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Epigallocatechin Gallate Adjuvant Immunomodulatory Therapy for Hypersensitivity Pneumonitis

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

The current research aims to investigate the management of Hypersensitivity Pneumonitis (HP), a respiratory ailment affecting the lung parenchyma, particularly the alveoli, terminal bronchioli, and alveolar interstitium, due to a delayed allergic response. Various factors, such as microbial agents, animal and plant proteins, organic and inorganic compounds, and environmental substances, can contribute to respiratory issues. The inflammatory reaction in the alveolar mucosa is a hypersensitive response of type-3 (immune-complex-mediated) or type-4 (T-lymphocytes-mediated) that plays a role in the development of HP. Depending on the frequency and intensity of exposure to the causative antigens, the disease is categorized into acute, subacute, and chronic forms. The research shows the success for subacute and chronic forms of the disease as an alternative to corticosteroids. Although corticosteroids can provide relief for subacute and chronic HP as well as

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acute symptoms, they do not appear to alter the disease's long-term progression. As corticosteroids are only suitable for certain patients, there is a pressing need for an alternative treatment option to advance patient care.
Sea Buckthorn is known to exhibit various beneficial effects, such as antioxidative, cytoprotective, wound-healing, immunomodulatory, and cardioprotective properties, among others. In contrast, Epigallocatechin gallate (EGCG) possesses antioxidant properties and can also mitigate the production of inflammatory cytokines, which are responsible for inducing inflammation in the body. The study postulates that incorporating EGCG alongside deciduous plant extracts could serve as a supplementary agent for HP treatment. Consequently, the research confirms the efficacy of the test medications in managing HP. It is essential to continue exploring alternative treatments and novel therapies to address the multifaceted aspects of HP and improve patients' overall quality of life. By broadening our understanding of the disease and potential treatment approaches, medical professionals can develop more personalized and effective interventions tailored to each patient's needs. Further investigations into the interplay between various therapeutic agents and their mechanisms of action will undoubtedly pave the way for more advanced and comprehensive strategies for combating HP.
The study focuses on the Toluene di-isocyanate induced Hypersensitivity Pneumonitis rats with the prophylactic as well as therapeutic dose of the Sea Buckthorn berries extract along with the Epigallocatechin gallate for around 2.5 months. The paper also shows the various parameters on which the research has been successfully conducted. The research confirms the effectiveness of the test medications in modern HP treatment. This gives a basis for replacing the steroid therapy and opt for the better prolonged lifestyle.

Keywords: Extrinsic allergic alveolitis; sea-buckthorn berries; Epigallocatechin gallate; corticosteroid; pharmacological action.

1. INTRODUCTION

Extrinsic Allergic Alveolitis (EAA), also referred to as Hypersensitivity Pneumonitis (HP), is a respiratory ailment caused by a delayed allergic reaction that primarily affects the lung parenchyma, including the alveoli, terminal bronchioli, and alveolar interstitium [1]. Those at the highest risk of developing this condition are workers in various settings exposed to organic dust, particularly farmers and breeders. There are multiple substances linked to EAA, such as avian dust, mould, paint, catalyst, sugar cane dust, hay dust, mushrooms, rat or gerbil urine, tobacco, water used in heating and cooling systems, cork dust, plastic residue, epoxy resin, enzyme detergents, and wheat mould or dust.

The severity of symptoms, clinical presentation, and prognosis of the syndrome can vary significantly based on factors like the specific causative agent, the duration of exposure, individual host variables, and the properties of the antigen involved. It is crucial to identify and address these factors to effectively manage and prevent the development of EAA. Further research and awareness among professionals in high-risk occupations are essential for better understanding and mitigating the impact of this respiratory condition [2]. When inhaled allergens trigger lymphocytic inflammation in the peripheral airways and adjacent interstitial tissue, they often cause acute exacerbations of the condition. Monocytes rapidly gather and develop into foamy macrophages, leading to the formation of granulomas throughout the lung. Ultimately, this process results in interstitial fibrosis, characterized by a radiographic appearance similar to a honeycomb [3]. The substances responsible for triggering the condition can originate from various sources such as bacteria, fungi, animal proteins, or chemically reactive materials. Occupational exposures have reduced due to better identification and management of these issues. However, several challenges persist:

a) Significant exposures are currently occurring within households and are associated with factors such as elevated indoor mold levels, contaminated humidifiers, and the presence of pet birds.
b) The contribution of macrophages and CD8+ cytotoxic cells to immunopathogenesis remains uncertain. There is a likelihood that similar immunologic processes are shared with other interstitial lung disorders, including idiopathic pulmonary fibrosis.

c) The disease often spreads in environments containing airborne gram-negative endotoxins. Brief exposure can result in fever and cough, while prolonged exposure may lead to chronic bronchitis and emphysema. Workers exposed to various contaminants are at risk of developing chronic obstructive pulmonary disease (COPD), a neutrophilic condition, or both HP and COPD [4,5].

1.1 Pathogenesis

When inhaled, antigens with a diameter below 5 µm can penetrate the lung parenchyma, travel through the lymphatic system, and settle at the respiratory bronchioles level. HP is primarily confined to distal airways, with infiltration of inflammatory cells in alveoli and interstitial areas, elevated serum precipitating antibodies against the responsible antigens, and normal IgE and eosinophil levels as key clinical features. The alveolar mucosa mounts a hypersensitivity reaction, either type-3 (immune-complex-mediated) or type-4 (T-lymphocytes-mediated), which contributes to the development of HP [6].

The characteristic pathology of this condition involves interstitial mononuclear cell infiltration centered around the bronchioles. Tiny, poorly formed non-necrotizing epithelioid cell granulomas, along with widespread cellular pneumonitis and varying degrees of pulmonary fibrosis, are observed. In subacute hypersensitivity pneumonitis, granulomas, which are smaller than 150 µm in diameter compared to those in sarcoidosis, are frequently present in the bronchiolar wall and alveolar ducts. The predominant immune response involved in the disease's pathophysiology is a type-4 hypersensitive response mediated by T cells [7]. In acute HP, a type-3 immune response seems to play a significant role in pulmonary parenchymal inflammation, as indicated by high levels of antigen-specific precipitating IgG in the serum and an increase in lung neutrophils. Subacute and chronic HP, on the other hand, are characterized by a T-cell-mediated immunological response, with enhanced T-cell migration and the development of distinctive T-lymphocytic alveolitis [8].

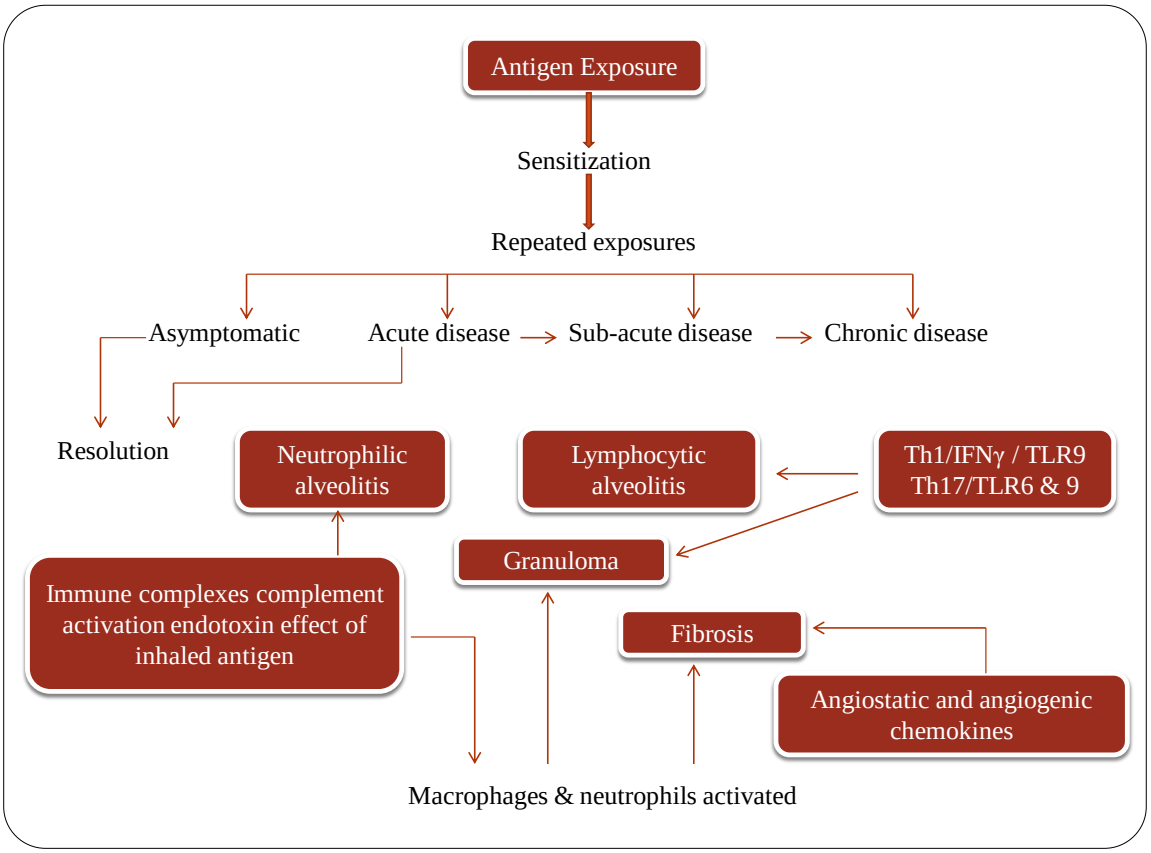


Fig. 1. Pathogenesis of hypersensitivity pneumonitis

1.2 Clinical Observation

Hypersensitivity Pneumonitis (HP) is clinically classified into acute, subacute, and chronic forms, depending on the frequency and intensity of exposure to the triggering antigens. The acute type usually occurs after brief and intermittent antigen exposure for hours or days. Initial symptoms in patients include fever, coughing, dyspnea, asthenia, and malaise, which can persist for up to a week after the causal substance exposure has ceased. Exposure to the allergenic environment or workplace might cause exacerbations when the individual returns to work. However, these exacerbations often cease once the individual has spent enough time away from the allergic environment or workplace. This time away allows the inflammatory response to subside, leading to symptom improvement [9]. Blood tests may reveal a slight increase in eosinophils, along with normal IgE levels. Subacute HP is a complex phenotype, challenging to diagnose, and arises from prolonged exposure to the triggering substance compared to the acute form. Clinically, the onset is subtle, characterized by a productive cough, dyspnea, and asthenia. Chronic HP develops due to continuous exposure to the pathogen, leading to ongoing inflammation, eventually resulting in permanent pulmonary fibrosis. The chronic variant presents with repeated low-grade fevers, wheezing episodes, and progressive dyspnea. Chest X-rays reveal visible diffuse interstitial fibrosis [10]. In the chronic form, exposure to the offending allergen for several months can lead to symptoms like dyspnea, cough, occasional mucopurulent sputum, anorexia, and weight loss. Despite avoiding the cause and administering steroids, the chronic variant may result in an irreversible condition that cannot be cured [5].

1.3 Remedy

The primary approach to managing HP is by avoiding the causative antigen. However, complete elimination is not always feasible due to difficulties in identifying the agent or potential significant lifestyle or job changes. In cases where allergen avoidance is impractical or does not provide full relief from symptoms, corticosteroid medication is recommended. Corticosteroids can be beneficial in treating subacute and chronic HP, as well as alleviating acute symptoms. However, it's important to note that these medications do not appear to influence the long-term progression of the disease. As such, alternative treatments and research efforts are needed to address the underlying mechanisms and find more effective strategies for managing HP in the long run [11]. A reasonable treatment plan involves administering oral prednisone at doses of 40 to 60 mg, or equivalent doses of other corticosteroids, for a few days to two weeks in acute HP, and for 4 to 8 weeks in subacute/chronic forms. Afterward, a gradual tapering to a maintenance dose of approximately 10 mg per day is recommended, with the option to discontinue corticosteroid treatment if the patient shows a particularly positive clinical response. However, it is important to be mindful that steroid medication might also dampen the immune system's regulatory immunological response. Supportive therapies include oxygen therapy, bronchodilators to widen the airways, and opioids to alleviate shortness of breath or chronic cough resistant to other treatments, especially if blood oxygen saturation is consistently below 90%. In some cases where the chronic form does not respond to corticosteroid treatment and progresses to progressive pulmonary fibrosis, lung transplantation should be considered as a potential intervention [5]. The main focus must be on reducing the oxidative stress and relaxing the airway muscles immediately. When avoiding the exposure to a known antigen becomes difficult, it becomes better to incorporate something as a preventive measure. The previous study conducted on seabuckthorn berries suggests that it has a potential as an antioxidant and anti-inflammatory agent. Furthermore, the epigallocatechin gallate has been proven as a healthy component to maintain the lung health within a limited dose intake. The following study focuses on replacing the traditional therapy with a combination therapy of Seabuckthorn berries extract, Epigallocatechin gallate and helping adjuvants such as bronchodilator, antiallergic agent and anti-fibrotic agent to minimize the extent of disease and lowering the risk at a faster pace.

1.4 Sea Buckthorn Berries

Sea Buckthorn (*Hippophae rhamnoides L*.; family Elaeagnaceae) is a deciduous plant thriving in temperate regions, including the Indian deserts of Ladhak, Lahaul, and Spiti. The Himachal Pradesh plant contains alkaloids, flavonoids, terpenoids, carbohydrates, phenolic compounds, saponins, tannins, glycosides, coumarin, and steroids, indicating its potential beneficial effects like antioxidant, cytoprotective, wound-healing,

immunomodulatory, and cardioprotective properties. Notably, the DPPH radical scavenging strategy showcased its antioxidant potential [12].

1.5 Epigallocatechin Gallate

EGCG, a complex compound, consists of a gallocatechol group and a gallate ester linked to a flavanol core (flavan-3-ols). These two gallocatechol rings grant EGCG potent antioxidant and chelating properties, contributing to its strong beneficial effects [13]. EGCG is a powerful compound with antioxidant, anti-inflammatory, antibacterial, and antiviral properties. Additionally, it can modify lipid metabolism by influencing specific pathways [14-16]. Inflammatory cytokines like Interleukin 1 (IL1β) and tumor necrosis factor-alpha (TNF-α) can induce inflammation in the body. However, EGCG possesses robust antioxidant properties and the capacity to mitigate this inflammatory response [17-19]. EGCG is a popular natural substance being extensively studied for its potential in preventing and treating various ailments due to its safety profile with no known side effects. However, its limited bioavailability necessitates higher doses to achieve therapeutic levels of this polyphenol molecule. The use of EGCG in treating both acute and chronic respiratory conditions is on the rise due to its wide array of anti-inflammatory, antioxidant, and anti-fibrotic properties. In a study, pretreatment with EGCG was found to decrease ICAM-1 expression and the levels of neutrophils and eosinophils in the bronchoalveolar lavage fluid (BALF). Moreover, EGCG reduced TNFα-induced NADPH oxidase activation, ROS production, MAPK phosphorylation, STAT3 phosphorylation, and activation transcription factor phosphorylation. These findings further support the potential therapeutic applications of EGCG in respiratory conditions [20]. A study demonstrated that administering EGCG one hour before intratracheal (i.t.) lipopolysaccharide (LPS) in mice with pulmonary inflammation resulted in reduced lung damage. Additionally, EGCG lowered neutrophil and macrophage counts in the lung, lung edema, myeloperoxidase (MPO) and protein kinase C activity. Furthermore, EGCG also decreased levels of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6. These findings indicate the potential of EGCG in mitigating inflammation and its detrimental effects in the lungs [21]. EGCG's therapeutic influence can also affect the efficacy of certain medications due to interactions. In patients with pulmonary fibrosis or interstitial lung disorders, EGCG reduced the bioavailability of the anti-fibrotic drug nintedanib [21,22].

2. MATERIALS AND METHODS

The research aims to investigate the immunomodulatory effects of Epigallocatechin gallate and Sea buckthorn berries extract on rats induced with Hypersensitivity Pneumonitis.

2.1 Animal Strain

The study utilized female Sprague Dawley rats, kept at a room temperature of 22 ± 3°C, relative humidity of 55 ± 5%, with a 12-hour light and 12-hour dark cycle. Throughout the study, the rats were provided ad libitum access to pelleted feed from Nutrivet Pvt. Ltd.

2.2 Induction of Disease

Toluene di-isocyanate (TDI) induced hypersensitivity pneumonitis (HP) is a condition characterized by inflammation of the lung tissue in rats due to exposure to TDI, a chemical compound commonly found in industrial settings. This condition represents an allergic response triggered by repeated inhalation of TDI fumes.

Upon exposure, TDI molecules can activate the immune system in susceptible rats, leading to an exaggerated immune response. This immune reaction involves the release of inflammatory molecules, resulting in damage to the lung tissue and the initiation of hypersensitivity pneumonitis. The affected rats may exhibit symptoms such as coughing, wheezing, labored breathing, and reduced physical activity.

Research suggests that the severity of TDI-induced hypersensitivity pneumonitis in rats can be influenced by factors such as the duration and concentration of TDI exposure, as well as individual variations in immune responses. Understanding the mechanisms underlying this condition is crucial for developing strategies to mitigate its effects and improve occupational safety standards.

In conclusion, Toluene di-isocyanate induced hypersensitivity pneumonitis in rats exemplifies an immune-mediated lung reaction resulting from repeated exposure to TDI fumes. Further research into its pathogenesis and potential therapeutic interventions could aid in safeguarding the health of industrial workers and enhancing workplace regulations.

The disease was induced in the rats through nostrils at the dose of 10µL of 0.3% TDI for 15 days.

2.3 Experimental Investigation

The Sea Buckthorn berries extract and EGCG were administered following the standard protocol, and the animals were closely monitored for signs, symptoms, and weekly body weight changes. The disease induction was performed using Toluene Diisocyanate through the nostrils. The study compared the treatment outcomes with the standard Prednisone treatment, to the treatment involving Sea Buckthorn extract, EGCG, and additional supportive agents like bronchodilators and antiallergic agents. The groups were divided as the preventive (before inducing the disease) and curative (after development of the disease) doses. The dose was fixed as per the OECD Guideline 420 (Seabuckthorn berries extract= 500 mg/kg and EGCG as per the human dose = 5 mg/kg).

1. **Erythrocyte Sedimentation Rate:** The collected blood sample was placed in a tall, thin test tube, and the rate at which red blood cells settled or sank to the bottom of the tube was measured. The measurement was recorded in mm/hr.
2. **Neutrophilia:** Neutrophilia refers to an elevated neutrophil count in the blood, exceeding the normal reference range for absolute neutrophil count. It can be observed in various conditions, including infections, inflammations, and neoplastic processes.
3. **Haematological Tests:** Hematological tests, also known as blood tests, are a group of laboratory analyses that provide valuable insights into the health and functioning of the blood and its components. These tests play a crucial role in diagnosing, monitoring, and managing a wide range of medical conditions, including anemia, infections, bleeding disorders and blood cancers. Haematological tests involve the examination of different components of the blood, such as red blood cells, white blood cells, platelets and plasma, to assess their numbers, structure and function. One of the most common haematological tests is the Complete Blood Count (CBC). It measures the number of red blood cells (RBCs), white blood cells (WBCs) and platelets in a given volume of blood. The CBC also provides information about the concentration of haemoglobin, a protein in red blood cells responsible for carrying

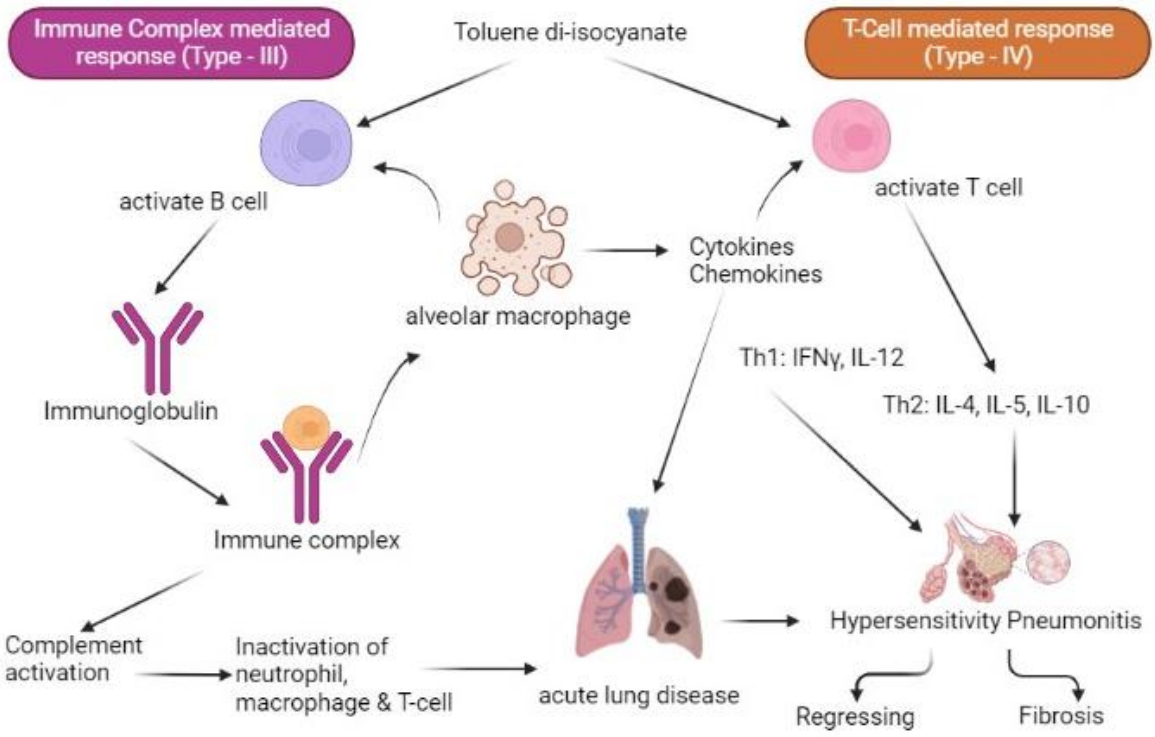


Fig. 2. Toluene di-isocyanate induced hypersensitivity pneumonitis

oxygen, and hematocrit, the proportion of blood that is composed of RBCs. Abnormalities in these parameters can indicate various conditions, such as anemia (low RBC count), infection (increased WBC count), or dehydration (elevated hematocrit). Another essential haematological test is the blood smear examination. In this test, a thin layer of blood is spread on a microscope slide and stained with special dyes to visualize the different blood cells. This allows for the identification of abnormalities in the shape, size and number of blood cells. Blood smear examination is particularly useful in diagnosing conditions like leukemia, where abnormal WBCs can be observed. Hemoglobin electrophoresis is used to diagnose various types of hemoglobinopathies, such as sickle cell disease and thalassemia. This test separates and identifies types of haemoglobin in the blood, allowing for the detection of abnormal variants.

Hematological tests are instrumental in diagnosing and monitoring blood cancers, such as leukemia, lymphoma and multiple myeloma. A bone marrow aspiration and biopsy may be performed to examine the bone marrow, where blood cells are produced. This procedure aids in diagnosing the presence of cancerous cells, evaluating their characteristics and determining the disease's stage and progression. The erythrocyte sedimentation rate (ESR) is a non-specific test used to detect inflammation in the body. During inflammation, certain proteins cause red blood cells to clump together and settle faster than normal. The rate at which the red blood cells settle is measured and reported as the ESR. Furthermore, flow cytometry is a sophisticated technique used to analyze the characteristics of individual cells within a blood sample. This method is commonly used in diagnosing and classifying various blood disorders, especially leukemia and lymphoma. In conclusion, haematological tests are invaluable tools in modern medicine, providing essential information about the health and functioning of the blood and its components. These tests aid in the diagnosis, monitoring, and management of a wide range of medical conditions, from anemia and infections to bleeding disorders and blood cancers.

The blood samples were collected in sterile tubes coated with anticoagulant and the complete blood count was done using the automated cell analyzer.

2.4 ELISA

2.4.1 TNF-α

100µl/well of standards and samples were added to the plates. The plates were then sealed and incubated for 2 hours at a temperature between 18-25°C. After incubation, the plate was aspirated and washed four times with Wash Buffer. Antibody solution was added to each well and incubated for an additional hour at the same temperature mentioned before. The plate was rewashed four times with wash buffer. Next, a diluted Streptavidin-HRP solution was added to each well, and the plate was sealed and incubated for 30 minutes. Following this, TMB Substrate solution was added, and the plate was incubated in the dark for another 30 minutes. Positive wells exhibited a bluish color. The reaction was stopped by adding the stop solution to each well, turning the color from blue to yellow. Finally, the absorbance was recorded at 450 nm within the next 30 minutes.

2.4.2 IL-6

Diluted biotin-conjugated detection antibody was added to all the wells, followed by the addition of standards and samples. The sealed plates were then incubated at 37°C for 2 hours. After incubation, the plates were washed four times with wash buffer. Next, a dilute Streptavidin-HRP solution was added to each well and incubated for an additional 30 minutes at the same temperature as mentioned before. The plates were washed four times with the wash buffer once again. Then, TMB Substrate solution was added, and the plates were incubated as done previously for TNF-α determination. Finally, the absorbance was read at 450 nm within 30 minutes of stopping the reaction.

3. RESULTS

3.1 Erythrocyte Sedimentation Rate

The study's ESR results revealed that TDI significantly increased fibrinogen levels, which were subsequently reduced with treatment. Additionally, TDI was found to induce inflammation in tissues, indicated by notably elevated ESR levels.

3.2 Neutrophilia

The diseased animals exhibited an elevated neutrophil count, resulting in an increased Absolute Neutrophil Count (ANC). However, after treatment with the test drugs, ANC showed a tendency towards normalization.

3.3 Haematological Tests

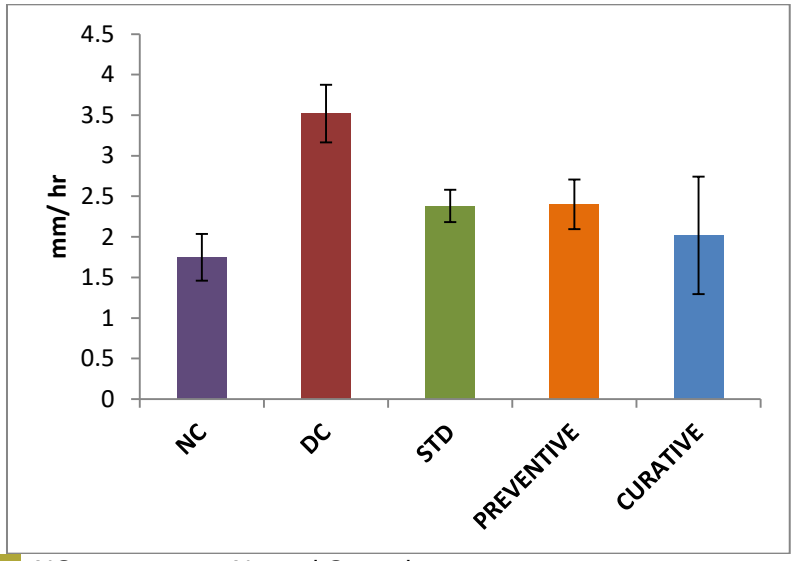
The comprehensive blood analysis, performed using an automated cell analyzer, included parameters such as WBC, LYM, MID, GRAN, RBC, HGB, HCT, MCV, MCH, MCHC, RDWCV, RDWSD, PLT, MPV, PDW, PCT, and PLCR. The results indicated significant differences between the diseased group and the drug-treated group of animals. It was observed that the higher doses of both preventive and curative test drugs were more effective compared to the lower doses in both groups.

3.4 ELISA

In the diseased groups of rats, IL-6 and TNF-α levels exhibited significant increases, indicating a strong association between their release and the extent of lymphocytosis in the lungs of rats with hypersensitivity. The upregulation of IL-6 is linked to the development of inflammatory responses, particularly characterized by type-4 hypersensitivity in the lungs of the animals. Consequently, both IL-6 and TNF-α release showed elevated levels in rats with both acute and chronic forms of hypersensitivity.

4. DISCUSSION

Corticosteroids, while valuable in managing inflammation and immune-related conditions, can also lead to a range of potential side effects. These effects can vary in severity and duration depending on factors like dosage, duration of use, and individual susceptibility. Short-term side effects might include increased appetite, weight gain, fluid retention, and mood swings. Sleep disturbances, elevated blood pressure, and elevated blood sugar levels can also occur. Long-term or high-dose use of corticosteroids may contribute to more serious complications, including osteoporosis, muscle weakness, thinning of the skin, and increased vulnerability to infections. Adrenal suppression is a significant concern. Prolonged use can hinder the adrenal glands' ability to produce cortisol naturally, necessitating a gradual tapering process to prevent adrenal insufficiency upon discontinuation. This treatment is also limited for pregnant or breastfeeding women, someone with chickenpox or measles, patient of liver cirrhosis, cardiovascular disease, epilepsy, diabetes, stomach ulcer, osteoporosis, etc.



NC	Normal Control
DC	Disease Control
STD	Standard (Corticosteroid)
PREVENTIVE	SeaBuck thorn extract(500 mg/ kg) + EGCG (5 mg/kg)
CURATIVE	SeaBuck thorn extract (500 mg/ kg) + EGCG (5 mg/kg)

Fig. 3. Erythrocyte Sedimentation Rate

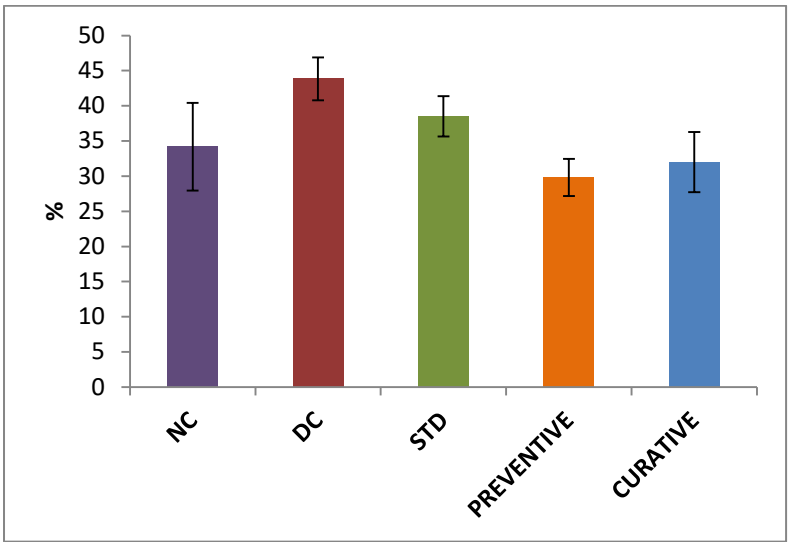


Fig. 4. Neutrophil count

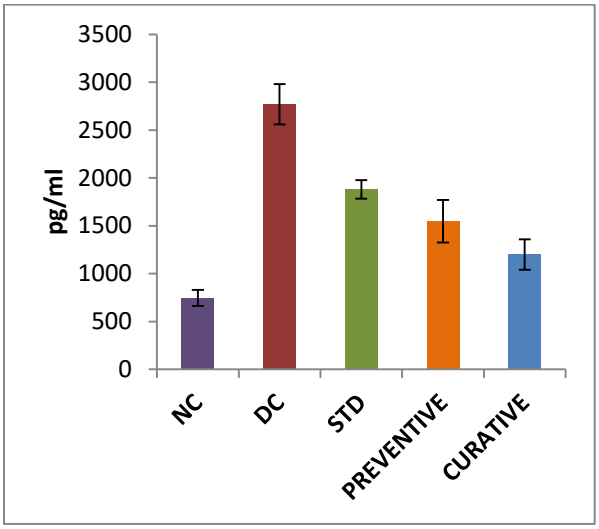


Fig. 5. IL-6

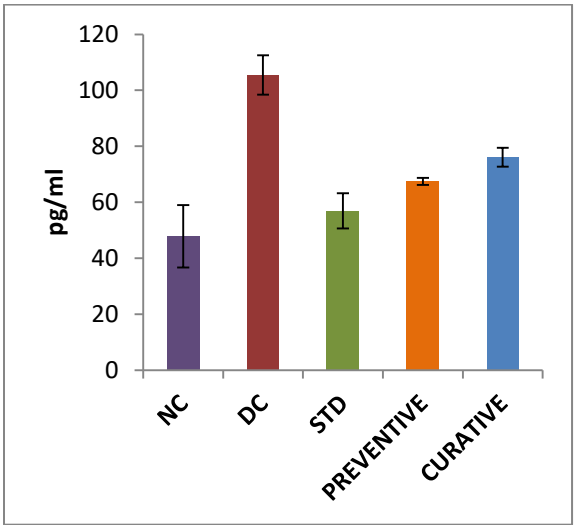


Fig. 6. TNF-α

The research specified in the paper can serve as an alternate treatment as well as prophylaxis. Since, there is only a specific treatment, i.e. corticosteroid, is available for the disease. It is high time to opt for the alternate sources as it becomes difficult to identify and limit the exposure to the causative agent or antigens. For which the prophylactic dose is the best alternative for a healthy and prolonged lifestyle. Also, the study shows better effectiveness with helping agents like bronchodilators and antifibrotic agents.

5. CONCLUSION

Given the complexity of hypersensitivity pneumonitis (HP), there is a crucial need for precise and meticulous diagnostic criteria and validation. The condition shares similarities with other interstitial lung diseases (ILDs), and its underlying mechanisms remain unclear. Chronic HP has shown limited response to anti-fibrotic medications, while some individuals cannot receive conventional corticosteroid treatment, emphasizing the necessity for improved HP therapies.

The study's findings highlight the potential benefits of EGCG and sea buckthorn in treating various disorders. Sea Buckthorn extract exhibits anti-inflammatory, antioxidant, anti-fibrotic, and anti-remodeling properties, making it a potential treatment or preventive measure for acute and

chronic respiratory conditions. EGCG can be used alongside the extract as a supplemental agent, as their therapeutic effects do not interfere with each other.

The research confirms the effectiveness of the test medications in modern HP treatment. However, a significant challenge lies in monitoring the interaction between the test drugs and supportive medications like bronchodilators and anti-allergic agents, considering each patient's genome, as certain genetic polymorphisms may impact efficacy and side effects in vulnerable individuals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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