and spleen tissues abnormalities like eosinophil infiltration, hyperplasia, disturbance of mucosal epithelium, macrophages with hemosiderin, edema etc. While Levocetirizine, barbaloin (100mg/kg), barbaloin (200mg/kg), Levocetirizine +barbaloin improved the abnormalities.

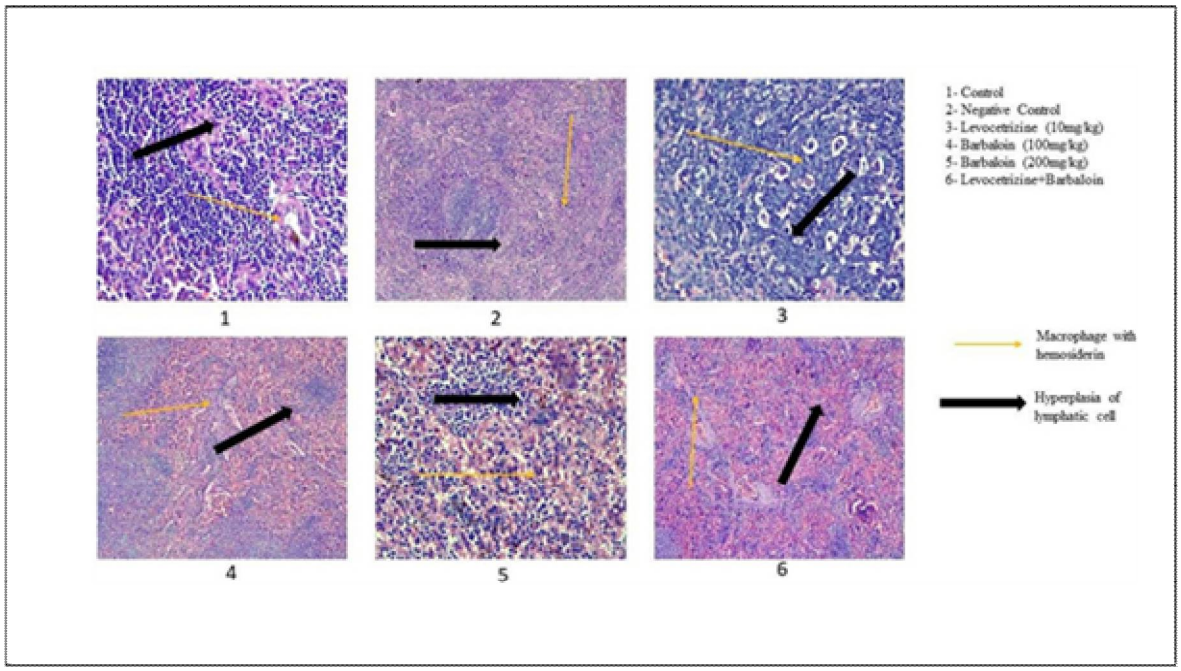

Fig 13. Effect of barbaloin treatment on ova induced alteration in spleen histopathology on day 22.

In this study, it was found that melatonin significantly improved the diagnostic parameters of AR, including the total nasal symptom score, behavioural changes, serum ovalbumin-specific IgE, IL-4, histamine level, NO level, antioxidant level (MDA,CAT,GSH) level, spleen weight and histological abnormalities. Acute toxicity study was performed according to OECD 425 guidelines for 14days (LD50 >2000mg/kg).There were behavioural changes such as alertness, restlessness, pain response, food intake, water intake found to be normal. During the course of the 14-day trial, no deaths, convulsions, or tremors were noticed. Dose was

selected on the basis of 1/10th of LD50 and which is found 200mg/kg, and one lower dose (100mg/kg) was taken for the further investigation.
This study employs a qualitative scale of Wen and Avinc'sal devised by rating rats' nasal problems. On a scale from 0 to 3, nose symptoms such as sneezing and rubbing were graded. Rats exposed too albumin were scored for allergic rhinitis in the current investigation. It was shown that nasal symptoms only appeared in AR control rats, indicating that the ovalbumin model has been successfully validated to cause allergic rhinitis. Physical examination revealed that barbaloin, Levocetirizine, and Levocetirizine + barbaloin all caused mild to moderate itching and sneezing. In the barbaloin and Levocetirizine groups, it was shown that allergic rhinitis symptoms were reduced [6]. The behavioral response of anxiety during experimentally induced allergic rhinitis was investigated. Together with the present results, these studies show that anxiety is a feature of respiratory allergic processes that occurs during the differences phases and when antigens are involved. In essence, allergic rhinitis (AR) is an IgE-mediated immune-inflammatory reaction. Numerous studies have suggested that peripheral inflammatory signal for cytokines from the nose, such as IL-1, IL-6, TNF-, GM-CSF, and IL-5,IL-13, and IL-4, can enter the central nervous system through potential "neural pathways" (olfactory and trigeminal nerves), "cellular pathways," and "humoral pathways," causing neuroinflammation, oxidative stress, and neurotransmitter disturbances in the brain. By using the raised plus test, this study examines the anxious behavior of animals suffering from allergic rhinitis. In contrast to the anxiety-like behavior seen in the allergic rhinitis group, significant improvement was observed in Levocetirizine, barbaloin, and Levocetirizine plus barbaloin groups [13].
This study unequivocally showed that the group that received H1antihistaminic Levocetirizine had altered locomotor activity. The G-protein-family histamine H1-receptors that the H1 antihistaminic target behave as inverse agonists rather than antagonists. The older first-generation H1-antihistamines easily penetrate the brain, causing sedation, weariness, drowsiness, and poor focus and memory. In this investigation, the actophotometer was utilized to measure the sedation caused by Levocetirizine, which was further improved by the combination of Levocetirizine and barbaloin. The negative effects brought on by Levocetirizine alone can be countered by combining it with barbaloin [14]. IgE plays a key role in causing an allergic reaction. It reduces affinities for receptors FcεR2 and however FcεR1 having specificity to form a complex with them. The surface of the various antigen-presenting cells, such as mast cells, basophils, monocytes, and dendritic cells, has a -chain of the tetrameric FcR complex, which IgE binds to and activates degranulation. IgE, on the other hand, has a very short half-life of a few days and exists in free form. The induction and maintenance of inflammatory immune disorders like AR were significantly aided by elevated IgE levels and cytokines released by infiltrated inflammatory cells. Previous studies and literature survey have demonstrated a relationship between high serum IgE levels and allergy diseases like atopic dermatitis, bronchial asthma, and AR. After antigen specific IgE was stimulated via an intranasal challenge with OVA in the current study, a raised level of serum IgE was found, and treatment with barbaloin had a protective effect in reducing the elevated serum IgE [15].
Mast cells has a significant role in the induction of AR through the control of FcεR1, which led to the release of antigens to IgE. Histamine, β-hexosaminidase, cytokines (including IL-4, IL-5, IL-13, and IL-17), chemokines, and derivatives of arachidonic acid are released as a result, induction of AR. The significance of IL-4 in the development and maintenance of allergy disorders through the differentiation of Th2 cells has been well-documented. By stimulating the growth of mast cells, eosinophil activation, modulating adhesion molecule control, and encouraging neutrophil and eosinophil chemotaxis, cytokines produced by Th2 cells promote allergic inflammation, which is likely a factor in inflammatory disorders. Treatment with barbaloin at both the doses100 and200mg showed a significant reduction of IL-4 concentration in nasal lavage, thereby suggesting that barbaloin is an inhibitor of IL-4 during rhinitis thus further symptoms of allergic rhinitis were prevented [3]. Additionally, Th2 cytokines activate eosinophils, which triggers the release of ROS and pro-inflammatory mediators. The advancement of inflammation in the allergic airway has been linked to increased ROS generation, which causes an oxidative influx in the airway and lung tissue. Researchers have established that elevated ROS generation harms the phospholipids in cell membranes, impairing membrane function and causing harm to cells and tissues. Lipid peroxidation, which displays elevated levels of MDA and is related with diminished antioxidant defense enzymes, reflects the structural breakdown of unsaturated fatty acids in lipid membranes. Inhibiting the over production of ROS is therefore advantageous in the management of allergic disorders. In the current study, barbaloin administration also resulted in a significant reduction in MDA levels, which may have an anti-inflammatory effect via reducing ROS formation. Previous studies and literature survey have demonstrated that patients with eosinophilic sinusitis have considerably lower levels of SOD activity in their epithelial mucosa. Decreased GSH levels are also linked to enhanced allergic responses by causing T cells of the Th1 to Th2 type. In the present work, rats given OVA revealed down-regulated expression of SOD, and GSH. These

OVA-induced changes were significantly inhibited when treated with barbaloin, demonstrating its antioxidant activity [10]. The serum of sensitized mice was also examined for histamine, the main mediator of the early-phase allergic response. Animals that had been exposed to OVA had higher levels of histamine than those in the control group. Barbaloin dosage-dependently decreased the blood level of histamine after treatment [12].

In the current investigation, serum nitrite levels in AR control mice have significantly increased. Another crucial mediator in allergic inflammatory reactions like AR is NO. NO increases the generation of cytokines like TNF- in nasal mucosa and activates G protein centration. Additionally, NO promotes eosinophil chemotactic activity and increases eosinophil recruitment. The increased serum NO levels were found to be associated with allergic rhinitis (AR) and decreased in barbaloin and Levocetirizine treated group [15]. An organ that is primarily impacted by immune-inflammation is the spleen. The relative spleen weight of the AR control mice in the current study significantly increased as compared to normal mice, which is a sign that immunological inflammation has been induced. This idea is also supported by a histological investigation of spleen tissue, which shows that OVA-induced AR animals have a high level of lymphocytes cells and macrophages with hemosiderin. Barbaloin therapy considerably reduced the effects of allergen-induced changes on spleen weight and its histology [3].Histological examination of the spleen and nasal tissues, revealed that OVA-induced AR mice are connected to the significant presence of lymphocytes and macrophages with hemosiderin in the spleen region and eosinophil infiltration, hyperplasia, and disturbances in mucosal epithelium nasal tissues. Barbaloin treatment considerably slowed down the development of spleen and nasal histopathological alterations brought on by allergens [3].

CONCLUSION

Barbaloin considerably reduces nasal symptoms, improves pathological alternation in nasal and spleen tissue, and prevents allergic reactions by suppressing the production of mediators including IgE, IL-4, histamine, and NO, according to the studies shown above. By lowering MDA levels and raising SOD and GSH levels, it also has the anti-oxidant potential. No sedative or anxiety-like behavior was noticed during the tested barbaloin administration. Thus, it is anticipated that barbaloin can be developed as a medication for therapeutic application because the current investigation demonstrates the protective action of barbaloin against ovalbumin-induced allergic rhinitis.

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CONFLICT OF INTEREST

Authors declared no conflict of interest.

ETHICAL STATEMENT

Before the commencement of study, permission from Institutional Animal Ethics Committee was obtained.

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