Saccharomyces boulardii Ameliorate the Inflammation in Ovalbumin-induced Atopic Dermatitis in Mice Model

M. Phil Thesis

by

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AUTHOR'S DECLARATION

I hereby declare that the thesis titled **"*Saccharomyces boulardii* Ameliorate the Inflammation in Ovalbumin-induced Atopic Dermatitis in Mice Model"** submitted to the Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad for the award of degree of Master of Philosophy in Pharmacy (Pharmacology) is the result of research work carried out by me under the supervision of **Dr. Muhammad Khalid Tipu** during the period 2022-2024. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship. I am aware of the terms copyright and plagiarism. I shall be responsible for any copyright violation found in this work.

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ALLAH Almighty who taught me with pen and taught things that I knew not. My humble gratitude to my beloved parents and family who supported me in every walk of life and my best friend who believed in me and helped me throughout my research.

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Acknowledgements

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Parveen Akhtar Buttar

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List of Abbreviations

Abbreviations	Description
AMPs	Antimicrobial Peptides
AD	Atopic Dermatitis
Bregs	Regulatory B cells
CAT	Catalase
C. difficile	*Clostridium difficile*
Concurrent-PBT	Concurrent treatment with probiotic
DC	Disease Control
DCs	Dendritic Cells
EH	Eczema Herpeticum
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
EO	Eosinophils
E coli	*Escherichia coli*
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FAO	The United Nations Food and Agriculture Organization
GSH	Glutathione
GST	Glutathione-S-Transferase
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HDPs	Human Defense Peptides
H Pylori	*Helicobacter pylori*
IBD	Inflammatory Bowel Disease
IDECs	Inflammatory dendritic epidermal cells
ILCs	Innate lymphoid cells
ILCregs	ILC regulatory cells
IFN- γ	Interferon gamma
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8

IL-12	Interleukin-12
IgE	Immunoglobulin E
IBS	Irritable Bowel Syndrome
JAKs	Janus kinases
LCs	Langerhans Cells
LYM	Lymphocytes
MDA	Malonaldehyde
MRSA	Methicillin-resistant *Staphylococcus aureus*
MAP kinase	Mitogen-activated protein
MC	Molluscum Contagiosum
MO	Monocytes
NEC	Necrotizing enterocolitis
NE	Neutrophils
NF-κB	Nuclear factor kappa B
NO	Nitric Oxide
PDE4	Phosphodiesterase 4
PPAR-c	Peroxisome proliferator-activated receptor gamma
PLT count	Platelets count
PC	Positive Control
Post PBT	Post-treatment with probiotic
Pre PBT	Pre-treatment with probiotic
PARC	Pulmonary and Activation- Regulated Chemokine
SD	Seborrheic Dermatitis
S aureus	*Staphylococcus aureus*
TSLP	Thymic Stromal Lymphopoietin
TARC	Thymus and Activation- Regulated Chemokine
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor Alpha
WBCs	White Blood Cells

Abstract

The current study aimed to investigate the immunomodulatory and anti-inflammatory role of *Saccharomyces boulardii* in Ovalbumin-induced Atopic Dermatitis (AD) in murine model. AD is a life-long disease so there is need for a therapy which uplifts the standard of living and reduce the side effects. The current treatment for AD has many limitations including compromised quality of life, low compliance, lack of cost effectiveness and reported adverse effects. The probiotic *S. boulardii* under study managed to reduce inflammation caused by allergen and AD progression by immune regulation and preventing barrier disfunction. The disease induced by ovalbumin was reversed by *S. boulardii* showing promising results through behavioral parameters, improvements in clinical scores and spleen index. Serum biochemistry, hematological parameters and serum IgE investigation by ELISA further showed reduction in inflammation in groups treated with probiotic. Histological examination revealed reduction in tissue infiltration, decreased collagen accumulation and infiltration of immune cells in treated groups as compared to the diseased group. The level of serum bilirubin and creatinine was measured along with the histological changes in liver and kidney to observe the effect of Dexamethasone (5 mg/kg) in mice model. Furthermore, TNF-α/NF-κB signaling pathway activated in AD was regulated in treatment groups and *S. boulardii* also maintained oxidant/antioxidant balance. This study concludes that *S. boulardii* could be an effective novel treatment for AD that could reduce inflammation with increased safety and improved quality of life.

CHAPTER 1

INTRDUCTION

1. INTRODUCTION

1.1. Background

Atopic dermatitis is defined as inflammatory skin disease characterized by interference with the normal functioning of skin affecting protective barrier integrity and function and moisture holding capability of the skin. It can sometimes be referred to as eczema and is genetically transmitted and can affect any age group but often affected by this condition are newborn and children. It lasts for years and is often a recurring condition.

1.1.1. World Allergy Organization

An inflammatory skin condition called atopic dermatitis (AD) is characterized by intense itching and recurring skin lesions. Epidermal barrier abnormalities, IgE production in response to many allergens in the environment, and immunological dysfunction caused by an unbalanced Th2 response are all characteristics of AD. Children are most affected by AD, a long-term reactive skin condition that causes blisters, rashes, peeling, itchiness and discomfort. Moreover, the condition frequency coexists with asthma and allergic hay fever because the patients are hypersensitive to allergens causing rhinitis and food allergies (Shershakova *et al.*, 2015).

Within the afflicted skin, AD displays biphasic response. Early stages are characterized by itching, red patches or elevated regions with fluid from scratching. Microscopic examination indicates a noticeable enlargement between skin cells as well as a rush of immune cells, primarily lymphocytes and DCs. Not only this, eosinophils, basophils, and mast cells can be seen around vessels, along with some lymphocytes and monocytes macrophages. Chronic lesions show thicker skin with probable elevated regions as a result of persistent scratching; these lesions are sometimes lichenified and called prurigo nodules or papules (Wang *et al.*, 2007).

1.1.2. Prevalence

Investigations into AD over many years have mostly concentrated on how common this disease is among people of all the age groups. Approximately one quarter of children who have AD go on to develop the disorder throughout adulthood, either via ongoing symptoms or recurrence following symptom-free intervals. On the other hand, roughly 75% of AD cases in children spontaneously disappear before puberty. The patterns of AD

occurrence show variability between individuals of different ages and geographical locations. In advanced German research, it was found that the incidence of AD was lower in adults (3.6%) than in children (10.35%). Understanding the epidemiology of AD and its major risk factors has relied heavily on data on international frequency collected over a 12-year time.

Prevalence studies show the role of AD on an individual's morbidity, longevity, and standard of life. There are many factors such as age, gender, socioeconomic variables, region, and race all have a different influence on the intensity of the disease. The prevalence of AD in youngsters appears to be steady, according to research from Denmark, Sweden, and the UK, albeit social and demographic factors can create variations. For example, as compared to girls, boys under the age of one had a higher risk as compared to girls. The study's goal is to compile data on AD among adults, teens, and kids throughout the world. Between 2009 and 2019, there was a considerable worldwide variation in the appearance of AD. To quantify prevalence, several techniques have been used, including the ISSAC survey, Hanifin and Raika's criteria, doctor diagnosis, and parental interviews. However, there are just a few incidence statistics available, mostly from China, Germany, Denmark etc. which is the limitation. Numerous risk factors for AD are consistent with its main pathogenic components, including weakened immunological control, genetic abnormalities, and reduced function of the skin barrier. Skin pH and moisture are disturbed by alterations such as FLG deficiency. AD occurrence is influenced by environmental variables including air pollution and climate. There is also evidence of immunological dysregulation linked to both obesity and food sensitivities.

Studies are conducted geographically in many advanced countries America, Africa, Europe, Asia, and Oceania. Children from Sweden reported the largest occurrence and children from Tunisia has the lowest with frequency ranging greatly. From 2009 to 2014, Norway experienced a rise in the prevalence of AD in children under six, but children's AD incidence was steady in Denmark and Sweden. In various age categories in Germany, the frequency was 1.7% and the recurrence rate was 2.4% the widespread distribution and incidence of AD have shed important light on its worldwide effect and different risk factors. The interplay of the illness with environmental, genetic, and immunological

components highlights how complicated it is and every aspect has to be studied. Knowing these epidemiological factors can help manage and prevent AD, improving the standard of life for people who are affected (Hadi *et al.*, 2021).

Studies sows that by 2022, 223 million people suffered from atopic dermatitis, with 43 million of them being children, underscoring the disease's relatively higher occurrence in this age range. Atopic dermatitis can not only produce itching, irritation and peeling effect on skin but also negatively impact a child's growth and employsment prospects and result in social stigma. Additionally, it commonly acts as a risk factor for disease including breathing problems, hay fever, and food related allergies. Although AD does not cause this but occur concurrently in patients with such hypersensitivity. Atopic dermatitis frequently coexists with physiological problems such as social exclusion, depressive disorders and anxiety in adults. Financial difficulties and restrictions relating to work might also occur. Numerous studies have been done on the intricate pathophysiology of atopic dermatitis, which involves immunology, genes, the function of the skin barrier, and external variables. In treating severe instances, advancements have been made. Data on frequency, severity, distribution by region, triggers from the environment, requirements for therapy and methods of treatment, however, are still severely lacking. The integration of fundamental understandings of the causes with improved healthcare, innovative therapies, and prevention measures depends on closing this knowledge gap (Arents *et al.*, 2023).

1.2. Pathogenesis

AD is a disease which is caused by many underlying factors. The variations in the symptoms of AD suggest that there must be variations in the pathogenesis of the disease. Many genetic and immune factors are involved in the development of this chronic skin condition along with the environmental factors that will initiate reaction by exposing to the specific allergen. The mutations in the genes such as FLG coding for filaggrin and other protein mutations are responsible for the dysfunctioning of the skin barrier, as result of which easy entry of allergen and microbes occur causing AD development. Moreover, the immune function is also affected in AD. There is Th2 mediated response and the cytokines released show Th2 dominance which include IL-4, IL-5, IL-13 and IL31. Th2 dominance causes the barrier dysfunction, attraction of eosinophils to the site of allergen

entry, IgE production. Also, Th2 cytokines cause the increase in the level of JAK/STAT. JAKs and PDE4 worsen AD indirectly while IgE act directly and cause pruritis and chronic skin inflammation (Vakharia and Silverberg, 2019).

In AD, the skin barrier function is reduced or lost completely dur to many factors such as mutations of proteins such as filaggrin that disrupt the barrier integrity and other proteins such as claudin that cause tight junction abnormalities. This results in enhanced entry of allergens and microbes in the body. Once the pathogens enter, they attract immune cells to the site of entry and cytokines and chemokines produce excessive immune response. Upon pathogen entry, TLRs are activated initially which causes cascade of reactions in the body and leading to inflammation and allergic symptoms in the skin region and AMPs are depleted. As a result of decrease in the levels of AMPs, the allergens enter more easily in the body further worsening the symptoms of AD.

The majority of AD patients showing lack of filaggrin protein also show an increase in the IL-4, IL5, IL13 and IL-22 and IL-31. All these cytokines have shown a link with deficiency of filaggrin and claudin. Studies have also shown that in addition to barrier abnormalities, Th2 dominance is also observed in the AD patients due to increased levels of cytokines observed in the serum. IgE level is also measured and has shown a sharp increase in most of the cases of AD. The most common harmful bacteria observed in almost all the cases of AD is *S. aureus.* These further damages the already compromised skin barrier through the production of serine protease (Leung and Guttman-Yassky, 2014).

Filaggrin is an important protein maintain the structure and normal function of the skin by forming a skin barrier. If there is mutation in the gene coding this protein, this results in pathological conditions such as Atopic Dermatitis and many others. Ichthyosis vulgaris is genetically transmitted skin condition in which there is development of dry peeling skin because there is problem with PR filaggrin as it is not stored properly and hence skin is not hydrated sufficiently. Also, the lamellar bodies that contain substances such as lipids and form the protective layer of skin are not performing appropriately. There is deficiency of filaggrin protein which leads to not only barrier dysfunction but also alter the skin pH because in the process of stratum corneum formation, the filaggrin is metabolized to amino acids and other degradation products which render skin slightly

acidic. This increase in pH due to lack of filaggrin cause allergens and pathogens to enter the body and disturb normal barrier function. Tight junctions are also disturbed. All these changes lead to invasion of allergens, which bind to immune cells on specific receptors. Immune cells such as DCs, mast cells, eosinophils and macrophages are activated and over excitation of immune response takes place. Hydration of skin is lost and itchy, peely skin is produced. Urocanic acid and pyrrolidone carboxylic acid make skin acidic and do not allow the colonization of *S. aureus.* But in the absence of these, the bacteria enters and the serine protease of bacteria causing inflammation and allergy as shown in Figure 1.1. (Irvine *et al.*, 2011).

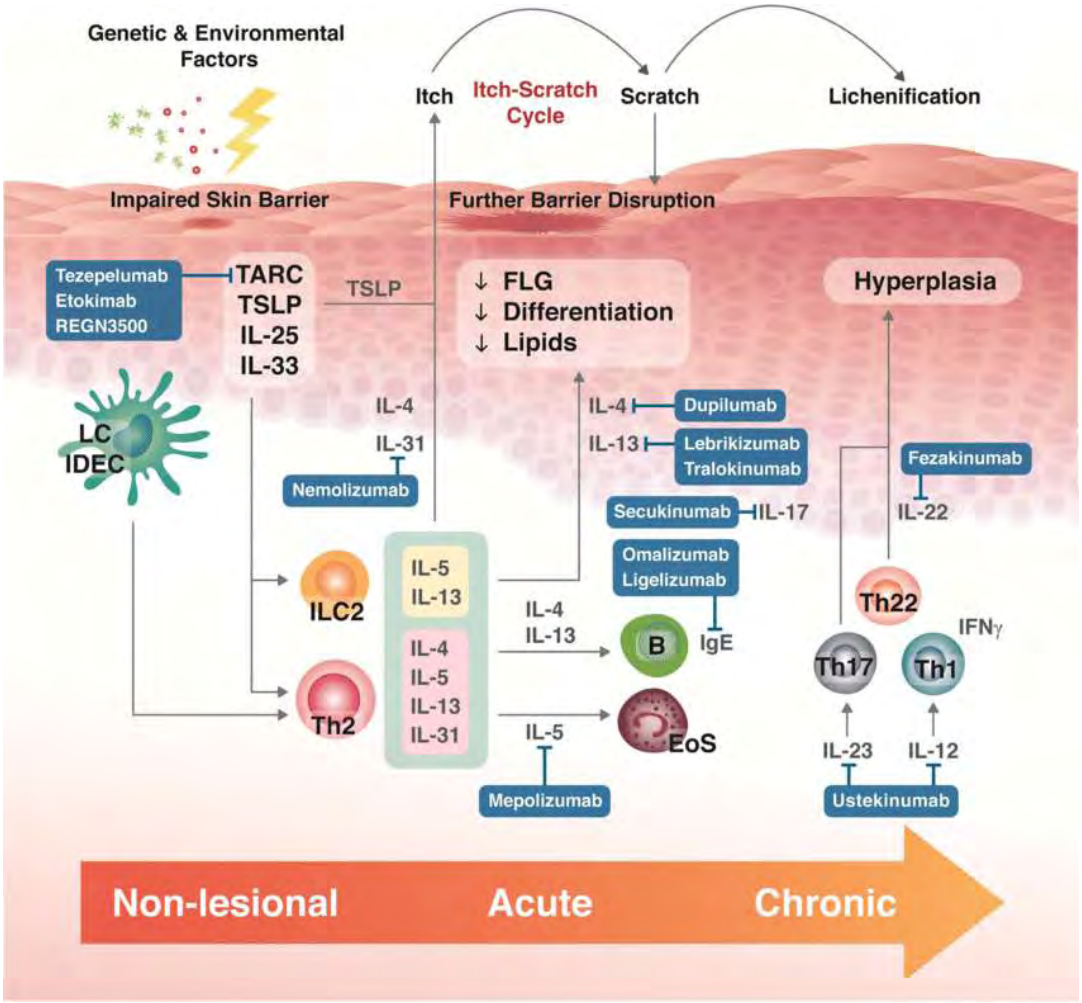

Figure.1.1. Immunological pathogenesis involved in Atopic Dermatitis (Chu, 2021).

1.3. Risk Factors of Atopic Dermatitis

1.3.1. Immune mechanisms

The immune mechanisms of AD show that it occurs in two phases. First there is acute phase in which Th2 mediated response is observed with an abundance of IL4, IL13, TSLP and eosinophil levels. After this the chronic phase begins which is long-lasting and is marked by Th1/Th0 where there is abundance of IL5, IFN-γ, IL-12, IL-5, and GM-CSF.

Innate immunity plays an important role in preventing infections. Some antimicrobial peptides are released in the skin of healthy individuals that inhibit the penetration of harmful bacteria such as *S. aureus,* the main culprit in AD. But once the disease is developed, antimicrobial peptides are replenished on the skin and hence the bacterial colonization of *S. aureus* can be observed everywhere on the skin.

1.3.2. Relation of AD with other allergies

Many other allergies, especially food allergies, have shown a link with AD. Studies have shown that more than half of the patients with AD reported other allergies prior to the development of AD. However, it is suggested that they are not the cause of AD but have shown the potential of developing AD soon. Cutaneous reactions are observed in many allergies, they do not cause AD but might itching and irritation might worsen the underlying AD disease.

The allergies which are observed in many children before development of AD are lactose intolerance, wheat allergy, hypersensitivity from eggs, peanuts, mites, pollen and pets.

1.3.3. Genetic factors

Genetic factors have been found to be linked to occurrence of AD. Family history and presence of disease in identical twins is quite common. FLG gene that codes for the Filaggrin protein is mutated in almost half of the patients 50% suffering from AD. Filaggrin is pivotal in normal functioning of skin as it is involved in the formation of stratum corneum. Not only this, but in the formation of stratum corneum, filaggrin is metabolized to amino acids and other metabolites. These metabolites create an acidic condition on the skin preventing penetration and colonization of harmful microbes. Along with the maintenance of pH, it also provides hydration to the skin. In addition to FLG gene mutation, mutations in SPINK5/LEKT1 genes are also observed in AD.

1.3.4. Impaired barrier function

Healthy intact skin barrier is important functioning of the body and decreasing the risk of allergies. But some factors such as filaggrin mutation led to dysfunctioning of the normal barrier allowing the invasion and colonization of harmful bacteria such as *S. aureus* on the skin. Due to abnormal barrier functioning, the risk of other allergies is also increased especially asthma and allergic rhinitis because the allergens directly come in contact with the epidermal layers and due to impaired barrier, they enter the body and immune cells are activated. The over activation of immune cells produces excessive response and development of diseases.

1.3.5. Environmental factors and microbial exposure

Environmental conditions and exposure to microbes also have an impact on development of allergic conditions and AD. Many factors such as climate including temperature extremes, urbanization leading to poor air quality and availability of unhealthy and processed food, improper hygiene, less breastfeeding time and early weaning time, lack of exercise and bad lifestyle, obesity and tobacco smoking all have led to increased incidence of diseases and AD. Studies have suggested that the exposure of children to the natural environment such as farms and using unprocessed food such as natural fruits, vegetables and unpasteurized milk will lead to healthy immune response and a healthy life free from allergies and diseases.

If proper hygiene is maintained, it leads to healthy body and barrier functioning and less exposure of allergens such as harmful bacteria and fungi are important in the management of AD. this is shown in Figure 1.2. (Nutten, 2015).

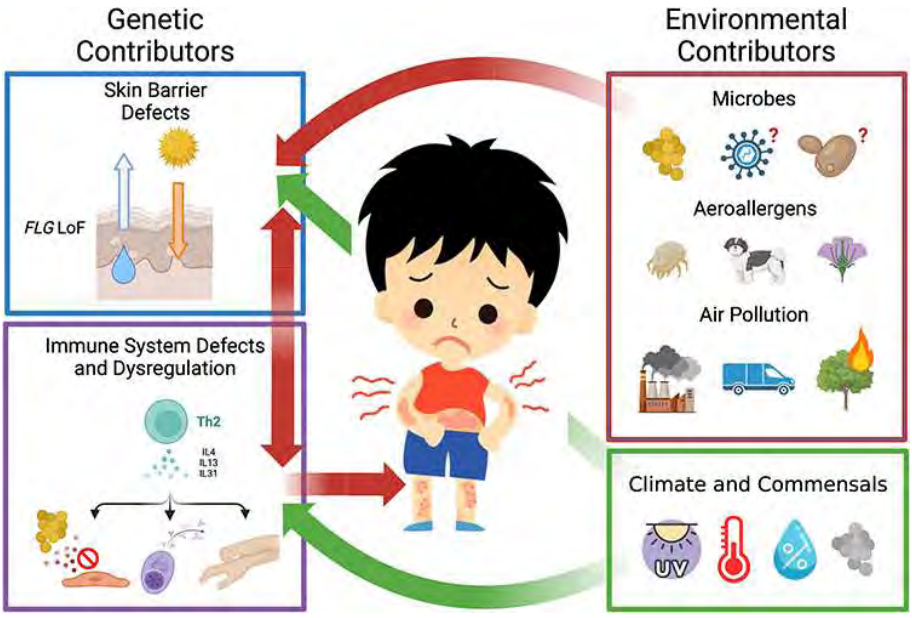

Figure.1.2. Various mechanisms involved in the development of Atopic Dermatitis (Chong *et al.*, 2022).

1.4. Immune Mechanism of AD

AD is a complex medical condition involving various underlying mechanisms. Barrier dysfunction being the most important because this is where allergy begins. In AD, due to dysfunctioning of skin barrier, the allergen comes in contact with the skin causing interaction with the immune cells and activating them effectively. The mutations in the genes are responsible for this impaired barrier. Filaggrin protein plays pivotal role in normal functioning of barrier which is lost by FLG gene mutations. As a result, there is lack of antimicrobial peptides, loss of hydration, imbalance of skin pH and colonization of *S. aureus* along with irritation and itching. In addition to filaggrin, there are other protein performing similar functions which includes involicrin and loricin. All these genes collectively put up a normal barrier function. Mutations in any of the gene causes disruption of the barrier function and IgE mediated immune response. IL-1 axis is disturbed leading to the release of proinflammatory cytokines such as IL-1β and IL-18. One the immune system is activated, there is exaggeration of the immune functioning because inflammatory molecules are attracted to the region. Serine protease is also depleted, which normally was involved in desquamation, wound healing, tissue repair and scar formation (Kezic *et al.*, 2012).

With the dysfunction of the skin barrier, harmful microbes invade and form colonies on the skin. This invasion leads to the activation of immune cells and release of proinflammatory cytokines which include IL-6, IL-18, IL-23, GM-CSF, and TNF-α and hence the appearance of AD symptoms. In early AD, the mechanism involved is the activation of Th17 which release IL-17, this leads to the activation of Th2 and this further leads to release of proinflammatory cytokines mainly IL-4. So, in early AD, Th2 mediated cytokines produce inflammatory symptoms. The variations in the symptoms of AD worldwide suggest that there are variations in the underlying mechanisms as well. In Asia, AD observed mainly is due to Th17 activation which leads to activation of Th22 and symptomatically like that of psoriasis.

Upon invasion with the allergen, keratinocytes release chemokines to attract the immune cells and over excitation of immune system. The immune cells that are attracted and move to the site of inflammation are DCs, T cells, macrophages, eosinophils and mast cells. The chemokines are substances or signals that attract specific immune cells, these include eotaxin which attracts eosinophils and lead to IgE production, MCP 1 and 4, attracting monocytes and macrophages, PARC attracting DCs and T cells and TARC attracting T cells mainly. Chemokines bind to CCR3 receptors and produce response. The level of eotaxin-3 and TARC in serum gives the estimation that how severe the AD has been developed (Chieosilapatham *et al.*, 2021).

The role of TSLP, IL-25, and IL-33 in the development and initiation of atopic dermatitis is studied. TSLP, IL-25, and IL-33 are the cytokines that are generally produced from the keratinocytes and attract the immune cells such as DCs and T cells and cause the activation of T cells towards a Th2 mediated response. TSLP directly activates the Th2 response and IL25 and IL-33 are also responsible for the Th2 dominance. As result Th2 mediated cytokines are released which are IL-4, IL-5, and IL-13. These are proinflammatory cytokines involved in the development of AD lesions, itching, swelling, dryness, and other symptoms of atopic dermatitis. IL-25 and IL-33 are also responsible for itching and irritation development and TSLP plays pivotal role in depletion of antimicrobial peptides that inhibit the colonization and invasion of harmful bacteria on the surface of the skin. IL-31 is another cytokine that by acting on its specific receptors

produce atopy associated symptoms that is genetic predisposition for causing hypersensitivity in individuals (Roan *et al.*, 2019).

When the microbes and allergens invade the body, immune cells such as DCs, cells and macrophages move towards the site of allergen entry and get activated by binding allergen on their surface. When mast cells are activated, they release histamine. Histamine produces its effect either by acting on the sensory neve ending and creating a sensation of itching and irritation or by slowing down or inhibiting the production of proteins that are responsible for the maintenance of the skin surface as well as the barrier formation. As result, surface integrity is further compromised in AD. The role of many interleukins in AD is well-established. IL-4 and IL-13 will attract the eosinophils which are the main culprit in AD and an increase in the level of eosinophils is observed in nearly all the patients. IL-22 is responsible for the growth and development of skin cells, but the major drawback is that they dysregulate the barrier integrity and hence the function. Healthy skin cells are responsible for the formation of antimicrobial peptides that prevent the entry of allergens in the body. These are also called human defense peptides (HDPs) which include cathelicidin LL-37, hBDs, S100A7, and RNase 7. In AD, these antimicrobial peptides are depleted from the surface of the skin and hence the normal defense is no more effective.

In AD, there is mutations in the genes coding for proteins such as filaggrin that maintain barrier integrity, absence of antimicrobial peptides facilitating easy allergen exposure, production of cytokines that dysregulate or overexcites the immune system. All these mechanisms are studied in detail in recent decades because there is lack of AD-specific drug. To overcome this lack of specific drug, therapies can be developed by targeting these specific mechanisms as shown in Figure 1.3. (Chieosilapatham *et al.*, 2021).

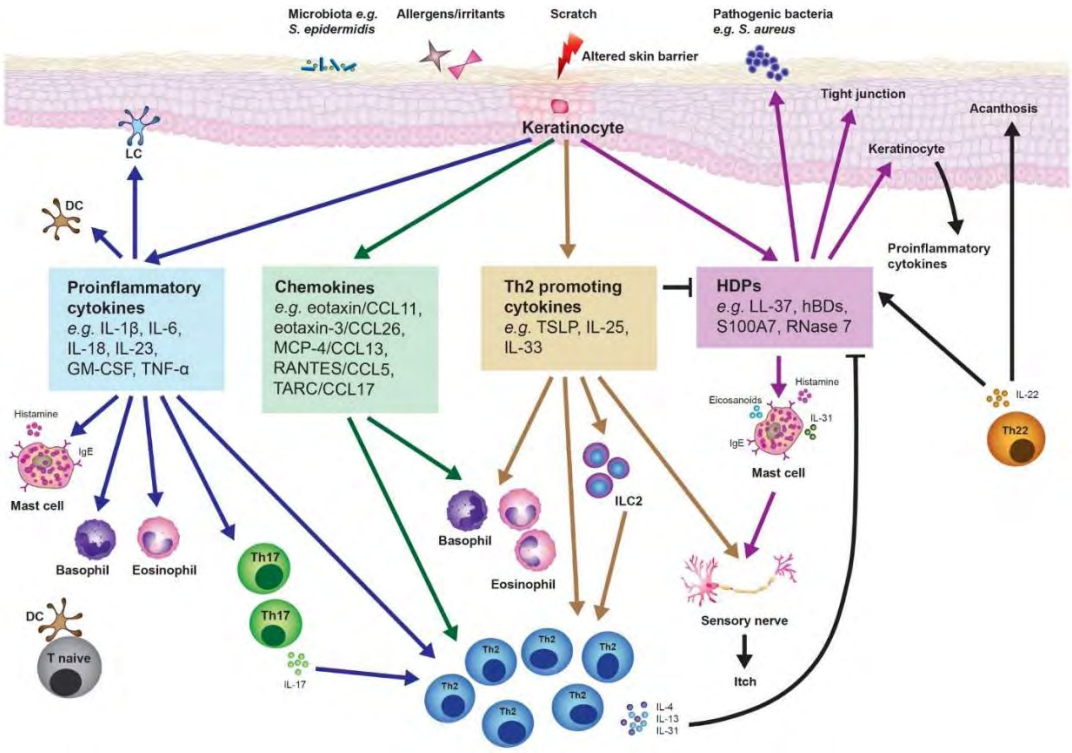

Figure.1.3. The role of inflammatory mediators released from keratinocytes in the development of Atopic Dermatitis (Chieosilapatham *et al.*, 2021).

The mechanism and pathology involved in AD development has been studied over the course of time and studies showed that in AD, there is an imbalance of Th1/Th2, and Treg cells are also involved. The immature DC is converted into mature DC and tolerogenic DC. Then there is the involvement of Th2 and Th1 and Treg cells. Th2 is considered the main culprit in IgE associated AD. The upregulation of Th2 causes a boost in the level of IgE, there is an imbalance of the Th1 and Th2 leading to release of pro inflammatory cytokines and increase in the level of IL4 and IL13. The pathogenic Treg cells are also playing the role to develop symptoms of AD. Many drugs are used in recent years for the treatment of AD. These mainly include topical corticosteroids and Dupilumab which is a monoclonal antibody. Dupilumab blocks the IL4 and IL13 release and hence count er the symptoms of AD.

TSLP is also pivotal in understanding the pathology of atopic dermatitis. It is not only involved in the maturation of DC but also differentiation of T cells into Th2 cells. As a result, cytokines such as IL4, IL5 and IL13 are released as a result there is development

of AD. Moreover, TSLP is also involved in maintaining the integrity of the skin barrier and also in the itching process. TSLP is present on different parts of body such as lungs but its presence on the skin epithelial cells is involved in the pathology of AD (Cianferoni and Spergel, 2014).

ILCs are the immune cells that are also involved in the development and worsening of AD. The subtype type 2 ILC2s is the main culprit in AD. Being activated by IL33 and TSLP, the ILC2s is involved in the Th2 cytokines especially IL5 production further worsening the AD symptoms. When there is barrier dysfunction, ILCs kick in and hence there is development of AD.

TLRs are the receptors involved in the recognition of microorganisms such as bacteria and fungi and then activate the immune response in the body. They are present in different regions such as DCs and skin cells. Their main function is the membrane barrier regulation and upon barrier dysfunction, they are involved in immune response by activating Th2 mediated allergic response.

Inflammasomes are the protein complexes that are also involved in immune regulation. When activated, they cause a release of proinflammatory cytokines playing a crucial role in AD. barrier dysregulation allows the entry of harmful microbes in the body, as result of which immune system is activated and inflammatory mediators are released in the body.

Filaggrin protein which is important in barrier regulation. The mutations in filaggrin leads to dysfunction of skin barriers and activation of innate immune response (Yang *et al.*, 2020).

Development of AD is initiated when an allergen encounters the skin upon which the immune system is activated. AD is mainly associated with Th2 dominated response and release of inflammatory mediators. In these ILCs is significant. Moreover, there is involvement of DCs, basophils and eosinophils. Many cells are involved to put up the development of AD.

DCs are quite popular for their involvement in AD. There are different types of DCs such as LCs which include CD127+, CD1a+, and FcεRI+ and dermal dendritic cells which include CD1c+, CD11c+, and FcεRI+. FcεRI+ role is important as it is involved in the pathogen uptake. Another type of DCs is IDECs which includes CD1a+ and CD206+ but it is only present in cases where inflammation and JaK kinases 1 and 2 signaling is

involved in the development of IDECs. So, inhibitors of JaK kinases hold promise for the treatment of AD. Another type Plasmacytoid DCs is pivotal for inhibiting inflammatory responses, but it vanishes in AD and hence there is severe skin allergy in AD.

ILCs and ILCregs are considered as the potential targets for the treatment of AD. For this purpose, the mechanism by which they contribute to AD is quite important. ILCs generally contribute to Th2 mediated response and release of IL4, IL5 and IL13 and suppression of arginine methyltransferase. ILCs are activated by not only TSLP but also IL25 and IL33. Interaction of ILCs with mast cells produces IL5 dependent response and with that of basophils produce IL4 dependent response. ILCregs regulate the innate immune system by generally stopping the overreaction of the immune system towards incoming allergens.

AD involves both innate and adaptive immunity. T cells are activated in response to an allergen, mainly the Th2-dominatimg effect is produced and IgE is released along with, any other allergy mediators. IgE again binds to FcεRI on skin and further activation of T cells occurs. Moreover, the histamine released also binds to histamine specific receptors and leads to irritation, itching and swelling.

In AD, B cells contribute to adaptive immunity. Upon activation, B cells differentiate into plasma cells and memory B cells. As the name suggests, memory B cells are responsible for keeping a record for future contact with the same allergen and plasma cells produce the antigen specific antibodies. B cells play a role in pathogenesis of AD by releasing IgA, IgG and IgE antibodies and IL4 and IL13 cytokines. Bregs are involved in the immune regulation by preventing over stimulation of the immune system. Rituximab is an antibody whose mechanism of action is removal of B antibodies from the body. This has proved to be an effective drug for management of AD.

The role of T cells by production of IgE, IL4, IL5 and IL13 and imbalance of Th2/Th1, also the role of B cells, role of TSLP, DCs, ILCs, filaggrin mutations, TLRs are important in pathogenesis of AD. some antimicrobials are also produced by skin cells. All these are the potential targets for the development of drugs for managing AD and holds a promising future through research and innovation (Kuo *et al.*, 2013).

1.5. Therapeutic Approaches of AD

AD is a severe skin condition but unfortunately there is no definite cure for this chronic illness. Only symptomatic and palliative treatments are given. Many corticosteroids whether oral or topical preferentially topical are given. Moreover, some immunosuppressants and biologics are the talk of town for management of AD. Mostly, for preventing or managing the symptoms moisturizers are applied on the skin for dryness and itch control. Inhibiting the contact with the allergen is the best cure but once the person has been exposed to the allergen, symptomatic treatment kicks in. for sever cases immunomodulators and biologics are used mostly. There is need for the proper drugs and medications for the treatment of diseases.

1.5.1. First-line therapies

Patient education is the first line therapy provided to individuals. In the management of AD, physicians with different specializations work together to bring a proper treatment plan for the patients. Mostly moisturizers and allergen avoidance are the best for long-term benefits. Topical corticosteroids are currently most common for usage and management of AD, however, the side effects of these topical corticosteroids cannot be avoided. Calcineurin inhibitors such as Tacrolimus and Pimecrolimus are also used for treating AD.

1.5.2. Second-line therapies

In AD, superinfections are quite common because there is mostly colonization of *S. aureus* on the skin. Antibiotics, antiseptics, antivirals and antifungals are used for bacterial, viral and fungal infections but the risk of microbial resistance cannot be avoided, so careful monitoring is important. Antihistamines are also used because histamine, which when released in the body produce severe itching and irritation. PDE4 inhibitors such as crisaborole is also used as a second-line defense.

Probiotics such as *Lactobacillus* can also be used in the treatment of AD.

1.5.3. Third-line therapies

When AD is not managed despite the medications mentioned above, then biologics are used. They are the advanced treatment used in AD and the biologics show varied results in the management of AD. Some of the therapies currently being used in the management of AD are Dupilumab which focuses on reduction of Th2 mediated cytokines release and

targets IL-4 receptor, Lebrikizumab and tralokinumab focus on IL-13, Nemolizumab targets on IL-31 and hence cures itching, Fezakinumab targets IL-22, Ustekinumab targets IL-12 and IL-23, Etokimab targets IL33 and Tezepelumab targets TSLP (Kader *et al.*, 2021).

1.6. Emerging Therapeutic Small Molecules for AD

The prevalence and non-availability specific drugs in AD has gained importance in recent decades. Many drugs are being discovered and clinical trials are under way to solve this biological problem. JAK kinase 1 and 2 involvement has gained scientists interest, and many JAK kinase inhibitors are currently under study for treating AD. These include Baricitinib which is JAK1/2 inhibitor, Abrocitinib and Upadacitinib being JAK1 inhibitors and Gusacitinib being JAK/SYK inhibitor are potential drugs. Adrifort binds to H4 receptor inhibitor and prevents histamine binding, Serlopitant and Tradipitant block neurokinin 1 receptors and Apremilast inhibit PDE4. All these show promising results in the treatment of AD. Recently, phototherapy is also gaining importance in the cure of AD.

1.7. Clinical Presentation

AD manifests itself on various parts of the body especially joint, back of knees, inside armpits, hands, feet, and eyelids etc.

Based on the complexity of symptoms, atopic dermatitis can be divided into 3 phases:

1) Acute Phase: it manifests itself as vesicles filled with fluids and having a crusty appearance leaving a yellow film behind on the top layer of the skin.
2) Subacute Phase: it presents as dry patches, firstly a pinkish red appearance on the skin that is replaced ultimately by a flat-topped lesion that are associated with irritation, slightly raised temperature (calor) and swelling (tumor).
3) Chronic Phase: prolonged irritation and scratching leads to the skin thickening and rough and leathery patch formation and lichenification.

In addition to classification into phases AD can also be classified in order of the pattern of appearance of the disease as:

1) Persistent AD: as the name suggests, symptoms of dermatitis appear continuously at every hospital visit from the beginning of time till 7 years old. It includes 19% of AD population.

2) Intermittent AD: irregular appearance not following persistent nor remission AD patterns and covers 38% disease population.
3) Remission AD: covers 43% of disease patient and manifests as disappearance of symptoms after 2 years of life.

For the proper management of dermatitis and improvement of the quality of life, it is necessary to identify the proper phase as well as the pattern followed by the disease.

1.8. Diagnostic Criteria

For the diagnosis of the disease family history and physical examination are of importance. The criteria for the diagnosis of AD used extensively is Hanifin and Rajka Criteria. This includes pruritis, chronic and chronically relapsing dermatitis and personal or family history as major criteria and many minor criteria. Its presence of it was a turning point for the diagnosis of AD but the presence of so many criteria became its limitation. Using these criteria as a baseline the UK working party developed the diagnostic criteria of AD that consisted of 1 major and 5 minor criteria to be met. With the development of this criteria, the need for lab findings was rendered unnecessary. But the inability of this criteria to be used in infants and neonates was a hinderance in its applicability. So, it overcome this hurdle, American Dermatology consensus conference revised the original Hanifin and Rajka criteria and developed a criteria to be used in all age groups including neonates, pediatrics, adults and geriatrics.

1.8.1. Exclusion of diseases in the diagnosis of AD

There are some skin diseases that may be mistaken as AD. Sometimes they may coexist with AD but sometimes they lead to false diagnosis. For making a diagnosis, all these conditions should be ruled out. They include:

SD is an inflammatory condition that appears as white, off-white or yellowish greasy surface. It affects oily skin parts affecting oily parts of the face and scalp and sleep pattern is not affected by this. AD and SD are difficult to differentiate, often leading to faulty diagnosis. Sometimes these conditions may co-exist, but SD generally lasts within 2 years of life and quality of life is less compromised as compared to AD. Psoriasis is another inflammatory condition that form scales on the skin. This condition is characterized by the formation of a dead skin layer on the skin and mainly affects nail area. The probability of psoriasis should also be ruled out before diagnosing AD which is

comparatively severe condition and affects the quality of life. Contact dermatitis is commonly mistaken as AD. It also appears as a rash and redness on the skin that may be followed by blistering and lesion formation. The basic difference with AD is that it appears only on the site of contact with the allergen. The physicians must observe closely to distinguish between the two.

Scabies is another skin condition caused by *Sarcoptes scabiei.* In this, the mites burrowing can be observed on the skin and its eggs and faeces worsen the condition. By eradicating mites, this condition can be cured but it often leads to false diagnosis. Pityriasis alba is a skin condition in which there is formation of round areas lighter than the normal color of skin and sometimes they are misunderstood as AD lesions. Keratosis pilaris involves the formation of scales on the body where these scales do not hurt but their appearance is often misunderstood as early AD. It does not alter quality of life unlike AD. Ichthyosis vulgaris often also appears as scaly skin disease due to filaggrin problems and skin appears to be extremely dry. Similarly, dermatographism is a condition in which lesions appear on the skin and it is often considered AD which is an incorrect diagnosis (Siegfried and Hebert, 2015).

1.9. Biomarkers

Currently, there is no well-grounded biomarker for AD. IgE is considered as a potential biomarker for AD because its level is generally raised in this specific disease condition. There is a limitation for its use as a biomarker because studies have shown that in some confirmed cases where there is severe AD, IgE level is not raised in the body. Sometimes IgE level is raised after the disease course and sometimes it is not raised at all. Similarly, sometimes the level of IgE is raised when AD is not present. Many disease conditions have shown raised IgE values which includes some inflammatory diseases, parasitic diseases and even certain cancers. Due to all this situation IgE is not considered an effective biomarker for AD. With the advent of research, many new cytokines such as proinflammatory cytokines and T lymphocytes have been discovered that may be used as biomarkers of AD. Similarly, advancement in the field of chemokine immunobiology has led to new likely biomarkers for AD. AD can develop very early in life even in neonates. It starts with mild scaling on the body near elbows and knees. Then, there is rash and redness formation usually due to dilation of capillaries in the affected areas which is

followed by itching and irritation. The scaling and redness then travel to face, scalp, and other regions of body except the nose and also reached legs. The skin gets rough and even more scaly because of itching and scratching behavior. Scratching makes things even worse and leads to several infections. In early life, AD manifests itself as rough skin that has been subjected to successive scratching and lichenification and appearance as dead skin. In this age group AD signa are observed in the curved of body like on elbows and neck creases, on hands and feet and even under the eyes causing Dennie-Morgan folds under the eye. There are excessive excoriations in which the child excessively picks his skin and as a result there is broken skin that is creating more itching and redness. At this stage, there are recurrent AD symptoms and quality of life is highly compromised. In adulthood, AD continues as itchy scaly skin, especially face is involved. Over the years, there is development of macular brown ring on the neck of the affected person and there is swelling ang rashes and lesion marks on the body (Health, 2017).

1.9.1. Hallmark essential features of AD

Itching is a characteristic feature present in AD patients. Although it is the most common and significant feature for diagnosing AD, its presence is not good for the patient. Because when there is excessive rubbing and scratching in an area the skin becomes broken and more affected. Sometimes the rubbing is not under the control of the patient and occurs while sleeping and compromising his quality of life. Many factors are responsible for further deteriorating the patient's condition such as clothing, emotional and psychological stress, extremely hot temperatures, sweating and excoriation. One the scratching phenomenon occurs, the skin around the wound area also become sensitive and there is hyperkinesis and the remaining skin is also affected leading to spread of AD.

1.9.2. Other skin features commonly associated with AD

Many diseases that are confused with AD are discussed above. Some of the conditions are further explained as follows:

Pityriasis alba is a skin condition in which there is formation of red scaly pattern on the skin that leaves a permanent mark on the skin. It usually affects the upper parts of body and when the skin is exposed to UV rays from sun, they turn into marks that are usually lighter than the normal skin color. Keratosis pilaris is a skin condition which gives a rough appearance to skin and as a result misleading diagnosis of AD is made. In this

condition, there is excessive skin cell proliferation and dead skin cells are not removed from the body and a result scaling like pattern is observed on the skin, but the scales are neither itchy nor they hurt. Ichthyosis vulgaris is a skin condition that is generally associated with AD because it mostly leads to AD and is considered as early symptoms of AD and it also worsens the AD symptoms if it coexists with the much worse AD. In this condition there is appearance of scales on lower extremities of body and giving fish-like characteristic to skin mainly occurring due to filaggrin problems. Dermatographias is a skin condition that occurs immediately due to rubbing and itching of the skin. It generally lasts within 30 minutes but the lesions can be confused with AD because there is a rash on the skin which sometimes is accompanied with the swelling and redness which gives false AD appearance (Berke *et al.*, 2012).

1.10. AD-Associated Complications

1.10.1. Bacterial infections

In AD, the microbiota is completely disturbed. The most important microbiota disturbed is of colon and ultimately of the skin. The skin microbiota is very important in maintaining normal barrier functioning and keeping it healthy, fresh and free from infections. Once the skin microbiota is disturbed and reduced in AD patients, there are increased chances of *Staphylococcus aureus* colonization on the skin. The *S. aureus* colonies are reported in nearly all the patients with AD. But such patients may not necessarily have *S. aureus* infection, they are just easy victims of it. Similarly, those having colonies of *S. aureus* have increased chances of developing MRSA infections.

1.10.2. Viral infections

When the patient is suffering from AD, there are many chances of developing many superinfections that worsen AD symptoms in patients further compromising quality of life. Herpes simplex virus sometimes superinfects AD patients leading to EH. Although its incidence is only 20% but it a very serious condition sometimes leading to death in patients. Similarly, MC is another skin condition in which there is the formation of pinkish bead-like structures on the skin which are highly itchy. They may turn white over time and then leave permanent scars on the skin. If this condition is deteriorated, it is called Molluscum Eczema in which additional AD lesions are formed on the skin

surrounding these pinkish white beads and giving even bumpier and rough skin which is painful.

1.10.3. Fungal infections

When the skin is broken and has weeping lesions, this is an opportunity for tinea and yeast etc to easily invade the skin causing long-lasting fungal skin infections. Generally, they do not affect skin but when there is skin rash and unevenness and open wounds, these fungi can easily grow and worsen the AD symptoms.

1.11. AD-associated Comorbidities

Currently, AD is a topic gaining importance from researchers and some comorbidities are linked to AD which include hyperactivity disorders and sleep disturbance. Sleeping disorders are very common with AD patients and compromise the quality of life in them. Other disorders such as anxiety and depression have also been observed after prolonged AD symptoms. Many children have shown signs of anemia and headache. Anemia is developed because there are many bleeding wounds. Moreover, the patient body mass index is also affected. The bone mineral density is also affected because sometimes the skin infection is so worse and deep that it reaches bone where it affects it badly and even the absorption of minerals and nutrients is also affected. In recent decades, studies have been conducted which show a link of AD with diabetes and obesity. There are studies suggesting the link between CV and AD, but this is a highly controversial topic. Some although some studies suggest that AD leads to hypertension and stroke, many deny this speculation saying that CV is not caused by AD but by the accompanied asthma which was also reported in those studies. Since this topic is gaining so much importance, many studies are under way to check the comorbidities which can be linked to AD (Avena-Woods, 2017).

1.12. Probiotics

Figure 1.4. shows the mechanism of probiotics once they come in contact with gut.

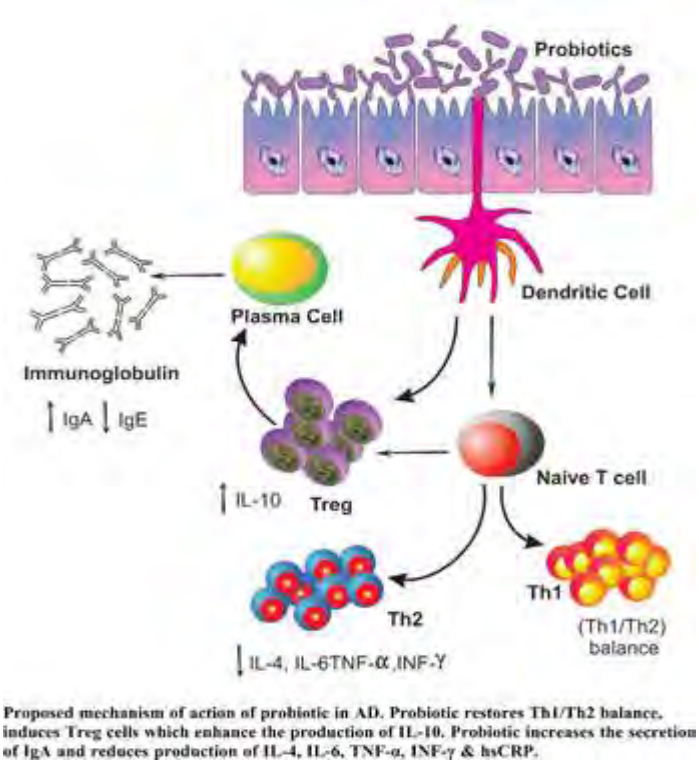

Figure.1.4. Mechanism of action of probiotics in the treatment of Atopic Dermatitis (Huidrom, 2021).

According to WHO and FAO "Probiotics" are the living microorganisms that when administered in the body provide the desired beneficial effects to the host. They regulate the immune system by providing a barrier for pathogen absorption. The mechanism of action is that they bind with the gastrointestinal cells and a as results pathogens are competitively eliminated from the body. Probiotics include strains of *Lactobacillus* and *Bifidobacterium* and *Saccharomyces* mostly, but also *E coli* and *Bacillus clausii* (Wieërs et al., 2020). In general, the intestinal microbiota is regulated by many factors including food intake, genetic and environmental factors and exposure to the probiotics if any. The role of intestinal microbiota is being recognized in many diseases which include common diarrhea, IBD, IBS, *H. pylori* infections and NEC (Sniffen *et al.*, 2018). Probiotics play a very important role in maintaining homeostasis in the gut and regulating the immune system by various mechanisms which include competitive binding of the probiotic to the potential receptor sites o0f pathogenic organisms, production of mucin to prevent the invasion of harmful strains, production of antimicrobial peptides, production of short chain fatty acids and maintaining the proper functioning of tight junctions as shown in

figure 1.4. All these processes provide protection from harmful organisms and regulate the immune system. Probiotics play an important role in the management of allergic diseases. The immature DC differentiates into mature DC and tolerogenic DC. Probiotics maintain Th1/ Th2 balance downregulating Th2 response which ultimately leads to reduced IgE production and managing allergic symptoms. Probiotics have beneficial effects in pregnancy because the mother's microbiota could reach the fetus and can impact its growth and development (Anania *et al.*, 2022). (Figure 1.5)

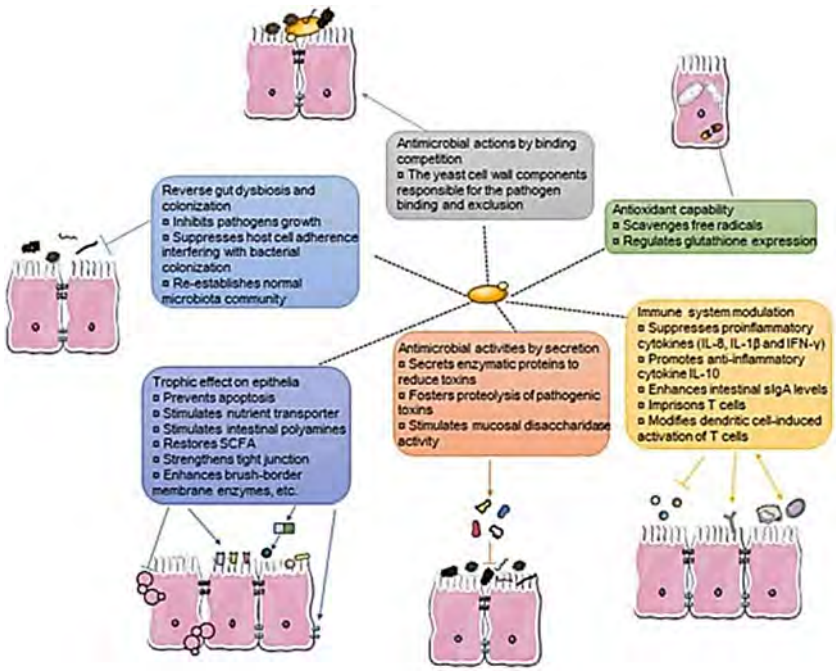

Figure. 1.5. Mechanism of action of *S. boulardii* (Cui et al., 2022).

Saccharomyces boulardii is a yeast whose beneficial health effects have been studied in past decades and it has proved to be highly effective in gastrointestinal diseases especially those in which the inflammatory pathway is involved as shown in figure 1.5. Its use has been reported in common diarrhea but in recent years, its use in IBD has been studied and it has proved its effective role in controlling and managing IBD. *S. boulardii* inhibits inflammation by regulating NF-κB and MAP kinase. NF-κB in an important inflammatory mediator. It is present in cytoplasm in inactivated form bound to its inhibitor IκB. Once the stimulus arrives, it is converted to its activated form NF-κB which translocate into the nucleus causing proinflammatory gene transcription including TNF-α and IL-8. Unlike many corticosteroids that only non-specifically inhibit this

inflammatory mediator, *S. boulardii* plays an important role by inhibiting NF-κB signaling at various steps (Albensi, 2019). MAP kinase once activated MAPK like ERK1/2 and JNK, p38 involved in growth, apoptosis and inflammation respectively. *S. boulardii* plays a significant role in regulating inflammation by acting at various steps in MAP kinase and NF-κB signaling cascade. This evidence provides a strong base for *S. boulardii* to be used in inflammatory diseases. It has been used recently in a number of inflammatory conditions such as IBD, EHEC, EPEC, *C. difficile, Shigella* and *Salmonella* infections by inhibition of NF-κB signaling, IL8 secretion and regulating MAP kinase signaling. In IBD, *S. boulardii* has shown unique mechanism of activating PPAR-c and entrapment of T cells in mesenteric lymph nodes. Both these mechanisms aim to reduce inflammation. In case of gram-negative bacteria, *S. boulardii* dephosphorylates LPS by its protein proteases and prevent many infections from harmful bacteria and improve the quality of life as shown in Figure 1.6 (Pothoulakis, 2009).

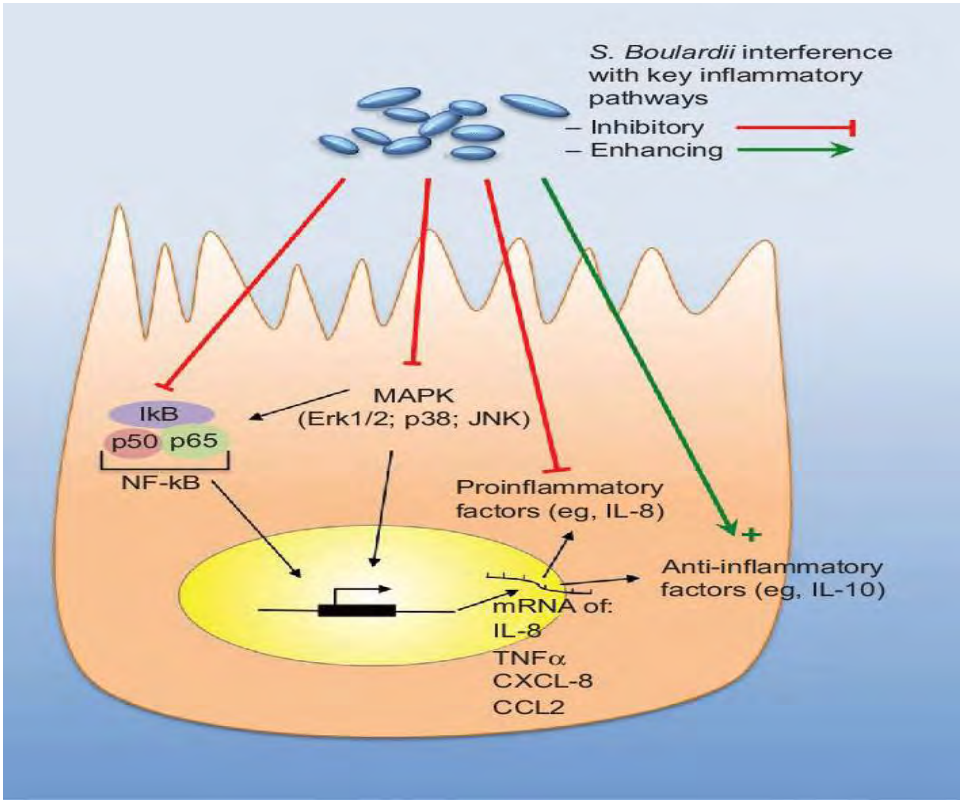

Figure.1.6. Summary of actions of *S. boulardii* against inflammatory pathways in the GIT (Stier and Bischoff, 2016).

1.13. Problem Statement

Atopic Dermatitis is a severe skin condition but unfortunately there is no definite cure for this chronic illness. Only symptomatic and palliative treatments are given. There is need for the proper drugs and medications for the treatment of diseases.

1.14. Rationale of the Study

Probiotics have reported antioxidant, anti-inflammatory and immunomodulatory properties. This study will exploit whether it can protect against ovalbumin-induced Atopic dermatitis.

1.15. Aim

The study aims to evaluate whether probiotic *Saccharomyces boulardii* can exert therapeutic potential against Ovalbumin-induced Atopic Dermatitis.

1.16. Objectives

- Establishment of Ovalbumin-induced Atopic Dermatitis in mice model.
- Evaluation of hematological parameters and serum biochemistry.
- Evaluation of antioxidants (GSH, GST, Catalase) and oxidative stress markers (MDA and NO).
- Histopathological analysis (skin, liver, kidneys, spleen and colon) for evaluation of role of probiotic in Ovalbumin-induced Atopic Dermatitis.
- IHC evaluation of NF-κB, TNF-α, IL-1β in different organs.
- Evaluation of serum IgE by ELISA.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

The chemical reagents used in this study are Ovalbumin (Sigma-Aldrich St. Loius, MO) (grade V), Aluminum hydroxide (Alum), Normal Saline (1000 ml), Dexamethasone, Chloroform/ Ketamine, Formalin (10%), Phosphate Buffered Saline (PBS) (1000 ml), Autoclaved Distilled Water, Glutathione (GSH), Hydrogen peroxide (H_2O_2), Potassium hydrogen phosphate (KH_2PO_4), Dipotassium hydrogen phosphate (K_2HPO_4), Ferric chloride ($FeCl_3$), Sodium hydrogen phosphate (NaH_2PO_4), Disodium hydrogen phosphate (Na_2HPO_4), Sodium chloride (NaCl) , Potassium chloride (KCl) Ascorbic acid, Ethanol, Thiobarbituric acid (TBA), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DNTB), 1 Cloro 2,4 dinitrobenzene (CDNB), Trichloroacetic acid (TCA) . Albumin, globulin, creatinine and bilirubin kits for serum biochemistry were purchased from TERSACO, Switzerland. ELISA kit for Immunoglobulin (IgE) analysis was purchased from the Fine Biotech (Fn) China.

2.1.2. Apparatus and equipment

Cleaned, dried glassware apparatus was used to prepare solutions. Beakers (50 ml, 100 ml, 1000 ml), Volumetric flask, Glass Vials, Stirrer, Glass slides, Sterile syringes (1ml, 3ml), Micropipettes (10µL, 100 µL, 1000 µL), Micropipette tips, Eppendorf tubes (1.5 ml, 2ml), Falcon tubes (15 ml, 50 ml), Ethylenediaminetetraacetic acid (EDTA) tubes, Aluminum foil, Tissue paper, Cotton, Paper tape, Surgical gloves, Face masks, Tagging tape, Plastic animal cages, Feeding bottles, and thermometer, were used. Hotplate, Weighing Balance (RADWAG Wagai Elektroniczne), Homogenizer, Centrifuge (Hermlegmbh Z326K, Germany), Dissection box, 96 well plate spectrophotometer, Water distillation apparatus, Refrigerator and Cryofreezer (-20°C) were used.

2.1.3. Software's

Image J software was used for quantification of histopathological and immunohistochemistry images. GraphPad prism was used to plot the graphical results. Bio render (14 days free trial version) with permission was used to graphically illustrate the drug mechanism. End Note was used to add references.

2.1.4. Animals

Albino BALB/c male mice (4-6) weeks, weighing 25-30 g, were used in the study. Male mice were included in the study to avoid variations in the results due to hormonal cycle in female mice. Mice were procured from National Institute of Health Sciences, Islamabad, Pakistan, and were used only once for the current study. Animals were acclimatized for about 1 week in the animal house under standard conditions for 12hour light and dark cycle, in plastic cages divided according to groups. The temperature was maintained at 23±2°C and during the complete study animals were provided with fresh food and free access to water. The experimental protocol was prior approved by the bio-ethical committee of Faculty of Biological Sciences, Quaid i Azam University, Islamabad (approval # BEC-FBS-QAU 2023-499). The authority expressed no reservations or any such conflict. Vigilant efforts were made to keep the number of animals at a minimum and prevent any harm to the animals.

2.2. METHODS

2.2.1. Study design

The study consists of 28 days including sensitization and challenge. Animals were grouped into six groups, each containing five mice (n=5/ group). The number of mice in each group was carefully determined considering statistical and bioethical parameters. The grouping was done randomly without any preference.

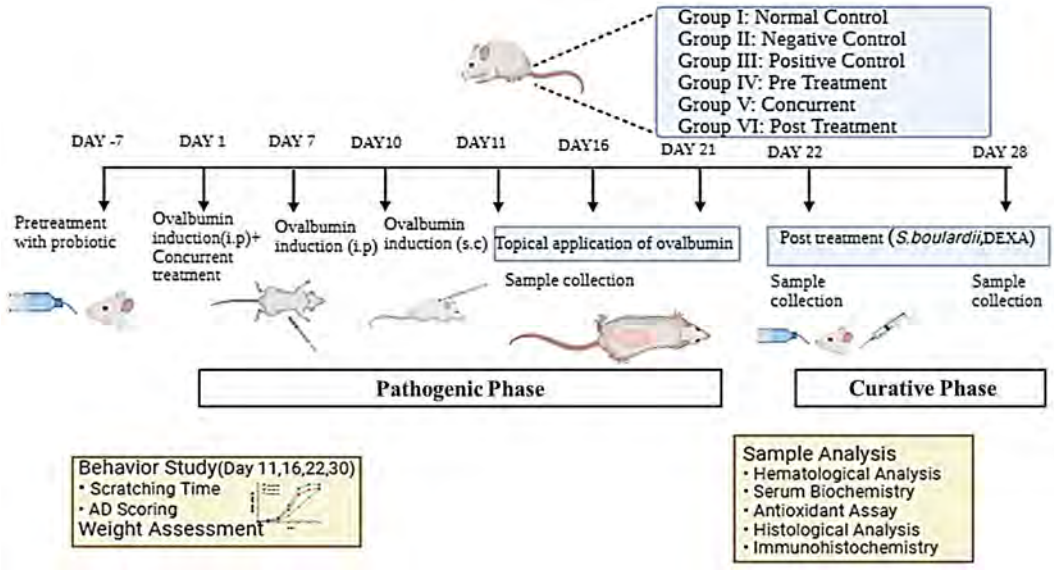

Figure 2.1. Schematic representation of *in vivo* study design. (Software: Biorender-14 days free trial version).

Table 2.1. Grouping of animals in Pathogenic phase.

Group	Induction Criteria
Control (group I)	Animals in this group received only normal saline topically for ten days from day 11-21.
Disease Control (group II)	Animals in this group received i.p. ovalbumin on day 1 and 7, S.C. on day 11. After that the animals were challenged with topical ovalbumin from day 11 to 21.
Pre-PBT (group IV)	Animals in this group received probiotic *Saccharomyces boulardii* orally seven days prior to the first dose of ovalbumin sensitization and received it throughout this phase.
Concurrent-PBT (group V)	Animals in this group started receiving probiotic *Saccharomyces boulardii* orally from day 1 of sensitization.

Table 2.2. Grouping of animals in Curative phase.

Control (group I)	After application of normal saline for ten days, the animals did not receive any treatment.
Disease Control (group II)	After sensitization and challenge by ovalbumin, the animals did not receive ant treatment.
Positive Control (group III)	Animals were given Dexamethasone 5 mg/kg from day 21-28 after sensitization and challenge by ovalbumin.
Pre-PBT (group IV)	*Saccharomyces boulardii* 1×10^9 CFU/ml/day which was given orally seven days before the sensitization was continued in the treatment phase and lasted on day 28 final day of treatment.
Concurrent-PBT (group V)	*Saccharomyces boulardii* 1×10^9 CFU/ml/day which was given concurrently with sensitization and challenge was continued in the treatment phase till day 28.
Post-PBT (group VI)	After sensitization and challenge, *Saccharomyces boulardii* 1×10^9 CFU/ml/day was given orally from day 21-28.

2.2.2. Ovalbumin sensitization

The mice were sensitized with 10 µg of ovalbumin (Grade V) mixed with 4 mg Alum (Alumin um hydroxide) in a volume 150 µL on day 1 and 7 through i.p. route and on day 10 through S.C. route in the loose skin behind neck (Na *et al.*, 2020).

2.2.3. Challenge through ovalbumin

The mice were epicutaneously challenged with ovalbumin (Grade V) 10 µg in 50 µL PBS on the dorsal skin of mice daily for 10 days from day 11-21 (Na *et al.*, 2020).

2.2.4. Preparation of dose of Probiotic

The dose 1×10^8 CFU/ml/day of *Saccharomyces boulardii* was prepared from the commercially available sachets (Mazhar *et al.*, 2023).

2.2.5. Microscopic evaluation of *Saccharomyces boulardii*

To evaluate the structure of *Saccharomyces boulardii,* lactophenol cotton blue staining was performed. The structure was observed microscopically (Hossain *et al.*, 2020).

2.3. Behavioral Parameters

2.3.1. Assessment of body weight

During the study period, each mice was weighed using a digital weighing balance from induction to final day of sampling, or days 11, 16, 22 and 28 to note any significant variations in body weights between diseased groups and treatment groups (Kim *et al.*, 2008). After division into groups, every mice was weighed in all the groups to observe weight gain or loss during the 28 days model.

2.3.2. Assessment of relative spleen weight

The spleen index was calculated by dividing the spleen weight with total body weight and then multiplying it by 100.

Spleens were collected from every mouse after euthanization and weighed on the digital balance to observe the variations in its weight (Kukreti *et al.*, 2023).

2.3.3. Assessment of relative kidney weight

The relative kidney weight was calculated by dividing the kidney weight with total body weight and then multiplying it by 100.

Kidneys were collected from every mouse after euthanization and weighed on the digital balance to observe the variations in its weight (Mossa *et al.*, 2015).

2.3.4. Assessment of relative liver weight

The relative liver weight was calculated by dividing the liver weight with total body weight and then multiplying it by 100.

Livers were collected from every mouse after euthanization and weighed on the digital balance to observe the variations in its weight (Lazic *et al.*, 2020).

2.3.5. Assessment of scratching behavior

The scratching behavior was evaluated on days 11, 16, 22 and 28 immediately after the topical application on the dorsal skin of the mice by placing the animals in the observation cages (Zhu *et al.*, 2015). The animals were recorded for 1 minute and the scratching was observed on the back of ears, dorsal surface of skin and hind paws afterwards through video recordings (Köchling *et al.*, 2017).

2.3.6. Assessment of AD lesions

To understand the progression of disease in AD model, clinical scoring was done. The method followed for evaluating clinical score for Atopic dermatitis called SCORAD was reported as (Kim *et al.*, 2012). The scores were evaluated as 0-3, where 0 showed no symptoms, 1, 2 and 3 showed mild, moderate, and severe symptoms respectively. The scores were allotted based on signs and visible disease symptoms such as edema, scarring, dryness, scaling, erythema, hemorrhage and erosion on the dorsal skin of mice. This assessment was carried out on days 11, 16, 22 and 28 (Kim *et al.*, 2012).

2.4. Hematological Analysis

2.4.1. Collection of blood and serum

For the collection of blood and serum, animals were sacrificed on the day 11, 16, 22 and 28. After anesthetization with ketamine, cardiac puncture was performed with 3ml syringes. The blood samples (1ml per mice) obtained were collected in ethylenediaminetetraacetic acid (EDTA) tubes to preventing blood coagulation for hematological assay. Blood was obtained from remaining animals in eppendorf tubes (1.5ml) which after clotting was allowed to be centrifuged at 5000 rpm for 10 min and yellowish transparent serum was collected from the top in the separate eppendorf tubes and stored at -20°C in Cryofreezer. This serum was used for performing assays such as serum biochemistry and IgE determination (Thakur *et al.*, 2019).

2.4.2. Total blood count

For evaluation of the effect of ovalbumin induced inflammation and anti-inflammatory effects of the treatment groups, a total blood count hematological assay was carried out. Blood was collected by cardiac puncture in EDTA tubes and the effect of ovalbumin, Dexamethasone and all three treatment groups was determined on blood parameters such as WBCs, lymphocytes, monocytes, eosinophils, basophils, and neutrophils according to the reported procedure (Hong *et al.*, 2014).

2.4.3. Serum biochemistry

To observe the effect of Ovalbumin and especially Dexamethasone on vital organs in the body, albumin, globulin, total protein, bilirubin, and creatinine were evaluated in the serum. Albumin and globulin tests were performed by using TERSACO kits and total protein was calculated from the obtained data to observe the effect of all the groups especially as the globulin IgE was raised due to allergic disease (Gong *et al.*, 2020). The level of bilirubin (Eken *et al.*, 2006) and creatinine (Chen *et al.*, 2015) was also determined as renal and hepatic functions are reported to be compromised with Dexamethasone treatment.

2.5. Collection of Organs

For the collection of organs, animals were sacrificed on the day 11, 16, 22 and 28. After anesthetization with ketamine, cardiac puncture was performed, and animals were dissected for collecting samples. Skin, spleen, colon, liver, and kidneys were collected from animals in eppendorf tubes (2 ml) and Falcon tubes (15 ml). the organs were fixed in 10% Formalin solution at room temperature (Kim *et al.*, 2017). These samples were used for histological analysis and immunohistochemistry. Similarly, the samples were obtained from remaining animals and then they were homogenized with 1ml PBS and centrifuged at 6000 rpm for 10 min and supernatant was collected from the top in the separate Eppendorf tubes and stored at -20°C. This supernatant was used for performing assays such as assessment of biochemical parameters (Sharma *et al.*, 2020).

2.6. Histological Analysis

2.6.1. Hematoxylin and eosin staining

Histological changes in different tissues were evaluated by histopathological analysis after ovalbumin induced allergic disease. Skin tissues were collected and epidermal and dermal thickness was measured by Image J as reported (Sharma *et al.*, 2020). Spleen, colon, liver and kidney tissues were also collected as reported in (Hernandez *et al.*, 2020, Heymann *et al.*, 2009, Jung *et al.*, 2019) respectively. All the tissues were removed, rinsed with normal saline, and then maintained in fixative solution (10% formalin) overnight. Tissue samples were allowed to dehydrate the following day with alcohol and a xylene replacement. The specimens had paraffin in them and then stained with H & E by appropriate technique. The infiltration of inflammatory cells was quantified in skin, spleen, colon, liver, and kidney.

2.6.2. Masson's trichrome staining

The deposition of collagen in the skin tissues weas determined by Masson trichrome staining as reported (Suvik and Effendy, 2012). The slides were prepared according to the protocol by deparaffinizing by immersing them in a series of concentrations of 100% xylene and ethanol. After that, they were incubated with Weigert's iron hematoxylin, Biebrich scarlet-acid fuchsin, phosphomolybdic-phosphotungstic acid and aniline blue. After washing with 1% acetic acid, collogen deposition was observed as blue staining, nuclei and cytoplasm as dark red or purple and red or pink respectively (Kim *et al.*, 2021).

2.7. Immunohistochemistry (IHC)

The anti-inflammatory activity of *Saccharomyces boulardii* was further assessed by the immunohistochemical analysis of skin tissue as reported (Qiao *et al.*, 2020). Immunohistochemistry of spleen, colon and liver was also performed. The antibodies corresponding to the relevant markers involved in inflammation were used; NF-κB, TNF-α and IL-1β (Zhu *et al.*, 2020). Tissue sections were fixed in 10% formalin and then paraffin, sectioned and deparaffinized. Enzymatic antigen retrieval was carried out and after that the tissues were incubated overnight with protein kinase K, primary antibodies, and normal goat serum (5%) and incubated. Following incubation, PBS was used to wash the primary antibodies, Anti-rabbit secondary antibodies were used, with the tissues were

then incubated with ABC for 1 hour in a humid environment. The slides were rinsed and stained with 3'3 diaminobenzidine (DAB) to see the generated antigen-antibody combination. To protect the slides, excess DAB was removed, and mounting medium was used to apply coverslips (Kim *et al.*, 2022).

2.8. Assessment of Biochemical Parameters

2.8.1. Determination of NO

Nitric oxide (NO) is synthesized from nitric oxide synthase (iNOS) which has physiological functions, but excessive occurrence contributes to pathological reactions. The Griess method was used for the determination of the level of nitric oxide in all the five tissues collected that include skin, spleen, colon, liver and kidney after homogenization and centrifugation. This required sulphanilamide and N-naphthyl-ethylenediamine and wavelength of the reaction mixture was measured at 540 nm through spectrophotometer when a purple color complex appears (Sun *et al.*, 2003).

2.8.2. Determination of malondialdehyde (MDA)

The level of oxidants such as MDA is raised in allergic disease conditions such as atopic dermatitis. MDA is the final product of lipid peroxidation (Sivaranjani *et al.*, 2013). To evaluate MDA it was made to react with the TBA in acidic conditions and resulted in the formation of pink complex which was measured at 535 nm with spectrophotometer (Jain *et al.*, 1989).

2.8.3. Determination of *glutathione* (GSH)

GSH is an important biological marker for easing oxidative stress and modulating immune responses. In pathological condition, the GSH is depleted from body and hence it is an important tool in determining the level of oxidative stress in body (Nuhu *et al.*, 2020).

Griffith method was used to determine its level in all five organs. The reaction of GSH by 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) gives 5′-thio-2-nitrobenzoic acid (TNB). Yellow color appeared and the rate of reduction was measured by spectrophotometer at 412 nm (Ammar *et al.*, 2022).

2.8.4. Determination of *glutathione S-transferase* (GST)

(Habig *et al.*, 1974) method was used to determine the GST level in skin, spleen, colon, liver and kidney. The mixture contained phosphate buffer, reduced glutathione, 1-chloro-

2,4-dinitrobenzene (CDNB) and homogenate sample in such a way that total volume is 2 ml (Khan *et al.*, 2012). The absorbance was measured at 340 nm spectrophotometer (Katerji *et al.*, 2019).

2.8.5. Determination of catalase

Under normal physiological conditions, catalase plays an important role in the defense mechanism of the body because it neutralizes H_2O_2. In the allergic disease model, the level of catalase is diminished in the body and hence compromised immune defense (Reynaert *et al.*, 2007). Catalase level was determined through enzymatic method by using 340nm wavelength on spectrophotometer. Peroxidic reaction involves ethanol, hydrogen peroxide and catalase and the absorbance of NADH produced in this reaction is measured (Van Lente and Pepoy, 1990).

2.9. Assessment of IgE by ELISA (Enzyme-linked immunosorbent assay)

ELISA was carried out as reported by (Kim *et al.*, 2017) to observe the inhibitory effect of Saccharomyces boulardii on the IgE production in ovalbumin-induced atopic dermatitis. Serum was collected from the blood sample after centrifugation. at 5000 rpm for 10 min (Kim *et al.*, 2022). The commercially available kit for IgE ELISA was obtained from Fine Biotech (Fn).

2.10. Statistical Analysis

Data obtained from was expressed as mean (n=5) ± Standard deviation. Data from pathogenic phase was subjected to two-way analysis of variance (two-way ANOVA) using LSD test for comparison. Data from curative phase was analyzed using a one-way analysis of variance (one-way ANOVA) followed by Duncan's multiple range test. For the results to be considered statistically significant, the level of significance was kept 0.005 (Kim *et al.*, 2005).

CHAPTER 3

RESULTS

3. RESULTS

3.1. Lactophenol Cotton Blue Staining

Lactophenol cotton blue staining was performed indicating thick-walled oval cellular structure.

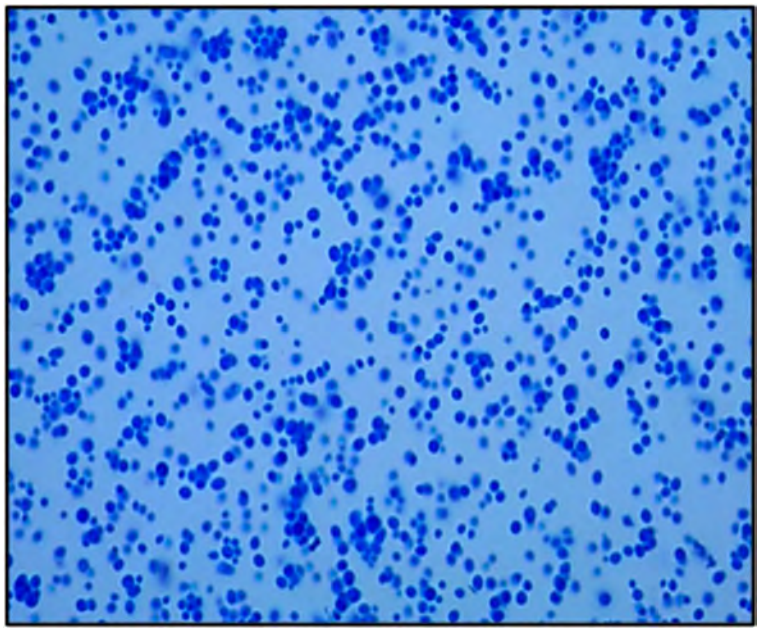

Figure 3.1. Microscopic evaluation of *Saccharomyces boulardii* by Lactophenol Cotton Blue Staining on 40X.

3.2. Assessment of Body Weight

The results of weight variation in the pathogenic and curative phase are shown in the Figure 3.2. There is no significant difference in the weights of animals in various groups on day 11 of the study.

However, on day 16, 22 and 28, there is a significant ($p<0.001$) decrease in the body weights of disease control group sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 22, treatment with Pre-PBT ($p<0.01$), Concurrent ($p<0.05$) and on day 28, treatment with PC ($p<0.001$), Pre-PBT ($p<0.01$) and Concurrent ($p<0.05$) significantly increased total body weight when compared to DC group.

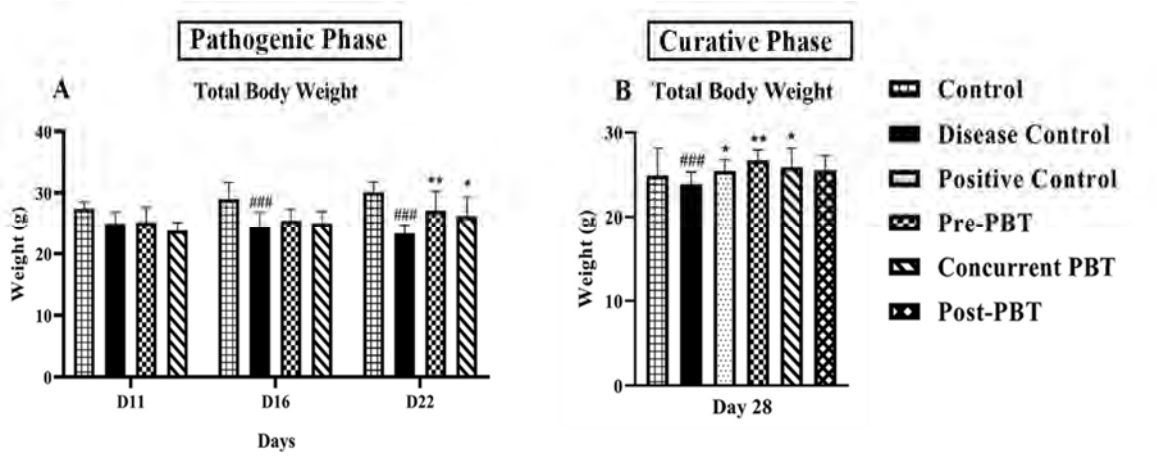

Figure 3.2. The effect of *S. boulardii* on Total Body Weight in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.3. Assessment of Relative Spleen Weight

The results of relative spleen weight in the pathogenic and curative phase are shown in the Figure 3.3. On day 11, 16, 22 and 28, the relative spleen weight was found to be significantly ($p < 0.001$) higher in the disease control animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I).

On day 11, treatment with pre-PBT ($p < 0.01$), Concurrent ($p < 0.05$), on day 16, treatment with pre-PBT ($p < 0.01$) and Concurrent ($p < 0.01$) and on day 22, treatment with pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) significantly increased relative spleen weight when compared to DC group. On and on day 28, treatment with PC ($p < 0.001$), pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and post-PBT ($p < 0.05$) significantly increased relative spleen weight when compared to DC group.

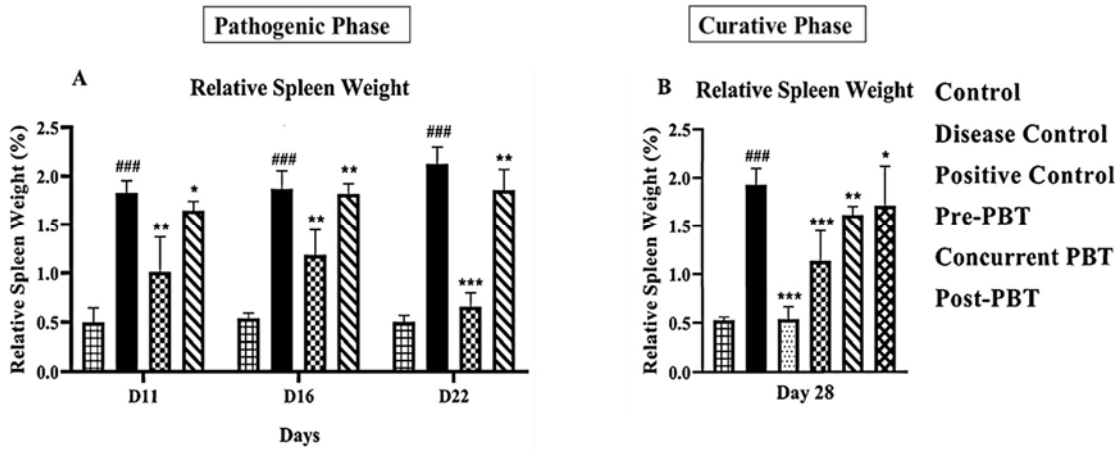

Figure 3.3. The effect of *S. boulardii* on Relative Spleen Weight in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group*

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3.4. Assessment of Relative Kidney Weight

The results of relative kidney weight in the pathogenic and curative phase are shown in the Figure 3.4. On day 11, 16, 22 and 28, the relative kidney weight was found to be significantly ($p < 0.05$) higher in the disease control animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). There is no significant difference in the relative kidney weights of animals in various treatment groups as compared to disease control group throughout the study.

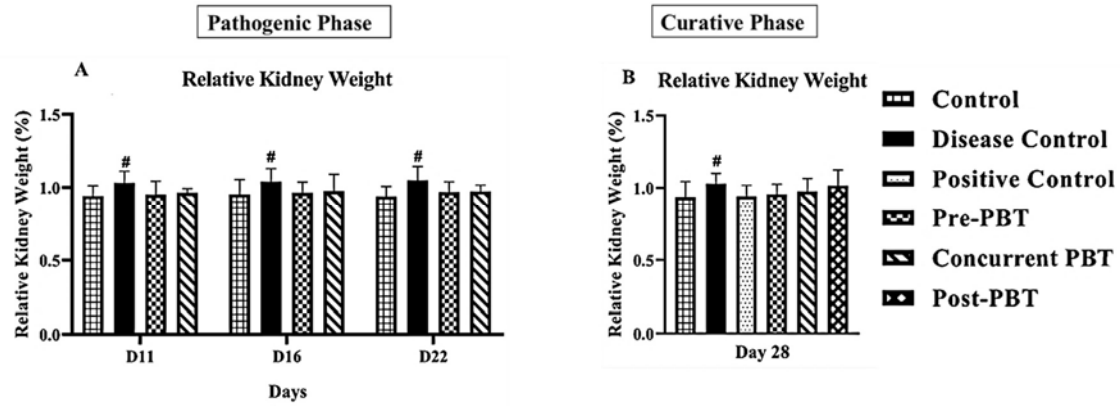

Figure 3.4. The effect of *S. boulardii* on Relative Kidney Weight in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.5. Assessment of Relative Liver Weight

The results of relative liver weight in the pathogenic and curative phase are shown in the Figure 3.5. There is no significant difference in the relative liver weights of animals in various groups on day 11, 16 and 22 of the study. Relative liver weight was found to be significantly ($p < 0.01$) in Pre-PBT, ($p < 0.05$) in Concurrent-PBT and ($p < 0.05$) in Post-PBT lower than that of animals in negative control group sensitized and challenged by ovalbumin (group II).

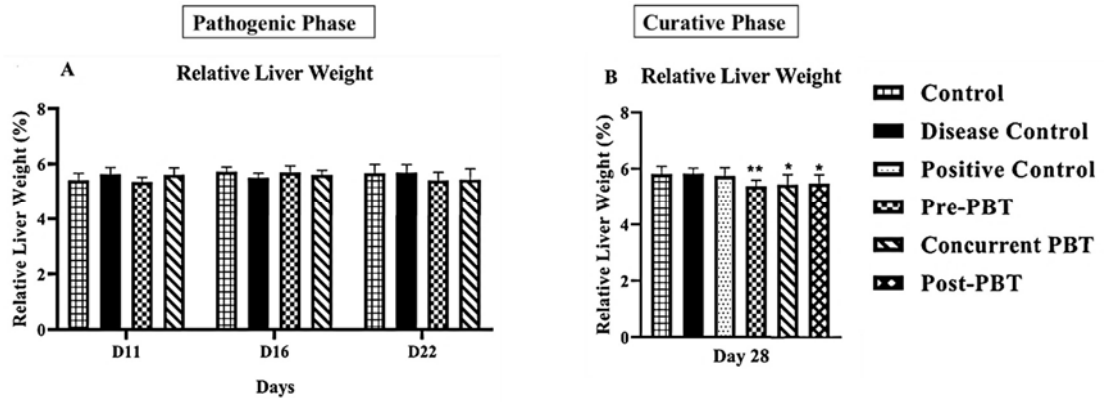

Figure 3.5. The effect of *S. boulardii* on Relative Liver Weight in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.6. Assessment of Scratching Behavior

To determine the severity of atopic dermatitis, the scratching time of animals was recorded in seconds for a total of 60 seconds. Animals with severe inflammation show an increase in the scratching time. On days 11, 16, 22 and 28, the scratching time was found to be significantly ($p < 0.001$) higher in the animals sensitized and challenged by ovalbumin in the DC group (group II) than that of animals in control group (group I). On day 11, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.001$), on day 16, treatment with Pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and on day 22, treatment with Pre-PBT ($p < 0.01$), Concurrent ($p < 0.01$) significantly increased scratching when compared to DC group. On and on day 28, treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) significantly increased scratching time when compared to DC group. (Figure 3.6)

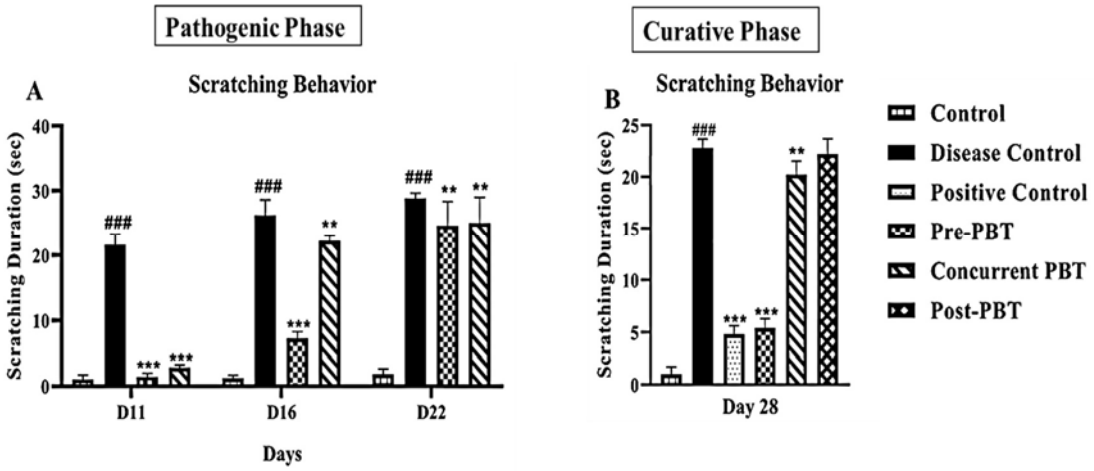

Figure 3.6. The effect of *S. boulardii* on Scratching Behavior in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.7. Assessment of AD Lesions (SCORAD)

To determine the severity of atopic dermatitis, the animals were given scores based on visible signs and symptoms (redness and inflammation). Animals with no sign and symptoms were given '0' and animals with severe inflammation, bleeding and swelling were allotted '4'.

On day 11, there was no significant difference between the animals in any group in terms of AD Scoring. On days 16, 22 and 28, the value of scoring was found to be significantly ($p < 0.001$) higher in the animals sensitized and challenged by ovalbumin in the DC group (group II) than that of animals in control group (group I). On days 16, the value of scoring was found to be significantly ($p < 0.001$) in Pre-PBT and ($p < 0.001$) in Concurrent-PBT lower than that of DC group and on day 22, value of scoring was found to be significantly ($p < 0.001$) Pre-PBT and ($p < 0.01$) in Concurrent-PBT lower than that of DC group.

On day 28, the value of scoring was found to be significantly ($p < 0.001$) in PC, ($p < 0.001$) in Pre-PBT, ($p < 0.05$) in Concurrent-PBT lower than that of animals in the DC group sensitized and challenged by ovalbumin (group II). (Figure 3.7)

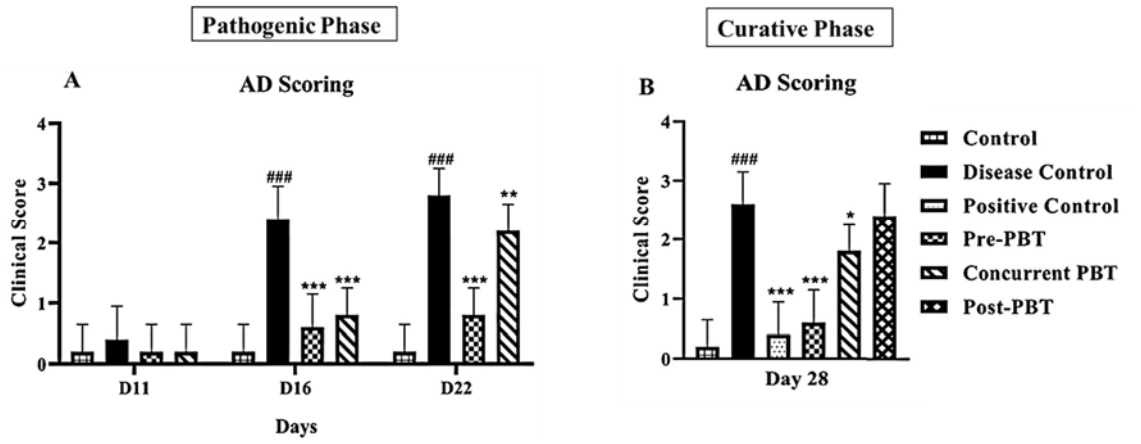

Figure 3.7. The effect of *S. boulardii* on AD Scoring in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.8. Representative Images of Atopic Dermatitis

The representative images of AD are given below in Figure 3.8. These images indicated that ovalbumin sensitization and challenge caused formation of skin rashes and lesions while treatment groups especially PC and Pre-PBT reversed the effect of ovalbumin in mice model.

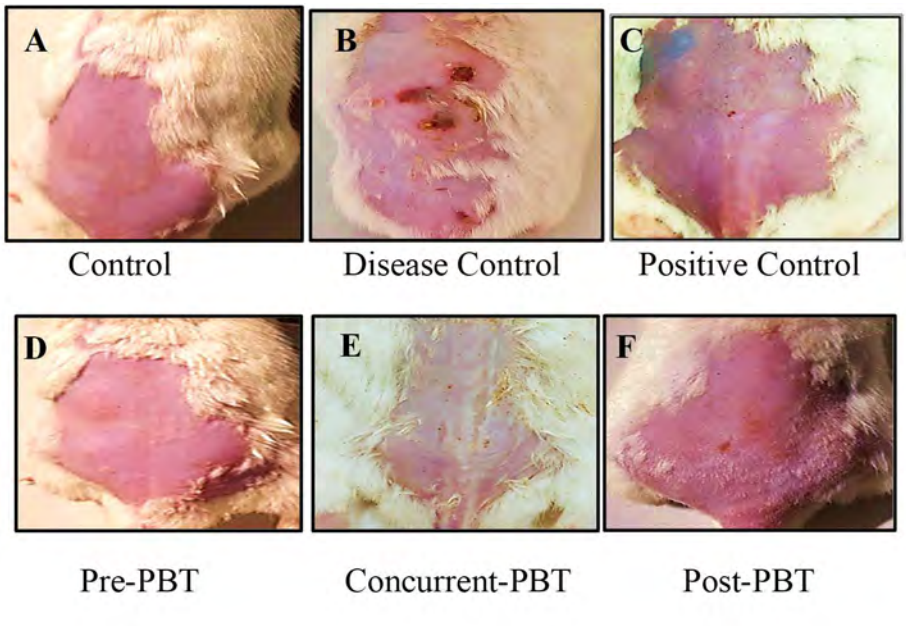

Figure 3.8. Representative images of Ovalbumin-induced Atopic Dermatitis **(A-F)** in mice model. Effect of Probiotics and Dexamethasone on the dorsal surface of the mice.

3.9. Effect of *S. boulardii* on Ovalbumin-induced Splenomegaly

The representative images of spleen are shown in Figure 3.9. below. These images indicated that the ovalbumin sensitization and challenge caused splenomegaly while the treatment groups especially PC and Pre-PBT reversed the effect of ovalbumin on spleen in mice model.

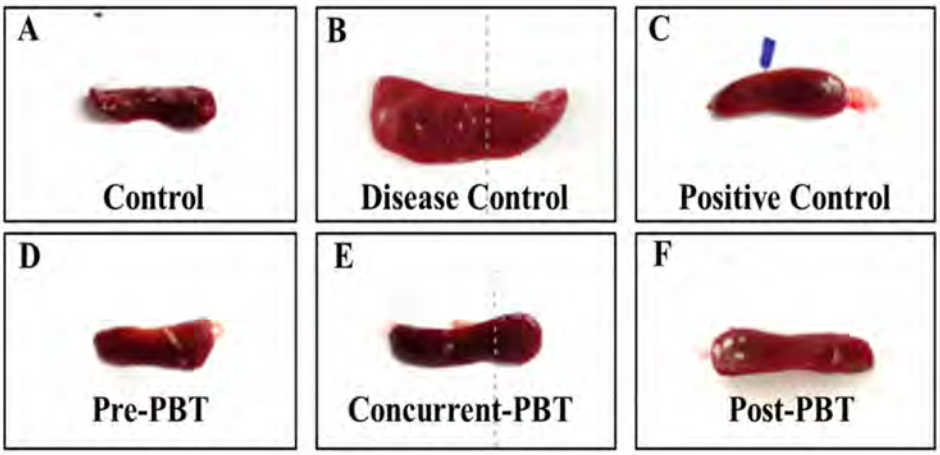

Figure 3.9. Representative images of effect of spleen on Ovalbumin-induced Atopic Dermatitis **(A-F)** in mice model. Probiotic groups and Dexamethasone reversed splenomegaly caused by ovalbumin sensitization and challenge.

3.10. Total Blood Count

The results of total blood count in the pathogenic and curative phase are shown in Tables 3.1 and 3.2 below.

On day 11, 16, and 22, the level of WBCs was found to be significantly ($p < 0.001$) higher in the disease control animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 11, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.001$), on day 16, treatment with Pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and on day 22, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) significantly increased the level of WBCs when compared to DC group.

On day 11, 16, and 22, the level of PLT count was found to be significantly ($p < 0.001$) higher in the disease control animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 11, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.001$), on day 16, treatment with Pre-PBT ($p < 0.001$) and

Concurrent ($p < 0.001$) and on day 22, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) significantly increased the level of PLT count when compared to DC group.

On day 11, 16, and 22, the level of NE was found to be significantly ($p < 0.001$) higher in the disease control animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 11, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$), on day 16, treatment with Pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and on day 22, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) significantly increased the level of NE when compared to DC group.

On day 11, 16, and 22, the level of LYM (%) was found to be significantly ($p < 0.001$) higher in the disease control animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 11, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$), on day 16, treatment with Pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and on day 22, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.05$) significantly increased the level of LYM when compared to DC group.

On day 11, 16, and 22, the level of MO (%) was found to be significantly ($p < 0.001$) higher in the disease control animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I).

On day 11, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.001$), on day 16, treatment with Pre-PBT ($p < 0.001$) and Concurrent ($p < 0.001$) and on day 22, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.001$) significantly increased the level of MO when compared to DC group.

On day 11, 16, and 22, the level of EO (%) was found to be significantly ($p < 0.001$) higher in the disease control animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 11, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.001$), on day 16, treatment with Pre-PBT ($p < 0.001$) and Concurrent ($p < 0.001$) and on day 22, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.001$) significantly increased the level of EO when compared to DC group.

Table 3.1. Effect of *S. boulardii* on the Total Blood Count of the Pathogenic Phase of Ovalbumin-induced Atopic Dermatitis on days 11, 16 and 22.

GROUPS	DAY	WBCs (10^3/µL)	Platelet Count (10^3/µL)	Neutrophils (%)	Monocytes (%)	Eosinophils (%)
Control	11	5.41±1.43	531.4±22.41	30.99±0.92	4.33±0.20	3.56±0.07
	16	5.08±0.68	504.4±3.286	30.34±0.77	4.48±0.32	3.64±0.21
	22	5.004±1.2	502.8±3.114	30.46±0.48	4.56±0.25	3.75±0.36
Disease Control	11	10±1.3$^{###}$	1304.2±26.5$^{###}$	36.65±0.7$^{###}$	5.85±0.1$^{###}$	7.19±0.2$^{###}$
	16	10±1.8$^{###}$	1348.8±18.6$^{###}$	37.14±1.2$^{###}$	5.96±0.2$^{###}$	7.6±0.23$^{###}$
	22	11±1.1$^{###}$	1390.6±20.3$^{###}$	39.20±1.6$^{###}$	5.81±0.4$^{###}$	7.8±0.26$^{###}$
Pre-PBT	11	4.6±0.3***	711.2±46.89***	30.52±0.7***	4.67±0.2***	3.9±0.02***
	16	5.3±0.4***	718±24.96***	32.72±4.9***	4.85±0.2***	4.2±0.20***
	22	6.7±0.7**	712.6±10.87***	31.24±0.7***	4.86±0.2***	4.1±0.12***
Con current-PBT	11	5.8±0.3***	803.6±5.128***	38.43±1.39**	4.87±0.2***	4.4±0.17***
	16	6.8±0.6***	821.2±22.29***	38.68±1.96**	5.10±0.1***	4.7±0.19***
	22	9.1±0.7**	1210.6±94.9**	37.44±0.92**	5.4±0.11***	4.9±0.17***

Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way using LSD test for comparison. (*) p < 0.05, (**) p < 0.01 and (***) p < 0.001 indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

On day 28, the level of WBCs was found to be significantly ($p<0.001$) higher in the disease control group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On and on day 28, treatment with PC ($p < 0.05$), pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and post-PBT ($p < 0.01$) significantly increased the level of WBCs when compared to DC group. On day 28, the level of PLT count was found to be significantly ($p<0.001$) higher in the disease control group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On and on day 28, treatment with PC ($p < 0.05$), pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and post-PBT ($p < 0.05$) significantly increased the level of WBCs when compared to DC group. On day 28, the level of neutrophils (NE%) was found to be significantly ($p<0.001$) higher in the disease control group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On and on day 28, treatment with PC ($p < 0.05$), pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and post-PBT ($p < 0.05$) significantly increased the level of WBCs when compared to DC group. On day 28, the percent of lymphocytes (LYM%)

was found to be significantly ($p<0.001$) higher in the disease control group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On and on day 28, treatment with PC ($p < 0.001$), pre-PBT ($p < 0.001$) and Concurrent ($p < 0.001$) and post-PBT ($p < 0.001$) significantly increased the level of WBCs when compared to DC group.

On day 28, the level of monocytes (MO%) was found to be significantly ($p<0.001$) higher in the disease control group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On and on day 28, treatment with PC ($p < 0.001$), pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and post-PBT ($p < 0.05$) significantly increased the level of WBCs when compared to DC group. On day 28, the level of eosinophils (EO%) was found to be significantly ($p<0.001$) higher in the disease control group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On and on day 28, treatment with PC ($p < 0.001$), pre-PBT ($p < 0.001$) and Concurrent ($p < 0.001$) and Post-PBT ($p < 0.05$) significantly increased the level of WBCs when compared to DC group.

Table 3.2. Effect of *S. boulardii* and Dexamethasone on the Total Blood Count of the Curative Phase of Ovalbumin-induced Atopic Dermatitis on day 28.

GROUPS	WBCs (10^3/µL)	Platelet Count (10^3/µL)	Neutrophils (%)	Monocytes (%)	Eosinophils (%)
Control	5.648±1.76	533.4±10.54	30.17±0.59	4.47±0.09	3.53±0.04
Disease Control	10.76±1.118$^{###}$	1375.4±16.75$^{###}$	39.96±0.79$^{###}$	5.82±0.17$^{###}$	7.59±0.25$^{###}$
Positive Control	9.32±0.15*	1317.6±52.61*	37.03±0.96*	4.40±0.06***	3.81±0.11***
Pre-PBT	5.72±0.65***	529.6±9.343***	31.74±4.35***	4.56±0.12***	3.77±0.06***
Con current-PBT	8.7±0.254**	1243.2±47.23**	35.71±1.41**	4.83±0.10**	7.29±0.72***
Post-PBT	8.82±0.76**	1313.2±49.48*	36.06±0.73*	5.42±0.17*	7.414±0.48*

Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.11. Serum Albumin

On days 11 and 16, the level of serum albumin was found to be significantly ($p<0.01$) lower in the animals sensitized and challenged by ovalbumin in the disease control group (group II) than that of animals in control group (group I) and on day 22 and 28, the level of serum albumin was found to be significantly ($p<0.001$) lower in the animals sensitized and challenged by ovalbumin in the DC group (group II) than that of animals in control group (group I). On days 11, 16 and 22, the level of serum albumin was found to be comparable in the Pre-PBT (group IV) and Concurrent-PBT (group V) to that of animals in DC group sensitized and challenged by ovalbumin (group II) and animals in the control group (group I). On day 28, the level of serum albumin was found to be significantly ($p<0.05$) higher in PC group and significantly ($p<0.01$) lower when compared to animals in the DC group sensitized and challenged by ovalbumin (group II) and animals in the control group (group I). (Figure 3.11).

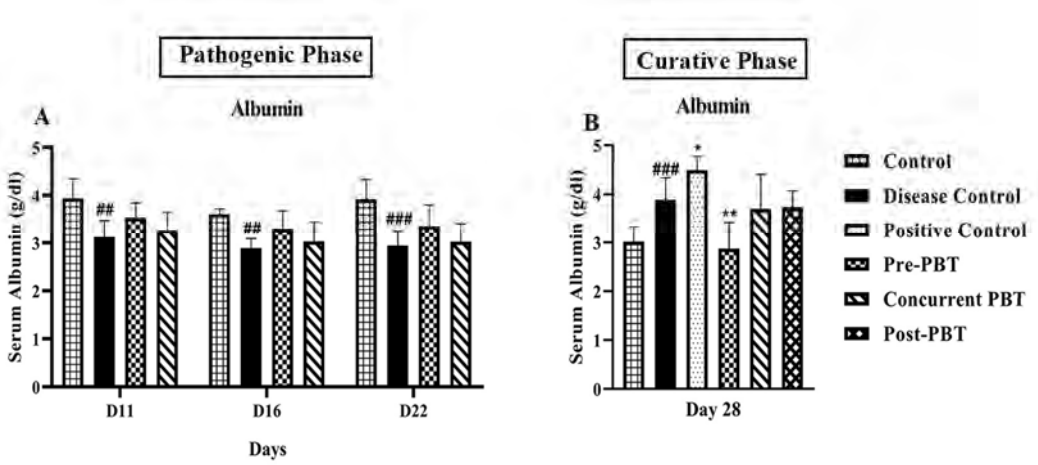

Figure 3.11. The effect of *S. boulardii* on Serum Albumin in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.12. Serum Globulin

On day 11, 16, 22 and 28, the level of globulin in serum was found to be significantly ($p<0.001$) higher in the negative control group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 11,

treatment with Pre-PBT ($p < 0.01$), Concurrent ($p < 0.01$), on day 16, treatment with Pre-PBT ($p < 0.01$) and Concurrent ($p < 0.01$) and on day 22, treatment with Pre-PBT ($p < 0.01$), Concurrent ($p < 0.05$) significantly decreased the level of globulin in serum when compared to DC group. On day 28, treatment with PC ($p < 0.05$), Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly decreased the level of globulin in serum when compared to DC group. (Figure 3.12)

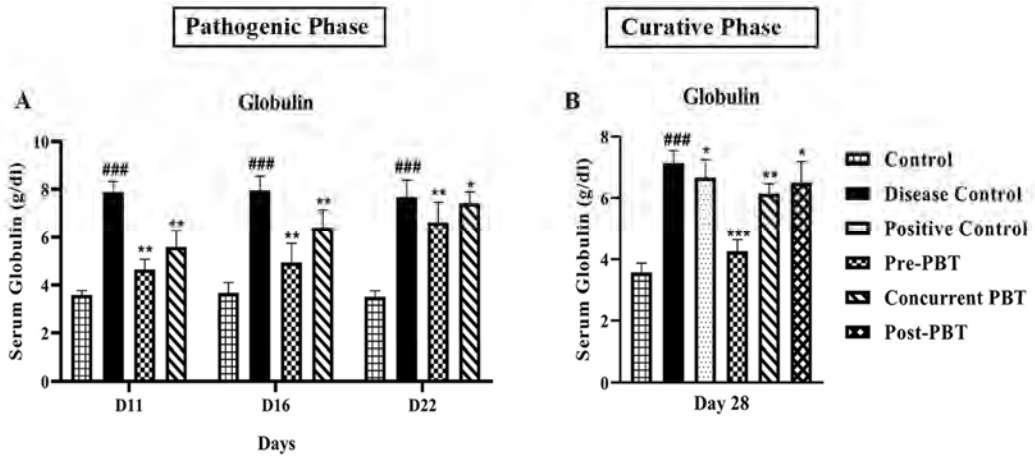

Figure 3.12. The effect of *S. boulardii* on Serum Globulin in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.13. Serum Total Protein

On day 11, 16, 22 and 28, the level of total protein in serum was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 11, treatment with pre-PBT ($p < 0.001$), Concurrent ($p < 0.001$), on day 16, treatment with pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and on day 22, treatment with pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) significantly decreased the level of total protein in serum when compared to DC group. On day 28, treatment with PC ($p < 0.05$), pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) and post-PBT ($p < 0.05$) significantly decreased the level of total protein in serum when compared to DC group. (Figure 3.13)

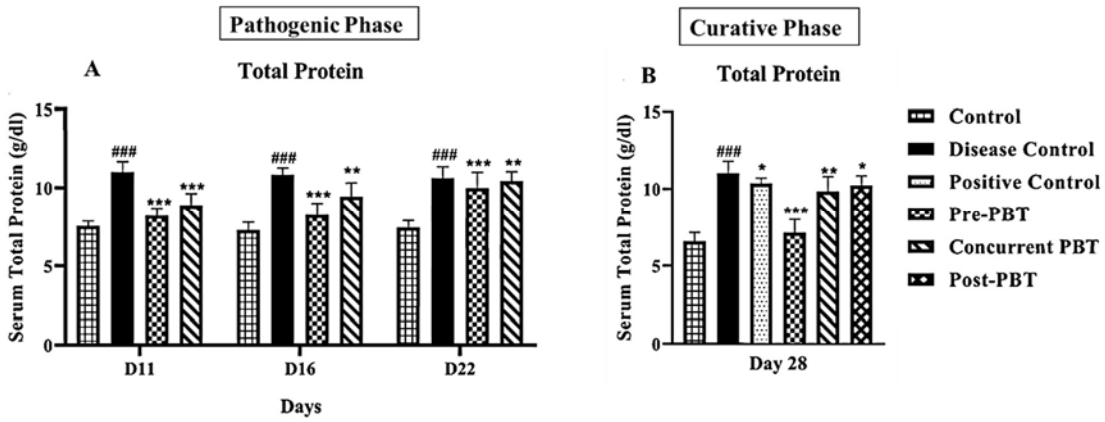

Figure 3.13. The effect of *S. boulardii* on Serum Total Protein in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) p < 0.05, (**) p < 0.01 and (***) p < 0.001 indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.14. Serum Creatinine

On day 11, 16, 22 and 28, creatinine level in serum was found to be significantly ($p<0.001$) higher in the DC group animals than that of animals in the control group. On day 11, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.001$), on day 16, treatment with Pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and on day 22, treatment with Pre-PBT ($p < 0.01$), Concurrent ($p < 0.05$) significantly decreased creatinine level in serum when compared to DC group. On day 28, treatment with PC ($p < 0.01$), Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly decreased the level of creatinine in serum when compared to DC group. (Figure 3.14)

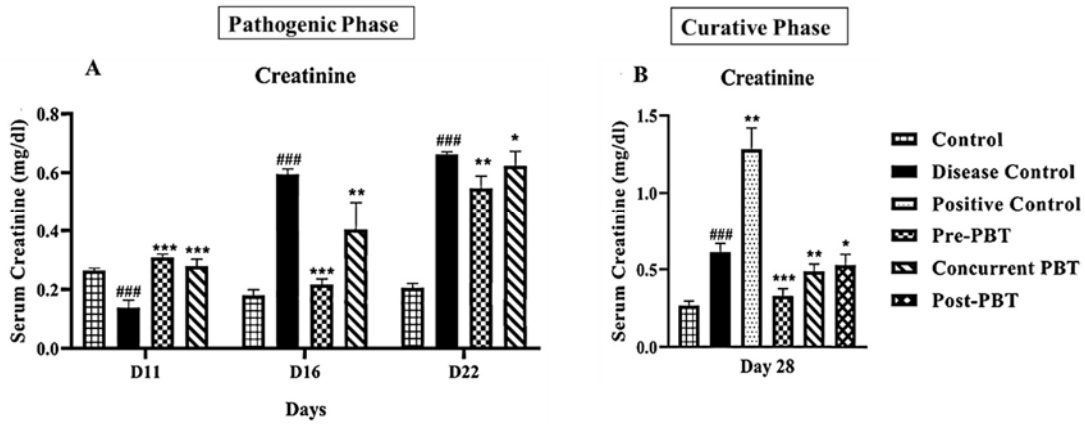

Figure 3.14. The effect of *S. boulardii* on Serum Creatinine in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison.*

3.15. Serum Bilirubin

On day 11, 16, 22 and 28, the level of bilirubin in serum was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 11, treatment with pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$), on day 16, treatment with pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and on day 22, treatment with pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) significantly decreased the level of bilirubin in serum when compared to DC group. On day 28, treatment with PC ($p < 0.01$) significantly increased and treatment with pre-PBT ($p < 0.01$) and Concurrent ($p < 0.05$) significantly decreased the level of bilirubin in serum when compared to DC group. (Figure 3.15)

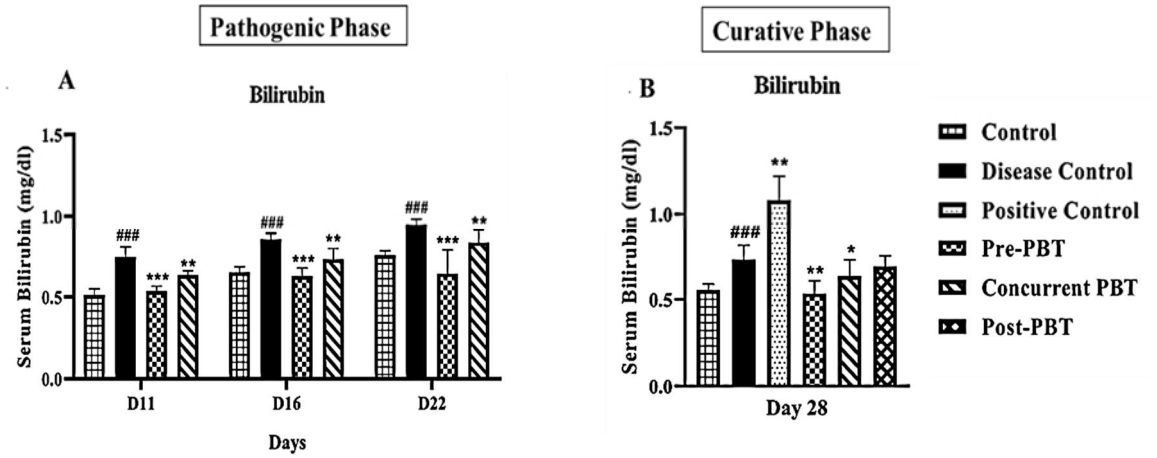

Figure 3.15. The effect of *S. boulardii* on Serum Bilirubin in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.16. H and E Staining of Skin (10X)

H and E staining of skin in pathogenic phase is shown in Figure 3.16. These images indicated cellular infiltration, changes in epidermal and dermal thickness and destruction of tissues in skin sections of the DC group while this infiltration was reversed in the treatment groups.

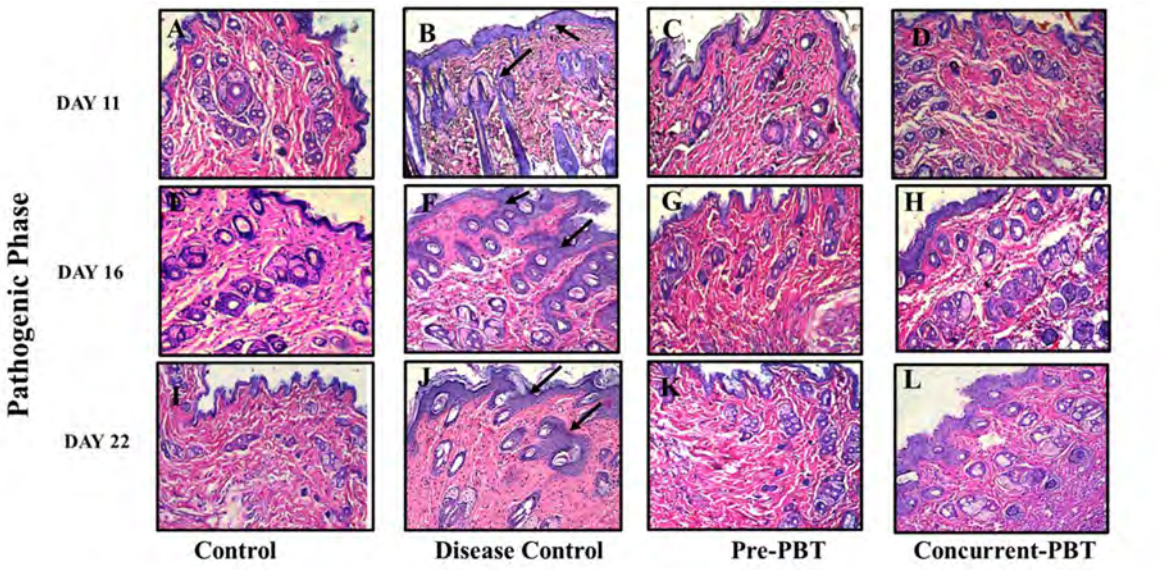

Figure 3.16. Hematoxylin and eosin staining (10X). The effect of *S. boulardii* on histopathological changes in Pathogenic phase on days 11, 16 and 22 **(A-L)** in Ovalbumin induced Atopic Dermatitis in mice.

H and E staining of skin in curative phase is shown in Figure 3.17. These images indicated cellular infiltration, changes in epidermal and dermal thickness and destruction of tissues in skin sections of the DC group while this infiltration was reversed in the treatment groups.

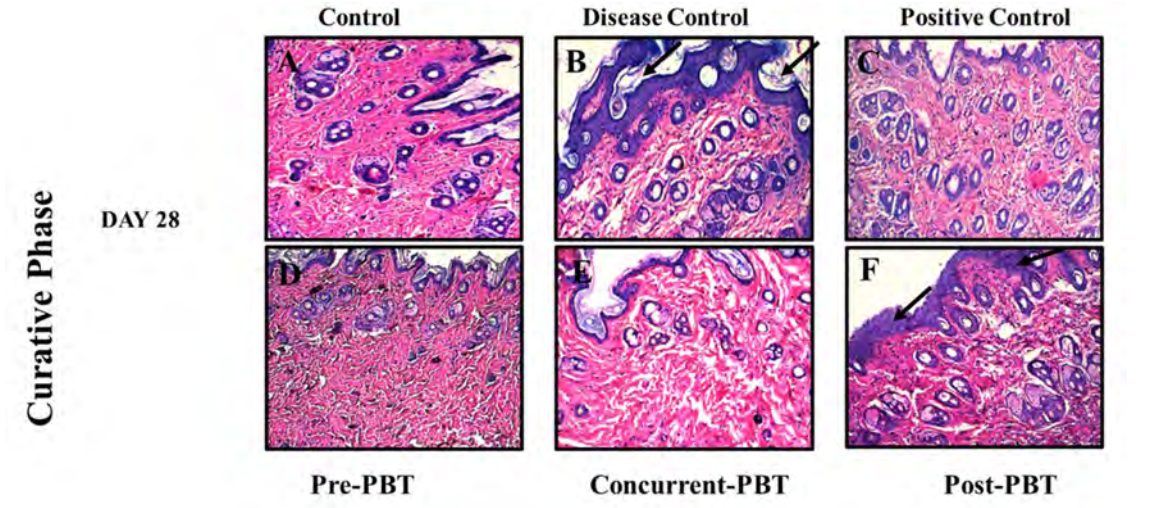

Figure 3.17. Hematoxylin and eosin staining (10X). The effect of *S. boulardii* on histopathological changes in Curative phase on days 11, 16 and 22 **(A-F)** in Ovalbumin induced Atopic Dermatitis in mice.

3.18. Skin Cells Infiltration

The H and E staining of skin was quantified by measuring the cell infiltration in the different groups. On day 11, 16, 22 and 28, the inflammatory cell infiltration was found

to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 11, treatment with pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$), on day 16, treatment with pre-PBT ($p < 0.01$) and Concurrent ($p < 0.01$) and on day 22, treatment with pre-PBT ($p < 0.01$), Concurrent ($p < 0.01$) significantly decreased in infiltration when compared to DC group. On day 28, treatment with PC ($p < 0.001$), pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) and post-PBT ($p < 0.05$) significantly decreased in infiltration when compared to DC group. (Figure 3.18)

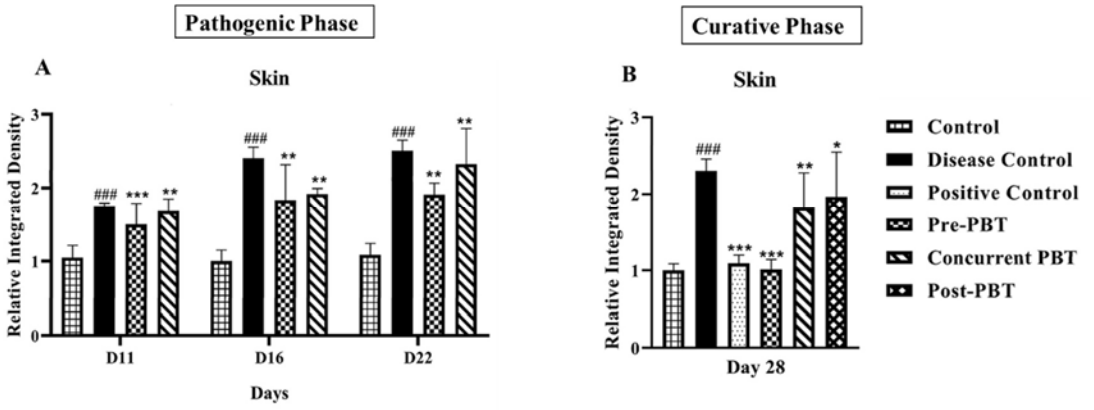

Figure 3.18. The effect of *S. boulardii* on Infiltration of inflammatory cells in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.19. Measurement of Epidermal Dermal Thickness

The H and E staining of skin was used in the measurement of epidermal and dermal thickness in the different groups. On day 11, the epidermal and dermal thickness was found to be significantly ($p<0.01$) higher in the DC group than that of control group and on days 16, 22 and 28, the epidermal and dermal thickness was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 11, treatment with Pre-PBT ($p < 0.05$), on day 16, treatment with Pre-PBT ($p < 0.01$) and Concurrent ($p < 0.05$) and on day 22, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) significantly decreased in epidermal and dermal thickness when compared to DC group. On day 28, treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p <$

0.01) and Post-PBT ($p < 0.05$) significantly decreased in epidermal and dermal thickness when compared to DC group. (Figure 3.19)

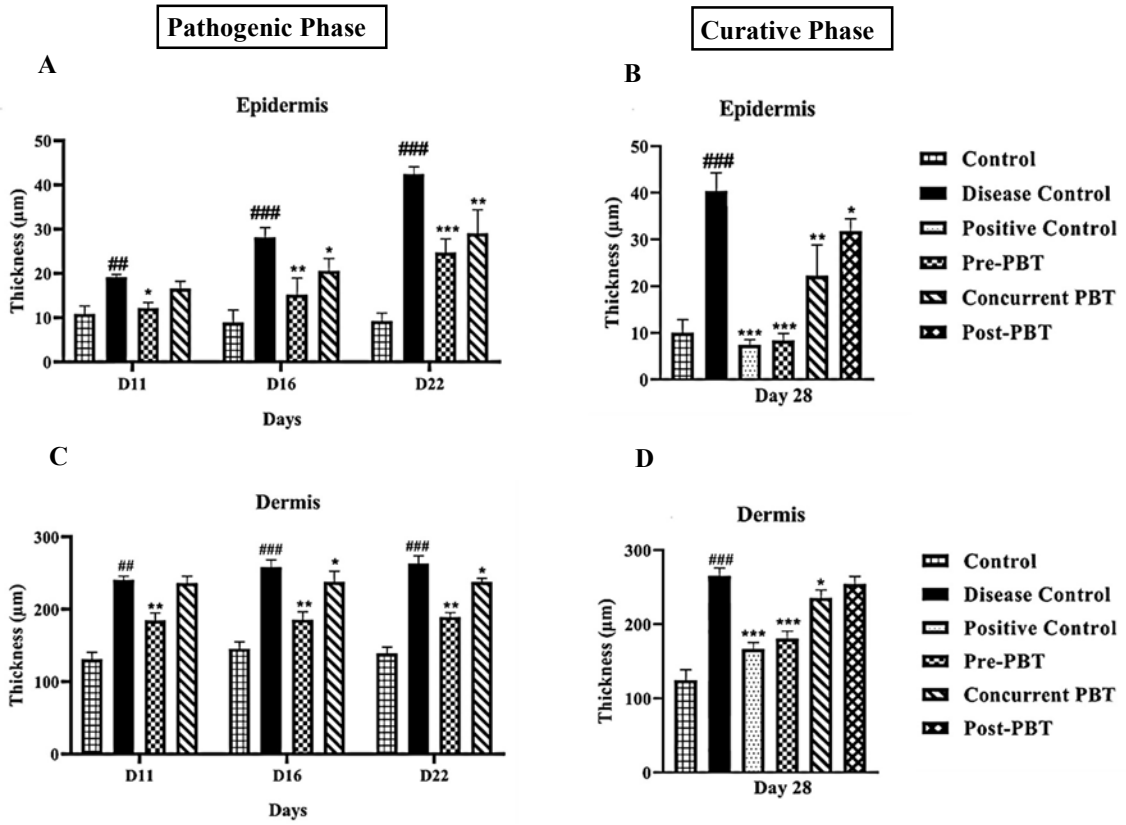

Figure 3.19. The effect of *S. boulardii* on epidermal thickness in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** and dermal thickness in Pathogenic phase on days 11, 16 and 22 **(C)** and Curative Phase on day 28 **(D)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.20. H and E Staining of Spleen (10X)

The H and E staining of spleen in pathogenic phase is shown in Figure 3.20. These images indicated cellular infiltration and destruction of tissues in spleen sections of the DC group while this infiltration was reversed in the treatment groups.

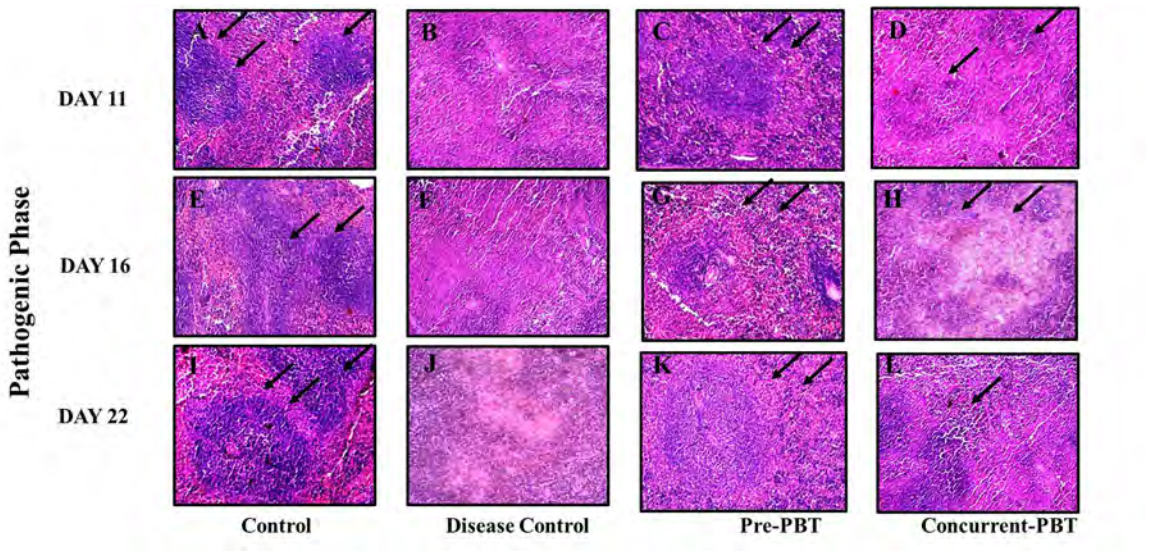

Figure 3.20. Hematoxylin and eosin staining (10X). The effect of *S. boulardii* on histopathological changes in Pathogenic phase on days 11, 16 and 22 **(A-L)** in Ovalbumin induced Atopic Dermatitis in mice.

H and E staining of spleen in curative phase is shown in Figure 3.21. These images indicated cellular infiltration and destruction of tissues in spleen sections of the DC group while this infiltration was reversed in the treatment groups.

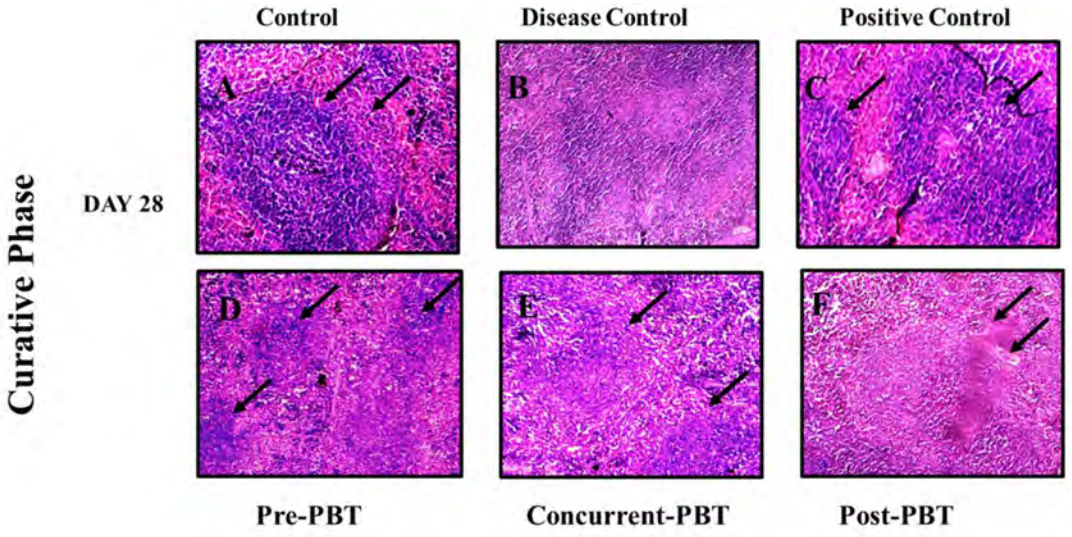

Figure. 3.21. Hematoxylin and eosin staining (10X). The effect of *S. boulardii* on histopathological changes in Curative phase on days 11, 16 and 22 **(A-F)** in Ovalbumin induced Atopic Dermatitis in mice.

3.22. Spleen Cells Infiltration

The H and E staining of spleen was quantified by measuring the cell infiltration in the different groups. On day 11, 16, 22 and 28, the inflammatory cell infiltration was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged

by ovalbumin (group II) than that of animals in the control group (group I). On day 11, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$), on day 16, treatment with Pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and on day 22, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) significantly decreased in infiltration when compared to DC group. On day 28, treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly decreased in infiltration when compared to DC group. (Figure 3.22)

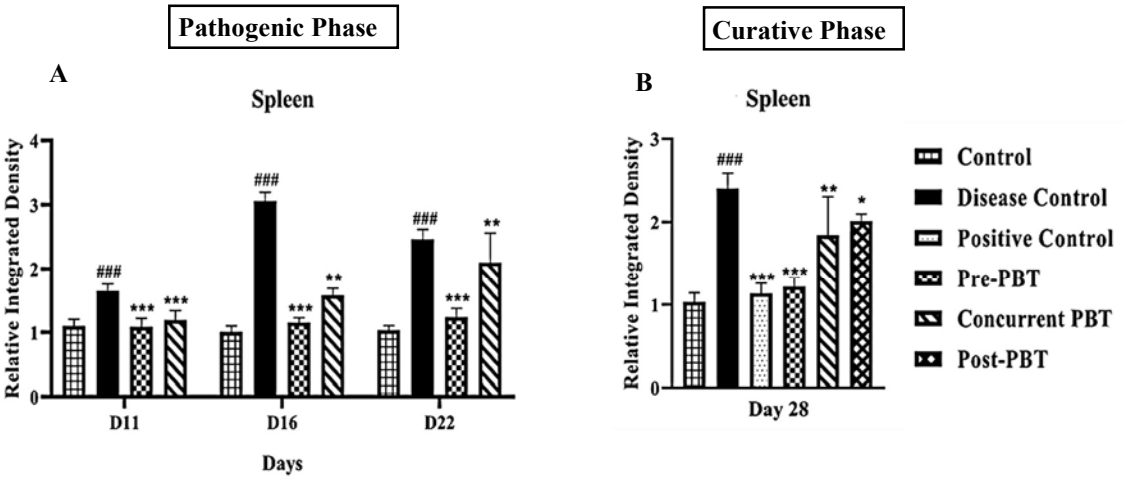

Figure 3.22. The effect of *S. boulardii* on Infiltration of inflammatory cells in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.23. H and E Staining of Colon (10X)

The H and E staining of colon in pathogenic phase is shown in Figure 3.23. These images indicated cellular infiltration and destruction of tissues in colon sections of the DC group while this infiltration was reversed in the treatment groups.

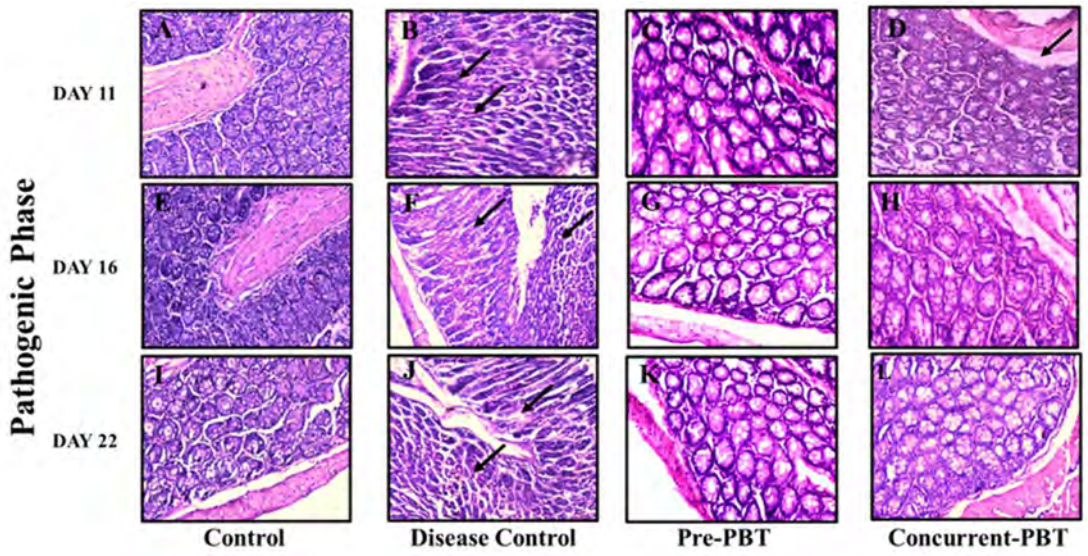

Figure 3.23. Hematoxylin and eosin staining (10X). The effect of *S. boulardii* on histopathological changes in Pathogenic phase on days 11, 16 and 22 **(A-L)** in Ovalbumin induced Atopic Dermatitis in mice.

H and E staining of colon in curative phase is shown in Figure 3.24. These images indicated cellular infiltration and destruction of tissues in colon sections of the DC group while this infiltration was reversed in the treatment groups.

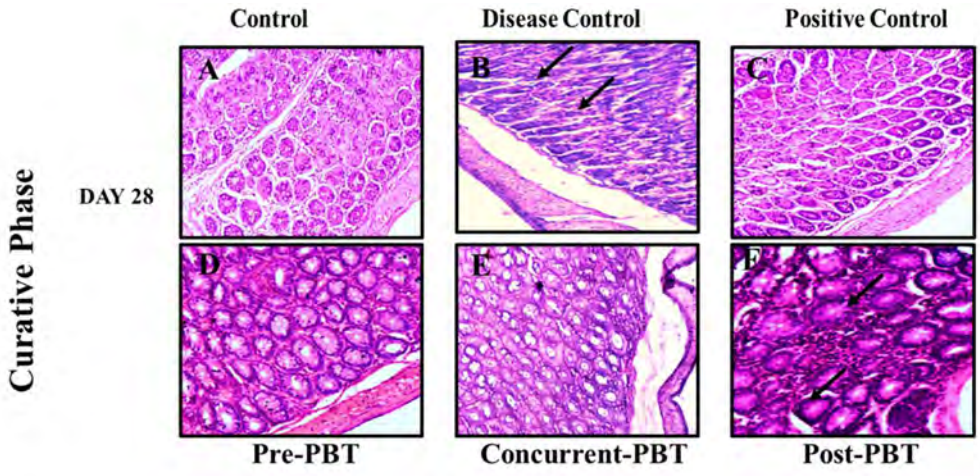

Figure 3.24. Hematoxylin and eosin staining (10X). The effect of *S. boulardii* on histopathological changes in Curative phase on day 28 **(A-F)** in Ovalbumin induced Atopic Dermatitis in mice.

3.25. Colon Cells Infiltration

The H and E staining of colon was quantified by measuring the cell infiltration in the different groups. On day 11, 16, 22 and 28, the inflammatory cell infiltration was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged

by ovalbumin (group II) than that of animals in the control group (group I). On day 11, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$), on day 16, treatment with Pre-PBT ($p < 0.01$) and Concurrent ($p < 0.01$) and on day 22, treatment with Pre-PBT ($p < 0.01$), Concurrent ($p < 0.01$) significantly decreased in infiltration when compared to DC group. On day 28, treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly decreased in infiltration when compared to DC group. (Figure 3.25)

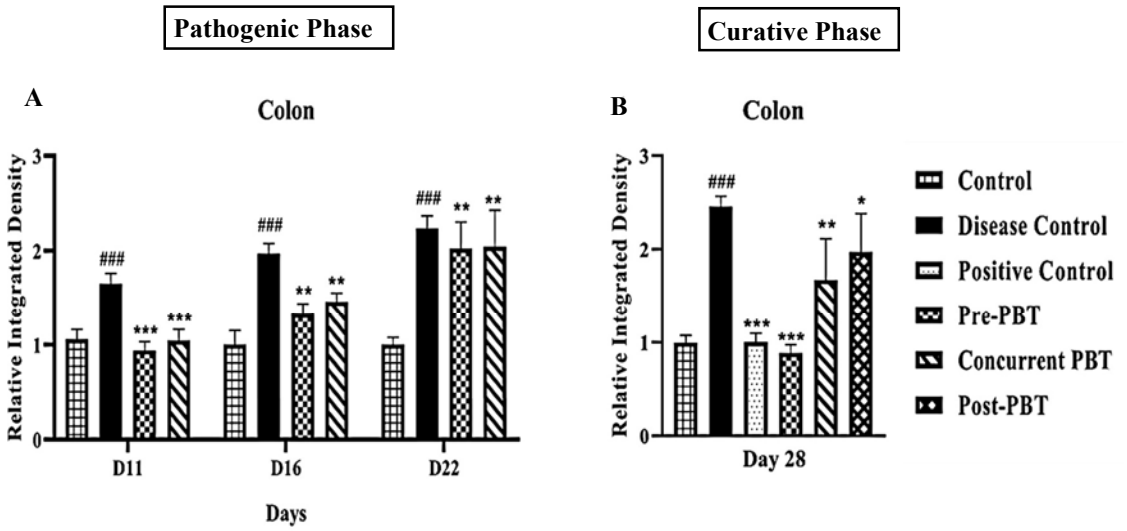

Figure 3.25. The effect of *S. boulardii* on Infiltration of inflammatory cells in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.26. H and E Staining of Liver (10X)

The H and E staining of liver in pathogenic phase is shown in Figure 3.26. These images indicated cellular infiltration and destruction of tissues in liver sections of the DC group while this infiltration was reversed in the treatment groups.

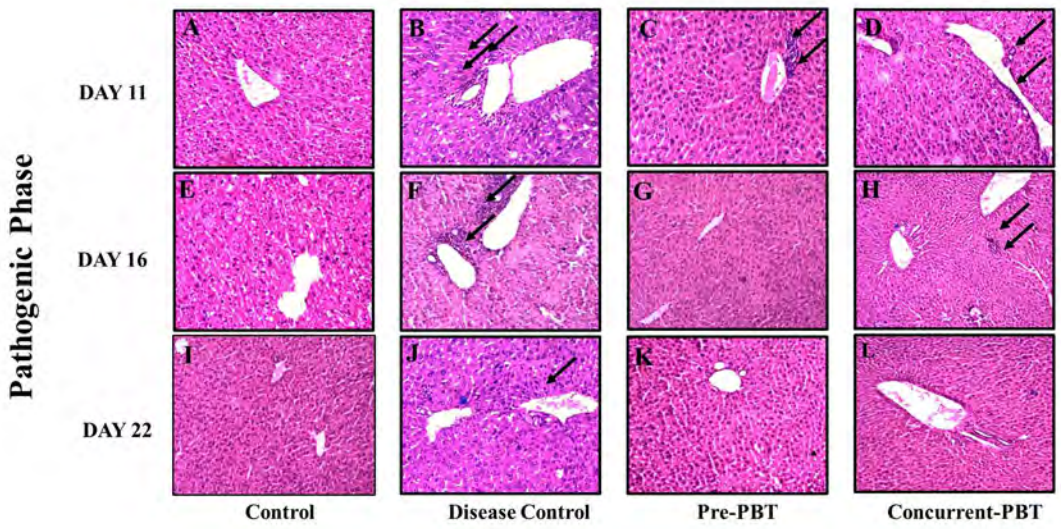

Figure 3.26. Hematoxylin and eosin staining (10X). The effect of *S. boulardii* on histopathological changes in Pathogenic phase on days 11, 16 and 22 **(A-L)** in Ovalbumin induced Atopic Dermatitis in mice.

The H and E staining of liver in curative phase is shown in Figure 3.27. These images indicated cellular infiltration and destruction of tissues in liver sections of the DC group while this infiltration was reversed in the treatment groups.

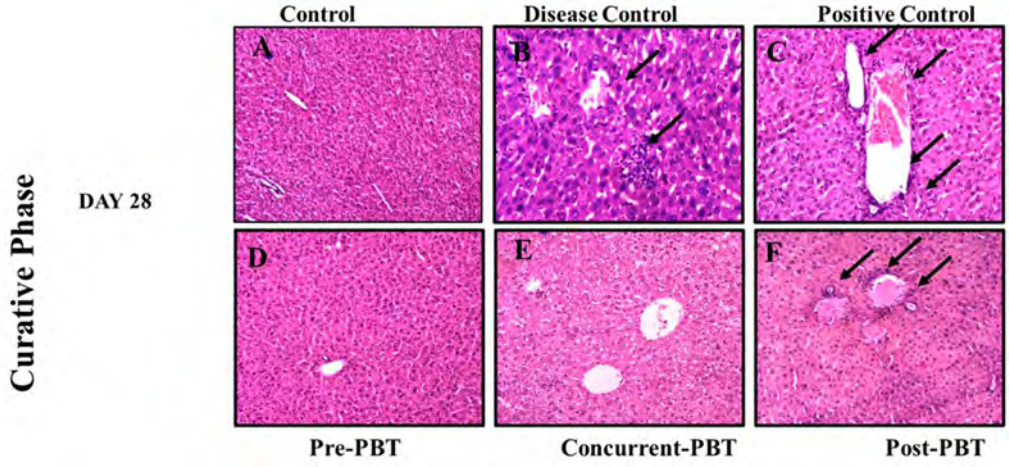

Figure. 3.27. Hematoxylin and eosin staining (10X). The effect of *S. boulardii* on histopathological changes in Curative phase on day 28 **(A-F)** in Ovalbumin induced Atopic Dermatitis in mice.

3.28. Liver Cells Infiltration

The H and E staining of liver was quantified by measuring the cell infiltration in the different groups. On day 11, 16, 22 and 28, the inflammatory cell infiltration was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). On day 11,

treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.001$), on day 16, treatment with Pre-PBT ($p < 0.001$) and Concurrent ($p < 0.001$) and on day 22, treatment with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.01$) significantly decreased in infiltration when compared to DC group. On day 28, treatment with PC ($p < 0.05$), Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.001$) and Post-PBT ($p < 0.01$) significantly decreased in infiltration when compared to DC group. (Figure 3.28)

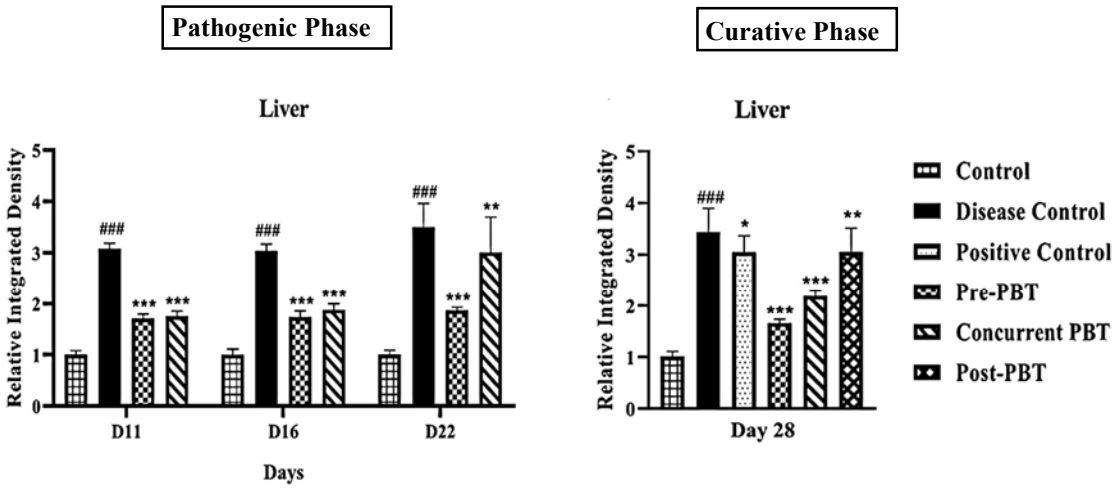

Figure 3.28. The effect of *S. boulardii* on Infiltration of inflammatory cells in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.29. H and E Staining of Kidney (10X)

The H and E staining of kidney in pathogenic phase is shown in Figure 3.29. These images indicated cellular infiltration and destruction of tissues in kidney sections of the DC group while this infiltration was reversed in the treatment groups.

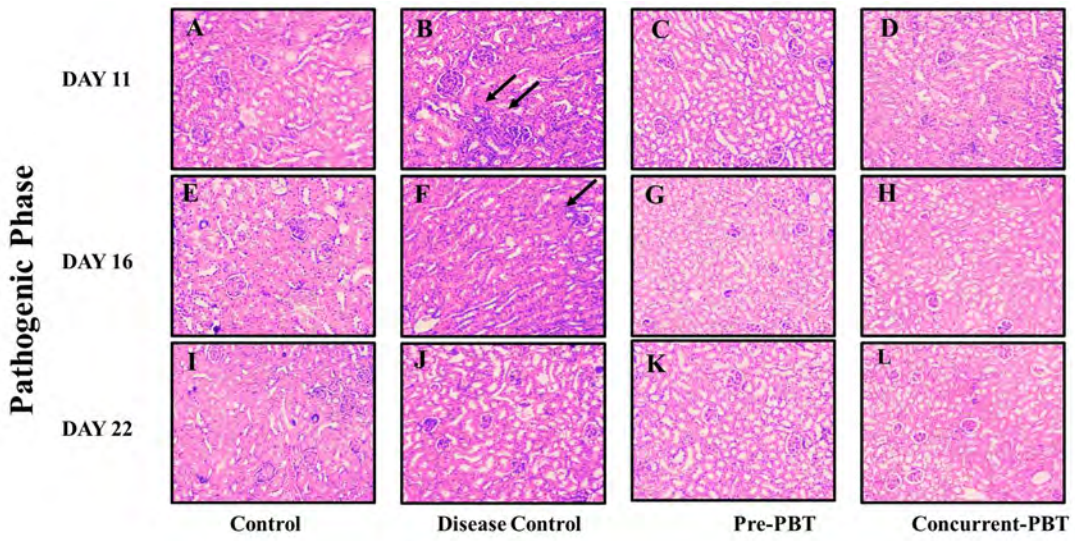

Figure 3.29. Hematoxylin and eosin staining (10X). The effect of *S. boulardii* on histopathological changes in Pathogenic phase on days 11, 16 and 22 **(A-L)** in Ovalbumin induced Atopic Dermatitis in mice.

The H and E staining of liver in curative phase is shown in Figure 3.30. These images indicated cellular infiltration and destruction of tissues in kidney sections of the DC group while this infiltration was reversed in the treatment groups.

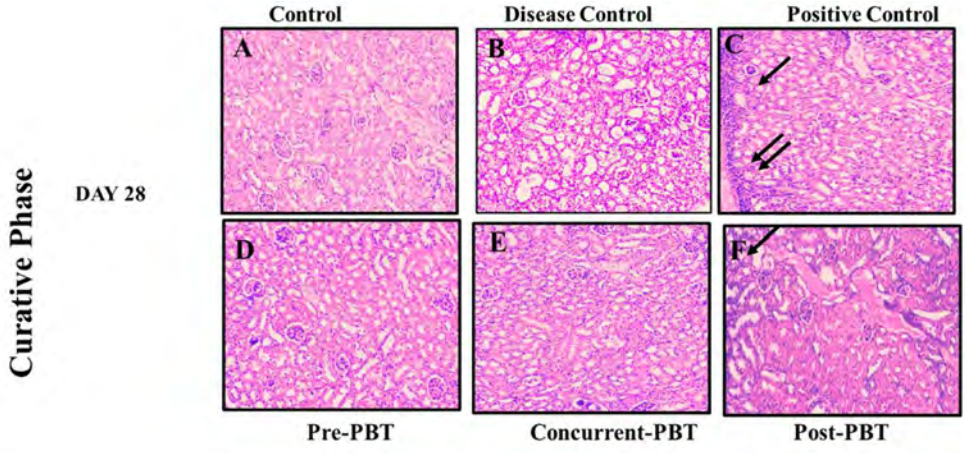

Figure 3.30. Hematoxylin and eosin staining (10X). The effect of *S. boulardii* on histopathological changes in Curative phase on day 28 **(A-F)** in Ovalbumin induced Atopic Dermatitis in mice.

3.31. Kidney Cells Infiltration

The H and E staining of kidney was quantified by measuring the cell infiltration in the different groups. On day 11, 16, 22 and 28, the inflammatory cell infiltration was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin than that of animals in the control group (group I). On day 11, treatment

with Pre-PBT ($p < 0.001$), Concurrent ($p < 0.001$), on day 16, treatment with Pre-PBT ($p < 0.001$) and Concurrent ($p < 0.01$) and on day 22, treatment with Pre-PBT ($p < 0.01$), Concurrent ($p < 0.01$) significantly decreased in infiltration when compared to DC group. On day 28, treatment with PC ($p < 0.05$), Pre-PBT ($p < 0.001$), Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly decreased in infiltration. (Figure 3.31)

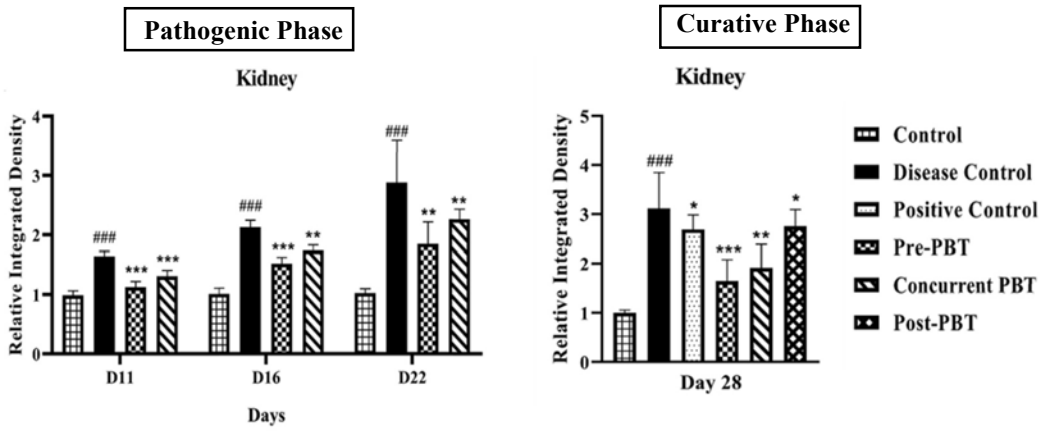

Figure 3.31. The effect of *S. boulardii* on Infiltration of inflammatory cells in Pathogenic phase on days 11, 16 and 22 **(A)** and Curative Phase on day 28 **(B)** in Ovalbumin induced Atopic Dermatitis in mice.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way and One-way ANOVA respectively using LSD test for comparison.*

3.32. Masson's Trichrome Staining

Masson's Trichrome staining of skin on day 28 is shown in Figure 3.32. These images indicated fibrosis and collagen deposition in skin sections of the DC group while this fibrosis was reversed in the treatment groups.

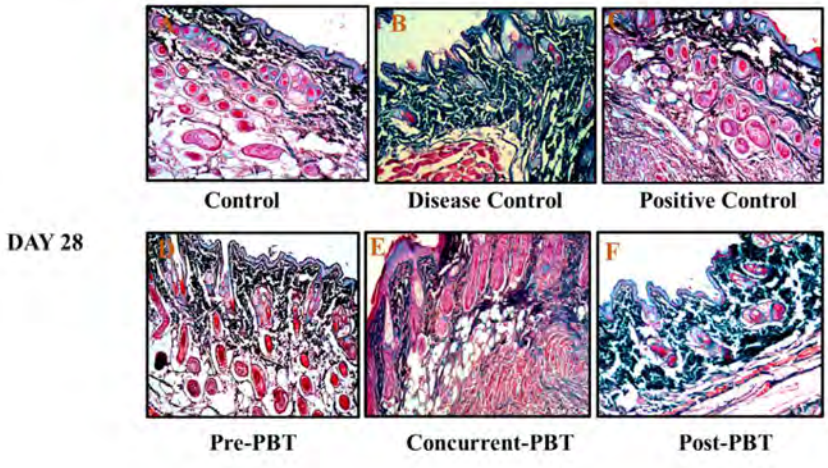

Figure 3.32. Trichome Staining sections of Skin (10X). The effect of *S. boulardii* on histopathological changes in Ovalbumin-induced Atopic Dermatitis in skin tissues of mice on day 28.

3.33. Relative Expression of TNF-α in Skin

Immunohistochemistry was done to examine the relative expression of TNF-α to access the inhibitory potential of Probiotic on ovalbumin-induced Atopic Dermatitis in skin sections. The relative expression of TNF-α was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly decreased relative expression of TNF-α when compared to DC group. (Figure 3.33)

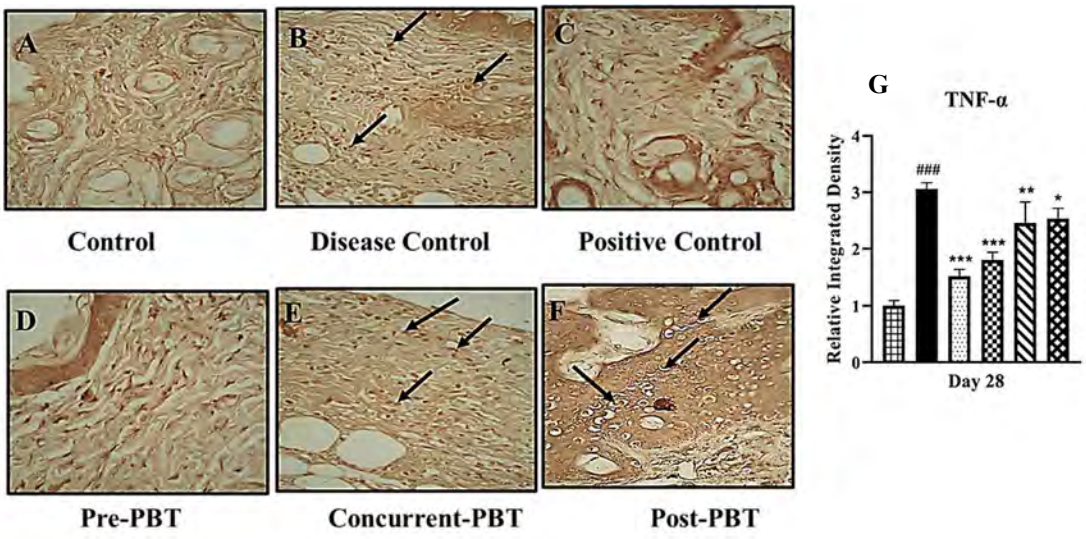

Figure 3.33. Visual representation of effect of *S. boulardii* on TNF-α expression in skin sections **(A-F)** and the relative expression measurement **(G)** on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.34. Relative Expression of NF-κB in Skin

Immunohistochemistry was done to examine the relative expression of NF-κB to access the inhibitory potential of Probiotic on ovalbumin-induced Atopic Dermatitis in skin sections. The relative expression of NF-κB was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT (p

< 0.01) and Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly decreased relative expression of NF-κB when compared to DC group. (Figure 3.34)

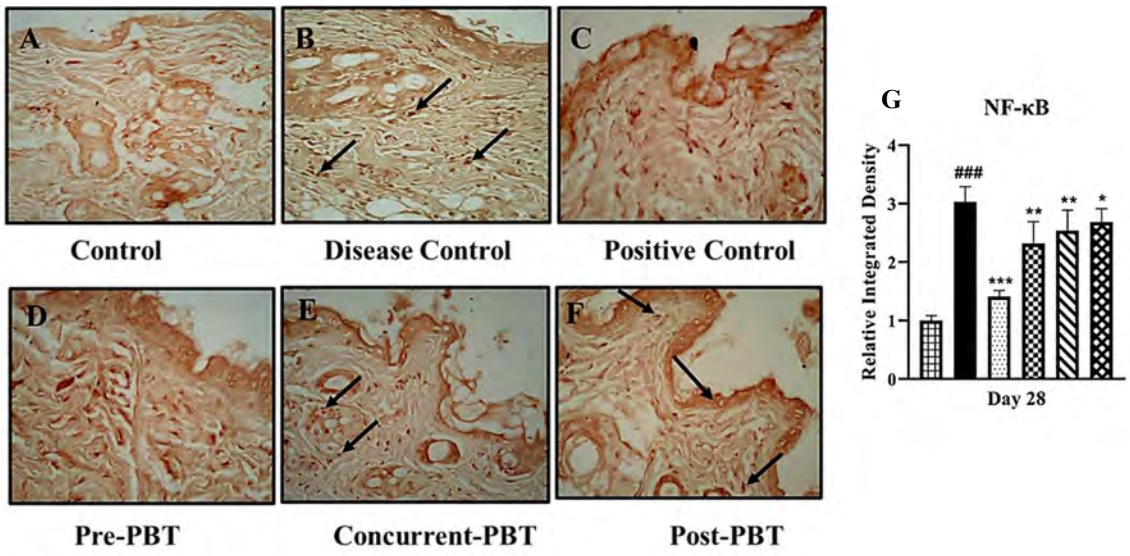

Figure 3.34. Visual representation of effect of *S. boulardii* on NF-κB expression in skin sections **(A-F)** and the relative expression measurement **(G)** on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.35. Relative Expression of IL-1β in Skin

Immunohistochemistry was done to examine the relative expression of IL-1β to access the inhibitory potential of Probiotic on ovalbumin-induced Atopic Dermatitis in skin sections. The relative expression IL-1β was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.001$) and Post-PBT ($p < 0.05$) significantly decreased relative expression of IL-1β when compared to DC group. (Figure 3.35)

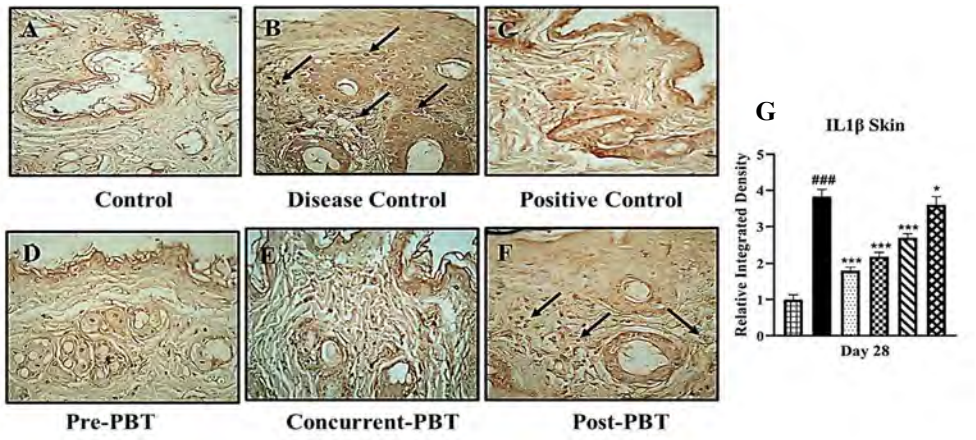

Figure 3.35. Visual representation of effect of *S. boulardii* on IL-1β expression in skin sections **(A-F)** and the relative expression measurement **(G)** on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison.*

3.36. Relative Expression of TNF-α in Spleen

Immunohistochemistry was done to examine the relative expression of TNF-α to access the inhibitory potential of Probiotic on ovalbumin-induced Atopic Dermatitis in spleen sections. The relative expression of TNF-α was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.05$) significantly decreased relative expression of TNF-α when compared to DC group. (Figure 3.36)

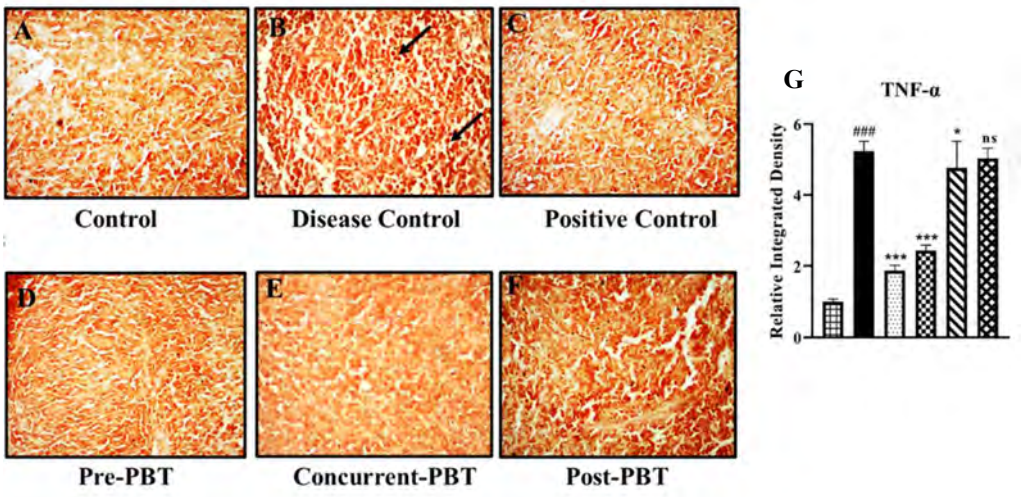

Figure 3.36. Visual representation of effect of *S. boulardii* on TNF-α expression in spleen sections **(A-F)** and the relative expression measurement **(G)** on day 28.

3.37. Relative expression of NF-κB in spleen

Immunohistochemistry was done to examine the relative expression of NF-κB to access the inhibitory potential of Probiotic on ovalbumin-induced Atopic Dermatitis in spleen sections. The relative expression of NF-κB was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.001$) and Post-PBT ($p < 0.05$) significantly decreased relative expression of NF-κB when compared to DC group. (Figure 3.37)

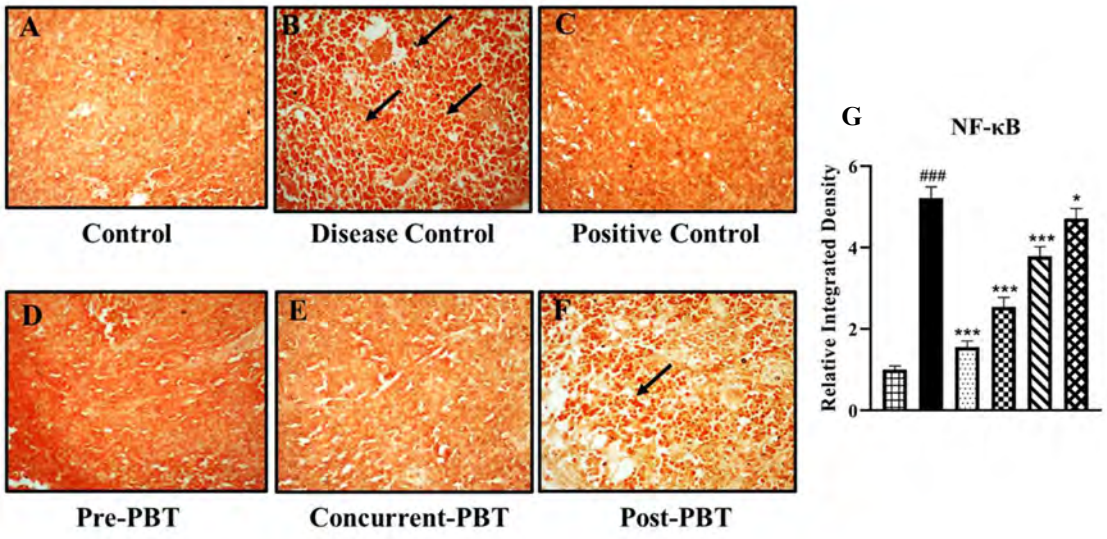

Figure 3.37. Visual representation of effect of *S. boulardii* on NF-κB expression in skin sections **(A-F)** and the relative expression measurement **(G)** on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.38. Relative Expression of IL-1β in Spleen

Immunohistochemistry was done to examine the relative expression of IL-1β to access the inhibitory potential of Probiotic on ovalbumin-induced Atopic Dermatitis in spleen sections. The relative expression IL-1β was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p <$

0.01) and Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly decreased relative expression of IL-1β when compared to DC group. (Figure 3.38)

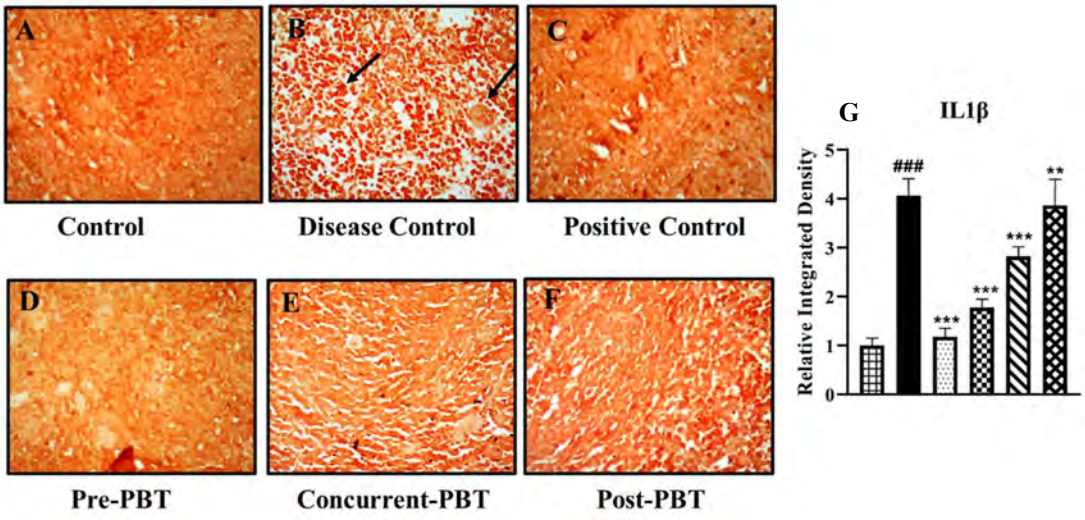

Figure 3.38. Visual representation of effect of *S. boulardii* on IL-1β expression in spleen sections **(A-F)** and the relative expression measurement **(G)** on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.39. Relative Expression of TNF-α in Colon

Immunohistochemistry was done to examine the relative expression of TNF-α to access the inhibitory potential of Probiotic on ovalbumin-induced Atopic Dermatitis in colon sections. The relative expression of TNF-α was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$), Concurrent-PBT ($p < 0.001$) and Post-PBT ($p < 0.05$) significantly decreased relative expression of TNF-α when compared to DC group. (Figure 3.39)

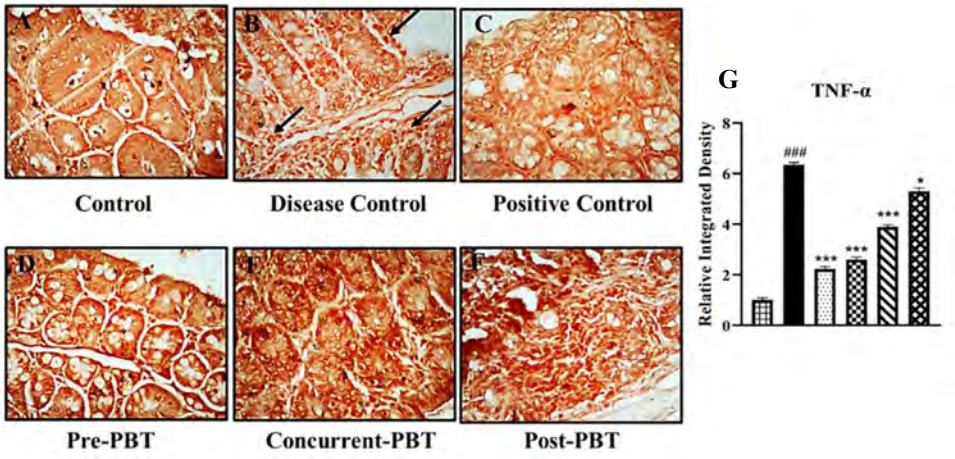

Figure 3.39. Visual representation of effect of *S. boulardii* on TNF-α expression in colon sections **(A-F)** and the relative expression measurement **(G)** on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) p < 0.05, (**) p < 0.01 and (***) p < 0.001 indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.40. Relative Expression of NF-κB in Colon

Immunohistochemistry was done to examine the relative expression of NF-κB to access the inhibitory potential of Probiotic in colon sections. The relative expression of NF-κB was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$). Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.01$) significantly decreased relative expression of NF-κB. (Figure 3.40)

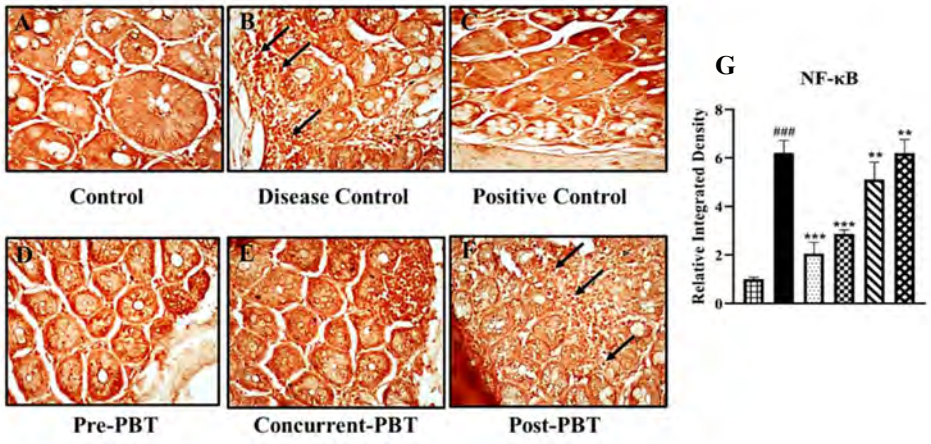

Figure 3.40. Visual representation of effect of *S. boulardii* on NF-κB expression in colon sections **(A-F)** and the relative expression measurement **(G)** on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison.*

3.41. Relative Expression of IL-1β in Colon

Immunohistochemistry was done to examine the relative expression of IL-1β to access the inhibitory potential of Probiotic on ovalbumin-induced Atopic Dermatitis in colon sections. The relative expression IL-1β was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.001$) and Post-PBT ($p < 0.01$) significantly decreased relative expression of IL-1β when compared to DC group. (Figure 3.41)

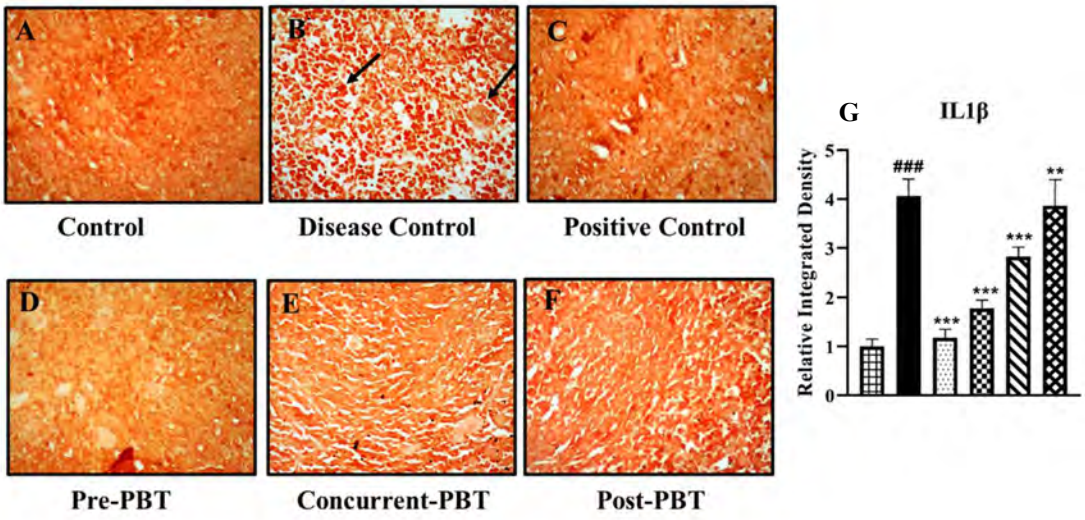

Figure 3.41. Visual representation of effect of *S. boulardii* on IL-1β expression in colon sections **(A-F)** and the relative expression measurement **(G)** on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.42. Relative Expression of TNF-α in Liver

Immunohistochemistry was done to examine the relative expression of TNF-α to access the inhibitory potential of Probiotic on ovalbumin-induced Atopic Dermatitis in liver sections. The relative expression of TNF-α was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.001$) and Post-PBT ($p < 0.01$) significantly decreased relative expression of TNF-α when compared to DC group. (Figure 3.42)

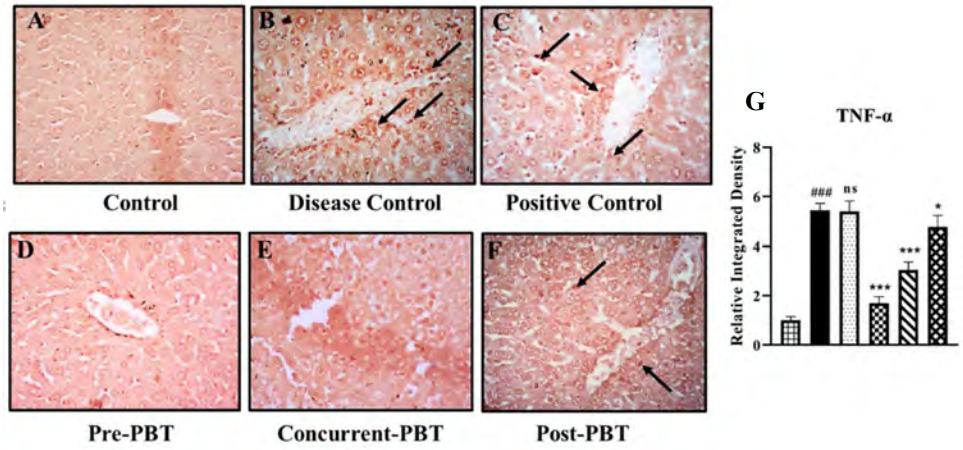

Figure 3.42. Visual representation of effect of *S. boulardii* on TNF-α expression in liver sections **(A-F)** and the relative expression measurement **(G)** on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) p < 0.05, (**) p < 0.01 and (***) p < 0.001 indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.43. Relative Expression of NF-κB in Liver

Immunohistochemistry was done to examine the relative expression of NF-κB to access the inhibitory potential of Probiotic on ovalbumin-induced Atopic Dermatitis in liver sections. The relative expression of NF-κB was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.05$), Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.001$) and Post-PBT ($p < 0.05$) significantly decreased relative expression of NF-κB when compared to DC group. (Figure 3.43)

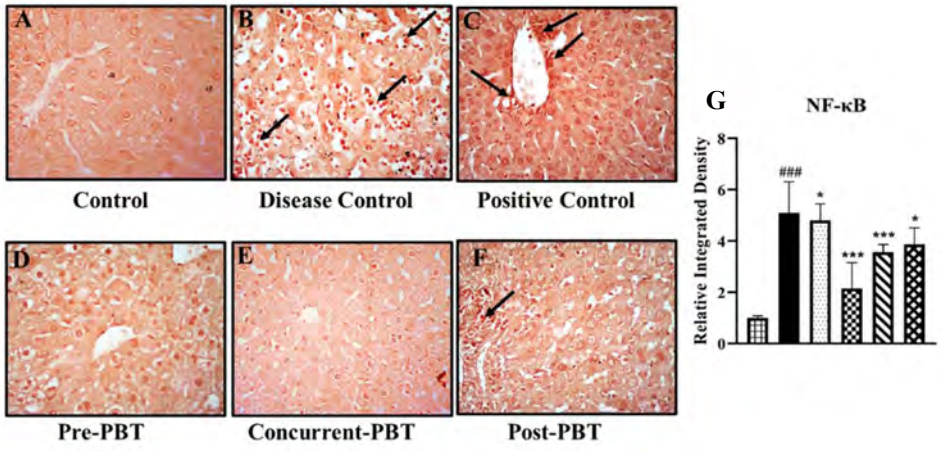

Figure 3.43. Visual representation of effect of *S. boulardii* on NF-κB expression in liver sections **(A-F)** and the relative expression measurement **(G)** on day 28.

3.44. Relative Expression of IL-1β in Liver

Immunohistochemistry was done to examine the relative expression of IL-1β to access the inhibitory potential of Probiotic on ovalbumin-induced Atopic Dermatitis in liver sections. The relative expression IL-1β was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.001$) significantly decreased relative expression of IL-1β when compared to DC group. (Figure 3.44)

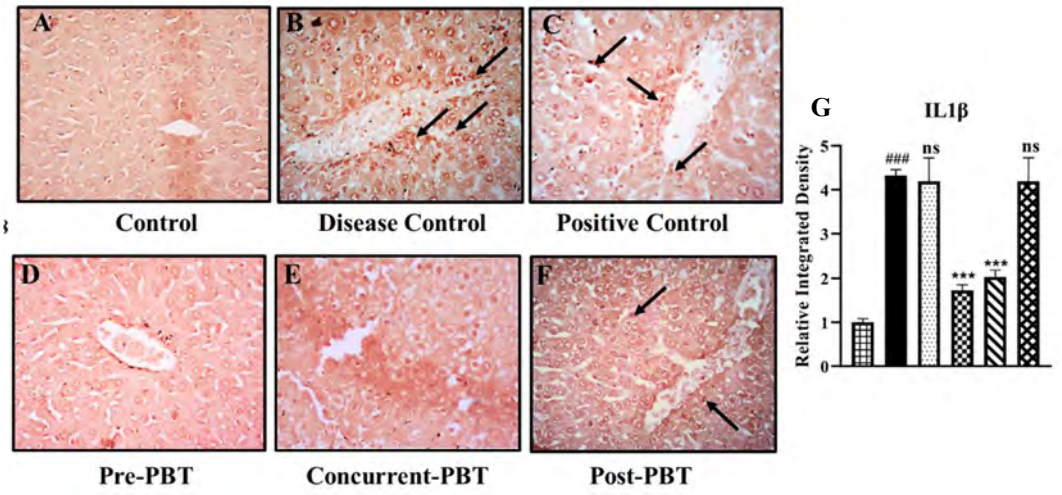

Figure 3.44. Visual representation of effect of *S. boulardii* on IL-1β expression in liver sections **(A-F)** and the relative expression measurement **(G)** on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.45. Assessment of Biochemical Parameters in Skin

The level of antioxidants and oxidative stress markers was evaluated in pathogenic and curative phases in skin, spleen, colon, liver, and kidneys. The effect of treatment groups was evaluated as compared to the DC group.

The level of antioxidants and oxidative stress markers was evaluated in pathogenic in skin is shown in Figure 3.45. The effect of treatment groups was evaluated as compared to the DC group. The level of antioxidants was found to be significantly ($p<0.001$) lower, and level of oxidative stress markers was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of

animals in the control group (group I). Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) significantly increased antioxidants level such as GSH, GST and CAT on day 11, 16 and 22 when compared to DC group. Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) significantly decreased level of MDA on day 11, 16 and 22 when compared to DC group. Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) significantly decreased level of NO on day 11, 16 and treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.05$) significantly decreased level of NO on day 22 when compared to DC group. (Figure 3.45)

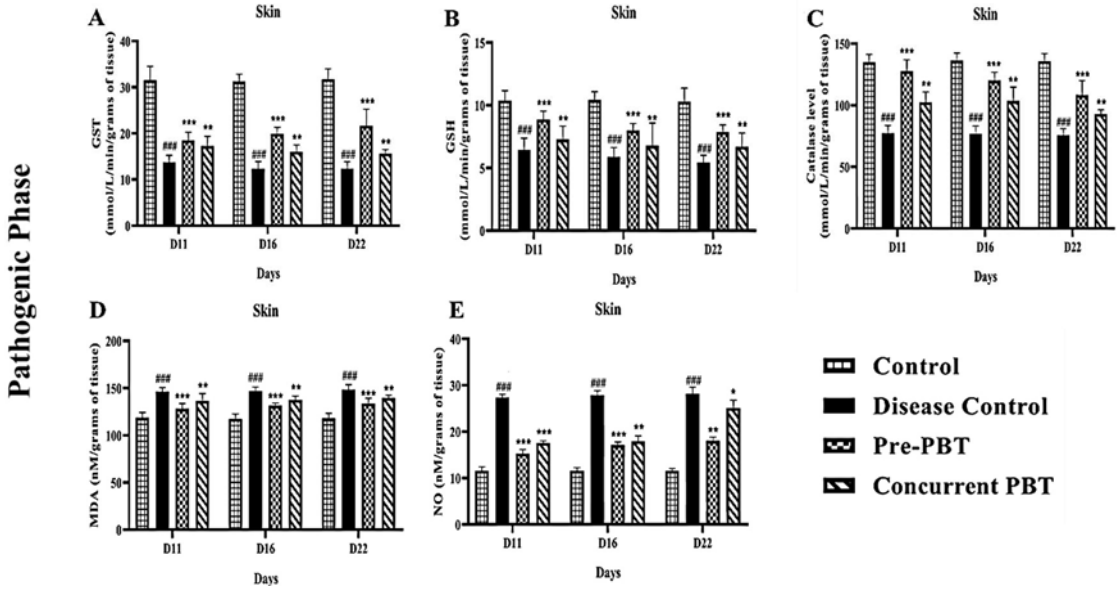

Figure 3.45. Effect of *S. boulardii* on levels of antioxidants and oxidative stress markers. GST **(A)**, GSH **(B)**, CAT **(C)**, MDA **(D)** and NO **(E)** in skin on days 11, 16 and 22.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

The level of antioxidants and oxidative stress markers was evaluated in curative in skin is shown in Figure 3.46. The effect of treatment groups was evaluated as compared to the DC group. The level of antioxidants was found to be significantly ($p<0.001$) lower, and level of oxidative stress markers was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$), Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly increased antioxidants level such as GSH, GST and CAT on day 28 when compared to DC group. Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$), Concurrent-PBT ($p < 0.01$) and Post-PBT ($p <$

0.05) significantly decreased level of oxidative stress markers such as MDA and NO on day 28 when compared to DC group. (Figure 3.46)

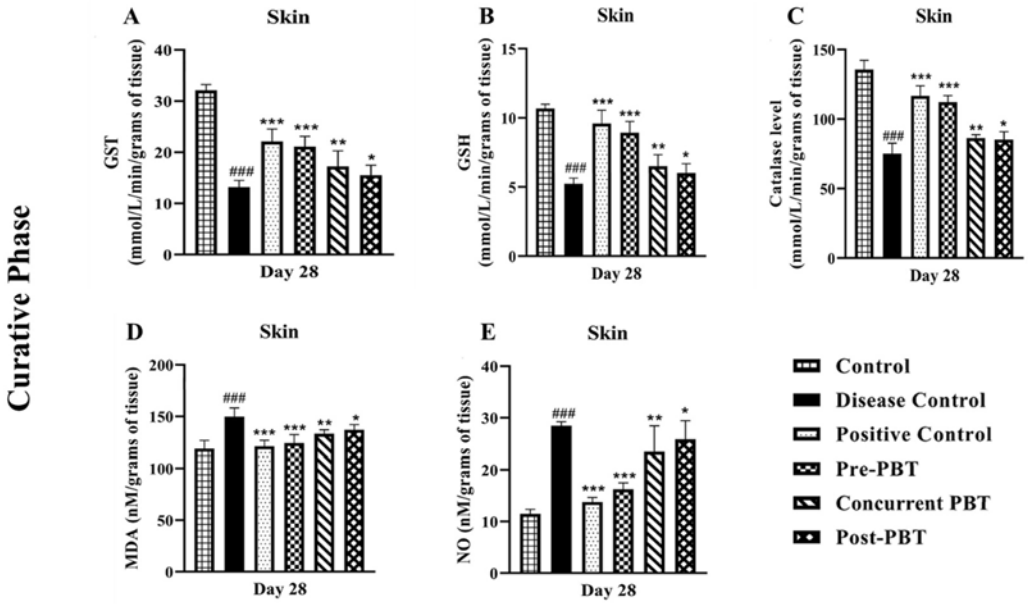

Figure 3.46. Effect of *S. boulardii* on levels of antioxidants and oxidative stress markers. GST **(A)**, GSH **(B)**, CAT **(C)**, MDA **(D)** and NO **(E)** in skin on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.47. Assessment of Biochemical Parameters in Spleen

The level of antioxidants and oxidative stress markers was evaluated in pathogenic in spleen is shown in Figure 3.47. The effect of treatment groups was evaluated as compared to the DC group. The level of antioxidants was found to be significantly ($p<0.001$) lower, and level of oxidative stress markers was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) significantly increased antioxidants level such as GSH, GST and CAT on days 11, 16 and 22 when compared to DC group. Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) significantly decreased level of oxidative stress markers such as MDA and NO on days 11, 16 and 22 when compared to DC group. (Figure 3.47)

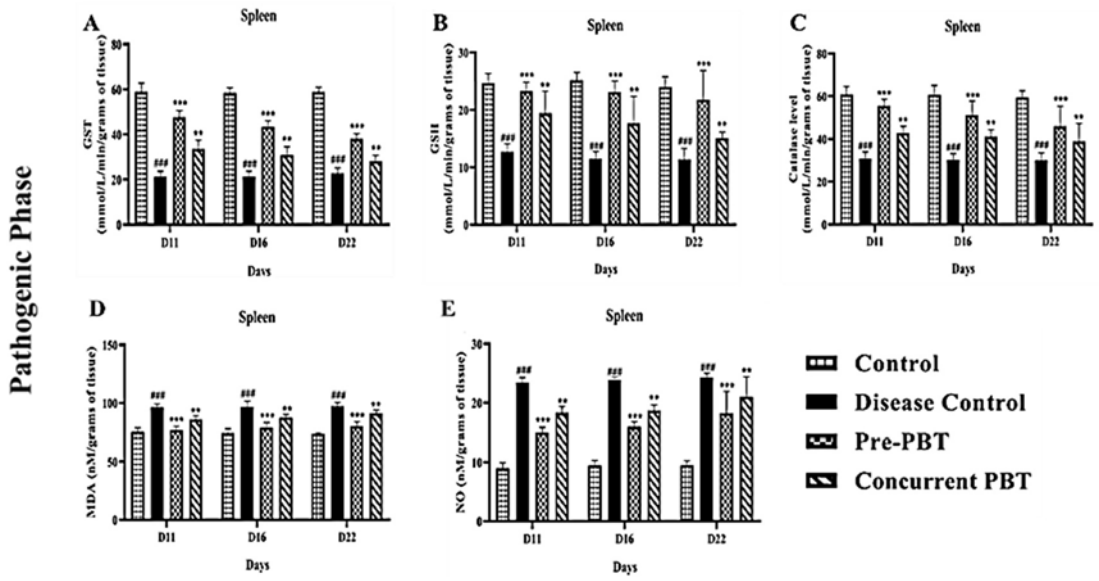

Figure 3.47. Effect of *S. boulardii* on levels of antioxidants and oxidative stress markers. GST **(A)**, GSH **(B)**, CAT **(C)**, MDA **(D)** and NO **(E)** in spleen on days 11, 16 and 22.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way using LSD test for comparison. (*) p < 0.05, (**) p < 0.01 and (***) p < 0.001 indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

The level of antioxidants and oxidative stress markers was evaluated in curative in spleen is shown in Figure 3.48. The effect of treatment groups was evaluated as compared to the DC group. The level of antioxidants was found to be significantly ($p<0.001$) lower, and level of oxidative stress markers was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$), Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly increased antioxidants level such as GSH, GST and CAT on day 28 when compared to DC group. Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$), Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly decreased level of oxidative stress markers such as MDA and NO on day 28 when compared to DC group.

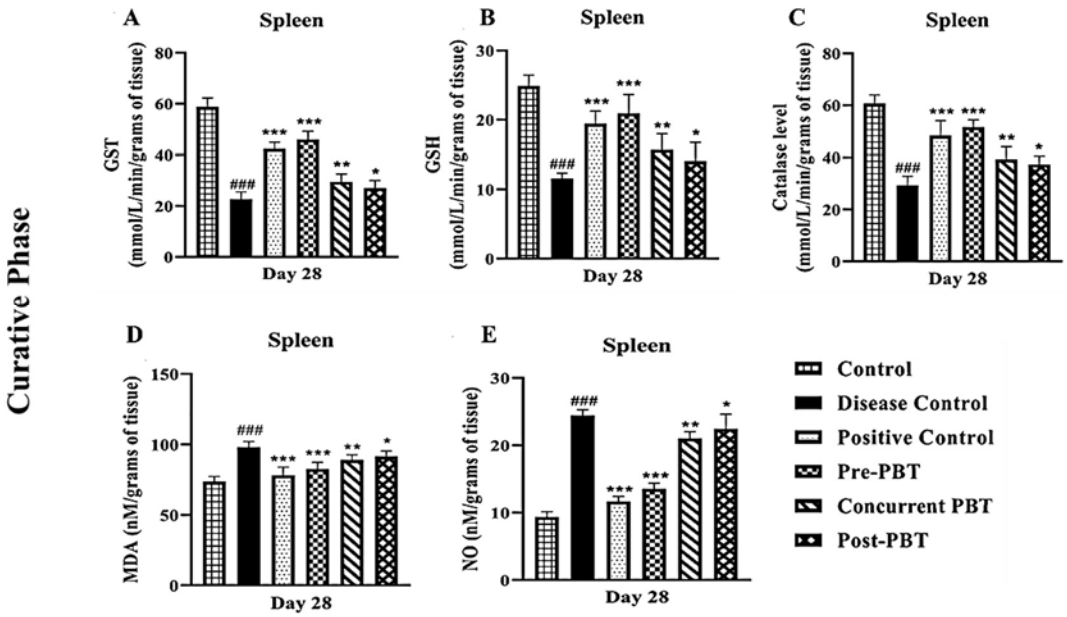

Figure 3.48. Effect of *S. boulardii* on levels of antioxidants and oxidative stress markers. GST **(A)**, GSH **(B)**, CAT **(C)**, MDA **(D)** and NO **(E)** in spleen on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) p < 0.05, (**) p < 0.01 and (***) p < 0.001 indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.49. Assessment of Biochemical Parameters in Colon

The level of antioxidants and oxidative stress markers was evaluated in pathogenic in colon is shown in Figure 3.49. The effect of treatment groups was evaluated as compared to the DC group. The level of antioxidants was found to be significantly ($p<0.001$) lower, and level of oxidative stress markers was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) on day 11, Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.05$) on days 16 and 22 significantly increased the level of GST. Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) on days 11 and 16, Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.05$) on day 22 significantly increased the level of GSH. Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.05$) on days 11, 16 and 22 significantly increased the level of CAT. Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) significantly decreased level of oxidative stress markers such as MDA and NO on days 11, 16 and 22 when compared to DC group.

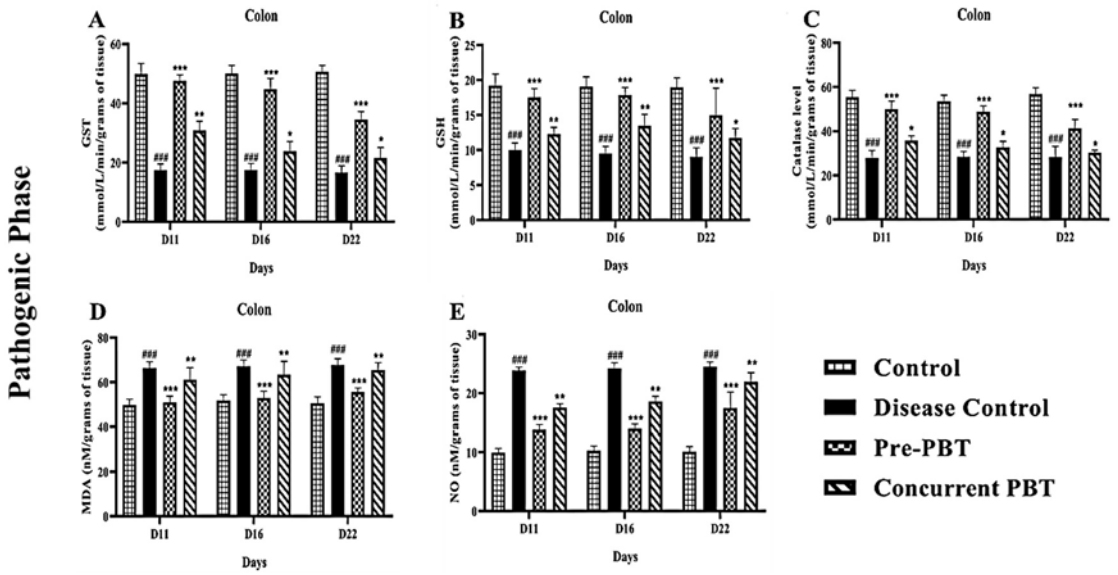

Figure 3.49. Effect of *S. boulardii* on levels of antioxidants and oxidative stress markers. GST **(A)**, GSH **(B)**, CAT **(C)**, MDA **(D)** and NO **(E)** in colon on days 11, 16 and 22.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way using LSD test for comparison. (*) p < 0.05, (**) p < 0.01 and (***) p < 0.001 indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

The level of antioxidants and oxidative stress markers was evaluated in curative in colon is shown in Figure 3.50. The effect of treatment groups was evaluated as compared to the DC group. The level of antioxidants was found to be significantly ($p<0.001$) lower, and level of oxidative stress markers was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$), Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly increased antioxidants level such as GSH, GST and CAT and significantly decreased level of oxidative stress markers such as MDA and NO on day 28 when compared to DC group.

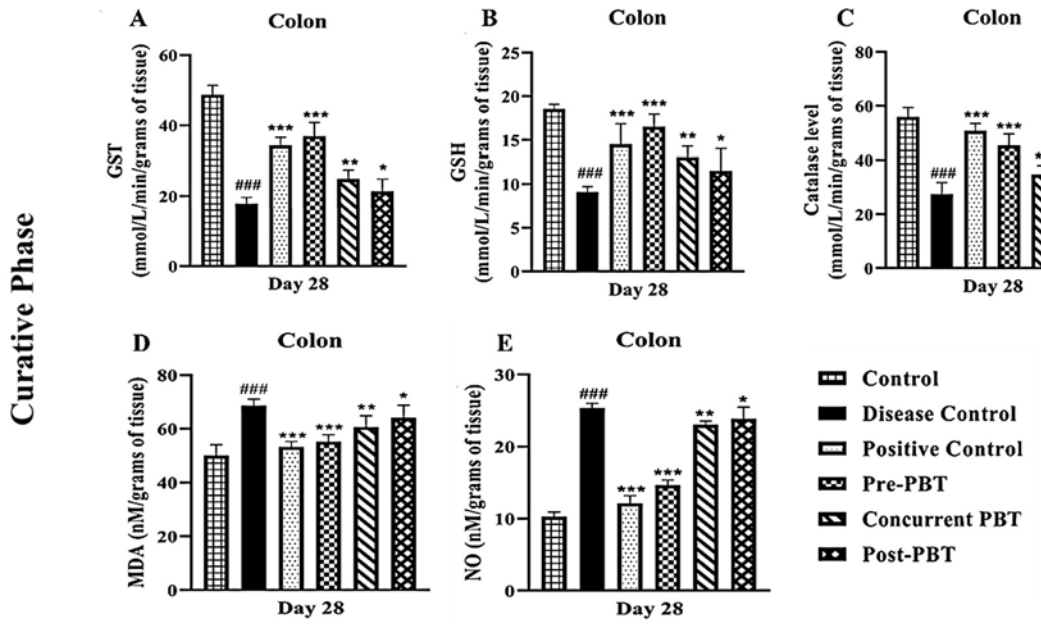

Figure 3.50. Effect of *S. boulardii* on levels of antioxidants and oxidative stress markers. GST **(A)**, GSH **(B)**, CAT **(C)**, MDA **(D)** and NO **(E)** in colon on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) p < 0.05, (**) p < 0.01 and (***) p < 0.001 indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.51. Assessment of Biochemical Parameters in Liver

The level of antioxidants and oxidative stress markers was evaluated in pathogenic in liver is shown in Figure 3.51. The effect of treatment groups was evaluated as compared to the DC group. The level of antioxidants was found to be significantly ($p<0.001$) lower, and level of oxidative stress markers was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) on days 11,16 and 22 significantly increased the level of GST. Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) on day 11, Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.05$) on days 16 and 22 significantly increased the level of GSH and CAT when compared to DC group. Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) significantly decreased level of oxidative stress markers such as MDA and NO on days 11, 16 and 22 when compared to DC group.

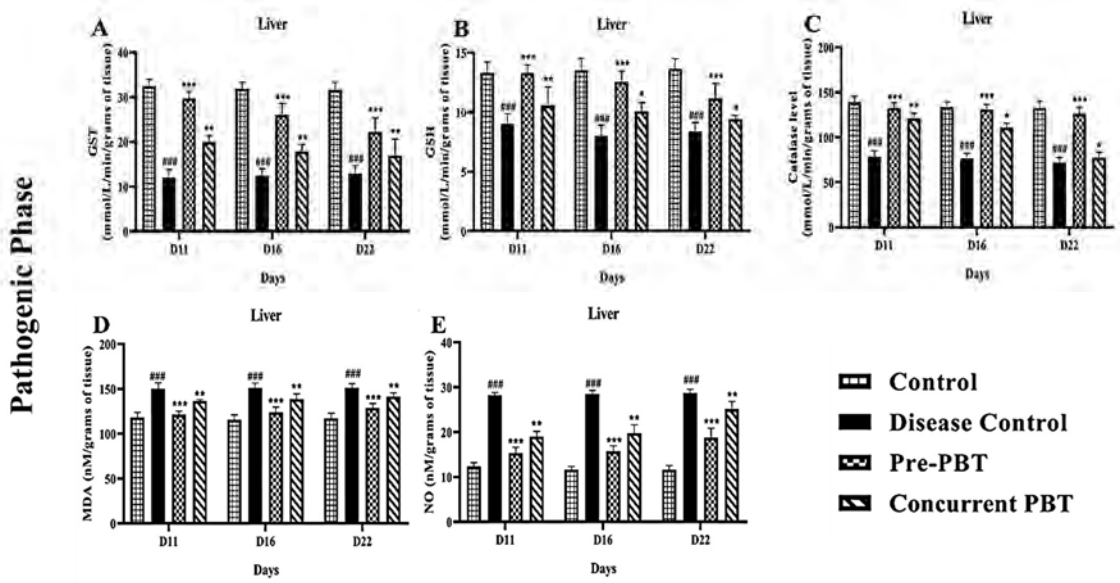

Figure 3.51. Effect of *S. boulardii* on levels of antioxidants and oxidative stress markers. GST **(A)**, GSH **(B)**, CAT **(C)**, MDA **(D)** and NO **(E)** in liver on days 11, 16 and 22.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

The level of antioxidants and oxidative stress markers was evaluated in curative in liver is shown in Figure 3.52. The effect of treatment groups was evaluated as compared to the DC group. The level of antioxidants was found to be significantly ($p<0.001$) lower, and level of oxidative stress markers was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$), Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly increased antioxidants level such as GSH, GST and CAT and significantly decreased level of oxidative stress markers such as MDA and NO on day 28 when compared to DC group.

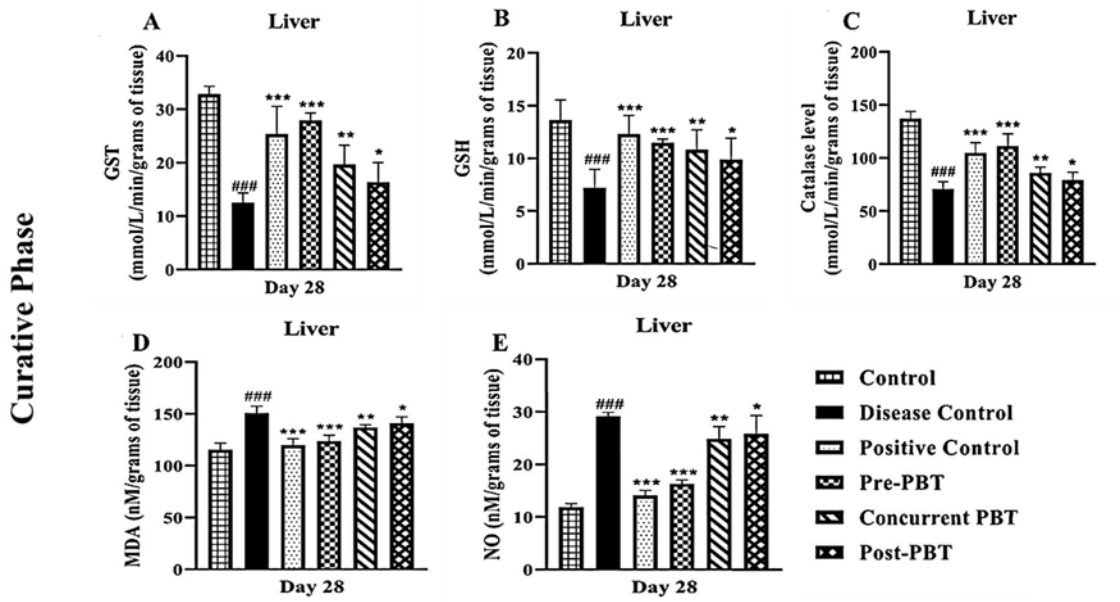

Figure 3.52. Effect of *S. boulardii* on levels of antioxidants and oxidative stress markers. GST **(A)**, GSH **(B)**, CAT **(C)**, MDA **(D)** and NO **(E)** in liver on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) p < 0.05, (**) p < 0.01 and (***) p < 0.001 indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.53. Assessment of Biochemical Parameters in Kidney

The level of antioxidants and oxidative stress markers was evaluated in pathogenic in kidney is shown in Figure 3.53. The effect of treatment groups was evaluated as compared to the DC group. The level of antioxidants was found to be significantly ($p<0.001$) lower and level of oxidative stress markers was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) on days 11,16 and 22 significantly increased the level of GST and CAT. Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) on days 11 and 16, Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.05$) on day 22 significantly increased the level of GSH when compared to DC group. Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) significantly decreased level of oxidative stress markers such as MDA and NO on days 11, 16 and 22 when compared to DC group.

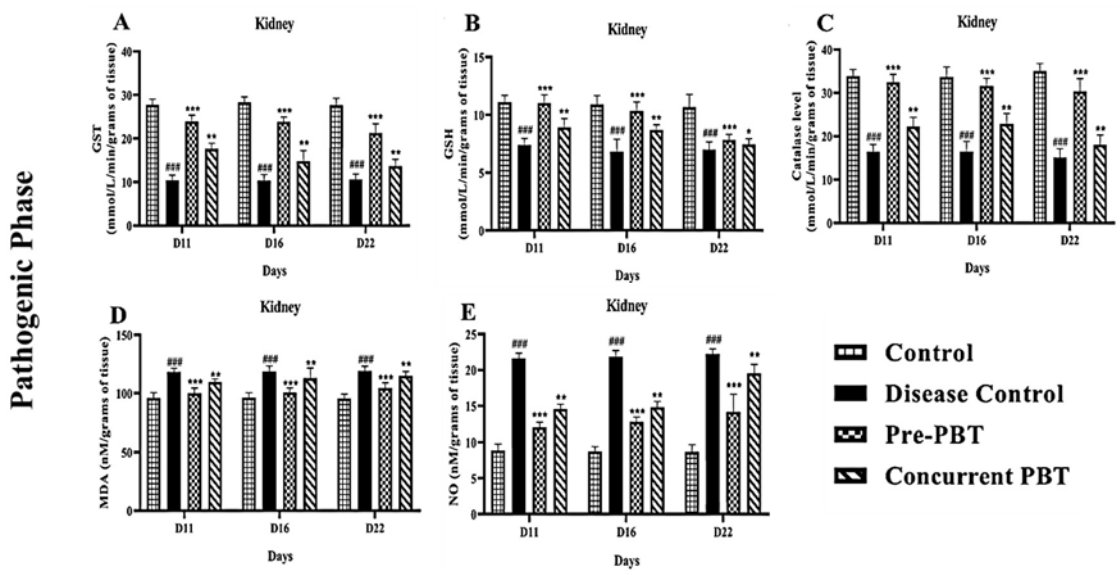

Figure 3.53. Effect of *S. boulardii* on levels of antioxidants and oxidative stress markers. GST **(A)**, GSH **(B)**, CAT **(C)**, MDA **(D)** and NO **(E)** in kidneys on days 11, 16 and 22.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by Two-way using LSD test for comparison. (*) p < 0.05, (**) p < 0.01 and (***) p < 0.001 indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

The level of antioxidants and oxidative stress markers was evaluated in curative in kidneys is shown in Figure 3.54. The effect of treatment groups was evaluated as compared to the DC group. The level of antioxidants was found to be significantly ($p<0.001$) lower, and level of oxidative stress markers was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$), Concurrent-PBT ($p < 0.01$) and Post-PBT ($p < 0.05$) significantly increased antioxidants level such as GSH, GST and CAT and significantly decreased level of NO on day 28 when compared to DC group. Treatment with PC ($p < 0.001$), Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) significantly decreased level of MDA on day 28 when compared to DC group.

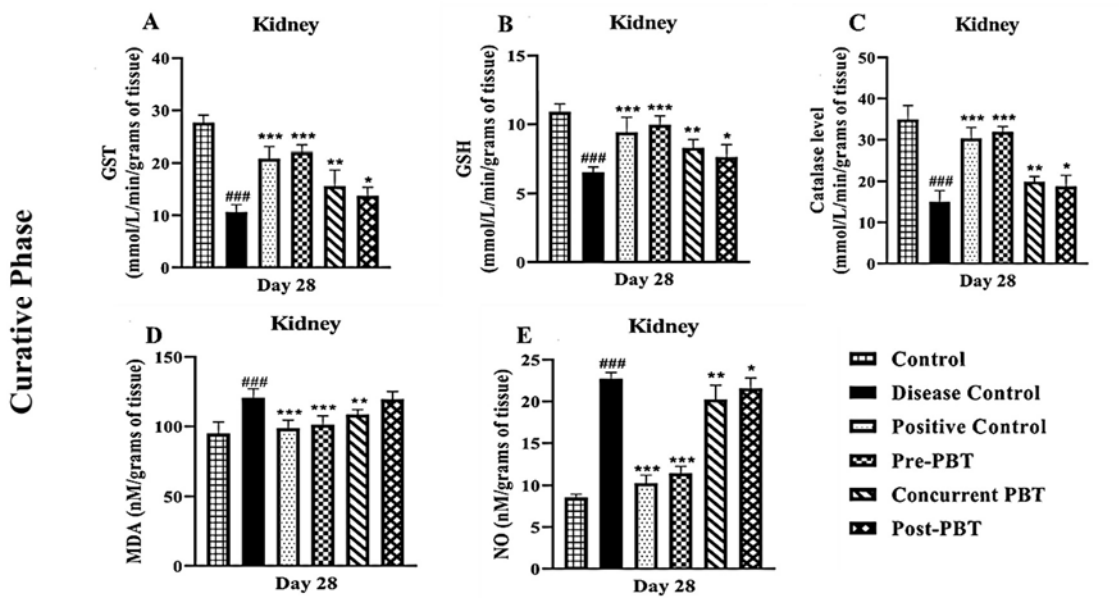

Figure 3.54. Effect of *S. boulardii* on levels of antioxidants and oxidative stress markers. GST **(A)**, GSH **(B)**, CAT **(C)**, MDA **(D)** and NO **(E)** in liver on day 28.
Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

3.55. Assessment of IgE through ELISA Kit

The level of IgE was evaluated in pathogenic phase on day 22 and curative phase on day 28 as shown in Figure 3.10. The effect of treatment groups was evaluated as compared to the DC group. The level of IgE was found to be significantly ($p<0.001$) higher in the DC group animals sensitized and challenged by ovalbumin (group II) than that of animals in the control group (group I). Treatment with Pre-PBT ($p < 0.001$) and Concurrent-PBT ($p < 0.01$) significantly decreased the level of IgE on day 22. Treatment with PC($p < 0.001$), Pre-PBT ($p < 0.001$), Concurrent-PBT ($p < 0.01$) AND Post-PBT ($p < 0.05$) significantly decreased level of IgE when compared to DC group.

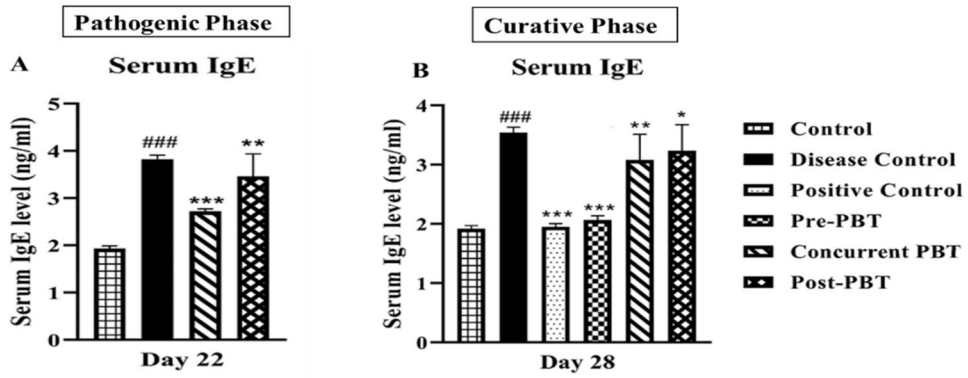

Figure 3.55. Effect of *S. boulardii* on level of IgE in serum. Serum IgE on day 22 **(A)** and Serum IgE on day 28 **(B).**

Note: *Data sets were expressed as mean±S.D (n=5), analyzed by One-way ANOVA respectively using LSD test for comparison. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates significant difference of each treatment compared to the Disease Control group. (###) indicates comparison of Disease Control with the Control group.*

CHAPTER 4

DISCUSSION

4. DISCUSSION

The current study was aimed to observe the anti-inflammatory and immune regulating effect of probiotic *Saccharomyces boulardii*. Atopic Dermatitis model induced by ovalbumin administration. AD is a complex disease whose cure is still not developed. Symptomatic treatment is given to cope with the disease and corticosteroids and immunosuppressants are still in use.

For this purpose, there is a need for novel treatment by understanding the underlying mechanism of AD for the proper management of this disease so that the quality of life can be improved (Feldman *et al.*, 2019). The literature study showed that *S. boulardii* has been proved to be an effective anti-inflammatory agent having antimicrobial, enzymatic, metabolic, antioxidant and antitoxin activity (Billoo *et al.*, 2006).

In the current study, Atopic Dermatitis was induced by Ovalbumin and the symptoms of redness, scaling, swelling, and erosion was visible (Na *et al.*, 2020). However, the progression of the disease was reversed by the oral administration of *S. boulardii.* Clinical Atopic Dermatitis scoring, scratching behavior and assessment of spleen index, relative liver and kidney weights were carried out to determine the effectiveness of the response to the treatment (Rossbach *et al.*, 2016). In the current study, the behavioral parameters were improved markedly in the treatment groups especially the pre-PBT group receiving probiotic seven days prior to the administration of ovalbumin. The Dexamethasone group animals also show improvement in behavioral parameters. The body weights of all the animals in each group were measured during acclimation and on day 11, 16, 22 and 28. Dexamethasone suppressed the inflammation. This suppression affects the metabolic rate and leads to weight loss while PBT flourished the gut microbiota and lead to slightly increased in body weight.

Relative liver and kidney weights remained unaltered indicating that no environmental factor or hepatotoxicity was induced. (Kim *et al.*, 2012). The hematological parameters were determined showing marked leukocytosis in the Ovalbumin diseased group and PBT-treated groups showed marked decrease in inflammation by reducing WBCs and the level of eosinophils (Kim *et al.*, 2022).

Serum biochemistry was carried out to evaluate alterations in total protein level and functioning of important organs such as liver and kidneys (Jo *et al.*, 2018). The results

showed altered level of proteins especially globulins level was raised in the diseased animals which the PBT-treated groups reverse this alteration in the order Pre-PBT, Concurrent-PBT and Post-PBT. Dexamethasone group also showed effective treatment. The level of serum creatinine was carried out to determine the effect on liver enzymes and creatinine level. The Dexamethasone group slightly altered the normal functioning of liver while kidney functioning did not alter significantly (Coavoy-Sánchez *et al.*, 2023). The PBT- treated groups did not alter the normal functioning of organs.

The histological study was conducted by performing H and E staining to provide detailed information about the histological changes of the tissues. Skin, spleen, colon, liver, and kidney tissues were analyzed to observe infiltration. Pre-PBT and Dexamethasone groups effective reduction in infiltration caused by Ovalbumin in skin, spleen, and colon tissues. Skin tissue histology was used to observe epidermal and dermal thickness. Ovalbumin induced atopic dermatitis significantly altered the thickness. The PBT-treated groups improved the results markedly by decreasing the epidermal and dermal thickness (Na *et al.*, 2020).

Unlike PBT-treated groups, the infiltration was increased markedly in the Dexamethasone group. Masson trichome staining showed enhanced collagen deposition and inflammatory cell infiltration in diseased skin tissue, whereas the treatment groups reversed the effect and suppressed the inflammation (Kim *et al.*, 2021).

The TNF-α/NF-κB signaling (Wu and Zhou, 2010) have significantly played their role in the development of atopic dermatitis attack. Many studies reported the downregulation of already mentioned signaling pathways alleviated the symptoms of AD, and reduced itching and redness. The Ovalbumin induction has markedly increased the level of expression of the TNF-α (Danso *et al.*, 2014), NF-κB (Ko *et al.*, 2022) and IL-1β (Yeung *et al.*, 2021) when analyzed by using immunohistochemistry of skin, spleen and colon. Treatment groups markedly decreased the expression level of these important markers of inflammation. However, Dexamethasone showed an increase in expression of markers in liver tissue.

Moreover, the ovalbumin induction significantly increased the oxidative stress level and compromised the antioxidant level of the body. The effect of PBT was investigated on the

level of NO and MDA. The PBT-treated groups showed reduction in the production of the NO and MDA significantly compared to the Ovalbumin group (Jia *et al.*, 2023).

The PBT groups significantly improved the antioxidant level in skin, spleen, colon, liver, and kidney tissues compared to Ovalbumin group. The level of GSH, GST and Catalase was evaluated in ovalbumin induced atopic dermatitis model as reported by (Sharma *et al.*, 2020). Ovalbumin administration significantly reduced the level of antioxidants in tissue homogenates obtained from all the organs. The ELISA analysis was performed to evaluate the level of IgE level in the serum (Wang *et al.*, 2007). The results of the analysis showed the significant increase in the level of IgE in diseased animals. However, the PBT- treated groups along with the Dexamethasone group showed improvement in the inflammation caused by Ovalbumin.

The results showed that intervention of PBT significantly improved the oxidative and antioxidant balance. Similarly, dexamethasone also improved this barrier. The order in which the PBT groups improved the inflammation are Pre-PBT, Concurrent-PBT and Post-PBT where Pre-group showed most effective results. To summarize the effect of ovalbumin in atopic dermatitis is that ovalbumin causes epidermal barrier disruption and activates keratinocytes which release IL-1β, IL-25, IL-33 and TSLP which then activate dendritic cells which stimulate Th2 cells for the production of IL-4, IL-5, IL-13, IL31 and IL33. This causes barrier dysfunction, attraction of eosinophils to the site of allergen entry, IgE production and causes pruritis and chronic skin inflammation and itching symptoms (Kim *et al.*, 2019). *S. boulardii* reverse the progression of the disease. The probiotics, when administered orally, provide the desired beneficial effects to the host. They regulate the immune system by providing a barrier for pathogen absorption. The mechanism of action is that they bind with the gastrointestinal cells and a as results pathogens are competitively eliminated from the body (Wieërs *et al.*, 2020).

This study shows that *S. boulardii* reduces chronic inflammation in ovalbumin-induced AD by lowering inflammatory cytokines, including TNF-α, IL-1β, and NF-κβ as well as decreased the level IgE. PBT provided protection against ROS by enhancing the level of antioxidants. *S. boulardii* improves the behavioral characteristics that were changed by Ovalbumin. Dexa is not safe at 5 mg/kg/day because it damages the liver's and kidneys' ability to function as antioxidants. Therefore, the research conducted thus far has

indicated that *S. boulardii* might be a potential addressing long-term inflammatory conditions such as AD. Therefore, more study is needed to precisely understand its mechanism and usage.

CONCLUSIONS

- Using an Ovalbumin-induced model of Atopic Dermatitis (AD), the study sought to examine Saccharomyces boulardii immune-regulating and anti-inflammatory properties.
- Ovalbumin caused AD, which manifested as symptoms, and S. boulardii treatment stopped the disease's advancement. Significant improvements were seen in behavioral indicators, particularly in the Pre-PBT group, indicating the efficacy of probiotic therapy.
- Hematological measures showed a reduction in white blood cells and eosinophils, as well as a decrease in inflammation in the PBT-treated groups.
- Serum biochemistry showed that, in contrast to Dexamethasone, PBT-treated groups were able to reverse changes in protein levels brought on by ovalbumin.
- Histological investigations showed that PBT effectively decreased thickness and infiltration in a range of tissues.
- All therapy groups dramatically decreased TNF-α/NF-κB signaling pathways, which are implicated in the pathogenesis of AD, apart from Dexamethasone in the liver.
- The injection of ovalbumin exacerbated oxidative stress, but PBT therapy decreased NO and MDA generation. PBT groups markedly increased antioxidant levels across a range of tissues, highlighting its effect on the balance between oxidative and antioxidant processes.
- Ovalbumin raised IgE levels, which are a sign of inflammation; however, PBT and Dexamethasone therapies reduced these levels. S. boulardii showed anti-inflammatory properties by lowering IgE and cytokine levels, indicating that it may be able to treat chronic inflammatory diseases like AD.

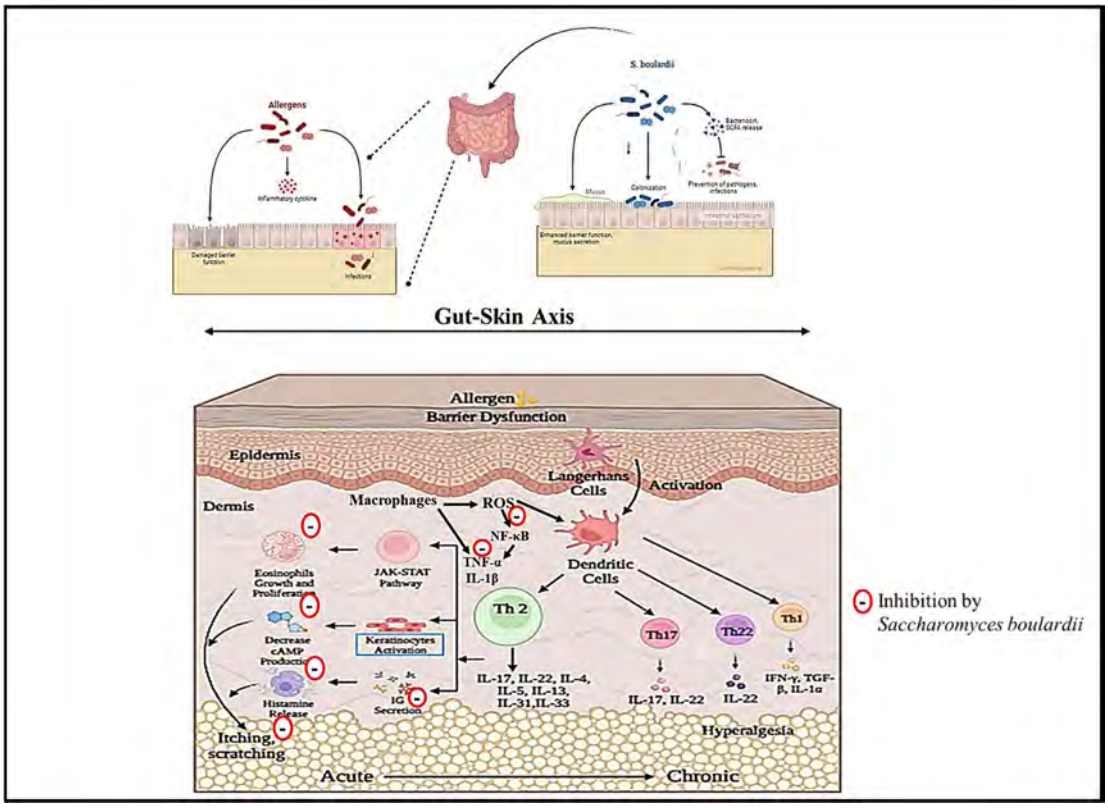

Figure 4.1. Proposed molecular mechanism of *S. boulardii* in ovalbumin-induced AD.

FUTURE PROSPECTIVES

- Conducting clinical trials to validate the effectiveness and safety of *Saccharomyces boulardii* in human subjects with AD. This step is important for translating preclinical findings into practical therapeutic applications.
- Investigating molecular mechanisms and underlying immunomodulatory effects of *Saccharomyces boulardii* as well as specific pathways, signaling molecules, and cellular interactions involved in the modulation of AD symptoms will enhance our understanding and may lead to more targeted therapies.
- Investigating the potential synergistic effects of *Saccharomyces boulardii* when combined with existing AD treatments or other immunomodulatory agents.

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Annexure I: Approval from Bioethics Committee

قائداعظم یونیورسٹی
QUAID-I-AZAM UNIVERSITY
Faculty of Biological Sciences
Bioethics Committee

No. #BEC-FBS-QAU2023-499 Dated: 30-05-2023

Ms. Parveen Akhtar Buttar
C/O Dr. Muhammad Khalid Tipu
Department of Pharmacy,
Faculty of Biological Sciences,
Quaid-I-Azam University, Islamabad
45320, Pakistan

Subject: - **"*Saccharomyces boulardii* Ameliorate the Inflammation in Ovalbumin-Induced Atopic Dermatitis."**

Dear Ms. Parveen Akhtar Buttar,

We wish to inform you that your subject research study has been reviewed and is hereby granted approval for implementation by Bio-Ethical Committee (BEC) of Quaid-i-Azam University, Your study has been assigned protocol **#BEC-FBS-QAU2023-499.**

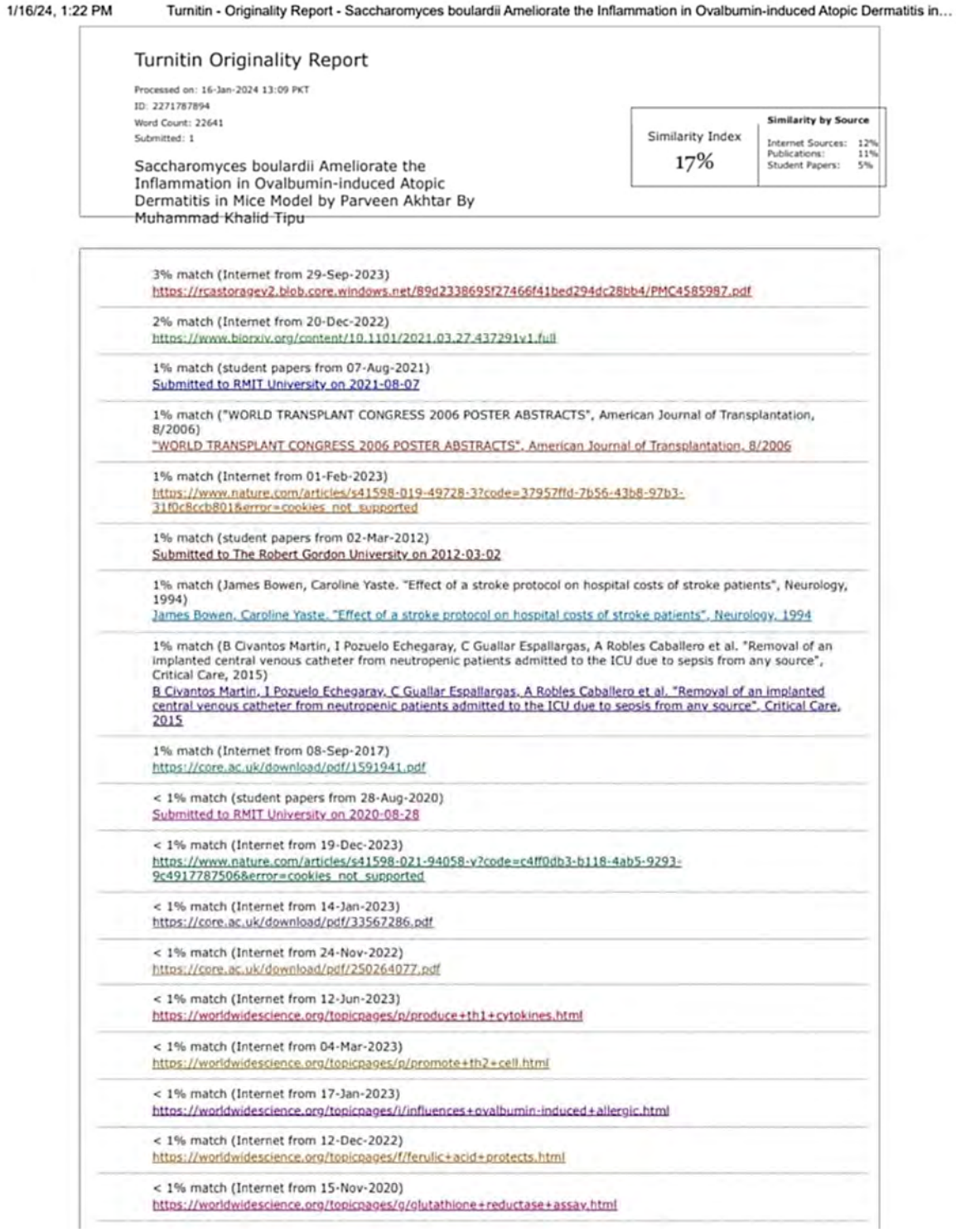
While the study is in progress, please inform us of any adverse events or new, relevant information about risks associated with the research. In case changes have to be made to the study procedure, the informed consent from and or informed consent process, the BEC must review and approve any of these changes prior to implementation.

Sincerely,

Prof. Dr. Sarwat Jahan
Department of Zoology

cc:
Dean, F.B.S

Annexure II: Turnitin Similarity Index Report



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