

**KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,
KUMASI, GHANA**

**IMMUNE SYSTEM MODULATORY EFFECTS OF STIGMASTEROL IN
MURINE MODELS**

By

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Pharmaceutical Sciences, College of Health Sciences in partial fulfilment of the
requirements for the degree of

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NOVEMBER, 2018

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any degree or diploma at Kwame Nkrumah University of Science and Technology, Kumasi or any other educational institution, except where due acknowledgment is made in the thesis.

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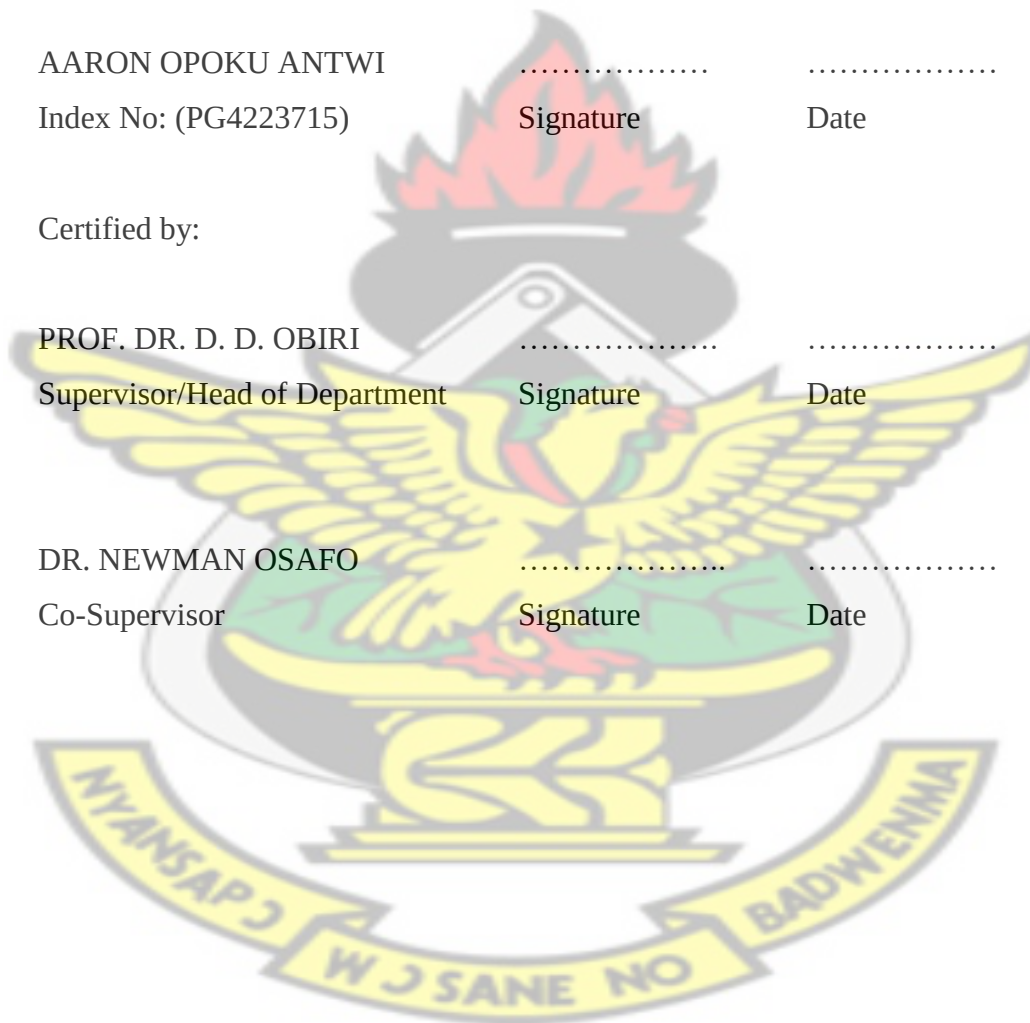
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I thank God for all his providence and mercies throughout this journey.

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DEDICATION

Dedicated to the memory of the best man I have ever known: Mr. Kwaku Antwi-Boasiako.

Your sacrifices were not and will never be in vain.

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ABSTRACT

Stigmasterol, a naturally occurring steroid alcohol belongs to a class of plant-derived compounds known as phytosterols. The therapeutic potential of stigmasterol in selected innate and adaptive immune responses, disease models of allergy as well as its immunotoxicity was assessed. The effects of stigmasterol on lipopolysaccharide (LPS)-induced febrile response, inflammatory cell proliferation, multiple organ damage and mortality were investigated. The anti-allergic potential of stigmasterol was assessed in immunogenic and non-immunogenic mast cell degranulation and in models of local and systemic mast cell-mediated responses. The suppressive effects of stigmasterol on compound 48/80-induced pruritus, 12-O-Tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation and its modulatory effects on chronic allergen-induced airway inflammation were also studied. The effects of stigmasterol on indices of general and immune system toxicity were also investigated. Stigmasterol at 10 - 100 mg/kg reduced LPS-induced fever response by $39.93 \pm 10.52\%$ - $77.27 \pm 6.25\%$. Lung and liver histopathology showed stigmasterol controlled LPS-induced organ damage. Stigmasterol at 50 and 100 mg/kg significantly protected mice from LPS-induced mortality. Stigmasterol suppressed active cutaneous anaphylactic reaction, recording $44.74 \pm 10.62\%$ - $52.38 \pm 7.87\%$ inhibition of concentration of extravasated Evans blue dye at 10 - 100 mg/kg. Pretreatment of mesenteric tissue mast cells with 0.04 - 0.16 $\mu\text{g/ml}$ of stigmasterol reduced ovalbumin-induced and C48/80-induced degranulation by $39.00 \pm 3.92\%$ - $22.90 \pm 4.60\%$ and $47.60 \pm 3.37\%$ - $24.5 \pm 2.79\%$ respectively. Stigmasterol offered significant protection from C48/80-induced systemic anaphylaxis. Survival rates of 16.66%, 33.33% and 66.67% were recorded in mice at 10, 50 and 100 mg/kg respectively. Stigmasterol at 50 and 100 mg/kg significantly inhibited compound 48/80-induced scratching behaviour by $34.89 \pm 6.41\%$ and $36.00 \pm 2.21\%$ respectively, relative to control. Skin histopathology of injected sites showed stigmasterol reduced mast cell proliferation and degranulation associated with C48/80-induced pruritus. Again, stigmasterol controlled inflammatory features such as ear skin oedema, neutrophilia, overexpression of serum $\text{TNF}\alpha$ and skin histopathological changes induced by topical TPA application. Stigmasterol exerted significant suppressive effects on ovalbumin-induced airway inflammatory damage. Stigmasterol reduced proliferation of circulating eosinophils, lymphocytes and monocytes while reducing peribronchiolar, perivascular and alveolar infiltration of inflammatory cells. Histopathology revealed a preserved lung architecture and reduced collagen deposition, an index of lung remodeling, in stigmasterol-treated guinea pigs. Expression of serum vascular cell adhesion molecule-1 (VCAM-1) and ovalbumin specific Immunoglobulin E (OVA sIgE) elicited by ovalbumin sensitization and challenge was significantly controlled in stigmasterol-treated guinea pigs. In the toxicity study, no mortality was observed in stigmasterol-treated rats. Stigmasterol did not suppress peripheral leukocyte count, T cell-mediated delayed-type hypersensitivity responses, lymphoid organ weights and spleen cellularity. Histopathology of selected lymphoid organs showed limited deleterious effects on lymphoid tissues of stigmasterol-treated rats. Taken together, stigmasterol proved effective in inhibiting key innate and adaptive immune responses. These immune-modulating properties and limited harmful effects on the immune system suggest stigmasterol holds great therapeutic potential in the development of new drugs for immune system diseases.

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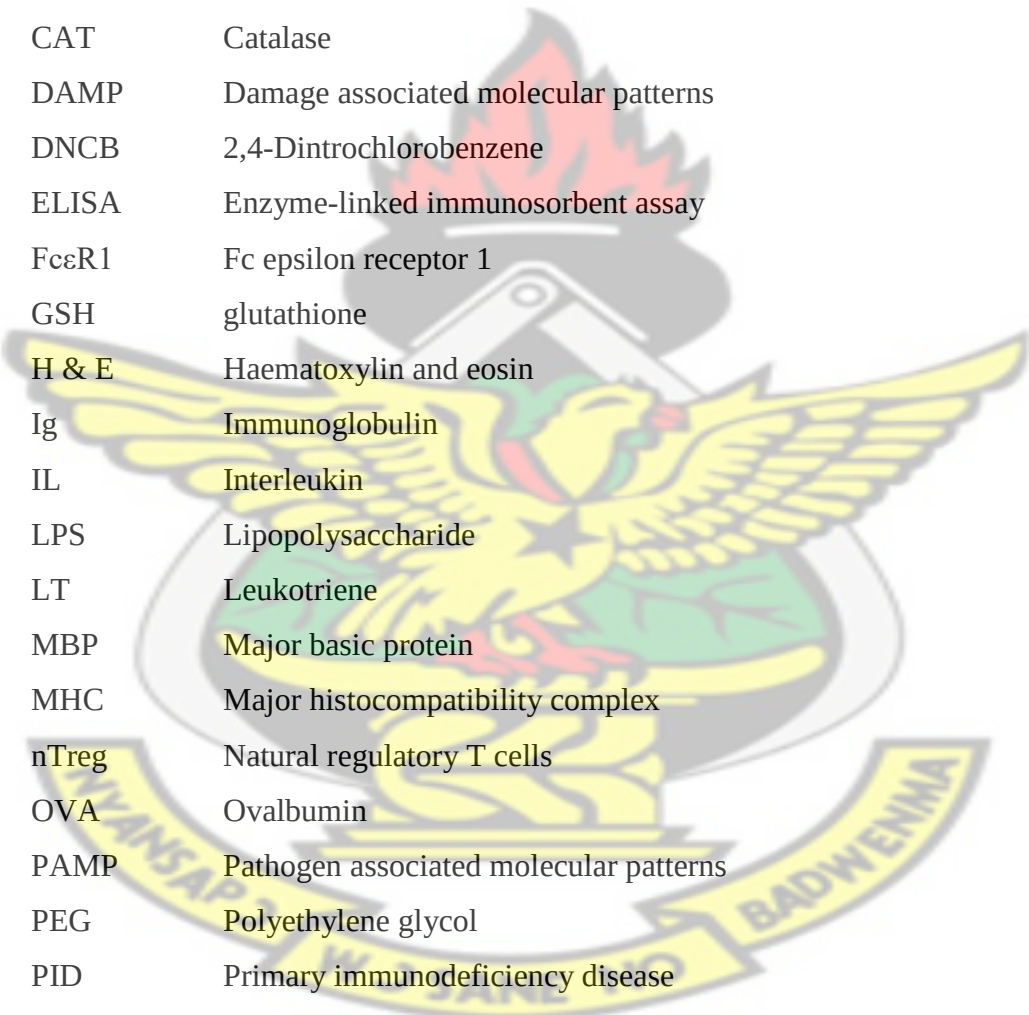
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ABBREVIATIONS

The logo of Kogi North University, Shomolu (KNUST) is a large, semi-transparent watermark in the background. It features a yellow eagle with spread wings perched on a green branch. Above the eagle is a red and white emblem. The text 'KNUST' is written in large, grey, sans-serif letters across the top. A yellow banner at the bottom contains the text 'KOGI NORTH UNIVERSITY SHOMOLU' and 'BADWENMA' on the right side.

ACA	Active cutaneous anaphylaxis
AHR	Airway hyper-responsiveness
ALT	Alanine transaminase
APC	Antigen presenting cells
AST	Aspartate transaminase
BALF	Broncho-alveolar fluid
BSA	Bovine serum albumin
CAT	Catalase
DAMP	Damage associated molecular patterns
DNCB	2,4-Dinitrochlorobenzene
ELISA	Enzyme-linked immunosorbent assay
FcεR1	Fc epsilon receptor 1
GSH	glutathione
H & E	Haematoxylin and eosin
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LT	Leukotriene
MBP	Major basic protein
MHC	Major histocompatibility complex
nTreg	Natural regulatory T cells
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PEG	Polyethylene glycol
PID	Primary immunodeficiency disease
PRR	Pathogen recognition receptors
RANTES	Regulated on activation, T cells expressed and secreted
ROS	Reactive oxygen species
SCID	severe combined immunodeficiency disorder
SOD	superoxide dismutase

T_H	Helper T cells
TLR	Toll-like receptors
$TNF\alpha$	Tumor necrosis factor alpha
TPA	12-O-Tetradecanoylphorbol-13-acetate
VCAM 1	Vascular cell adhesion molecule-1

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CHAPTER ONE

GENERAL INTRODUCTION

The immune system has presented 21st century scientists with unique challenges. Being the body's main defense system, studies have proven its active involvement in preventing diseases, fighting infections and surveillance of potentially cancerous cells (Warrington *et al.*, 2011; Kumar and Sharma, 2010; Finn, 2012). Hyperactivity and poor regulation of immune responses have however been linked to several conditions such as allergies, anaphylactic reactions and autoimmune diseases (Kemp and Lockey, 2002; Invernizzi and Gershwin, 2009).

1.1 PROBLEM STATEMENT

Data on prevalence of immune system disorders suggest that these conditions are on the rise globally, particularly in low and middle-income countries (To *et al.*, 2012; Lerner *et al.*, 2015). Worldwide, 300 million people suffer from asthma, 400 million from rhinitis with one tenth of the entire population having drug allergies, resulting in huge financial burden and cost of treatment (Pawankar, 2014). Similar trends have been recorded in Africa. Asthma, allergic rhinitis, atopic eczema and other allergic manifestations have seen a steady rise among African adolescents (Zar *et al.*, 2007). A report by Mbugi and Chilongola (2010) suggested that despite these worrying trends, scientific efforts in Africa remain almost exclusively towards traditional challenges such as infections and nutritional deficiencies.

Current treatment options for allergic diseases include antihistamines, mast cell stabilizers and glucocorticoids (Kun and Li, 2016). While the effectiveness of selective mediator antagonists in complex allergic manifestations has been questioned, the systemic unwanted effects of glucocorticoids, particularly with long term administration has been a major drawback to its use (Kuna *et al.*, 2016; Kun and Li, 2016). In an attempt to overcome these limitations, there has been renewed efforts towards novel immunomodulatory agents and treatment strategies. Immunotherapy represents one of the major successes of these efforts (Holgate and Polosa, 2008).

However, while the use of therapeutic antibodies, cytokines, soluble receptors and recombinant allergens in the management of immune disorders looks promising, it presents an obvious cost challenge to the patient (Hankin and Cox, 2014).

With the above challenges in mind, this study considers the potential of a naturally occurring compound, stigmasterol, as an effective, cheap and safe alternative in the search for novel immunomodulatory agents.

1.2 JUSTIFICATION

Like most conditions, health foods, plants and herbal medicines have been offered as dependable alternatives in the treatment of immune disorders (Kaminogawa and Nanno, 2004; Ma *et al.*, 2013). Advocates of natural remedies have long claimed they possess essential ingredients that can enhance the defense role of the immune system while regulating its detrimental reactions, thereby providing an overall beneficial immunomodulatory effect (Issazadeh-Navikas *et al.*, 2012). While debates still continue concerning their place in mainstream disease management, it cannot be denied that medicinal plants have provided several molecules that have become, or served as templates for developing novel compounds (Jantan *et al.*, 2015; Riyazzudin *et al.*, 2016). Understanding the role of natural compounds presents us with fantastic opportunities to understand the control of diseases better and to possibly use these compounds as motifs for developing more potent and effective alternatives. Phytosterols in particular have shown significant promise in the regulation of immune responses to achieve therapeutic effects in cancer and other inflammatory diseases (Moreau *et al.*, 2002; Yuk *et al.*, 2007). While there exist some reports of the effect of one such phytosterol, stigmasterol, there is a significant gap in knowledge concerning its activity. Research questions surrounding the therapeutic potential of stigmasterol in the treatment of allergic disorders, the scope of its activity, the mechanisms involved in its immunomodulatory effect and possible harmful effects, either general or immune system-specific, are addressed in this study.

1.3 AIM AND OBJECTIVES

The aim of this study is to evaluate the potential anti-allergic and other immunomodulatory effects of stigmasterol. The specific objectives of the experimental work are to:

- I. Assess the effect of stigmasterol on innate immune responses induced by lipopolysaccharide (LPS).
- II. Investigate the effect of stigmasterol on IgE and non-IgE mediated mast cell responses.
- III. Evaluate the effect of stigmasterol on compound 48/80-induced pruritus and 12-O-Tetradecanoylphorbol-13-acetate (TPA)-induced contact dermatitis.
- IV. Assess the effect of stigmasterol on allergen-induced chronic airway inflammation.
- V. Evaluate any potential immunotoxic properties of stigmasterol.



CHAPTER TWO

LITERATURE REVIEW

2.1 THE IMMUNE SYSTEM

The immune system is a complex collection of lymphoid organs, cells, humoral factors and cytokines. These interact as a unit with the principal function of host defense. The immune system is broadly divided into two parts based on the onset and specificity of its response (Parkin and Cohen, 2001).

2.1.1 INNATE IMMUNE SYSTEM

The innate immune system is a major division of the immune system responsible for the host's first-line defense against invading microbes. It is a rapid, relatively non-specific system of protection that recognizes and responds to pathogen invasion. It is made up of several chemical and physical barriers, humoral factors and a number of immune cells (Modlin, 2012). The cells of the innate immune system perform their surveillance functions by recognizing certain molecular structures on microbes referred to as pathogen associated molecular patterns (PAMPs) via specialized, germline encoded receptors known as pathogen recognition receptors (PRRs) (Peiser *et al.*, 2002; Underhill and Ozinsky, 2002). The innate immune system also recognizes self-molecules called danger-associated molecular patterns (DAMPs) from dead or damaged host cells (Martinon *et al.*, 2006).

2.1.1.1 Physical and chemical barriers

2.1.1.1.1 Skin

The skin, essentially the largest organ, provides the most formidable mechanical barrier protection of the host against environmental pathogens as well as non-pathogenic offenders. The thick stratified squamous epithelium renders the skin impenetrable to microbes (Elias, 2005). The epidermis is made up of layers of keratinocytes in its outermost surface which shed off, clearing pathogens in the process. The unique

‘resistant sheet’ structure of the stratum corneum is further re-enforced by the lipid rich extracellular matrix in which the corneocytes are embedded (Elias and Choi, 2005). Sebaceous glands and sweat glands located in the skin secrete sebum and sweat respectively thereby depositing antimicrobial contents such as lysozyme and dermicidin. These, in addition to antimicrobial proteins such as psoriasin, RNase 7 and LL-37 secreted by the epidermal region and the presence of cutaneous microflora such as propionobacteria and micrococcaeae on the surface of the skin contribute to its immune functions. Microflora are believed to fight pathogen either by competition for nutrients or directly by the secretion of antimicrobial molecules such as azelaic acid and penicillin (Wille and Kydonieus, 2003; Elias, 2007).

2.1.1.1.2 Mucous membranes

Mucous membranes serve as protective barriers in body cavities which open to the host’s environment typically in the reproductive, genitourinary and digestive systems (Sansonetti, 2004). These linings are made up of tightly bound non-keratinized, epithelial cells. The layer of mucus on epithelial surfaces aid in the trapping of pathogens and debris to be eventually cleared out by synchronous beating of epithelial cilia or shedding off of the rapidly dividing mucosal lining (Chilvers and O’Callaghan, 2000). The mucous complex is made up of mainly mucin, antimicrobial proteins such as cathelicidins and defensins and immunoglobulins which are all secreted in mucosal fluid (Bansil and Turner, 2017). Mucin the major macromolecule contained in mucus is a glycoprotein which, in addition to reported direct antimicrobial properties, oligomerizes to form mesh-like structure in-penetrable to pathogens (Sperandio *et al.*, 2015).

2.1.1.1.3 Normal microbiota

Normal flora or commensal organisms are non-pathogenic bacteria and yeast and to a lesser extent virus and protozoa distributed on the skin, digestive system, respiratory airways and throughout the body (Belkaid and Hand, 2014). They serve as a microbiological barrier to pathogenic invasion and infection forming a formidable symbiotic relationship with the host (Mazmanian *et al.*, 2008). Commensal organisms

suppress the thriving of pathogens by out-competing them for nutrients and colonization sites by a process known as colonization resistance (Buffie and Pamer, 2013). Again, these organisms may secrete chemicals that create an unfavourable condition for or are directly harmful to pathogens (Cash *et al.*, 2006).

2.1.1.2 Humoral factors

2.1.1.2.1 Acute phase proteins

During infections, microbial products stimulate macrophage and other cells to produce cytokines such as tumor necrosis factor alpha (TNF α) and interleukin 6 (IL-6) which activates liver cells to release an array of protein substances known as acute phase proteins (Quinton *et al.*, 2009). These proteins which include C-reactive protein, α 1 antitrypsin, α 2 macroglobulin, fibrinogen and serum amyloid A (SAA) protein contribute to the innate immune response by facilitating opsonisation of bacteria or apoptotic cells and activation of complement. A classic example is the binding of C-reactive protein to phosphorylcholine molecules in the cell wall of some bacteria to trigger the complement pathway (Black *et al.*, 2004). SAA is known to induce the trafficking of immune cells at the inflammatory site while fibrinogen is key in the trapping of microbes within blood clots (Esmon *et al.*, 2011).

2.1.1.2.2 Complement

Complement pathway is a complex cascading, humoral mechanism involved in innate immune system. It consists of protein or enzyme precursors (some acute phase proteins) which undergo proteolysis into active forms (Gasque, 2004). Based on the nature of activation, the complement system is divided into three main pathways namely; the classical, lectin or alternative (properdin) pathways (Kang *et al.*, 2009). Activation of any of these three pathways leads to the formation of terminal component protein fragments C3a, C3b, C5a and C5b which serve as the main effector functions or of the complement system. In an innate immune response, C3a and C5a sustain the ongoing inflammatory response by mediating vasodilation, oxidative burst of macrophages and neutrophils and degranulation of mast cells. C3b functions as an opsonin, binding to complement receptors on the cell surface of pathogens thereby enhancing phagocytosis

and clearing out of pathogens. C5b is known to mediate the formation of the Membrane Attack Complex, one of the main effector units of the complement pathway involved in bacteria lysis and inactivation of viruses (Dunkelberger and Song, 2010).

2.1.1.2.3 Antimicrobial peptides

Among the repertoire of microbe fighting elements of the innate immune system is a class of highly conserved and diverse proteins referred to as Antimicrobial Peptides (AMPs) (Nizet *et al.*, 2001). They exert their effects ranging from membrane disruption, interference with microbe protein synthesis and folding to cell membrane pore formation on bacteria, parasites and fungi (Brogden, 2005; Zasloff, 2002). Unlike other humoral components which are induced during infections, AMPs occur constitutively in several innate immune cells and tissues. They therefore scarcely occur in blood. There are 3 main classes of AMPs reported in literature; defensins, histatins and cathelicidins (De Smet and Contreras, 2005).

2.1.1.3 Cellular factors

2.1.1.3.1 Neutrophils (microphages)

Neutrophils are polymorphonuclear leukocytes which develop from CD34+ pluripotent stem cells in the bone marrow (Kobayashi *et al.*, 2005). They are the most abundant granulocyte in systemic circulation, representing about 50% of all leukocytes but with a short lifespan of about 1-2 days. Neutrophils are professional phagocytes and the foremost responder in inflammation and infection. They have multi-lobed nuclei with granules which stain poorly to histological stains. Neutrophil granules which contain effector antimicrobial substances and degrading enzymes can be divided into two types; primary azurophil granule and secondary specific granules. Azurophilic granules contain a host of antimicrobial peptides such as α defensins, cathepsin G, bactericidal/permeability increasing (BPI) protein and myeloperoxidase (Nathan, 2006). Lactoferrin, flavocytochrome b558, lysozymes and alkaline phosphatase from secondary granules also add to the repertoire of neutrophil-derived microbicidal components (Faurschou and Borregaard, 2003; Ward and Conneely, 2004). Activation

of the pathogen recognition receptors on neutrophils such as Toll-like receptors and C-type lectin receptors results in enhanced cell survival, phagocytosis, secretion of reactive oxygen species, chemokine and cytokine release and degranulation (Borregaard, 2010). These events contribute to the significant role of neutrophils in the innate immune system.

2.1.1.3.2 Macrophages

Macrophages develop from bone marrow-derived promonocytes which differentiate into circulating monocytes (Vega and Corbi, 2006). These monocytes migrate into tissues where they mature into specialized macrophages which are described according to their location. Macrophages reside throughout body tissues such as in the liver (Kupffer cells), lungs (alveolar macrophages) intestinal connective tissue (histiocytes) or lymph node macrophages (Epelman *et al.*, 2014). Macrophages are either mobile or stationary within tissues with the main function of acting as sentinels for inflammatory signals or pathogen invasion. Recruitment and signaling is mediated by neutrophil-derived chemotactic factors and complement (Silva, 2010). They also play crucial roles in tissue repair (You *et al.*, 2013) and antigen presentation (Delamarre *et al.*, 2005) with the latter being an important interface with adaptive immunity. Macrophages are efficient phagocytes of dead cells, debris and microbes and possess detection molecules for both pathogen and damage associated molecular patterns (Hume *et al.*, 2002). Intracellular signaling follows activation of these receptors resulting in the release of proinflammatory cytokines, nitric oxide, oxygen radicals and stimulation of the recruitment of other inflammatory cells to the site of inflammation (Serbina *et al.*, 2008).

2.1.1.3.3 Eosinophil

Eosinophils are key effector granulocyte cells which play critical roles in allergic immune responses and parasitic infections (Acharya and Ackerman, 2014). They have been found to perform additional functions in controlling the course of inflammatory reactions and interfacing between innate and adaptive immunity. Circulating eosinophils account for about 4% of peripheral leukocytes, with a larger number

resident in mucosal and lymphoid tissues (Shamri *et al.*, 2011). Eosinophils express a wide repertoire of receptors which detect signals and migrate to sites of inflammation. These include receptors for cytokines, growth factors, lipid mediators, chemokines (C-C chemokine receptor type 3 and C-X-C chemokine receptor 4) and complement (C5aR, FCεR11) (Muniz *et al.*, 2012). Activated eosinophils releases a number of cytotoxic peptides, chemokines and lipid mediators from its secondary crystalloid granules to perform effector functions. Contents may be released by one of three mechanisms namely piece-mill degranulation, exocytosis and cytolysis (Acharya and Ackerman, 2014). Cationic proteins, one of the main range of effector molecules include major basic protein (MBP), eosinophil peroxidase (EPO) (Lacy *et al.*, 2003), eosinophilic cationic protein (ECP) (Boix *et al.*, 2001) among others.

2.1.1.3.4 Mast Cells

Mast cells develop in the bone marrow where their maturation, differentiation and proliferation is mediated by the stem cell factor (SCF) and an array of cytokines (Dahlin and Hallgren, 2015). Human mast cells are classified into MC(T) and MC(TC) mast cells based on the protease composition of their secretory granules (Amin, 2012). The mast cells play a crucial role in inflammation and immune responses. Central to this is the high affinity FcεRI receptor expressed on mast cell surfaces. Mast cells participate in type 1 hypersensitivity reactions when allergens bind to IgE molecules attached to mast cell surfaces. Secretory granules release preformed inflammatory mediators such as prostaglandins, histamine and cytokines (Amin *et al.*, 2005). These, in addition to *de novo* synthesized mediators influence allergic responses such as urticaria, anaphylaxis, angioedema and allergic rhinitis (Krystel-Whittemore *et al.*, 2016).

2.1.1.3.5 Basophils

Basophils are granulocyte cells with characteristic cytoplasmic granules that stain with basophilic dyes. They develop from haematopoietic CD34+ pluripotent stem cells in cord blood and bone marrow (Arock *et al.*, 2002). The specific lineage is however unclear. They are structurally and functionally similar to mast cells and represent less than 1% of all blood leukocytes (Falcone *et al.*, 2006). Basophils express high affinity

immunoglobulin E receptors, FcεRI and are recognized mainly as participants in allergic immune reactions (Falcone *et al.*, 2000). Additionally, basophils express exotoxin receptors C-C receptor type 3 (CCR3) for eotaxin, CCL11 and other C-C chemokines as well (Iikura *et al.*, 2001).

2.1.1.3.6 Natural Killer (NK) cells

NK cells are lymphocyte-like immune cells with immense cytotoxic capacity which participates in both innate and adaptive immunity. Like lymphocyte cells they develop from the haematopoietic stem cells but do not undergo the gene rearrangement typical of T lymphocyte development (Andoniou *et al.*, 2005; Wang *et al.*, 2013). They develop from NK progenitor cells upon their interaction with the stromal cells of the bone marrow. NK cells respond to a cytokine environment such as IL-15 from monocytes and IL-12 from dendritic cells inducing IFN gamma which has direct antiviral properties. NK cells participate in early immune responses and are active against microbes including viruses and virus infected cells (Sun and Lanier, 2009). They exhibit a particularly significant activity against herpes virus and cytomegalovirus (Guma *et al.*, 2004; Kuijpers *et al.*, 2008). Overall, NK cells' effector mechanisms are mainly by the release of perforins pore forming proteins that destroy the target cells (Lanier, 2005). A less dominant mechanism is the inducing of intracellular proteases that lead to apoptosis of target cells (Chung *et al.*, 2008).

2.1.1.4 Pathogen recognition receptors

Pathogen recognition receptors are innate immune system receptors which perform sentinel functions for the host, by interacting with a wide range of foreign materials. They initiate an early immune response by mediating transcription of several inflammatory mediators. They consist of Toll-like receptors, RIG-I-like receptors, NOD-like receptors, and C-type lectin receptors (Takeuchi and Akira, 2010).

Toll-like receptors (TLRs) are a family of type 1 transmembrane or endosomal pathogen recognition receptors. They recognize a wide variety of pathogen associated components and danger associated molecules from host cells (Pasare and Medzhitov, 2004). TLRs possess ligand sensing domain with leucine rich repeats at its N terminal

end. Ten subtypes, TLR1-10, have been characterized in human and they are expressed on a host of cells ranging from phagocytes (neutrophils, macrophages, dendritic cells, monocytes), lymphocytes (B cell and T cells) and structural cells (fibroblasts, endothelial cells and epithelial cells) (Takeda *et al.*, 2003; Iwasaki and Medzhitov, 2004). TLR 1, 2, 4, 5, 6 and 10 subtypes are cell surface TLRs which recognize membrane and cell wall components of bacteria, mycobacteria, fungi and protozoa (Hayashi *et al.*, 2001). TLR3, 7, 8, and 9 occur in endosomal compartment and are tasked with recognizing nucleic acids from viruses (Alexopoulou *et al.*, 2001; Diebold *et al.*, 2004). Cells of innate immune system also express a number of other recognition receptors. Nucleotide binding, oligomerization domain (NOD) - like receptors are cytoplasmic pattern recognition receptors with characteristic nucleotide domains and are known to detect intracellular pathogens. They are grouped into subfamilies based on the type of N-terminal protein-interacting domains; NLRA, NLRB, NLRC, NLRP AND NLRX. Of these, two subtypes of the NLRC subfamily namely NLRC1 (NOD1) and NLRC2 (NOD2) are the most characterized and appear to be prototypical of the NLR family (Girardin *et al.*, 2003; Yang *et al.*, 2007). C-lectin type Receptors (CLR) exists as 17 subfamilies which all express the characteristic C-type lectin carbohydrate recognition domain, CTLD. They may be soluble or transmembrane and they recognize mannose or glucan carbohydrate structures. Dectin1 and Dectin 2 subfamilies being the most characterized and understood (Kingeter and Lin, 2012). The structure, classification and roles of other less prominent recognition receptors such as RIG-1-like receptors and AIM-2-Like receptors are also well documented in literature (Brubaker *et al.*, 2015).

2.1.2 ADAPTIVE IMMUNE SYSTEM

2.1.2.1 Cellular Immunity

Cellular immunity is one of the two arms of the body's adaptive immune system. In contrast to humoral immunity, whose main effector agents are antibodies, cellular immunity employs the functions of lymphocytes derived from the thymus (Takahama, 2006). These are referred to as T cells. T cells occur mainly in two subpopulations namely, T helper cells and T killer cells (Klein *et al.*, 2009). As the names suggest, T

helper cells (Cluster of differentiation 4 positive or CD4⁺ T cells) do not directly destroy antigens or antigen infected cells but activates other aspects of the immune system for targeted responses (Luckheeram *et al.*, 2012). The role of direct recognition and destruction of non-self antigens, infected or cancerous cells is performed by T killer cells (CD8⁺ or CD8 positive T cells) (Zhang and Bevan, 2011). Cellular immunity is critical in immune-surveillance and protection and mediates key immune responses such as delayed type hypersensitivity reactions and chronic inflammation.

2.1.2.1.1 Cellular components

T helper cells are thymus-derived lymphocyte cells that express the CD4 glycoprotein on their surface. Helper cells circulate in blood and lymph and reside in secondary lymphoid tissues where they recognize major histocompatibility complex class II (MHC II) complexes with antigen peptide fragments (Luckheeram *et al.*, 2012). T helper cells bind with T cell receptors (TCR) and upon activation triggers other aspects of the immune system such as natural killer cells, T killer cells and humoral responses to fight pathogen infection. CD4⁺ cells perform these specific functions by differentiating into various specialized subsets. TH1, TH2, TH17, regulatory T cells and to a lesser extent TH9 and TH22 (Zhu and Paul, 2010).

T killer cells (T cytotoxic killer cells) are CD8⁺ (express CD8 glycoprotein on their surface) thymus-derived lymphocytes which proliferate in the presence of IL-2 and play a critical role in cellular immunity (Zhang and Bevan, 2011). T killer cells attack and destroy cells infected with viral, protozoal and bacterial pathogens in response to MHC class I molecules (Kaeche and Cui, 2012).

Regulatory T (CD4⁺CD25⁺) cells are a subset of CD4⁺ T cells responsible for ensuring self-tolerance and suppressing autoimmunity (Miyara and Sakaguchi, 2007). nTReg cells, which represent about 10% of CD⁺ cells, mature in the thymus but may be formed in the periphery under the right antigen presenting conditions and cytokine environment such as the release of IL-2 (Schmitt and Williams, 2013). Recent studies have shown the most unique identifier for regulatory T cells in the forkhead box transcription factor (Foxp3) which is believed to be responsible for differentiating T cells into cells with regulatory roles (Schmitt and Williams, 2013). In cellular immunity, nTReg suppress

the induction, effector and memory functions of T helper cells, T killer cells as well as natural killer T cell cytotoxicity (Ray *et al.*, 2010).

Memory T cell differentiation is triggered as part of the T cell activation and proliferation signaling (Akondy *et al.*, 2017). Following neutralization of pathogen-infected, T killer cells are inactivated in favor of the development of memory T cells in mucosal lining of pathogen entry sites, secondary lymphoid tissues and the bone marrow (Chang *et al.*, 2014). They are continually maintained after the pathogen clearance due to the primary response by cytokine mediated cell division (interleukin 15) and cell survival (interleukin 7) (Judge *et al.*, 2002; Li *et al.*, 2003). In the event of a re-exposure of the host to the specific antigen, memory T cells develop into effector T cells which mount a quicker and more intense response. Clonal expansion in the first response produces more antigen specific T cells (MacLeod *et al.*, 2010).

2.1.2.2 Humoral Immunity

Following responses by the innate immune system, the activated adaptive immune system exerts its effect mainly through the humoral immune response. It comprises the activation and differentiation of B lymphocytes and production of a variety of immunoglobulins which ultimately provide a more targeted onslaught on invading pathogens (antigens). It occurs in two main phases, namely the activation phase and effector phase (Hardy and Hayakawa *et al.*, 2001).

2.1.2.2.1 Antibodies

Antibodies are heterodimeric glycoproteins produced by activated B lymphocytes and plasma cells in response to a specific antigen with which it is capable of reacting (Nutt *et al.*, 2015). Antibodies are produced in secondary lymphoid organs but are capable of recognizing and neutralizing antigens in all parts of the body once it is released into the bloodstream or mucosa. Though used interchangeably with the term 'immunoglobulins', not all immunoglobulins have reactive properties in response to antigens. Levels of gamma globulins yielded from electrophoretic separation of proteins were acutely elevated in immunized animals and could be reduced when neutralized with specific antigens. In 1939, a classic study by Kabat and Kiselius determined that

antibodies in serum were expressed as gamma globulins. Subsequent studies confirmed the structure, functions and subtypes (Schroeder and Cavacini, 2010).

Classes of Antibodies

Immunoglobulin M antibodies are the first to be produced upon B cell development and infection. They may occur as pentamers or monomers and serve as the main mediators of the primary humoral response. Naïve B cells express the low affinity monomeric IgM form but the pentameric form is produced upon B cell maturation or antigenic stimulation. The latter contains an additional glycoprotein chain, the J chain which enhances mucosal surface secretion. The early expression of IgM antibodies during B cell development results in early 'un-mutated' forms which are more poly-reactive than other isotypes formed after class switching. These IgM antibodies, known as natural antibodies are key in primary response to a number of antigens (Boes, 2002). IgM antibodies serve as 'parent' antibodies with the mu (μ) heavy chain which undergo class switching to produce antibodies of other classes after CD40 and cytokine interaction. They have a half-life of about 5 days and represent about 5-10% of antibodies. They are critical in fighting pathogens via opsonisation for destruction and activation of the complement system (Jayasekera *et al.*, 2007). They are the first antibody produced by foetus but they do not cross the placenta.

Immunoglobulin G antibodies are monomeric structured antibodies with a gamma (γ) heavy chain subtype. They are formed from class isotype switching upon CD40 and cytokine interaction with immunoglobulin M. It is known to be the predominant immunoglobulin class, representing about 80% of all serum antibodies and remains in circulation for about 21-23 days; the longest half-life of all antibody classes. IgG antibodies are known to be the main mediator of T cell-independent antigen responses and as well as memory cells-elicited secondary humoral responses. IgG antibodies play a key role in phagocytosis, neutralization of toxins and viruses, fetal protection and activation of the complement system (Kapur *et al.*, 2014). IgG antibodies are grouped into 4 main subclasses namely IgG1, IgG2, IgG3 and IgG4 which differ in antigen selectivity, structure and affinity for the Fc gamma receptor (Fc γ R) (Hogarth and Pietersz, 2012).

Immunoglobulin A class of antibodies represents those with the alpha (α) heavy chain subtype. They are formed from B lymphocytes in the bone marrow and lymphoid organs (Latiff and Kerr, 2007). IgM produced by these cells undergo class switching after interaction with CD40 and interleukin 10 (IL-10) to form IgA immunoglobulins. IgA antibodies account for about 20% of all serum immunoglobulins and have a half-life of 3-6 days (Latiff and Kerr, 2007). They exist as two subtypes namely IgA1 and IgA2 which show affinity for Fc α R receptor (CD89). Both subtypes however occur in equal proportions several body secretions (Woof *et al.*, 2004). Plasma IgA mostly occurs as monomeric IgA1. IgA, which is secreted in abundance in saliva, tears, colostrum, nasal secretion and urine, known as secretory IgA (sIgA) is polymeric (mostly dimeric) in nature. The secretory IgA which is abundant in mucosal surfaces possesses a j chain and the secretory component polypeptide chain. It plays a key role against toxins, viruses and bacteria in mucosal surfaces and provision of immunity in infant digestive tract. IgA antibodies are not known to activate the complement system (Woof and Kerr, 2006).

Immunoglobulin D antibodies are expressed in low concentrations in the serum. They have a low half-life of 3 days due to the susceptibility of the hinge region of the monomeric molecule to proteolysis. Circulating IgD antibodies represent about 0.2% of all serum antibodies, though their functions here are poorly understood. There however seems to be some information, though not exhaustive, about the membrane bound IgD subpopulations which occur along membrane bound IgM on mature B cells before antigenic stimulation. Here, they serve as transmembrane antigen receptors which assist in initiating immune responses (Chen and Cerutti, 2011).

Immunoglobulin E antibodies occur as monomeric molecules. They form about 0.002% of all serum antibodies, the least of all antibodies and have the lowest half-life of 2 days. CD40 and interleukin 4 (IL-4) interaction with IgM elicits a class switching to the epsilon (ϵ) heavy chain bearing isotype. Eosinophils are also known to induce class switching into IgE antibodies via the release of IL-13. IgE antibodies display affinity, although at varying degrees, for Fc ϵ RI and Fc ϵ RII (CD23) receptors. Despite having a low serum concentration, short half-life and low rate of synthesis, IgE antibodies are

very potent and critical to immune responses such as hypersensitivity reactions and responses to helminth infections (Winter *et al.*, 2000). Their potent activity is attributed to the high affinity for FcεRI expressed on mast cells, basophil, Langerhans cells and eosinophils. The increased expression of these receptors in response to allergen exposure and helminth infestation further contributes to the effectiveness of Immunoglobulin E-mediated responses. It however has low affinity for the FcεRII (CD23) which are normally distributed on naturally killer cells and platelets. Their main effector functions are mediating the release of inflammatory mediators and cytokines from mast cells and basophils in response to allergens and mediating cell-mediated cytotoxic responses which induce lysis of helminthic parasites (Winter *et al.*, 2000). IgE antibodies show no complement fixation and no placental transfer.

2.2 IMMUNE SYSTEM DISORDERS

2.2.1 ALLERGY AND HYPERSENSITIVITY

2.2.1.1 Asthma

Asthma is a disease of the airway associated with inflammation, airway obstruction, hyper-responsiveness and lung remodeling (Pynn *et al.*, 2012). It presents as acute bronchoconstriction, mucus hypersecretion resulting in airway obstruction, coughing, chest tightness and wheezing (Erle and Sheppard, 2014). Allergen-triggered asthma is the commonest type, accounting for about 90% of cases in children and 50% of adult cases (WHO, 2002). Pathogenesis of allergic asthma shows a strong genetic predisposition to IgE-mediated, TH2 driven immune responses (Hopkin, 2009). Indeed, asthma usually exists in patients along with allergic rhinitis and atopic eczema. This syndrome is known as the 'atopic triad'. Features of asthma are triggered by allergenic factors such as pollen, pet dander, mould, dust mites etc. However, symptoms may be exacerbated by non-allergenic factors such as cold and dry air, infections, exercise and pollutants (Malo *et al.*, 2004; Holgate and Davies, 2009).

2.2.1.2 Anaphylaxis

Anaphylactic reactions are acute, systemic allergic reactions with potentially fatal consequences. It is triggered by immunologic and non-immunologic factors and is characterized by a quick onset and progression and multiple organ involvement (Simons, 2008). Central to the pathogenesis of anaphylaxis is the interaction between immunoglobulin E (IgE) and the high affinity IgE receptors, Fc epsilon receptor 1 (FcεRI) (Rivera and Gilfillan, 2006). Allergen, food, medication and insect sting trigger either IgE or non IgE-dependent mechanisms. Immunologic mechanisms are initiated when immunoglobulin E antibodies are produced in response to host exposure to allergens and the subsequent binding of these antibodies to FcεRI receptors on the membrane surfaces of mast cells and basophils. Re-exposure of allergen leads to crosslinking of bound IgE and subsequent activation of mast cells and basophils (Simons, 2010). Other immune mechanisms involving activation of complement and coagulation pathways, immunoglobulin G and M, enhanced leukotriene production and participation of autoimmune factors have been reported (Kemp and Lockey, 2002). Non immunologic pathways, which are not known to involve in FcεRI/IgE interactions, such as drugs (opioids, vancomycin), exercise and cold air can activate mast cell and basophils to trigger anaphylaxis (Simons, 2010). Current management approaches focus on prompt emergency restoring of respiratory and cardiovascular impairment, removal of trigger and management of accompanying symptoms and co-morbidities. As such epinephrine, oxygen supply and IV fluids and to lesser extent antihistamines and corticosteroids all find use in its management (Simons and Simons, 2010; Lieberman *et al.*, 2010).

2.2.1.3 Allergic rhinitis (hay fever)

Hay fever is a heterogeneous allergic condition characterized by sneezing, palate pruritus, nasal congestion and rhinorrhea. It is usually accompanied by watery eyes conjunctival pruritus and post nasal drainage (Skoner, 2001). It is a subset of rhinitis, a more general disorder which may or may not be triggered by allergic pathways and accounts for 50% of all cases of rhinitis (Skoner, 2001). Allergic rhinitis may be seasonal, having occasional but prompt distinct symptoms in response to climatic

condition or local pollination seasons or perennial in nature, occurring for long periods (about 9 months in the year) in response to a single or multiple allergens (Sin and Togias, 2011). Allergic rhinitis occurs as a result of the host exposure to inhaled triggers such as pollen, animal dander, dust mites which trigger production of allergen specific immunoglobulins. Degranulation of mast cells following antigen re-exposure in sensitized individuals and release of mediators drive the early phase of the allergic response which occurs within minutes (Peters-Golden *et al.*, 2006; Togias, 2003). The late phase is characterized by a more dominant basophil involvement, recruitment and activation of eosinophils, lymphocytes and an increased expression of adhesion molecules. Management focuses on allergen avoidance and pharmacological interventions. Antihistamines via the H₁ antagonism show efficacy in controlling pruritus and sneeze reflexes. Decongestants constrict nasal blood vessel reducing incidents of exudate/edema in nasal passages. Topical corticosteroids and mast cell stabilizers have also been proven useful (Wallace *et al.*, 2008).

2.2.1.4 Atopic eczema

Atopic eczema, also known as atopic dermatitis is a chronic inflammatory disease of the skin caused by genetic predisposition, immunological and environmental factors. Clinically it presents as persistent and intermittent erythematous macules, papular lesions and severe pruritus (Dokmeci and Herrick, 2008; Nutt, 2015). In most instances, skin excoriations are worsened by scratching which further worsens skin damage. It occurs in childhood and may still be present in adulthood (Egawa and Weninger, 2015). Genetic linkages have been drawn with chromosome 5q 31-33 which contains genes for TH2 cell cytokines and other regions containing genes for FcεRI β chain and promoter genes for regulated on activation, normal T cell expressed and secreted (RANTES) (Forrest *et al.*, 1999). Genetic mutations in molecules engaged in barrier function affect their functions and contribute to atopic dermatitis. Deficiencies in ceramides, water carrying molecules in skin and filaggrin, a skin matrix protein precursor involved in terminal differentiation of epidermis, are key causal factors in atopic dermatitis (Irvine *et al.*, 2011; Imokawa, 2002). Severe dryness coupled with changes in lipid components in skin impair barrier function. Exposure of host to environmental factors such as

allergenic factors and superantigens from skin dwelling staphylococcus organisms are central to the disease pathogenesis (Baker, 2006). An abnormal production of pro-inflammatory cytokines by keratinocytes and the recruitment of IgE bearing mast cells and dendritic cells increase responsiveness to allergens. Lymphocyte recruitment into skin further worsens the inflammatory process (Kay, 2001). Treatment goals include skin hydration and moisturizing, removal of immune triggers and management of pruritus with antihistamines. Immunomodulatory interventions include oral and systemic anti-inflammatory steroids and immunosuppressive drugs like cyclosporine (Lipner *et al.*, 2000).

2.2.1.5 Allergic contact dermatitis (ACD)

Allergic contact dermatitis is an allergen-induced, lymphocyte-driven skin inflammation associated with eczematous eruptions, erythema, vesications, fluid filled papules and intense itching (Saint-Mezard *et al.*, 2004). Unlike atopic eczema, ACD occurs as a result of sensitization of the host through prior exposure to allergen and not a genetic predisposition (Saint-Mezard *et al.*, 2003). Re-exposure to or contact with the antigen, in a sensitized individual triggers a T cell (specifically CD8+ T cell)-mediated inflammation at the epidermal region at the site of contact and an array of innate and acquired immune responses (Coulter *et al.*, 2010). It follows a type IV/ delayed type hypersensitivity reaction and a TH1 cytokine profile. The sensitization phase which occurs about 4 h after exposure and may take up to 14 days, occurs when immune response inducing chemicals known as haptens infiltrate the epidermis and form immune complexes with host proteins and biochemically modified by skin enzymes (Divkovic *et al.*, 2005; Gerberick *et al.*, 2008). Non-specific activation of keratinocytes trigger chemokines which recruit Langerhans cells (Wang *et al.*, 2003). These then interact with antigen specific T cells which differentiate into memory cells. Re-exposure triggers activation of T cell memory by dermal antigen presenting cells (APCs). Triggers include poison ivy, poison oak and nickel (Budinger and Hertl, 2000).

2.2.2 IMMUNE DEFICIENCIES

2.2.2.1 Primary Immune Deficiency (PID)

Primary deficiency disorders are rare heterogeneous defects associated with loss or impaired function of aspects of the immune function (Al-Herz *et al.*, 2014). They are caused by either inherited or spontaneous genetic mutations which ultimately affect the development of the immune system (Lehman, 2015). PIDs, comprising over 200 disorders are generally classified based on the particular component of the immune system which is affected and are grouped under adaptive disorders (T cell, B cell, Combined Immune deficiency) and innate disorders (complement, phagocyte deficiencies etc.) (Samarghitean *et al.*, 2007). PIDs invariably lead to susceptibility to infections. Other manifestations include autoimmune conditions, granulomatosis and malignancies (Chapel *et al.*, 2008; Cunningham-Rundles, 2002).

2.2.2.1.1 T cell disorders/ Combined Immune disorders

Mutations in the genes responsible for the development, differentiation and maturation of T cells result in a decreased number or malfunctioning of T cells. It represents about 9% of all Primary Immune Deficiencies. Due to the role of T cells in activating B cells, T cell deficiencies are often accompanied by a loss of B cell function (Reust, 2013). T cell deficiencies clinically present as lymphopenia, pneumonia, gastrointestinal infections and opportunistic infections in the early years. Less severe forms of this combined deficiency include Wiskott-Aldrich syndrome, DiGeorge syndrome, ataxia-telangiectasia, and X-linked lymphoproliferative disease (McCusker and Warrington, 2011). Severe forms of this deficiency known as the Severe Combined Immune Deficiency SCID, are categorized based on the presence or otherwise of B cells.

2.2.2.1.2 Antibody deficiency disorders

These are a collection of adaptive disorders that are characterized by impaired B cell function. Aberrations in genetic material leads to defects in the maturation and differentiation of B cells and ultimately affects antibody production. It is the most common, contributing about 50% of all primary immune deficiencies (Bonilla *et al.*,

2005). B cell deficiencies present as low serum levels of immunoglobulins, vulnerability to infections particularly in the sinus and lungs to encapsulated organisms such as haemophilus influenza and streptococcus pneumonia and increased risk of malignancy (Conley *et al.*, 2009). Several specific disorders have been identified. X linked agammaglobulinaemia (XLA) occurs as a result of a mutation of the Bruton's tyrosine kinase gene which is critical in B cell development. The defect leads to a reduction in circulating antibodies and clinical manifestations that occur usually in the first 2 years of life (Bonilla *et al.*, 2005). Common variable immunodeficiency (CVID) manifests later than 10 years in the patient. It is also characterized by low antibody levels, sinopulmonary infections and an impaired response to immunization (Sneller, 2001). The most common antibody deficiency is the selective IgA deficiency. It is characterized by low serum levels of IgA in the presence of normal levels of IgG and IgM (Latiff and Kerr, 2007). While a majority of patients show no clinical manifestations, allergies and autoimmunity have been reported in a few (Pan-Hammarstrom and Hammarstrom, 2008).

2.2.2.1.3 Innate Defects

Innate deficiencies consist of defects in phagocytes, complement system, Toll-like receptors and leukocyte adhesion pathways (Notarangelo, 2010). Phagocyte disorders present as reduction in number and loss of function of neutrophils, monocytes and other phagocytic cells (Klein and Welte, 2010; Holland, 2010). Though immune system function is preserved to a large extent, the deficiency in the key cells result in certain disorders and susceptibility to particular infections. Lack of optimal phagocyte function leads to the thriving of catalase positive organisms and infections from *Staphylococcus aureus*, *Arspegillus* species and *Candida* species with respiratory tract and cutaneous infections dominating (Notarangelo, 2010). Complement deficiencies, which account for about 1-2% of all PIDs, present as various disorders depending on the specific protein fragment in the complement cascade which is affected (Unsworth, 2008). Defects to early components of the cascade, C1-C4 fragments, cause autoimmune systemic lupus erythematosus (SLE)-like symptoms and susceptibility to infections caused by capsulated bacteria (Walport *et al.*, 1998). Late fragment C5-C9 deficiencies

cause susceptibility to *Neisseria meningitidis* with diseases such as meningitis, sepsis and arthritis. Inherited defects in TLR signaling particularly adaptor molecules such as MyD88 and IRAK4 (IL-1 receptor associated kinase-4) cause susceptibility to several pathogens (von Bernuth *et al.*, 2008). Defects in specific cytokine functions are also implicated in deficiencies of the innate immune system.

2.2.2.2 Secondary Immune Deficiency (SID)

Secondary immune deficiencies are defects or dysfunctions in one or more components of the immune system as a result of another underlying process, external factors and primary infections. Often, these deficiencies can be reversed if the underlying cause is resolved. Malnutrition, infections and treatment-associated immunosuppression are the main causes of secondary immune deficiency (Chinen and Shearer, 2008a).

2.2.2.2.1 Malnutrition

Malnutrition is the commonest cause of secondary immune deficiencies worldwide (Rice *et al.*, 2000). This phenomenon, referred to as nutritionally acquired immune deficiency syndrome (NAIDS) is known to increase the prevalence, severity and mortality of infections, due to suppression of both the innate and adaptive immune system (Hughes and Kelly, 2006; Rodgers, 2011). Severe deficits in macronutrients form Protein Energy Malnutrition (PEM), especially during childhood, result in atrophy of lymphoid tissues, thymus involution and a resulting fall in circulating lymphocytes (França *et al.*, 2009).

2.2.2.2.2 Chronic infections

Long term infections place a burden on the immune system function (Lindblom *et al.*, 2008). Pathogens involved in significant immunosuppression include viruses (HIV, measles, herpes virus), mycobacteria, parasites (malaria, trypanosome) and superantigen producing bacteria (staphylococci, streptococci). The most common is the Human Immunodeficiency Virus (HIV) infection which causes Acquired Immune-deficiency Syndrome (AIDS) (Baliga *et al.*, 2008). In chronic infections, the presence of toxins and a high pathogen load during chronic infections leads to a hyper-

inflammatory state. The resultant tissue and organ damage makes the host vulnerable to secondary infection. Again, the persistent activation of the immune system could lead to a state of non-responsiveness and T cell anergy (Yi *et al.*, 2010).

2.2.2.2.3 Drug-induced immune deficiencies

Immune deficiencies can occur as a result of chronic corticosteroid use and as a side effect of drugs in cancer chemotherapy and maintaining immunosuppressive states (Steele, 2002). Corticosteroids serve as potent anti-inflammatory agents. They exert this effect by binding to glucocorticoid receptors and suppressing the gene transcription of pro-inflammatory proteins and stem cell production of inflammatory cells (Stahn and Buttgerit, 2008). The suppression result in reduction in lymphocyte cell counts, adhesion molecules, serum immunoglobulins and consequently a susceptibility to diseases. Immunosuppressive drugs such as cyclosporine used for controlling immune responses and avoiding transplant rejection, are implicated in immune deficiencies and make the host prone to opportunistic infections (Opelz and Dohler, 2009). Withdrawal of drug treatment invariably restores immune function. Chemotherapeutic agents such as cyclophosphamide and methotrexate, especially at high doses and after long term use, are associated with immunosuppression and an increased risk of bacterial infections (Saag *et al.*, 2008).

2.2.3 AUTO-IMMUNITY

Autoimmunity refers to a physiological or pathological immune response by auto-reactive cells and products of the immune system against host proteins known as auto antigens (Yamamoto, 2004). Auto reactive T lymphocytes and self-antigen recognizing antibodies known as auto-antibodies identify and initiate an immune response against the host's own tissues. Pathologic immune responses, due to genetic or acquired aberrations in immune mechanisms result in clinical disorders known as autoimmune diseases (Ramos *et al.*, 2015). One or more defects in immune regulatory mechanisms, clearance of apoptotic material and molecular mimicry of viral and bacterial proteins occurs in autoimmune diseases (Tanaka and Miyake, 2007; Cusick *et al.*, 2012).

There are a number of genetic, environmental and nutritional risk factors implicated in the pathogenesis of autoimmune diseases. Polygenic defects in genes encoding human leukocyte antigen, complement proteins, immunoglobulins, peptide transporter proteins and histocompatibility molecules increase the probability of the immunogenicity of auto antigens and the recognition of self-molecules to auto reactive T and B lymphocytes (Grimbacher *et al.*, 2016). Some aberrations confer a higher risk than others but the Human lymphocyte antigen is one of the best indicators of risk of autoimmune disease (Gough and Simmonds, 2007). Environmental factors directly cause and complement the genetic predisposition to immune disorders (Jörg *et al.*, 2016).

2.3 IMMUNOMODULATION AND DISEASE THERAPY

Immunomodulation refers to therapeutic interventions which alter immune responses. It may result in the enhancement of the immune responsiveness (immunostimulation), regulation of a particular response or muting of the immune system function (immunosuppression) (Gea-Banacloche, 2006). The regulatory adjustment of the immune system function has gained grounds in the treatment of allergies (Broide, 2009), autoimmune diseases (Kazatchkine and Kaveri, 2001), organ/tissue transplantation and cancer (Amano and Camara, 2013; Khalil *et al.*, 2016). Immunosurveillance of mutated cells, antigen recognition, antigen-antibody interactions, mediator release from immune cells, Toll-like receptor signaling and adaptive responses to allergens are considered viable targets for immunomodulators (Adorini *et al.*, 2004; Bascones-Martinez *et al.*, 2014; Ponce *et al.*, 2014). Current research is focused on the use of biologics such as cytokines, antibodies and synthetic drugs as immunomodulatory agents (Saxena *et al.*, 2012). An array of medicinal plants and plant-derived compounds have also shown promise in this respect (Kumar *et al.*, 2012). One such phytochemical is the steroid alcohol stigmasterol.

2.4 STIGMASTEROL

2.4.1 SOURCE AND CHEMISTRY

Stigmasterol, 3β -stigma-5,22-dien-3-ol ($3S,8S,9S,10R,13R,14S,17R$)-17-[($E,2R,5R$)-5-Ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H cyclopenta[a]phenanthren-3-ol is a naturally occurring steroidal alcohol with a molecular weight of 412.6908 g/mol ($C_{29}H_{48}O$) (Harborne, 1998). It occurs as a white or off-white powder with high melting point ($160 - 164^{\circ}C$), insoluble in water but readily soluble in non-polar solvents such as DMSO, hexane and propanol. Stigmasterol is widely distributed in nature alongside other phytosterols (Moreau *et al.*, 2002). They occur in nuts, vegetables and several medicinal plants. Structurally similar to animal cholesterol, stigmasterol is made up of a basic steroidal skeleton, a hydroxyl (OH) group attached to the ring A C3 atom and a double bond between the C5 and C6 atoms of ring B. This basic moiety is also a shared structure amongst phytosterols (Xu *et al.*, 2001). Stigmasterol varies from cholesterol and other closely related members of the phytosterol class such as β sitosterol, brassicasterol and campasterol, only by its aliphatic group attached at the C17 atom at ring D. Stigmasterol has a double bond at C22 and an ethyl attachment at C24. Due to the latter, stigmasterol is classified alongside β sitosterol as a '24 ethyl' sterol. It is a product of a squalene forming biosynthetic pathway similar to cholesterol and ergosterol producing pathways in animals and yeast respectively. It involves a pre-squalene pathway which begins with the synthesis of mevalonate from coenzyme A (Piironen *et al.*, 2000). Isopentenyl pyrophosphate (IPP), and farnesyl pyrophosphate (FPP) intermediates finally lead to squalene formation. Sterol methyl transferase 2 (SMT2) mediates a double methylation step which yields stigmasterol (Nes and Venkatramesh, 1999; Bouvier-Navé *et al.*, 1998).

2.4.2 PREVIOUS STUDIES ON STIGMASTEROL

2.4.2.1 Anti-infective and insecticidal properties

Stigmasterol isolated from *Caylusea abyssinica* demonstrated activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella thypimurium* (Edilu *et al.*, 2015). In a study by Ahamed *et al.* (2007) stigmasterol isolated from the *Grewia tiliaefolia* showed significant activity against clinical strains of *Pseudomonas aeruginosa* and *Klebsiella pneumonia*. Some anti-trypanosomal activity has also been reported. Stigmasterol showed activity, albeit weak, against blood forms of *Trypanosoma brucei* (Hoet *et al.*, 2007) while other studies have shown significant activity against trypanosome species by stigmasterol containing fractions. (Nweze, 2012; Rosas *et al.*, 2007). Some insecticidal activity has recently been reported. Stigmasterol, at concentrations above 0.5% has been reported to show activity against rabbit ear mites *Psoroptes cuniculi* (Nong *et al.*, 2017) while doses of 25 µg/ ml have been reported to be cytotoxic to *Spodoptera litura* cells (Huang *et al.*, 2009).

2.4.2.2 Anti-cholesterol properties

The lipid lowering properties of phytosterols is one of the most studied and most widely reported effects in literature (Ostlund, 2002; Normen *et al.*, 2004; Thornton *et al.*, 2011). Most studies point to competition with dietary and biliary cholesterol uptake in the intestine as a possible mechanism of action (Nissinen *et al.*, 2006; Calpe-Berdiel *et al.*, 2007). A meta-analysis of randomized control studies by Demonty *et al.* (2009) has established a dose-dependent relationship between phytosterol consumption and low density lipoprotein-cholesterol (LDL-C) reduction. Of the 84 trials which were reviewed, an 8.8% (95% CI: -9.4, -8.3) relative reduction in LDL-cholesterol concentrations, for an average daily phytosterol dose of 2.15 g was determined.

2.4.2.3 Anti-inflammatory and anti-allergic properties

Phytosterol mixture, containing 13% stigmasterol, fed to apo E knock-out mice reduced LPS-induced TNF α and IL-6 from isolated spleen cells (Nashed *et al.*, 2005). Orally administered phytosterol mixture containing stigmasterol (28.4%) reduced

carrageenan-induced paw oedema in mice (Navarro *et al.*, 2001). Garcia *et al.* (1999) showed anti-inflammatory effects of topically applied stigmasterol isolated from *Eryngium foetidum* against TPA-induced acute and chronic ear inflammation. A mixture of stigmasterol and β sitosterol isolated from *Bauhinia strychnifolia* was found to have promising anti-allergic properties. The mixture inhibited β -hexosaminidase release from Rat Basophilic Leukemia 2H3 (RBL-2H3) cells with an IC_{50} of 77.5 μ M (Bunluepuech *et al.*, 2013).

2.4.2.4 Anti-oxidant and anti-cancer activity

Stigmasterol has been shown to possess 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, superoxide and hydroxyl radical scavenging properties, with IC_{50} of 220 μ g/ml, 210 μ g/ml and 150 μ g/ml respectively (Shanthakumar *et al.*, 2013). Stigmasterol has also shown antioxidant properties *in vivo* in Erlich Ascites Carcinoma injected mice (Ghosh *et al.*, 2011) and in several other studies (Panda *et al.*, 2009; Ghosh *et al.*, 2014). In a study investigating the relationship between diet and ovarian cancer McCann *et al.* (2003) reported a reduced risk in women with high intake of stigmasterol (OR 0.42, 95% CI, 0.20-0.87). Stigmasterol at 5 mg/kg and 10 mg/kg significantly inhibited tumor weight and tumor cell count as well as other key parameters of disease progression in Erlich Ascites Carcinoma injected mice (Ghosh *et al.*, 2011). Orally administered stigmasterol, isolated from *Azadirachta indica*, at 200 mg/kg and 400 mg/kg three times a week for 16 weeks reduced tumor size and papilloma count in 7,12-dimethylbenz[a]anthracene (DMBA)-induced skin cancer in Swiss albino mice (Ali *et al.*, 2015).

2.4.2.5 Analgesic and anti-arthritic properties

Stigmasterol isolated from *Phyllanthus corcovandensis* has been reported to have analgesic properties (Santos *et al.*, 1995). Given intraperitoneally, at 10-100 mg/kg, stigmasterol significantly inhibited both the neurogenic and inflammatory phases of formalin-induced pain in male Swiss mice. A less potent response was however observed when stigmasterol was administered orally. Peres *et al.* (1998) determined an ID_{50} of 16 mg/kg for stigmasterol against acetic acid-induced nociception. These

findings are supported by a later investigation by Githinji *et al.* (2012), who identified stigmasterol as one of the major analgesic phytoconstituents in *Mondi whytei* against chemical-induced pain. Stigmasterol is also referred to as the ‘Wulzen anti-stiffness factor’ after discoveries made by Rosalind Wulzen and her colleagues. They discovered, that the then unknown sterol alleviated stiffness and joint calcification induced in guinea pigs by a special diet (Ross *et al.*, 1949). Studies by Gabay *et al.* (2010) and Chen *et al.* (2012) represent more recent efforts in investigating the anti-arthritic properties of stigmasterol. The former determined that pre-incubation of mouse chondrocytes and human osteoarthritis chondrocytes with stigmasterol inhibited IL-1 β -induced pro-inflammatory gene expression. Gabay and his colleagues investigated the potential anti-osteoarthritic properties of stigmasterol *in vivo*. Cartilage damage in rabbits induced by anterior cruciate ligament (ACL) transection was alleviated by intra-articular administration of stigmasterol.

2.4.2.6 Hypoglycaemic activity

Panda *et al.* (2009) recorded hypoglycaemic effect of stigmasterol isolated from the bark of *Buten monosperma*. Treatment of normoglycaemic Swiss albino mice with 1.3 and 2.6 mg/kg/day for 20 days reduced serum glucose concentration and increased insulin levels. In an alloxan-induced hyperglycaemia model, a 10 day and single dose oral treatment of diabetic Wistar rats with stigmasterol (10 and 50 mg/kg), isolated from *Bacopa monnieri*, both significantly reduced blood glucose levels (Ghosh *et al.*, 2014). Bioassay of isolated stigmasterol (*Pseuderanthemum palatiferum*) (0.25 and 0.5 mg/kg/d for 21 days) showed reduction in fasting blood glucose (FBG) levels and stimulation of insulin secretion in streptozocin-induced diabetic rats (Nualkaew *et al.*, 2015).

CHAPTER THREE

3.1 EFFECT OF STIGMASTEROL ON INNATE IMMUNE RESPONSES

3.1.1 INTRODUCTION

Stigmasterol and other closely related substances belong to a class of compounds referred to as phytosterols. These compounds have established and emerging health benefits including lipid lowering, anticancer and anti-inflammatory effects (Moreau *et al.*, 2002; Yuk *et al.*, 2007). Previous studies demonstrating the anti-inflammatory and anti-arthritic actions of stigmasterol suggests some modulatory effects on the immune system (Gabay *et al.*, 2010; Chen *et al.*, 2012). However, there still exists a significant knowledge gap with regards to the extent and specific role it plays in immune system disorders.

The innate immune system is made up of a variety of physical barriers, chemical and cellular components that form the first-line of defense for the body against foreign antigens and invading pathogens (Diacovich and Gorvel, 2010). The innate immune system has been shown to play a critical role in disease pathogenesis (Wickelgren, 2006; Moore and Freeman, 2008; Heneka *et al.*, 2015). Hyperactivity and imbalance in the immune system accounts for the underlying pathophysiology of several conditions such as rheumatoid arthritis, hypersensitivity reactions and septic shock (Shelley *et al.*, 2003; Invernizzi and Gershwin, 2009). Upon exposure to the pathogen, sentinel cells initiate immune responses aimed at containing and eliminating the invading pathogen. Stimulation of pathogen recognition receptors by pathogen-associated molecular patterns triggers the inflammatory response (Gay and Gangloff, 2007). Macrophage activation for instance, triggers the production of pro-inflammatory mediators such as nitric oxide and prostaglandins, increase in serum tumor necrosis factor alpha (TNF α), and interleukins such as IL-1 and IL-6 aimed at fighting perceived pathogen invasion. However, a persistent increase in the production of these factors can mediate tissue damage, hypo-perfusion and circulatory failure, progressive multiple organ failure and eventually death (Shapiro *et al.*, 2006; Kou *et al.*, 2011).

Lipopolysaccharide, LPS is a structural and functional component of the cell wall of gram negative bacteria made up of an outer part (O) polysaccharide, the core and a lipid component, lipid A (Skibbska *et al.*, 2006). LPS activates cells of the innate immune system by binding to Toll-like receptor 4, TLR4, and commences a hyper-inflammatory state similar to sepsis (Goraca *et al.*, 2013). Key aspects of innate immunity such as antigen presentation, pathogen recognition, phagocytosis and the subsequent release of inflammatory mediators have all received attention as possible drug targets in the development of immune-modulating drugs (Gong and Chen, 2003; Ripoll *et al.*, 2013).

In this section, the effects of stigmasterol on LPS-induced innate immune responses *in vivo* are investigated. It focuses on the potentially mitigating effect of stigmasterol on selected fatal and non-fatal immune responses in murine models after intraperitoneal LPS administration.

3.1.2 MATERIALS

3.1.2.1 Chemicals and reagents

Stigmasterol (95%), dexamethasone, polyethylene glycol (PEG), diclofenac sodium and lipopolysaccharide (LPS) (*Escherichia coli* 055: B5) were obtained from Sigma Aldrich (St. Louis, USA).

3.1.2.2 Animals

Wistar rats (180 - 220 g) and C57BL/6 mice (20 - 25 g) of either sex were obtained from Noguchi Memorial Institute for Medical Research (NMIMR), Legon, Ghana. Animals were kept under standard temperature and humidity (temperature $25 \pm 2^{\circ}\text{C}$ with a 12 h light-dark cycle) at the Animal house facility of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST. Experimental animals could access commercial chow and distilled water *ad libitum*. All protocols used in this study were approved by the Faculty of Pharmacy Ethics Committee and animal handling was done in compliance with Animal Welfare Regulations (USDA 1985; US Code, 42 USC § 289d) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002).

3.1.3 METHODS

3.1.3.1 LPS-induced pyrexia in rats

The basal rectal temperature of Wistar rats was recorded with a veterinary digital thermometer (Digi Vet SC 12, KRUSE Denmark). Rats were randomized into 7 groups (n = 5) and given the following treatments *via* the intraperitoneal route; Group 1, naïve control (saline 5 ml/kg), Group 2 (polyethylene glycol, PEG 5 ml/kg), Group 3 (diclofenac, 10 mg/kg), Groups 4 - 6 (stigmaterol 10, 50 and 100 mg/kg respectively), Group 7 (stigmaterol, 100 mg/kg). Subsequently, rats in Groups 1 and 7 received *via i.p.* Phosphate Buffered Saline (PBS) 5 ml/kg only, while the remaining animals received LPS (*Escherichia coli* 055:B5, 0.04 mg/kg *i.p.*) 30 min after drug treatment. Basal temperature readings for all treatments and LPS injections were done between 8.00 – 9.00 GMT to avoid temperature differences due to circadian rhythm. The change in rectal temperature in all rats was recorded 30 min after PBS or LPS injection and at subsequent 30 min intervals for 4 h. Pyrexia was monitored as the change in rectal temperature over the 4 h period and was presented in a time-course curve. The total pyrexia response or Thermal Index, T.I. was expressed as the area under the time-course curves (AUCs).

3.1.3.2 LPS-induced systemic inflammation in mice

C57BL/6 mice were randomly placed into 6 groups (n = 5) and treated intraperitoneally with either saline (5 ml/kg), PEG (5 ml/kg), dexamethasone (7 mg/kg) or stigmaterol (10, 50 and 100 mg/kg). Subsequently, naïve control mice received, *via i.p.* route, PBS 5 ml/kg only while mice in the other groups were challenged with LPS (*Escherichia coli*, 055:B50, 0.3 mg/kg *i.p.*). Mice were subjected to the following tests:

3.1.3.2.1 Neutrophil count

Mice were sacrificed by cervical dislocation 6 h after treatment. The jugular vein was quickly dissected and blood collected into EDTA tubes for blood neutrophil cell count. Normal saline (0.9% w/v NaCl, 7 ml) was injected into the peritoneal cavity of each mouse and the abdomen gently massaged. A mid-line incision was made and fluid aspirated with a Pasteur pipette into Eppendorf tubes. Neutrophil counts were

determined in blood and peritoneal fluid with an automated analyzer (Sysmex KX-21N, Sysmex America Inc., Illinois, USA).

3.1.3.2.2 Multiple organ damage

a) Histology

The lungs and liver of each mouse were harvested, stored in 10% buffered formalin and embedded in paraffin. For histological investigations, 3 μ m sections were cut, deparaffinized, dehydrated and stained with haematoxylin and eosin (H & E). The tissue sections were observed for inflammatory changes under light microscope, Leica DM2500 M (Leica Microsystems, Wetzlar, Germany). For assessment of lung tissue, a scoring method as previously described by Zare *et al.* (2008) with slight modifications was used. Briefly, the scoring system adopted was; 0, no cell; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2-4 cell layers deep; 4, a ring of cells >4 cell layers deep in the peribronchiolar and perivascular regions. Alveolar cell infiltration was assessed as; 0, no infiltrate or widening septa; 1, few infiltrates with widening septa; 2; obvious infiltrates with widening septa; 3, filled alveolar air spaces with thickened septa. Scores for peribronchiolar, perivascular and alveolar cell infiltration were summed into an 11-point composite score.

Liver tissues were observed for the occurrence of focal inflammation, mononuclear infiltrate, loss of normal architecture and necrosis. Acute liver injury was assessed using a composite inflammation and necrosis score as described by Siegmund *et al.* (2002). Briefly, lobular inflammation (0, no inflammation; 1, mild; 2, moderate; 3, severe), portal inflammation (0, no inflammation; 1, mild; 2, moderate; 3, severe) and necrosis (0, no necrosis; 1, < 10% of liver parenchyma; 2, 10 - 25% of liver parenchyma; 3, > 25% of liver parenchyma) were scored and summed up to determine the overall histopathology score. Five 3 μ m sections for each treatment group were scored by a trained blinded researcher.

b) Biochemical analysis

Mice were sacrificed by cervical dislocation 6 h after treatment. The jugular vein was quickly dissected and blood collected into plain capillary tubes for serum biochemical analysis for alanine transaminase, ALT and aspartate transaminase, AST. Briefly, blood collected into sterile capillary tubes was allowed to clot and centrifuged for 20 min at $1000 \times g$. Aliquots of serum were collected into sterile Eppendorf tubes. Levels of serum ALT and AST were determined using an automatic analyzer, ATAC 8000 Random Access Chemistry System (Elan Diagnostics, Smithfield, RI, USA).

3.1.3.3 LPS-induced systemic shock (mortality)

Control mice were pretreated with PEG (5 ml/kg) while test groups received either dexamethasone (7 mg/kg) or stigmasterol (10, 50 and 100 mg/kg). After 1 h, mice were injected with LPS (*Escherichia coli* 055:B5, 20 mg/kg i.p) previously established as the LD₁₀₀ in 12 – 26 h after challenge in preliminary studies. Mice were observed for mortality for 144 h (6 days).

3.1.3.4 Statistical analysis

Data is presented as mean $\pm$ standard error of mean (SEM). Data analysis was done using One-way analysis of variance (ANOVA). Multiple comparisons between treatment groups were performed using Dunnet's *post hoc* test. Mortality data is presented as survival curves and statistical differences compared to control were determined using Log-rank (Mantel-Cox) test. All statistical analyses were done using GraphPad for Windows version 6 (GraphPad Prism Software, San Diego, USA).

3.1.4 RESULTS

3.1.4.1 Effect of stigmasterol on LPS-induced pyrexia in rats

Pyrexia was monitored as the change in rectal temperature over the 4 h period and was presented in a time-course curve (Fig. 3.1.1A and 3.1.1C) while the total pyrexia response over 4 h or Thermal Index, T.I was expressed as the area under the time-course curve (Fig. 3.1.1B and 3.1.1D). No significant change in the rectal temperature in both naïve and stigmasterol only-treated rats was recorded (Fig. 3.1.1A and 3.1.1B). In PEG-

treated LPS-challenged rats, a significant and persistent change in basal rectal temperature was observed at 120 min with a maximal change of $2.38 \pm 0.24^{\circ}\text{C}$ (Fig 3.1.1A) and a total pyrexia, Thermal index of 431.40 ± 31.8 AUC units (Fig. 3.1.1B). Pretreatment with diclofenac 10 mg/kg significantly suppressed both the peak febrile response and the Thermal index to $0.26 \pm 0.21^{\circ}\text{C}$ and 74.43 ± 25.45 AUC units respectively compared to the naïve control (Fig. 3.1.1C and 3.1.1D). Similarly stigmasterol 10, 50 and 100 mg/kg significantly reduced the peak increase in basal rectal temperature to $1.30 \pm 0.21^{\circ}\text{C}$, $0.82 \pm 0.20^{\circ}\text{C}$, $0.74 \pm 0.22^{\circ}\text{C}$ respectively compared to the naïve control (Fig. 3.1.1C) significantly reducing the Thermal index to 259.20 ± 45.09 , 202.60 ± 25.22 , 98.08 ± 26.94 AUC units, respectively (Fig. 3.1.1D). Thus 39.93 $\pm$ 10.52%, 53.05 $\pm$ 5.84% and 77.27 $\pm$ 6.25% inhibition of LPS-induced pyrexia, respectively was obtained with 10 -100 mg/kg of stigmasterol.



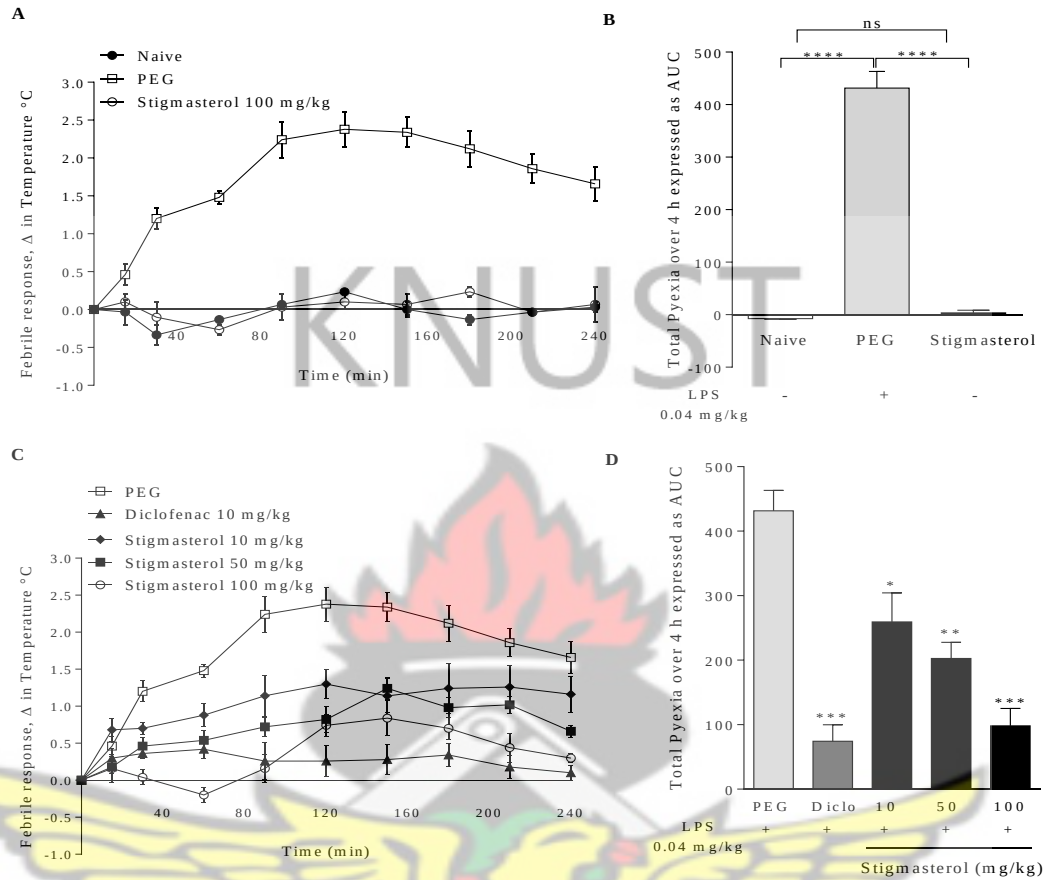


Fig. 3.1.1 Effect of stigmasterol on LPS-induced pyrexia. Wistar rats (180 – 220g) received either saline 5 ml/kg, PEG 5 ml/kg, diclofenac, Diclo 10 mg/kg or stigmasterol (10 - 100 mg/kg) and subsequently challenged 30 min after treatment with either PBS or LPS. Maximal pyrexia was monitored as change in rectal temperature measured 30 min post PBS/LPS injection and at subsequent 30 min intervals for 4 h (A and C). Total pyrexia was expressed as AUC of time course curves (B and D). Data is presented as mean $\pm$ SEM (n=5). ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 compared to PEG-treated control (one-way ANOVA followed by Dunnett's *post hoc* test).

3.1.4.2 LPS-induced systemic inflammation in mice

3.1.4.2.1 Effect of stigmasterol on neutrophil accumulation

Intraperitoneal administration of LPS to the PEG-treated mice yielded a ten-fold increase in neutrophil count in both blood (Fig. 3.1.2A) and peritoneal fluid (Fig. 3.1.2B) respectively above naïve levels. In the blood, the mean neutrophil count of $1.27 \pm 0.11 \times 10^3$ cells/ μ L in the naïve control mice was significantly increased to $10.85 \pm 1.14 \times 10^3$ cells/ μ L in the LPS-challenged PEG-treated mice. This count was reduced to $10.73 \pm 0.92 \times 10^3$ cells/ μ L, $7.46 \pm 1.08 \times 10^3$ cells/ μ L, and $5.83 \pm 0.69 \times 10^3$ cells/ μ L respectively in the stigmasterol-treated mice at 10, 50 and 100 mg/kg albeit insignificant at 10 mg/kg. Thus stigmasterol 50 and 100 mg/kg offered respectively $31.26 \pm 10.00\%$ and $46.23 \pm 6.44\%$ inhibition of neutrophil proliferation in blood (Fig. 3.1.2A). In the peritoneal fluid, neutrophil cell count in the naïve control significantly increased from $0.70 \pm 0.23 \times 10^3$ cells/ μ L to $7.65 \pm 0.81 \times 10^3$ cells/ μ L in the PEG-treated LPS-challenged mice. There was a significant inhibition of neutrophil proliferation of $51.40 \pm 9.07\%$ by stigmasterol administered at 100 mg/kg (Fig. 3.1.2B). However, the neutrophil counts in the peritoneal fluid on treatment with 10 and 50 mg/kg respectively of stigmasterol were $8.47 \pm 0.90 \times 10^3$ cells/ μ L and $7.68 \pm 0.88 \times 10^3$ cells/ μ L respectively and thus not statistically different from the PEG-treated LPS-challenged mice. As expected, dexamethasone significantly inhibited neutrophil proliferation in both blood and peritoneal fluid by $67.25 \pm 6.10\%$ and $57.73 \pm 3.82\%$ respectively.

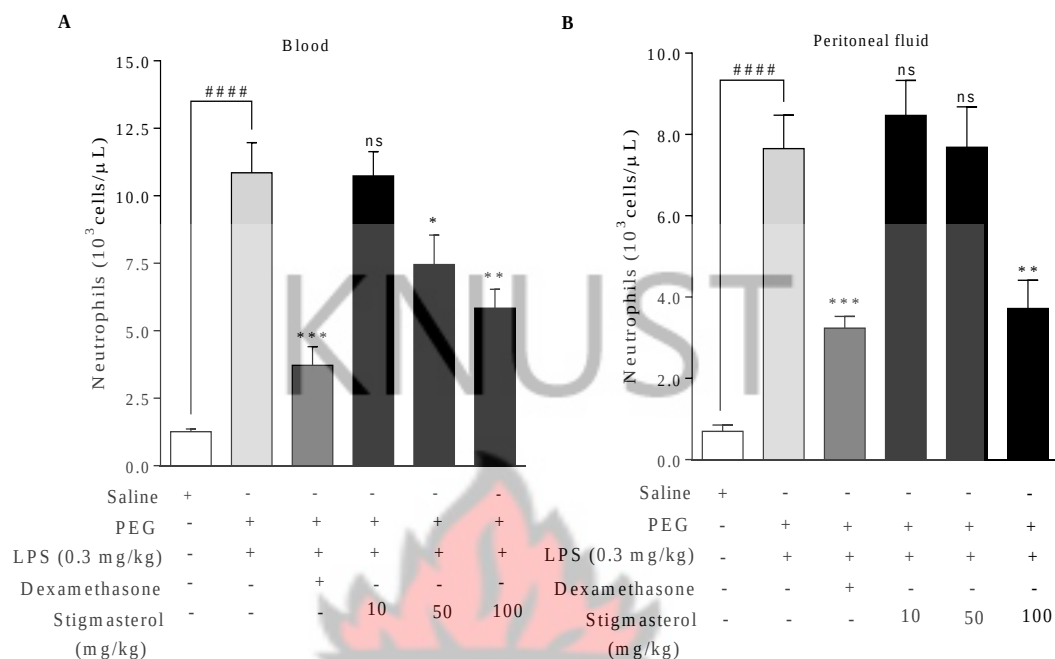


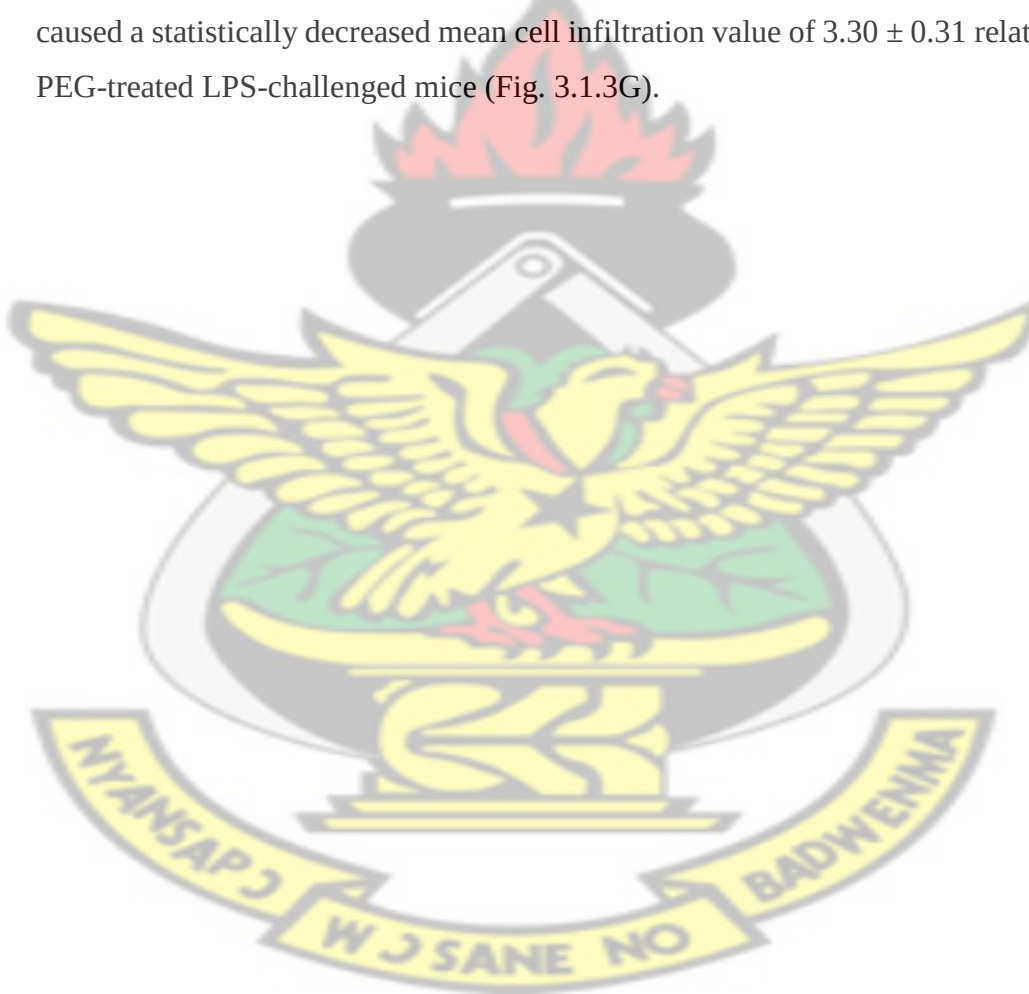
Fig. 3.1.2 Effect of stigmasterol on LPS-induced neutrophilia. C57BL/6 mice (20 – 25 g) received either saline 5 ml/kg (naïve group), PEG 5 ml/kg, dexamethasone 7 mg/kg or stigmasterol (10 – 100 mg/kg) 30 min before challenge with LPS (0.3 mg/kg, *i.p*) or PBS. Mice were sacrificed 6 h after challenge. Neutrophil cell count was obtained on blood collected by dissection of jugular vein (A) and peritoneal fluid collected by lavage with PBS (B). Data is expressed as mean $\pm$ SEM (n = 5). ***P<0.001, **P<0.01, *P<0.05 and ns (not significant) compared to PEG-treated control; ####P<0.0001 compared to naïve, saline-treated group (one-way ANOVA followed by Dunnett's *post hoc* test).

3.1.4.2.2 Multiple organ damage

a) Effect of stigmasterol on LPS-induced lung damage

Microscopic observation of lung sections showed features consistent with system inflammation-associated organ damage in PEG-treated LPS-challenged mice with severe airway tissue infiltration by inflammatory cells, leading to congestion of alveolar air spaces and peribronchiolar regions. Hypertrophy of bronchiole smooth muscle and desquamation of basement epithelium were observed as signs of progressive lung damage (Fig. 3.1.3B) as compared to the normal lung architecture in the saline-treated

naïve mice (Fig. 3.1.3A). On treatment with stigmasterol, lung sections showed improved lung architecture with little destruction of the epithelial linings and significant reduction on septal thickening in alveolar air spaces. A marked reduction in cell infiltration was also observed (Fig. 3.1.3D - F). On quantification, a statistically increased cell infiltration score of 9.20 ± 0.73 was obtained for the PEG-treated LPS-challenged mice relative to that of 1.60 ± 0.24 for the naïve control mice (Fig. 3.1.3G). Stigmasterol 10, 50 and 100 mg/kg showed significantly reduced cellular congestion and an overall improved lung tissue architecture with mean inflammation scores of 6.50 ± 0.54 , 4.60 ± 0.40 and 4.10 ± 0.42 respectively (Fig. 3.1.3G). Similarly, dexamethasone caused a statistically decreased mean cell infiltration value of 3.30 ± 0.31 relative to the PEG-treated LPS-challenged mice (Fig. 3.1.3G).



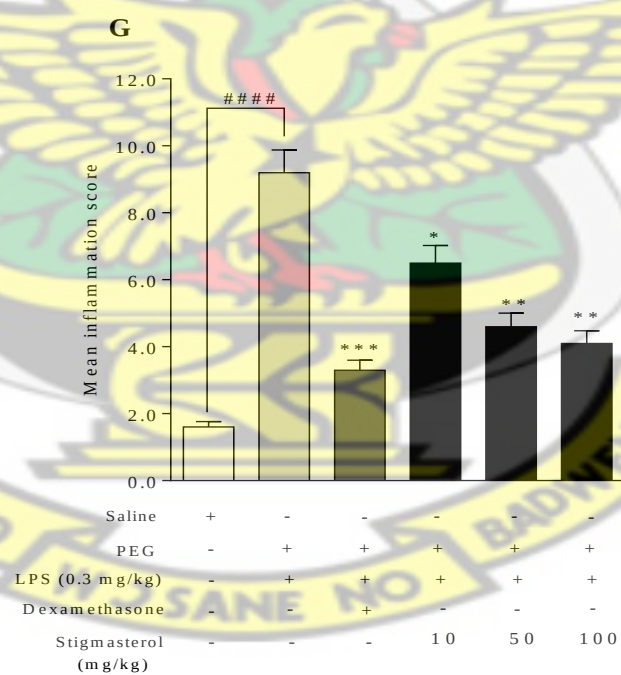
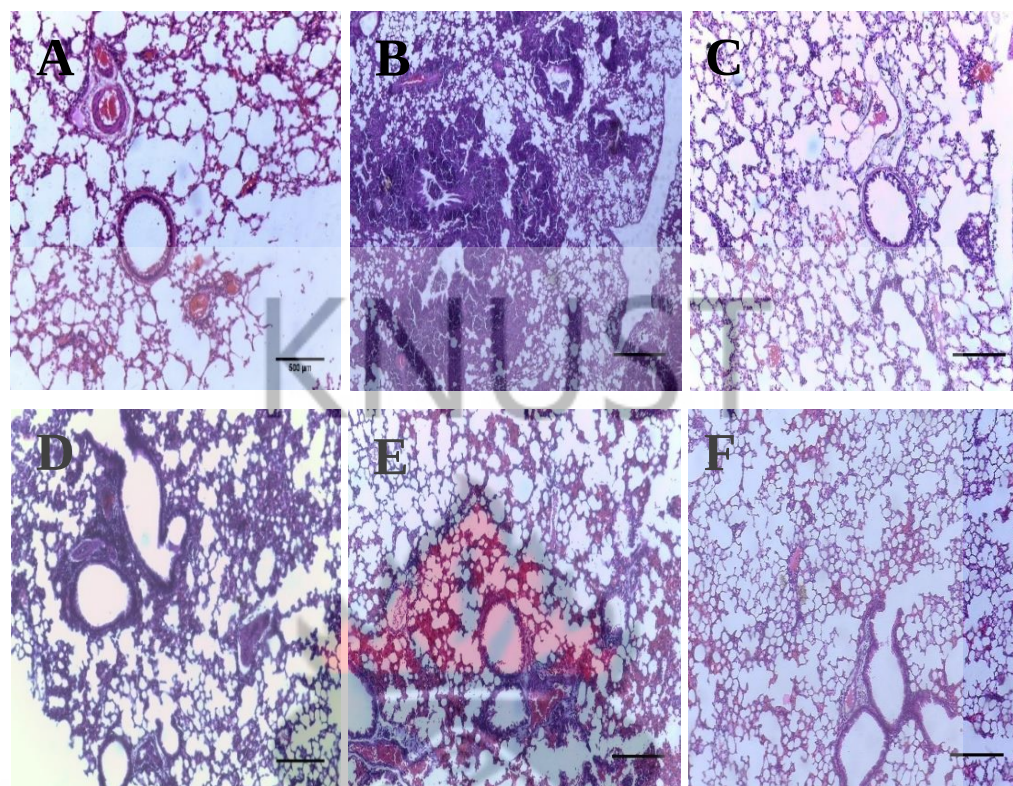


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Fig. 3.1.3 Effect of stigmasterol on LPS-induced lung damage. Mice received either (A) saline, 5 ml/kg, (B) PEG 5 ml/kg, (C) dexamethasone (Dex) 7 mg/kg or (D – F) stigmasterol (10 - 100 mg/kg) 30 min before challenge with LPS (0.3 mg/kg, *i.p.*) or PBS (0.1 ml, *i.p.*). Mice were sacrificed 6 h after challenge and the lungs fixed in 10% formalin and embedded in paraffin. Sections (3 μ m) were stained with H & E stain. Degree of lung inflammation was quantified using a slightly modified infiltration score described by Zare *et al.* (2008). Data is expressed as mean $\pm$ SEM (10 sections) (G). *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ compared to PEG-treated group; #### $P < 0.0001$ compared to naïve, saline-treated group (one-way ANOVA followed by Dunnett's *post hoc* test). Micron bar represents 500 μ m.

b) Effect of stigmasterol on LPS-induced liver damage

Naïve mice presented with a normal liver architecture with little or no cell infiltration, no necrosis and an intact and distinguishable hepatic lobule structure (Fig. 3.1.4A). In PEG-treated LPS-challenged mice, there was severe liver damage characterized by severe mononuclear infiltration, hepatic sinus expansion, severe degradation of liver structure and extensive necrosis in liver sections (Fig. 3.1.4B). Stigmasterol markedly controlled these features in pretreated mice (Fig. 3.1.4D - F) with moderate inflammatory cell infiltration at all doses and focal necrosis at 10 mg/kg. Dexamethasone showed significant inhibition of parameters of inflammatory damage and maintained liver architecture (Fig. 3.1.4C). Assessment of liver sections of PEG-treated LPS challenged mice showed a significantly increased mean histopathology score of 7.75 ± 0.4 relative to a mean score of 0.50 ± 0.20 for the naïve control group (Fig. 3.1.4G). Stigmasterol at 10, 50 and 100 mg/kg showed significant suppression of acute liver injury with mean histopathology scores of 5.50 ± 0.51 , 5.13 ± 0.43 and 3.9 ± 0.33 respectively. Likewise, dexamethasone controlled LPS-induced damage with a significantly reduced score of 2.75 ± 0.36 (Fig. 3.1.4G).

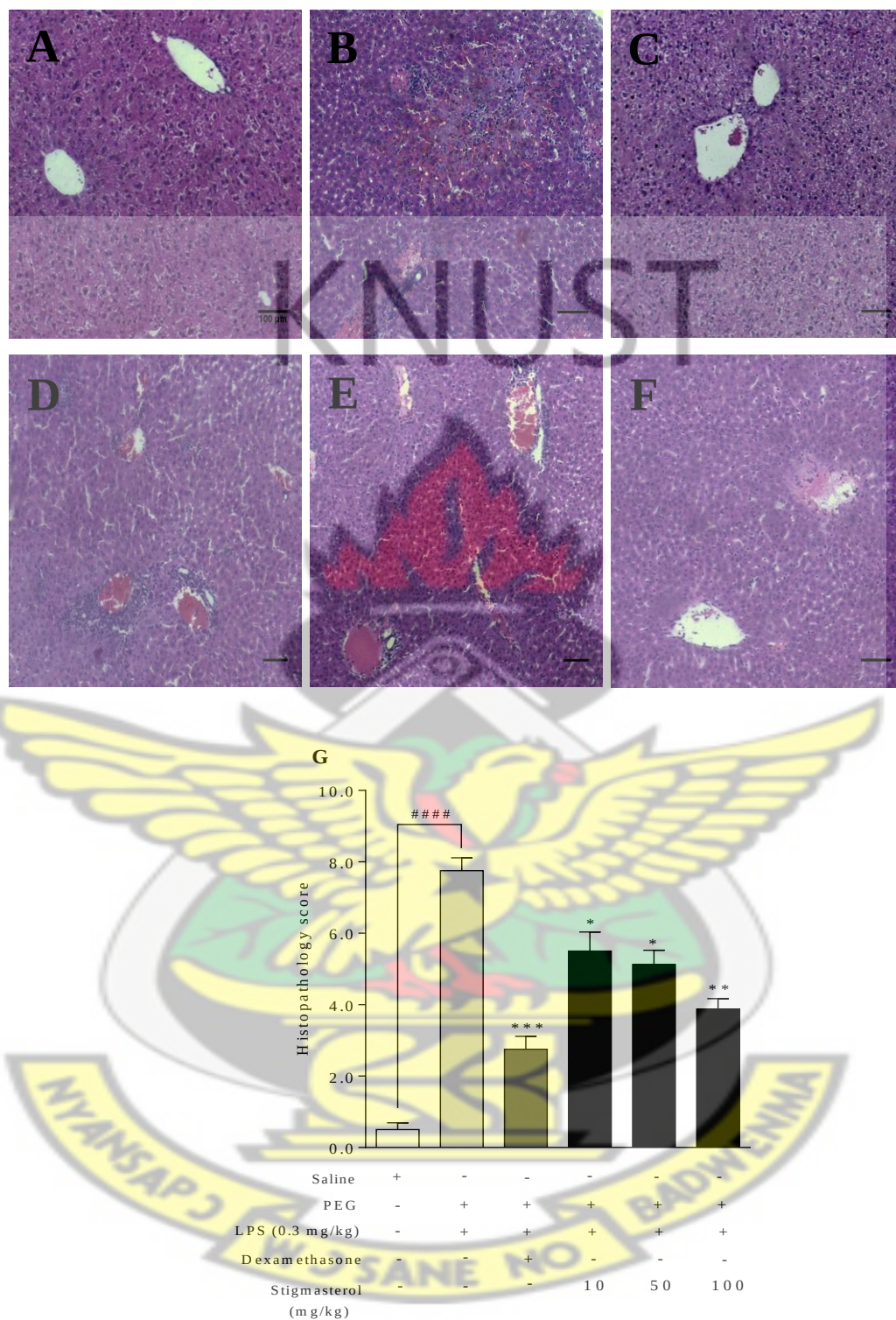


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Fig. 3.1.4 Effect of stigmasterol on LPS-induced liver damage. Mice received either (A) saline, 5 ml/kg, (B) PEG 5 ml/kg, (C) dexamethasone 7 mg/kg or (D – F) stigmasterol (10 - 100 mg/kg) 30 min before challenge with LPS (0.3 mg/kg, i.p) or PBS. Mice were sacrificed 6 h after challenge and the liver fixed in 10% formalin and embedded in paraffin. 3 μ m sections were stained with H & E stain for histological examination. Extent of liver injury was quantified using a composite inflammation and necrosis score described by Siegmund *et al.* (2002). Data is expressed as mean $\pm$ SEM (5 sections) (G). ***P<0.001, **P<0.01 and *P<0.05 compared to PEG-treated group; ####P<0.0001 compared to naïve, saline-treated group (one-way ANOVA followed by Dunnett's *post hoc* test). Micron bar represents 100 μ m.

c) Biochemical analysis

Effect of stigmasterol on serum alanine transaminase, ALT and aspartate transaminase, AST

The mean serum level of ALT in PEG-treated, LPS-challenged mice was 1003.00 ± 57.63 U/L, representing a seven-fold increase compared to levels in naïve mice of 141.10 ± 13.91 U/L (Fig. 3.1.5A). Stigmasterol (10, 50 and 100 mg/kg) significantly reduced the serum levels of ALT in the PEG-treated LPS-challenged mice to 646.70 ± 55.94 U/L, 538.50 ± 70.14 U/L, and 526.10 ± 39.52 U/L respectively giving $35.53 \pm 5.61\%$, $46.32 \pm 6.85\%$ and $47.55 \pm 3.90\%$ inhibition of ALT expression respectively (Fig. 3.1.5A). A similar trend was observed for AST. Statistically increased serum levels of AST in PEG-treated LPS-challenged mice of 1129.00 ± 63.32 U/L was obtained compared to levels recorded in naïve mice (225.20 ± 12.11 U/L) (Fig. 3.1.5B). Stigmasterol-treated mice showed significantly reduced LPS-induced AST expression. Stigmasterol at 10, 50 and 100 mg/kg recorded mean serum levels of 609.90 ± 57.00 U/L, 623.60 ± 67.92 U/L, and 495.61 ± 41.83 U/L representing $45.98 \pm 5.10\%$, $44.77 \pm 6.01\%$ and $56.15 \pm 3.68\%$ inhibition of serum levels of AST respectively (Fig. 3.1.5B). Treatment with dexamethasone showed $60.20 \pm 4.92\%$ and $67.77 \pm 2.71\%$ inhibition of the serum levels of ALT and AST respectively compared to PEG-treated, LPS challenged mice (Fig 3.1.5A and 3.1.5B).

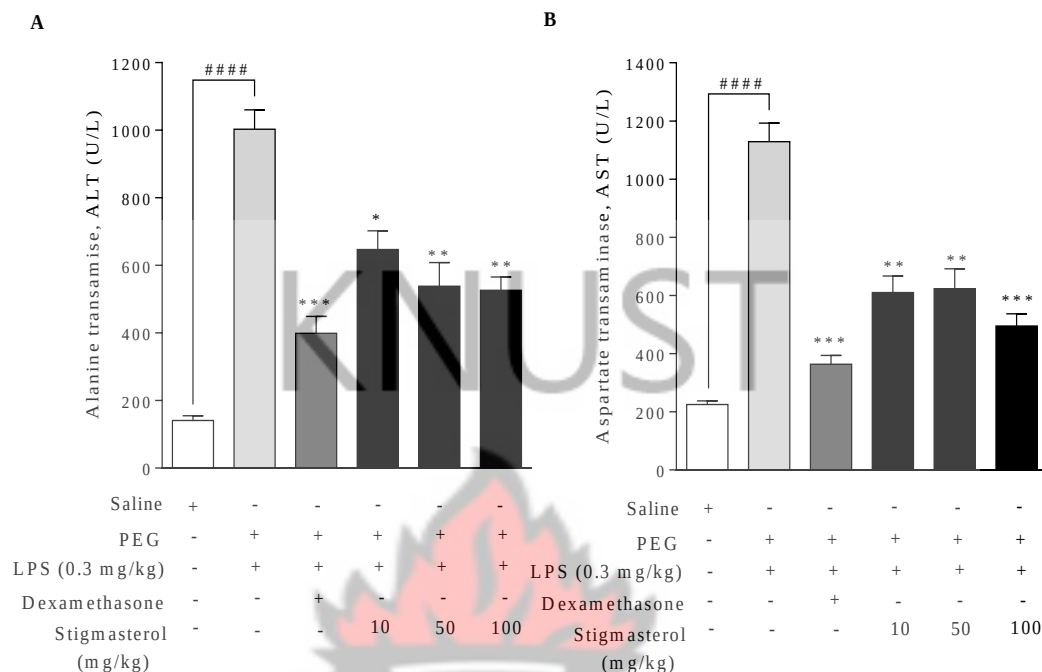


Fig. 3.1.5 Effect of stigmasterol on LPS-induced alanine transaminase (ALT) and aspartate transaminase (AST). C57BL/6 mice received either saline 5 ml/kg (naïve group), PEG 5 ml/kg, dexamethasone 7 mg/kg or stigmasterol (10 - 100 mg/kg) 30 min before challenge with LPS (0.3 mg/kg, *i.p*) or PBS. Mice were sacrificed 6 h after challenge and blood collected by dissection of jugular vein into plain capillary tubes for serum biochemical analysis for levels of (A) ALT and (B) AST. Data is expressed as mean enzyme levels (U/L) $\pm$ SEM (n = 5). *** P <0.001, ** P <0.01 and * P <0.05 compared to PEG-treated group; #### P <0.0001 compared to naïve saline-treated group (one-way ANOVA followed by Dunnett's *post hoc* test).

3.1.4.3 Effect of stigmasterol on LPS-induced systemic shock (Mortality)

Lethal dose of lipopolysaccharide (20 mg/kg *i.p.*) induced hypothermia, labored breathing and eventual death in C57BL/6 mice. Mortality due to fatal septic shock was recorded in all the PEG-treated LPS-challenged mice within 24 h (Fig. 3.1.6). Stigmasterol at 50 mg/kg and 100 mg/kg significantly delayed death and offered 40% survival in mice at the end of the six-day observation period (Fig. 3.1.6). There was no significant protection from LPS-induced mortality with the 10 mg/kg of stigmasterol

when compared to the control mice. Treatment with a single dose of dexamethasone (7 mg/kg) 30 min before LPS challenge offered 60% survival (Fig. 3.1.6).

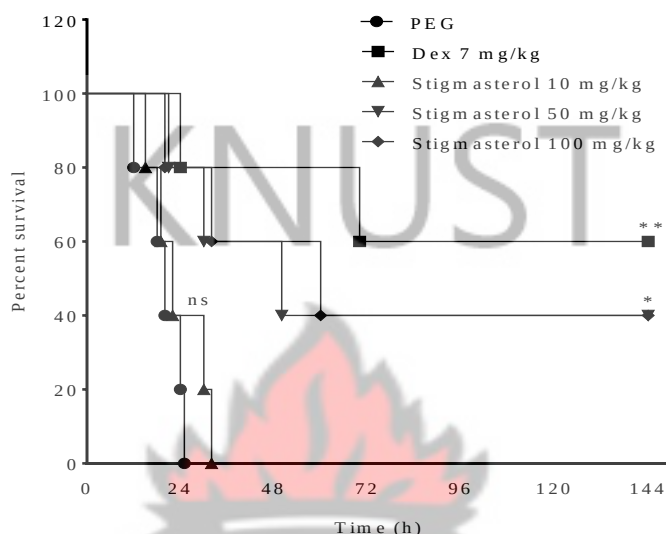


Fig. 3.1.6. Effect of stigmasterol on LPS-induced mortality in C57BL/6 mice. Fatal systemic shock was induced in mice by intraperitoneal injection of LPS, 20 mg/kg. Mice received either PEG 5 ml/kg, dexamethasone, Dex 7 mg/kg or stigmasterol 10, 50 and 100 mg/kg 30 min before LPS challenge. Mortality was monitored over 6 days. Data is presented as survival curves. Statistical differences compared to control (PEG) were determined using Log-rank (Mantel-Cox) test.

3.1.5 DISCUSSION

The inhibitory effect of stigmasterol on LPS-elicited, Toll-like receptor-mediated innate immune responses was investigated. Following lipopolysaccharide administration, the protective effect of stigmasterol on febrile responses, systemic inflammatory syndrome and ultimately endotoxin-induced mortality was assessed.

In this study, stigmasterol was shown to be effective in reducing febrile responses triggered by lipopolysaccharide. Peak and total pyrexia was significantly inhibited by pretreatment with stigmasterol at doses used. Host responses to bacterial infection are mostly mediated by the innate immune system via the action of a variety of cells, cytokines and pro-inflammatory mediators (Diacovich and Gorvel, 2010). Pathogen-

associated molecular patterns such as lipopolysaccharide stimulate cell surface pathogen recognition receptors on macrophages and dendritic cells, initiating a series of immune and inflammatory responses (Doyle and O'Neill, 2006). Though initially intended to contain the threat, an exaggerated immune response can be equally harmful to the host. Uncontrolled fever, haemodynamic changes, tissue damage and septic shock often occur as a result of the hyper-inflammatory state mediated by the innate immune system (Balk, 2000). Upon LPS-TLR4 binding, macrophages synthesize and release endogenous pyrogens namely IL-1, IL6 and TNF α into systemic circulation. The binding of these cytokines to the endothelium receptors in the brain and subsequent triggering of the arachidonic acid pathway releases PGE₂, the main mediator of the fever response (Dinarello, 2004; Anochie, 2013). Non-steroidal anti-inflammatory drugs, NSAIDS such as diclofenac disrupt this last step by inhibiting cyclooxygenase conversion of arachidonic acid to prostaglandin (Vane, 2000). The results therefore is not surprising as the antipyretic effect recorded for stigmasterol is consistent with literature. A study by Saadawi *et al.* (2012) demonstrated significant inhibition of prostaglandin and thromboxanes from LPS-stimulated whole blood by stigmasterol and suggested the inhibition of cyclooxygenase activity as a possible mechanism of action. Other closely related phytosterols have also been reported to have inhibitory effect on endogenous pyrogen release and cyclooxygenase expression (Medeiros *et al.*, 2007; Kim *et al.*, 2007).

LPS elicited a profound local and systemic influx of inflammatory cells and progressive organ damage, consistent with systemic inflammatory response syndrome. Macrophage activation triggers acute responses usually characterized by blood neutrophilia and neutrophil accumulation at the local inflammatory site (Holmes *et al.*, 2002). It was demonstrated that LPS yielded a ten-fold increase in both blood and peritoneal neutrophil counts in saline-treated control mice. Analysis of blood and peritoneal fluid, collected 6 h after LPS injection revealed inhibition of neutrophil flux. Perhaps this explains the protective effect observed against systemic organ damage. Sequestration of neutrophils and other inflammatory cells into target organs and the subsequent release of injurious chemicals and pro-inflammatory cytokines are implicated in LPS-induced organ failure (Cuenca *et al.*, 2006). Indeed, interventions such as modulation of

macrophage activities and neutralizing of pro-inflammatory cytokines have been shown to reverse endotoxin-induced organ damage and death (Liang, 2007; Yin *et al.*, 2010). It has been reported that the lung and liver are among the first organs to fail in both clinical and experimental septic shock (Andonegui *et al.*, 2003; Nessler *et al.*, 2012). The rapid accumulation of neutrophils in lung capillaries and the resulting injury to alveolar capillary partly accounts for this, making sepsis one of the major causes of acute respiratory distress syndrome, ARDS (Kim and Hong, 2016). The liver plays a key role in both initiation and amplification of multiple organ failure. With a greater proportion of all macrophages in the body resident in the liver as Kupffer cells, the organ plays a key role in scavenging bacteria and their toxins. As a result the liver is particularly exposed and susceptible to damage. The resulting impairment in its detoxification and 'clearing out' functions worsens the prognosis and increases the likelihood of mortality (Nessler *et al.*, 2012).

In this study, histopathology as well as biochemical parameters of inflammatory damage were controlled by stigmasterol. Profound mononuclear cell infiltration, necrosis and features consistent with acute liver inflammation and damage were observed in liver sections of saline-treated mice, with a corresponding rise in serum levels of ALT and AST. Pretreatment with stigmasterol suppressed features of liver damage and inhibited elevated levels of AST and ALT significantly. Similar protection was observed in the lung tissue. Stigmasterol controlled lymphocyte and polymorphonuclear leukocyte trafficking within alveolar airspaces and in the peribronchiolar regions while preserving lung architecture.

Stigmasterol, at 10 mg/kg prevented organ damage but did not significantly inhibit neutrophil recruitment. This is not surprising as it is consistent with literature and suggests the involvement of other mechanisms in LPS-induced organ damage. Indeed, antioxidant interventions have been shown to reverse organ damage and mortality via mechanisms independent of neutrophil recruitment (Lowe *et al.*, 2013; Zheng *et al.*, 2016). The antioxidant capacity of stigmasterol has been earlier reported (Panda *et al.*, 2009; Ghosh *et al.*, 2014). Organ damage occurs as a result of several factors which include but are not limited to neutrophil proliferation. Activation of transcription factors, adhesion molecule-aided transport of inflammatory cells into target organs, pro-

inflammatory cytokine release and oxidative stress are all involved in the eventual damage of target organs. Phytosterols are known to interfere with these processes (Gabay *et al.*, 2010; Melo *et al.*, 2010). While neutrophil accumulation is one of the earliest responses, suppression or interference with any of the later events, may account for the protective effect recorded.

In response to an overwhelming exposure to endotoxin, an exaggerated immune response could lead to mortality mostly due to hypovolemic shock and terminal organ failure. In this experiment, fatal doses of LPS administered to untreated mice, elicited death in 24 h. Stigmasterol at 50 and 100 mg/kg delayed death and showed significant inhibition of endotoxin-induced mortality.

Overall, these observed effects are consistent with the anti-inflammatory profile of phytosterols. Phytosterol mixtures have been shown to have significant modulatory effect on a wide array of immune responses such as gene transcription and mediator release and mechanisms of inflammatory cell recruitment (Gabay *et al.*, 2010; Melo *et al.*, 2010) as well as inhibition of intracellular signaling pathways associated with inflammation (Medeiros *et al.*, 2007). These mechanisms of stigmasterol action could account for the observed antipyretic effect and the significant inhibition of systemic inflammatory responses associated with Lipopolysaccharide-Toll-like receptor interactions.

3.1.6 CONCLUSION

Taken together, stigmasterol showed suppressive effect on potentially harmful innate immune responses induced by LPS.

3.2 EFFECT OF STIGMASTEROL ON MAST CELL-MEDIATED RESPONSES

3.2.1 INTRODUCTION

The mast cell has proven to be a very important part of the immune system since its discovery and characterization by Paul Ehrlich (Ehrlich 1878; Ghably *et al.*, 2015). As a key participant of the innate and adaptive immune responses, it possesses a wide range of cell surface receptors involved in mediation of allergic responses, pathogen recognition, auto-inhibition and the mast cell's own maturation and survival (Galli *et al.*, 2005; Krystel-Whittemore *et al.*, 2016). Its role in mediating Type I hypersensitivity reactions has been widely researched and reported on. Initial exposure of an individual to an antigen triggers an adaptive immune response involving the production of immunoglobulin E antibodies. They then bind to high affinity FcεRI receptors of the mast cell. Subsequent re-exposure leads to cross-linking of bound IgE and activation of the mast cell (Chinen and Shearer, 2008b). Degranulation of mast cell and release of pre-stored and *de novo* synthesized inflammatory mediators convey early and late phase reactions of mast cell-mediated allergic and inflammatory responses (Rao and Brown, 2008; Vo and Kim, 2013). Actions by these released mediators such as histamine to a large extent, cytokines and lipid mediators contribute to clinical signs of allergy such as dermatitis, rhinitis, pruritus, bronchoconstriction and systemic anaphylaxis in severe cases (Boden and Wesley Burks, 2011; Mygind, 2014). Not all mechanisms leading to degranulation of the mast cell involve prior production of IgE. Drugs such as clonidine, opioids and serotonin are known to elicit similar mast cell responses independent of IgE-FcεRI interactions (Lorenz *et al.*, 1998; Blunk *et al.*, 2004). Other endogenous inflammatory peptides and cationic drugs can also mediate these pseudo-allergic and anaphylactoid reactions. They are collectively referred to as basic secretagogues (McNeill *et al.*, 2015). These agents have become of immense clinical importance. A recent publication by McNeill *et al.* (2015), suggesting a dedicated receptor for pseudo-allergic reactions has further generated interest. Pathways and receptors mediating both IgE and non-IgE mast cell degranulation have become potential therapeutic targets in the development and screening of potential anti-allergic drugs. In all, current drugs in

use and under investigation against mast cell-mediated allergic reactions either target IgE distribution and FcεRI-IgE interactions (Kaplan *et al.*, 2017), molecular events leading to membrane disruption and degranulation (Yang *et al.*, 2013) or inhibition of mediator release and receptor binding, following mast cell activation and degranulation (Je *et al.*, 2014; Jie *et al.*, 2015). Naturally occurring compounds have shown great promise in this regard (Kimata *et al.*, 2000; Bae *et al.*, 2013; Balaji *et al.*, 2014). Indeed, the prototypical mast cell stabilizer, disodium cromoglycate was developed from the naturally occurring compound khellin derived from the plant *Amni visnaga* (Finn and Walsh, 2013). Stigmasterol is a naturally occurring steroid alcohol widely distributed in medicinal plants. Plant sterols are generally believed to have immune modulating effects and anti-allergic potential (Trautwein and Demonty, 2007).

This section assesses the potential anti-allergic property of stigmasterol by investigating its inhibitory effect on selected mast cell-mediated responses. It focuses on the effect of stigmasterol on immunogenic and non-immunogenic mast cell degranulation as well as local and systemic mast cell-mediated responses.

3.2.2 MATERIALS

3.2.2.1 Chemicals and reagents

Stigmasterol (95%), Compound 48/80, Evans blue dye, Toluidine Blue O stain, ovalbumin (OVA, Grade V) and polyethylene glycol, PEG were purchased from Sigma Aldrich (St. Louis, USA). Sodium cromoglycate was obtained from Ashford Laboratories (Macau, China). Bovine Serum Albumin, BSA, was purchased from PAA Laboratories (Germany).

3.2.2.2 Animals

ICR mice (25-30 g), Wistar rats and C57BL/6 mice (25-30 g) were obtained from Noguchi Memorial Institute for Medical Research, Legon - Ghana. Animals were housed at the Animal House facility of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST under standard temperature and humidity (temperature $25 \pm 2^{\circ}\text{C}$ with a 12 h light-dark cycle). Animals were allowed free access to commercial chow and distilled water. All protocols used in this study

were approved by the Faculty of Pharmacy Ethics Committee and animal handling was done in compliance with Animal Welfare Regulations (USDA 1985; US Code, 42 USC § 289d) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002).

3.2.3 METHODS

3.2.3.1 Active Cutaneous Anaphylaxis (ACA)

3.2.3.1.1 Sensitization and Challenge

ICR mice (25 - 30 g, n = 5) were immunized with 100 µl BSA (0.05 mg/ml, *s.c.*) on day 1 of the experiment. Immunization was repeated with a booster dose of 100 µl BSA (0.02 mg/ml *s.c.*) on day 14. Mice received an injection of 200 µl Evans blue dye (1% w/v) *via* the tail vein on day 21 and randomized into 6 groups for the following treatments intraperitoneally;

Group I: naïve control - normal saline (0.9% w/v) (5 ml/kg)

Group II: vehicle control - polyethylene glycol (PEG) (50% w/v) (5 ml/kg)

Group III: sodium cromoglycate (10 mg/kg)

Group IV – VI: stigmasterol (10, 50 and 100 mg/kg) respectively.

Under mild anaesthesia (pentobarbitone 30 mg/kg, *i.p.*) sensitized mice were challenged by inoculation with 100 µl BSA (0.1 mg/ml) on both pinnae using a 21-gauge hypodermic needle 1 h after drug treatment. Naïve animals (n = 5) were sham-sensitized with 100 µl normal saline (0.9% w/v) and challenged with 100 µl PBS only. All mice were euthanized 30 min after the antigen challenge with an overdose of ether. Each pinna was removed for area of dye extravasation determination.

3.2.3.1.2 Evans blue dye extraction

Evans blue dye was extracted from skin tissue by a method described by Je *et al.* (2014). Each pinna was placed in a stoppered glass tube containing 1 ml KOH (1M) and kept overnight at 37°C in an incubator. A 4 ml mixture of phosphoric acid and acetone (5:13) was added to each tube and shaken vigorously. The resulting mixture was centrifuged (C 257-120, Wagtech International, UK) at 1000 rpm for 15 min at 25°C. Absorbance of extracted dye from each pinna was measured at 620 nm with a microplate reader

(Synergy HI Multi-Mode, BioTek Technologies, Winooski, USA). Respective concentrations were determined from the standard calibration curve developed from serial dilutions of pure Evans blue dye.

3.2.3.2 Effect of stigmasterol on mast cell degranulation

3.2.3.2.1 Ovalbumin-induced (IgE-mediated) degranulation

Wistar rats were sensitized with intraperitoneal injection of ovalbumin (100 µl, 1% w/v) on days 1, 3, 5 and 12. One hour after the last injection, rats were sacrificed with an overdose of ether and a mid-line incision made at the peritoneal region. Mesenteric tissues were gently teased from intestinal walls and sections were gently placed in petri dishes (n = 5 mesentery sections) containing 10 ml Ringer Locke solution, and given the following treatments;

Group I: naïve control - (100 µl, PBS)

Group II: vehicle control - polyethylene glycol, PEG (100 µl, 50% w/v)

Group III: sodium cromoglycate (0.1 µg/ml)

Group IV - VI stigmasterol (0.04, 0.08 and 0.16 µg/ml) respectively

Mesentery tissue sections were incubated at 37°C for 15 min after respective treatments. Mesentery sections were then challenged with ovalbumin (100 µl, 1% w/v) and incubated again for 15 min. Naïve control tissue sections received a sham challenge with 100 µl PBS and were also incubated for 15 min. Tissues were then fixed in 10% formalin and stained with 1% Toluidine blue. After two cycles of washing in acetone and xylene tissues were mounted for microscopic observation and mast cell count. A minimum of 100 mast cells were counted from three different fields of each tissue section using a light microscope (Leica DM2500 M) and mast cell stability was determined by the equation.

$$\% \text{ mast cell degranulation} = \left[\frac{\text{no. of degranulated mast cells}}{\text{total no. of mast cells counted}} \right] \times 100$$

3.2.3.2.2 Compound 48/80-induced (non IgE-mediated) degranulation

Two male Wistar rats, without any prior sensitization were sacrificed with an overdose of ether. Mesentery tissues were collected using the same method described in section 3.2.3.2.1. Mesenteric tissues sections were gently placed in petri dishes (n = 5 mesentery sections) containing 10 ml Ringer Locke solution, and treated as follows;

Group I: naïve control - (100 µl, PBS)

Group II: vehicle control - polyethylene glycol, PEG (100 µl, 50% w/v)

Group III: sodium cromoglycate (0.1 µg/ml)

Group IV - VI: stigmasterol (0.04, 0.08 and 0.16 µg/ml) respectively

Mesentery tissue sections were incubated at 37°C for 15 min after respective treatments. Mesentery sections were then challenged with compound 48/40 (100 µl, 10 µg/ml) and incubated again for 15 min. Naïve control tissue sections received a sham challenge with 100 µl PBS and were also incubated for 15 min. Tissues were then fixed, stained and washed in the same manner as described in section 3.2.3.2.1. A minimum of 100 mast cells were counted from three different fields of each tissue section using a light microscope (Leica DM2500 M) and mast cell stability was determined by the equation.

$$\% \text{ mast cell degranulation} = \left[\frac{\text{no. of degranulated mast cells}}{\text{total no. of mast cells counted}} \right] \times 100$$

3.2.3.3 Compound 48/80-induced systemic anaphylaxis

Male C57BL/6 mice were randomly placed into 4 groups (n=6) and pre-treated as follows; polyethylene glycol (50% w/v) 5 ml/kg, stigmasterol 10, 50 and 100 mg/kg. Thirty (30) minutes later, anaphylaxis was induced in all animals by intraperitoneal injection of compound 48/80 (8 mg/kg). Mice were observed for 1 h post compound 48/80 injection. Time of death for each animal was recorded and computed as percentage survival. Total number of deaths due to anaphylactic shock was recorded in each group and expressed as percentage mortality.

3.2.3.4 Statistical analysis

Data is presented as mean $\pm$ standard error of mean (SEM). Data analysis was done using One-way analysis of variance (ANOVA). Multiple comparisons between treatment groups were performed using Dunnet's *post hoc* test. Mortality data is presented as survival curves and statistical differences compared to control were determined using Log-rank (Mantel-Cox) test. All statistical analyses were done using GraphPad for Windows version 6 (GraphPad Prism Software, San Diego, USA).

3.2.4 RESULTS

3.2.4.1 Effect of stigmasterol on ACA-mediated dye extravasation

Cutaneous challenge with bovine serum albumin induced a local inflammation marked by Evans blue dye extravasation in all the PEG vehicle-treated test mice. A significantly increased mean inflammatory reaction area of $7.79 \pm 0.68 \text{ mm}^2$ was observed in PEG-treated control mice relative to $0.06 \pm 0.02 \text{ mm}^2$ in the non-sensitized saline-treated naïve control (Fig. 3.2.1A). Mice pretreated with stigmasterol 30 min before antigen challenge showed markedly reduced active cutaneous anaphylaxis. Mean reaction areas of $4.93 \pm 0.62 \text{ mm}^2$, $4.57 \pm 0.53 \text{ mm}^2$ and $3.36 \pm 0.40 \text{ mm}^2$ were obtained for stigmasterol at 10, 50 and 100 mg/kg respectively (Fig. 3.2.1A) representing mean percentage inhibitions of $36.70 \pm 7.90\%$, $41.29 \pm 6.79\%$, and $56.88 \pm 5.15\%$ respectively of the active cutaneous anaphylaxis. A mean inflammatory reaction area of $4.64 \pm 0.46 \text{ mm}^2$ (Fig. 3.2.1A) was obtained on pretreatment with sodium cromoglycate inhibiting the dye extravasation area by $40.37 \pm 5.96\%$.

Quantification of the extravasated dye through absorbance measurements presented a similar trend. In the PEG-treated control mice a significant increase in mean concentration of dye to $0.32 \pm 0.02 \text{ mM}$ was obtained when compared to that for the naïve control of $0.005 \pm 0.0008 \text{ mM}$. At the doses of stigmasterol used the concentration of extracted dye were $0.18 \pm 0.03 \text{ mM}$, $0.19 \pm 0.01 \text{ mM}$ and $0.15 \pm 0.03 \text{ mM}$ respectively (Fig. 3.2.1B). Thus stigmasterol offered $44.74 \pm 10.62\%$, $40.08 \pm 4.59\%$ and $52.38 \pm 7.87\%$ reductions in the concentration of the extravasated dye respectively relative to PEG-treated control group at 10 - 100 mg/kg. Sodium cromoglycate reduced

extravasated dye concentration to 0.19 ± 0.02 mM representing a $39.95 \pm 7.00\%$ inhibition compared to the PEG-treated control group (Fig. 3.2.1B).

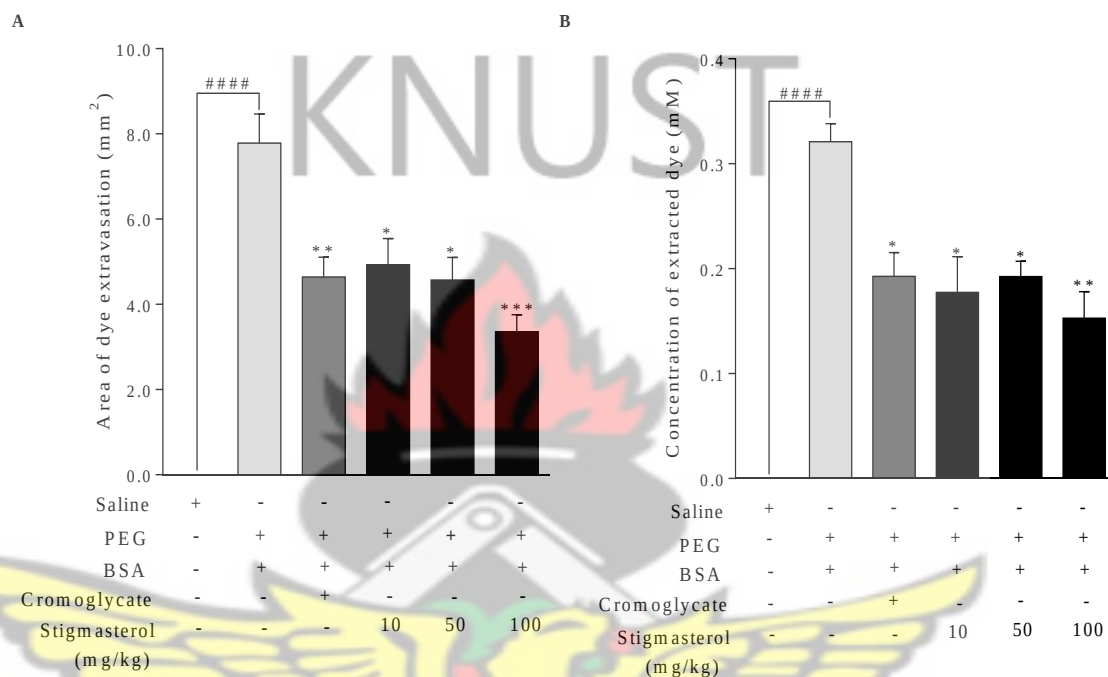


Fig. 3.2.1. Effect of stigmasterol on Active Cutaneous Anaphylaxis in mice. ICR mice (25 – 30 g) were sensitized on day 0 and day 14 with Bovine serum albumin (BSA) as previously described. Mice were randomized into 6 groups and treated with either normal saline 5 ml/kg, PEG 5 ml/kg, stigmasterol 10, 50 and 100 mg/kg or sodium cromoglycate 10 mg/kg on day 21. One (1) hour later each pinna of each mouse was inoculated with BSA solution. The area of dye extravasation was recorded 30 min after inoculation (A). Pinnae were removed and extravasated dye was extracted with a phosphoric acid/acetone (5:13) mixture. Specific concentrations were determined from a standard curve developed from serial dilutions of standard Evans blue dye (B). Data is expressed as mean area (A) or mean dye concentration (B) $\pm$ SEM (n = 10) ***P<0.001, **P<0.01, *P<0.05 as compared to saline-treated control; ####P<0.0001 compared to naïve, saline-treated group using One-way ANOVA followed Dunnet's *post hoc* test.

3.2.4.2 Effect on mast cell degranulation

3.2.4.2.1 Effect of stigmasterol on ovalbumin-induced degranulation

Naïve rat mesentery sections showed largely intact mast cells with very little incidents of degranulation (Fig. 3.2.2A). Microscopic observation revealed extensive incidents of either partial or total degranulation of mast cells in PEG-treated, ovalbumin-challenged mesentery sections. Observed fields showed significant membrane disruption, partial granule spillage and total rupture of the mast cells (Fig. 3.2.2B). These features were however significantly reduced in sodium cromoglycate-treated mesentery sections. Mast cells showed intact granules and undisrupted cell membranes (Fig. 3.2.2C). Similarly, pretreatment with stigmasterol (0.04 – 0.16 µg/ml) offered significant protection with treated tissue sections showing a low occurrence of mast cell degranulation and granule spillage (Fig. 3.2.2D - F). Quantification of degranulation was consistent with these observations (Fig. 3.2.2G). A percentage degranulation of $83.00 \pm 5.90\%$ was recorded for PEG-treated mesentery sections. This was significantly reduced in both stigmasterol and sodium cromoglycate-treated mesentery sections. Stigmasterol 0.04, 0.08 and 0.16 µg/ml recorded $39.00 \pm 3.92\%$, $29.60 \pm 3.45\%$ and $22.90 \pm 4.60\%$ percentage degranulation respectively (Fig. 3.2.2G) while a percentage degranulation of $20.60 \pm 2.99\%$ was recorded for sodium cromoglycate-treated mesentery tissues (Fig. 3.2.2G).

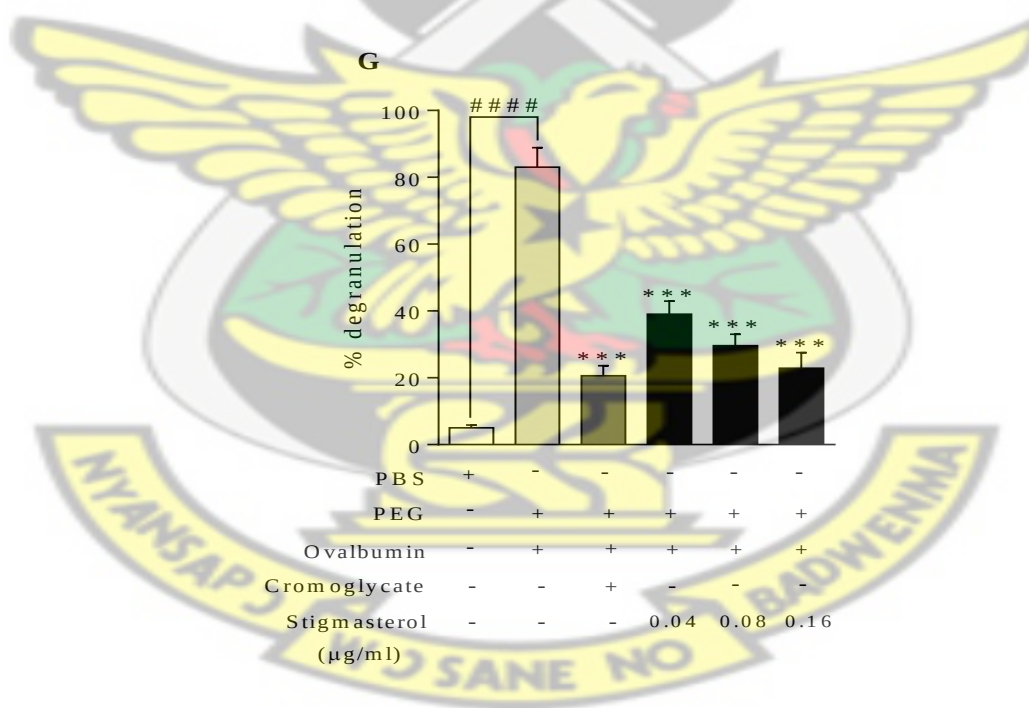
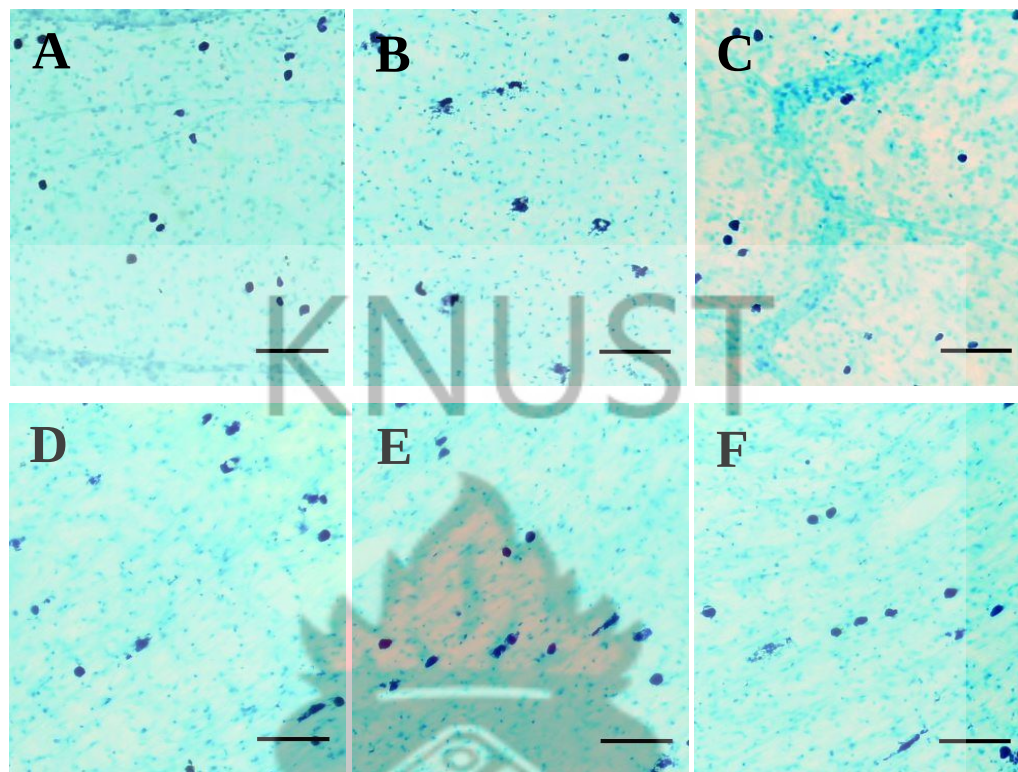


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Fig. 3.2.2 Effect of stigmasterol on ovalbumin-induced, IgE-mediated mast cell degranulation. Mesentery tissue sections were incubated with either PBS (naïve) (A), PEG (B), sodium cromoglycate (C) or stigmasterol 0.04 – 0.16 µg/ml (D - F) for 15 min at 37°C. Tissues were then challenged and incubated with ovalbumin (100 µl, 1% w/v) at 37 °C for another 15 min. Naïve control tissue sections received a sham challenge with 100 µl PBS and were also incubated for 15 min. Tissues were fixed and stained with 10 % buffered formalin and 1% Toluidine blue respectively. Mast cell counts were determined under light microscopy. Percentage degranulation of mast cells was quantified per a minimum of 100 mast cells from three different fields of each tissue section and values per each treatment are expressed as mean ± SEM (n = 5) (G) ***P <0.001 as compared to PEG-treated control and ####P < 0.0001 as compared to untreated/unchallenged naïve control using One-way ANOVA followed by Dunnet's *post hoc* test. Micron bar represents 100 µm.

3.2.4.2.2 Effect of stigmasterol on C48/80-induced degranulation

Mast cells observed in PBS-treated naïve mesentery sections were mostly intact with a few isolated incidents of degranulation (Fig 3.2.3A). Compound 48/80 challenge induced significant degranulation in PEG-treated mesentery tissue. Sections were characterized by widespread degranulation accompanied by marked granule spillage (Fig 3.2.3B). Sodium cromoglycate protected mast cells from compound 48/80-induced degranulation. A significant number of intact mast cells were seen in the observed fields (Fig 3.2.3C). A similar observation was made in stigmasterol-treated tissue sections (Fig 3.2.3D-F). Mast cell count and quantification of degranulation supported this observation (Fig 3.2.3G). Percentage degranulation of 78.70 ± 5.33 % recorded in PEG-treated mesentery tissues represented a significant increase above naïve levels (4.00 ± 0.95%). This was markedly reduced in stigmasterol-treated mesentery sections. 0.04, 0.08 and 0.16 µg/ml of stigmasterol recorded 47.60 ± 3.37%, 24.10 ± 2.78% and 24.5 ± 2.79% degranulation respectively. A percentage degranulation of 25.90 ± 2.99% was recorded for sodium cromoglycate-treated tissue sections (Fig 3.2.3G).

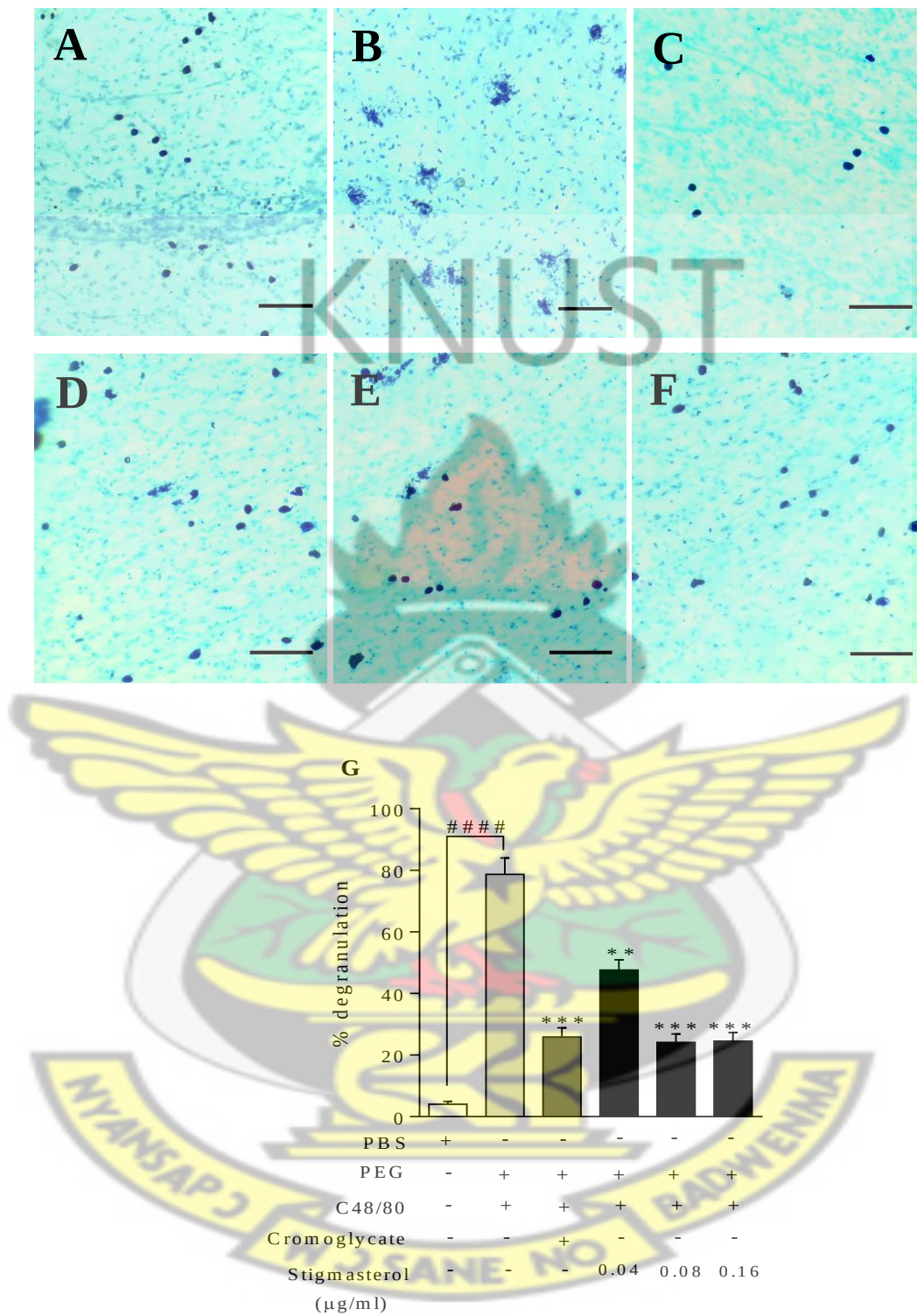


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Fig. 3.2.3 Effect of stigmasterol on C48/80-induced, non IgE-mediated mast cell degranulation. Mesentery tissue sections were incubated with either PBS (naïve) (A), PEG (B), sodium cromoglycate (C) or stigmasterol 0.04 – 0.16 µg/ml (D - F) for 15 min at 37°C. Tissues were then challenged and incubated with compound 48/80 (100 µl, 10 µg/ml) at 37°C for another 15 min. Naïve control tissue sections received a sham challenge with 100 µl PBS and were also incubated for 15 min. Tissues were fixed and stained with 10% buffered formalin and 1% Toluidine blue respectively. Mast cell counts were determined under light microscopy. Percentage degranulation of mast cells was quantified per a minimum of 100 mast cells from three different fields of each tissue section and values per each treatment are expressed as mean ± SEM (n = 5) (G) ***P <0.001 as compared to PEG-treated control and #####P < 0.0001 as compared to untreated/unchallenged naïve control using One-way ANOVA followed by Dunnet's *post hoc* test. Micron bar represents 100 µm.

3.2.4.3 Effect of stigmasterol on C48/80-induced systemic anaphylaxis

Compound 48/80 induced fatal anaphylaxis in PEG-treated mice. 100% mortality was recorded in the vehicle-treated group within 20 min post injection (Fig. 3.2.4). Stigmasterol significantly delayed anaphylaxis-induced death and improved survival rates as observed 1 h post C48/80 challenge. Stigmasterol at 10, 50 and 100 mg/kg offered 16.66%, 33.33% and 66.67% survival respectively (Fig. 3.2.4).

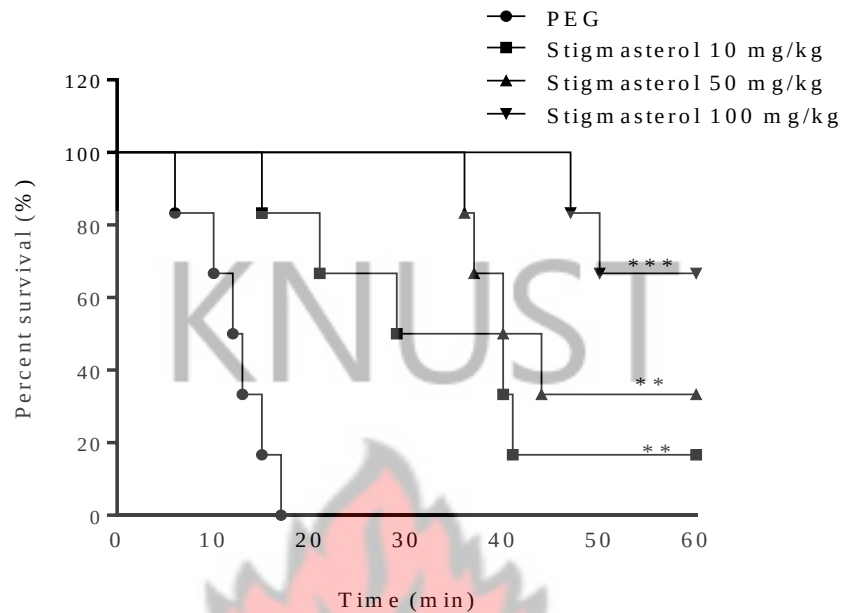


Fig. 3.2.4 Effect of stigmasterol on C48/80-induced mortality. Mice received either PEG (vehicle) 5 ml/kg or stigmasterol 10, 50 and 100 mg/kg 1 h prior to C48/80 injection. Fatal systemic shock was induced in mice by intraperitoneal injection of C48/80 (8 mg/kg). The time of death and survival rate within 1 h post-C48/80 challenge was recorded. Data is represented as survival curves. Statistical differences compared to vehicle control (PEG) were determined using Log-rank (Mantel-Cox) test.

3.2.5 DISCUSSION

Mast cells partake in allergic and inflammatory reactions via the release of stored or *de novo* synthesized mediators (Ogawa and Grant, 2007). Though novel views suggest a possible role of selective release of mediators, there is overwhelming evidence to show that mast cell activation and degranulation precedes signs of type I hypersensitivity and anaphylactoid reactions such as pruritus, angioedema, bronchoconstriction, hives and late phase inflammation. Research and development of new anti-allergy medications have therefore focused on immunoglobulin E interaction with the high affinity mast cell receptor FcεRI and the subsequent intracellular events which precede mast cell activation and degranulation (Kraft and Kinet, 2007; Harvima *et al.*, 2014).

In this study, stigmasterol at all doses inhibited dye extravasation in the Active cutaneous anaphylaxis (ACA) reactions. In this test, mice pre-sensitized with BSA were challenged with the antigen on their pinnae to initiate antigen-antibody reactions. The resulting mast cell degranulation leads to vasoactive mediator release, vascular permeability changes and subsequent extravasation of injected dye (Jeong *et al.*, 2006). Pretreatment with stigmasterol inhibited both the area and concentration of extravasated dye, signifying a marked inhibitory effect of allergic responses. The potential effect of stigmasterol on mast cell degranulation was therefore assessed.

Re-exposure of an atopic individual to antigen causes the crosslinking of immunoglobulin E molecules bound to the Fc ϵ RI of the mast cells. This is followed by activation of the mast cell leading to events such as degranulation, cytokine synthesis and release and the activation of the arachidonic acid pathway. Degranulation and histamine release mediates most of the immediate signs of allergy with the two latter events mediating the accompanying late phase inflammation (Metcalf *et al.*, 2009). Another pathway independent of IgE-Fc ϵ RI interactions has also been widely reported on. It is implicated in allergy-like responses to basic secretagogues (Seifert, 2015). Recent reports have pointed to Mrgprb2 receptor in mice and MRGPRX2, the human orthologue as receptors which mediate these responses collectively referred to as pseudo allergy (McNeill *et al.*, 2015). The clinical importance of this discovery has ignited interest in developing agents that may mitigate pseudo-allergic reaction by interacting with pathways involved in the mechanism of action of basic secretagogues or by blocking these novel targets.

This preliminary study revealed that stigmasterol showed protection against both ovalbumin and compound 48/80-induced mast cell degranulation. Again, stigmasterol improved survival and delayed compound 48/80-induced death. Taken together, it was observed that stigmasterol was effective in both mechanisms of mast cell degranulation. This suggests the mechanism of action of stigmasterol in the prevention of mast cell degranulation may not be dependent or may not be limited to IgE-Fc ϵ RI interactions.

3.2.6 CONCLUSION

Stigmasterol mitigated both IgE-dependent and non IgE-dependent mechanisms of degranulation and thus showed significant potential as an anti-allergic drug.

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3.3 EFFECT OF STIGMASTEROL ON CUTANEOUS ALLERGIC RESPONSES

3.3.1 INTRODUCTION

The skin is a large complex immunological organ that plays various roles in sensory functions, temperature control and host protection. Thus it is critical in the defense against pathogens and allergic responses (Heller, 2012). A complex interplay of barrier cells, resident and migratory immune cells and an array of inflammatory mediators makes the skin highly effective in its functions (Metz and Maurer, 2009; Cork *et al.*, 2009). Due to its unique functions, large surface area and exposure to the environment however, the skin is particularly vulnerable to environmental allergens, chemicals and pollutants (Bordon, 2017) and is one of the most affected organs in adverse drug reactions (French and Prins, 2013). Immune responses to these agents as well as ‘unchecked’ internal mechanisms account for a range of skin disorders including atopic dermatitis, contact dermatitis, urticaria, angioedema and psoriasis (Jenerowicz *et al.*, 2012; Zhou *et al.*, 2015). These disorders are collectively known as allergic skin diseases (ASD). Key in the pathogenesis of these allergic skin diseases are resident structural cells such as keratinocytes, skin mast cells and antigen presenting cells such as Langerhans cells and dendritic cells (Metz *et al.*, 2008; Tuchinda and Gaspari, 2010). After disruption of the epidermal layer and subsequent entry of allergens or irritants, these innate cells initiate a variety of early immune responses (Bowcock and Krueger, 2005; Rosenwasser, 2011). Consequently, activities of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF α) and interleukin-1 (IL-1), chemo-attractants like C-X-C motif chemokine ligand 8 (CXCL8, IL-8) and the migration of inflammatory cells into skin tissue promote a hyper-inflammatory state characterized by urticaria, eczema, pruritus and other dermatological changes (Tsicopoulos *et al.*, 2003; Fonacier *et al.*, 2010). Skin mast cells in particular, contribute to allergic skin diseases via both immunoglobulin E (IgE)-dependent and non-IgE-dependent mechanisms. The degranulation of the mast cell with the subsequent release of stored and *de novo* synthesized vasoactive amines, pro-inflammatory cytokines and lipid mediators

contribute to the underlying inflammation and clinical manifestations of most allergic skin diseases (Vo and Kim, 2013).

Pruritus is a common symptom in allergic skin disorders. Controlling scratching behaviour promotes faster healing of the epithelial barrier, enhances patient comfort and prevents secondary infections (Blauvelt *et al.*, 2003; Heller, 2012). While the release of pruritogens such as histamine and serotonin from mast cells is implicated in itch responses in skin diseases, reports on the effectiveness of antihistamines are conflicting and largely inconclusive (Rukwied *et al.*, 2000; Thurmond *et al.*, 2015). Several research findings have also demonstrated the ineffectiveness of mast cell stabilizers in controlling pruritus (Sugimoto *et al.*, 1998; Yamashita *et al.*, 2005; Dunford *et al.*, 2006) thus stressing the involvement of other pathways. Alternatives to the treatment of allergic skin diseases have become the subject of recent studies (Boneberger *et al.*, 2010; Rauh *et al.*, 2011). A number of studies have reported effectiveness for both topically applied and systemic plant-derived and other naturally occurring compounds (Otuki *et al.*, 2011; Park *et al.*, 2012; Joh *et al.*, 2014). Phytosterols in general have been shown to have significant modulatory effect on inflammatory gene transcription (Medeiros *et al.*, 2007), pro-inflammatory mediator release (Bouic, 2001) and generation of reactive oxygen species (Yuk *et al.*, 2007) and are therefore potential candidates for allergic skin disease therapy. Stigmasterol, a steroid alcohol, one of such phytosterols found in a number of medicinal plants, vegetables and nuts has acclaimed and proven immune-modulatory properties either alone or as a component of phytosterol mixtures (Trautwein and Demonty, 2007; Gabay *et al.*, 2010; Chen *et al.*, 2012).

This section assesses the suppressive effect of stigmasterol on compound 48/80-induced pruritic responses and TPA-induced skin inflammation.

3.3.2 MATERIALS

3.3.2.1 Chemicals and reagents

Stigmasterol (95%), dexamethasone, ketotifen fumarate, 12-O-Tetradecanoylphorbol-13-acetate (TPA), compound 48/80 (C2313), Polyethylene glycol (PEG), Toluidine blue O stain were obtained from Sigma Aldrich (St. Louis, USA). Bovine Serum Albumin (BSA) was purchased from PAA Laboratories (Marburg, Germany). Phosphate Buffered Saline (PBS) was purchased from Gibco (Karlsruhe, Germany). Rat TNF α ELISA quantification kit was purchased from MLBio Biotechnology Company Limited (Shanghai, China).

3.3.2.2 Animals

ICR mice (25 – 30 g) and Wistar rats (100 -120 g) of either sex were obtained from Noguchi Memorial Institute for Medical Research (NMIMR), Legon, Ghana. Animals were kept under standard temperature and humidity (temperature $25 \pm 2^{\circ}\text{C}$ with a 12 h light-dark cycle) at the Animal house facility of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST. Experimental animals were allowed access to commercial chow and distilled water *ad libitum*. All protocols used in this study were approved by the Faculty of Pharmacy and Pharmaceutical Ethics Committee and animal handling was done in compliance with Animal Welfare Regulations (USDA 1985; US Code, 42 USC § 289d) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002).

3.3.3 METHODS

3.3.3.1 Compound 48/80-induced pruritus

3.3.3.1.1 Induction of scratching behaviour

ICR mice (20 - 30 g, n = 5) were placed in Perspex observation chambers for 1 h to acclimatize before the experiment and randomized into 6 treatment groups as follows;

Group I: naïve control- normal saline (5 ml/kg, *i.p.*)

Group II: vehicle control- polyethylene glycol, PEG (50% w/v) (5 ml/kg, *i.p.*)

Group III: ketotifen fumarate (10 mg/kg, *p.o.*)

Group IV – VI: stigmasterol (10, 50, 100 mg/kg, *i.p.*) respectively.

Test mice received subcutaneous injections of 100 µg compound 48/80 (dissolved in 100 µl PBS) at the rostral part of the back 30 min after respective treatments, while naïve mice were given 100 µl PBS only. Immediately after injections, the mice were placed in the observation chambers and itch responses were recorded for 1 h. Scratching behaviour was observed and scored in accordance with the method described by Shafizadeh *et al.* (2014). Scratching was defined as a motor behaviour in which hind paws were applied repeatedly to the point of injection. A scratching series followed by a minimum of 1 s pause was considered as one scratching bout. Limb movements towards other parts of the body were considered as grooming and were excluded from the score.

3.3.3.1.2 Skin histopathology

Skin sections (2 mm²) from the site of injection were subsequently excised, stored in 10% w/v buffered formalin and embedded in paraffin. Sections of 3 µm thickness were cut, deparaffinized, dehydrated and stained with toluidine blue (1% w/v, pH 4.0) for assessment of mast cell proliferation (number of mast cells per square millimeter area of skin tissue) and percentage degranulation (number of degranulated mast cells per total cells in square millimeter area). Mast cell counts were performed by an independent observer. Morphometric analysis of skin area was performed with Image J analysis tool (version 1.50i, National Institutes of Health, USA).

3.3.3.2 TPA-induced dermatitis

Wistar rats of either sex (100 – 120 g, n = 5) were randomly placed in six groups and treated via the intraperitoneal route as follows;

Group I: naïve control- normal saline (5 ml/kg).

Group II: vehicle control- polyethylene glycol, PEG (50% w/v) (5 ml/kg)

Group III: dexamethasone (3 mg/kg)

Group IV – VI: stigmasterol (10, 50 and 100 mg/kg) respectively.

Test rats were challenged topically with 20 µg TPA (12-O-Tetradecanoylphorbol-13-acetate) dissolved in acetone (20 µg/ 20 µl) and applied on both inner and outer surfaces of each ear while naïve control rats received a topical application of acetone (20 µl)

only. Drug treatment and subsequent challenge were repeated in 24 h and 48 h for all groups. Rats were sacrificed by cervical dislocation 5 h after the last TPA or acetone challenge. Both ears of each rat were cut off and weighed. Increase in ear weight compared to control was considered as a measure of oedema. The jugular vein was quickly dissected and blood collected for the following determinations:

3.3.3.2.1 Neutrophil count determination

Blood was collected into EDTA tubes for neutrophil count with an automated analyzer (Sysmex KX-21N, Sysmex America Inc., Illinois, USA).

3.3.3.2.2 Serum TNF α

Blood was collected into sterile gel and clot activator tubes. Sera was separated by centrifugation (C 257-120, Wagtech International, UK) at 2000 rpm for 15 min at 20 °C. Aliquots of serum were collected into Eppendorf tubes and stored at -70 °C until needed. Serum levels of TNF α was quantified using Enzyme-linked immunosorbent assay (ELISA) kit according to the instructions prescribed by the manufacturer.

3.3.3.2.3 Histopathology

Excised ear tissues were stored in 10% w/v buffered formalin and embedded in paraffin. Sections of 3 μ m thickness were cut, deparafinized, dehydrated and stained with haematoxylin and eosin (H & E). The tissue sections were observed for inflammatory changes under light microscope, Leica DM2500 M (Leica Microsystems, Wetzlar, Germany). Three random fields for each skin section of randomly selected animals in each group were examined for leukocyte infiltration, skin and epidermal layer thickness. Morphometric analyses of skin and epidermal layer thickness were performed with Image J analysis tool (version 1.50i, National Institutes of Health, USA).

3.3.3.3 Statistical Analysis

Data is presented as Mean $\pm$ Standard error of mean (SEM). Data analysis was done using One-way analysis of variance (ANOVA). Multiple comparisons between treatment groups were performed using Dunnet's *post hoc* test. All statistical analyses

were done using GraphPad for Windows version 6 (GraphPad Prism Software, San Diego, USA).

3.3.4 RESULTS

3.3.4.1 Compound 48/80-induced pruritus

3.3.4.1.1 Effect of stigmasterol on C48/80-induced scratching behaviour

Intense scratching behaviour was induced by subcutaneous injection of C48/80. Itch responses such as hind limb scratching, biting and front limb rubbing of mainly the injected site (rostral back) and other parts of the body were observed in injected mice in which only specific hind limb rostral scratching sequences were recorded for consistency and to differentiate from normal grooming. Mean scratching bouts of 71.50 ± 4.22 was recorded in PEG-treated C48/80-injected control mice compared to mean counts in saline-treated naïve mice of 1.50 ± 0.76 (Fig. 3.3.1). Stigmasterol significantly reduced the mean scratching bouts to 36.50 ± 4.72 and 30.67 ± 2.28 thereby reducing the scratching intensity by 48.95 ± 6.61 % and 57.11 ± 3.18 % respectively when administered at 50 and 100 mg/kg in comparison with the C48/80-injected control mice. No significant inhibition was observed at 10 mg/kg with mean scratching bouts of 58.83 ± 5.04 (Fig. 3.3.1). Ketotifen significantly reduced the mean scratching bouts to 23.67 ± 2.57 (Fig. 3.3.1) representing 66.90 ± 3.59 % inhibition of scratching intensity.

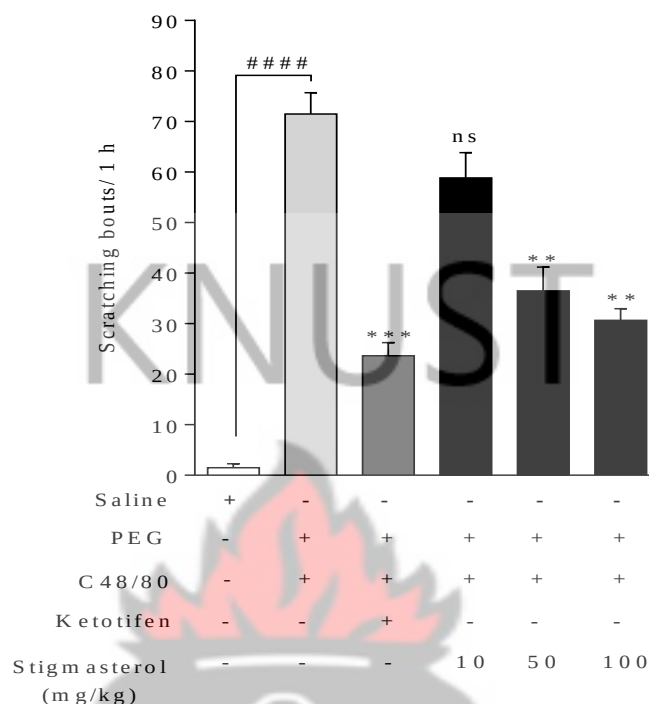


Fig. 3.3.1 Effect of stigmasterol on C48/80-induced scratching behaviour in mice. ICR mice received either normal saline (5 ml/kg), polyethylene glycol PEG (5 ml/kg), ketotifen (10 mg/kg) or stigmasterol (10, 50 and 100 mg/kg). Test mice received C48/80 (100 µg s.c.) in the rostral back region. Naïve mice were challenged with 100 µl PBS. Scratching behaviour was observed for 1 h as previously described. Data is expressed as mean scratching bout/ 1 h ± SEM, (n = 5). ***P < 0.001, **P < 0.01, ns is not significant as compared to PEG-treated control. #####P < 0.0001 as compared to saline-treated naïve control using One-way ANOVA followed by Dunnet's *post hoc* test.

3.3.4.1.2 Effect of stigmasterol on skin mast cell proliferation and degranulation

Skin sections from the rostral back of naïve ICR mice showed normal skin histology. Toluidine blue staining revealed sparse mast cell distribution within the dermal region. No mast cell clusters occurred and only limited mast cell degranulation was observed (Fig. 3.3.2A). Skin sections from PEG-treated control mice were however characterized by profound proliferation of mast cells within the dermal region. Several mast cell clusters and incidents of degranulation were observed throughout the skin section (Fig. 3.3.2B). These features were unchanged when mice were treated with 10 mg/kg

stigmasterol (Fig. 3.3.2D). Incidents of mast cell accumulation and degranulation were observed to be reduced in stigmasterol 50 and 100 mg/kg treated mice (Fig. 3.3.2E and 3.3.2F) as well as in ketotifen treated mice (Fig. 3.3.2C). The number of mast cells per millimeter square area of skin tissue, a measure of mast cell distribution, showed stigmasterol treatment significantly reduced mast cell proliferation at the injected site. In the naïve mice, a mean mast cell number per millimeter square area of skin of 18.13 ± 1.48 cells/mm² was significantly increased five-fold to 94.24 ± 7.53 cells/mm² when PEG-treated control mice were injected with C48/80 (Fig. 3.3.2G). This mean mast cell number was significantly reduced to 38.31 ± 8.07 cells/mm² and 23.56 ± 3.72 cells/mm² representing $59.35 \pm 8.56\%$ and $75.00 \pm 3.95\%$ inhibition of mast cell proliferation by treatment with 50 and 100 mg/kg stigmasterol respectively. Given at 10 mg/kg, stigmasterol reduced the mean mast cell number per millimeter square area of skin to 75.06 ± 4.08 causing an inhibition of $24.94 \pm 5.02\%$ in mast cell proliferation albeit insignificant when compared to the PEG-treated C48/80-injected control (Fig. 3.3.2G). On the other hand, ketotifen treatment recorded a mean of 44.31 ± 4.21 mast cells/mm² which represented a $52.98 \pm 4.47\%$ significant reduction compared to PEG-treated, C48/80-injected control (Fig. 3.3.2G). The percentage degranulation in the saline-treated naïve control mice was calculated to be $1.44 \pm 0.38\%$ which was significantly increased to $16.38 \pm 1.66\%$ in the PEG-treated C48/80-injected control mice (Fig. 3.3.2H). Treatment with 50 and 100 mg/kg stigmasterol significantly reduced the percentage degranulation respectively to $6.69 \pm 1.19\%$ and $4.56 \pm 0.90\%$ when compared to the PEG-treated C48/80-injected control. Thus respectively, $59.17 \pm 7.26\%$ and $72.15 \pm 5.49\%$ inhibitions in mast cell degranulation were obtained by treatment with 50 and 100 mg/kg stigmasterol. However, given at 10 mg/kg stigmasterol reduced the mean percentage degranulation to 14.06 ± 1.63 causing a suppression of $24.94 \pm 5.02\%$ in mast degranulation albeit insignificant when compared to the PEG-treated C48/80-injected control. Ketotifen reduced the mean percentage degranulation to $8.44 \pm 1.04\%$ representing an inhibition of $48.49 \pm 6.33\%$ compared to PEG-treated control (Fig. 3.3.2H).

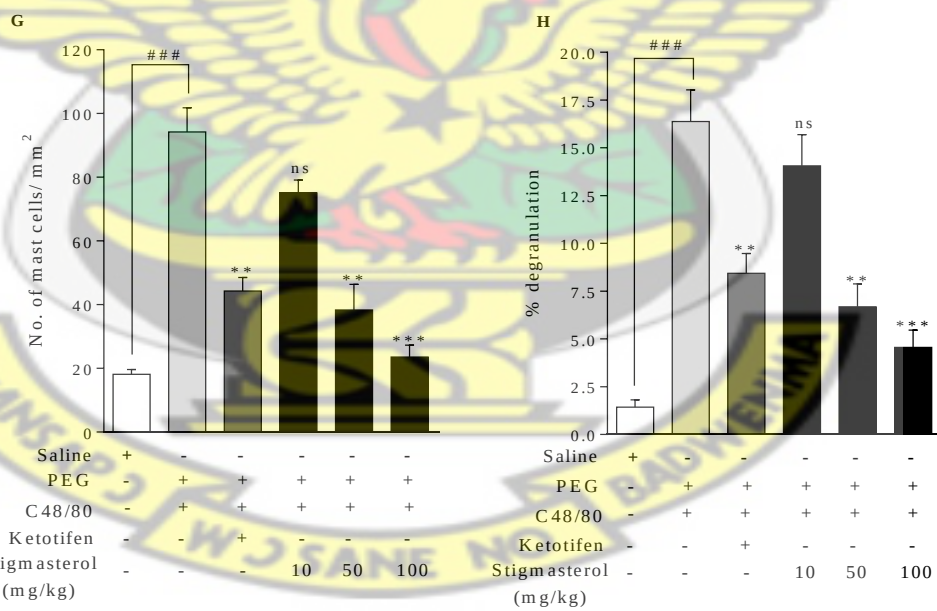
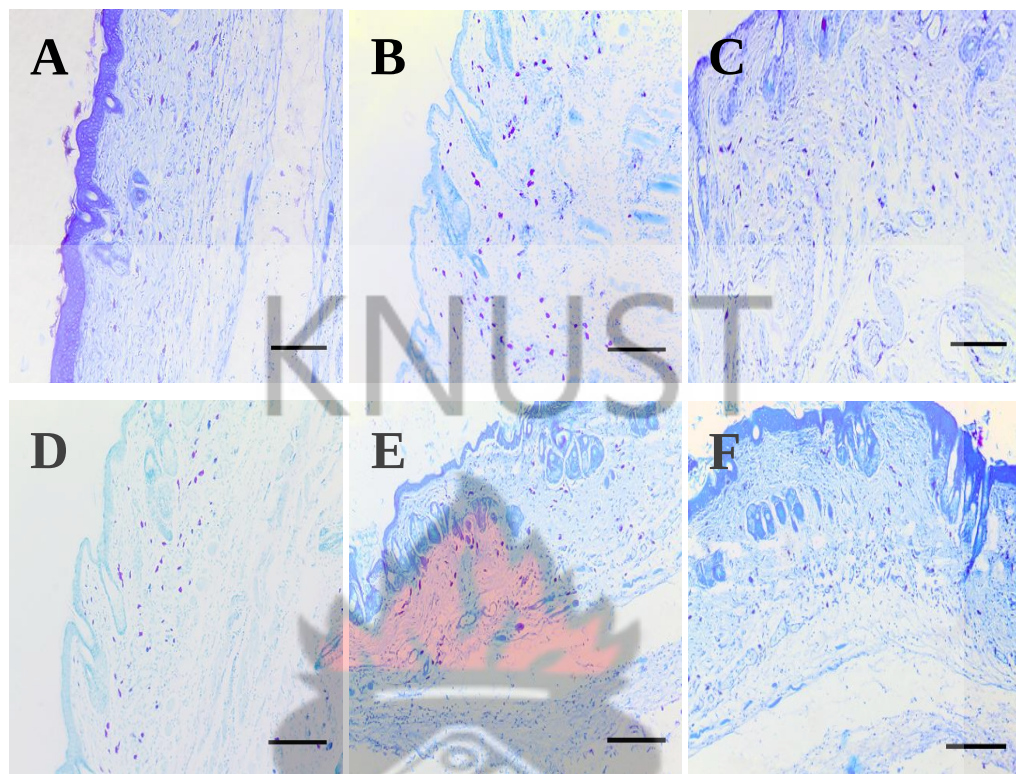


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Fig. 3.3.2 Effect of stigmasterol on C48/80-induced mast cell proliferation and degranulation. ICR mice were challenged with subcutaneous injection of C48/80 and scratching behaviour was observed for 1 h. Skin sections were excised from injected site and stored in 10% buffered formalin for histopathological assessment. 3 μ m section of skin were stained with Toluidine blue stain and observed for mast cell distribution and degranulation in normal saline 5 ml/kg (naïve group) (A), polyethylene glycol 5ml/kg (PEG) (B), ketotifen 10 mg/kg (C) and stigmasterol 10, 50 and 100mg/kg (D –F) treated groups. Data is expressed as mean mast cell number (cells/mm²) $\pm$ SEM (G) and mean percentage degranulation of mast cells $\pm$ SEM (H) ***P < 0.001, ** P < 0.01 and ns (not significant) as compared to PEG-treated, compound 48/80- challenged group. ###P<0.001 as compared to saline-treated, unchallenged (naïve) group using One-way ANOVA followed by Dunnet's *post hoc* test.

3.3.4.2 TPA-induced dermatitis

3.3.4.2.1 Effect of stigmasterol on ear oedema

Topical application of TPA for 3 days induced significant inflammation in rat ear skin observed as significant increase in ear thickness, dilation of auricular blood vessels and erythema in PEG-treated TPA-challenged rats (Fig. 3.3.3B) when compared to the naïve acetone-challenged rats (Fig. 3.3.3A). Stigmasterol at 10, 50 and 100 mg/kg caused a significant decrease in these inflammatory features (Fig. 3.3.3D - F). Similarly, inflammation was also significantly suppressed by dexamethasone (Fig. 3.3.3C). Ear weights were indexed as a measure of TPA-induced oedema in ear skin. Mean ear weight of 181.3 ± 3.98 mg was recorded for the PEG-treated TPA-challenged rats measured a 1.96-fold increase of 92.50 ± 2.50 mg for the naïve control (Fig. 3.3.3G). TPA-induced increase in ear weight was significantly abated by both stigmasterol and dexamethasone. Mean ear weights of 140.71 ± 8.52 mg, 113.80 ± 7.34 mg and 103.34 ± 4.22 mg (Fig. 3.3.3G) representing percentage inhibitions in ear oedema of $21.86 \pm 7.46\%$, $37.26 \pm 4.03\%$, $43.00 \pm 2.32\%$ respectively, compared to PEG-treated TPA-challenged control were obtained in the stigmasterol 10, 50 and 100 mg/kg-treated rats (Fig. 3.3.3G). In the group that received dexamethasone a mean ear weight of 107.51 ± 6.23 mg (Fig. 3.3.3G) causing a $40.71 \pm 3.42\%$ inhibition of ear oedema was obtained.

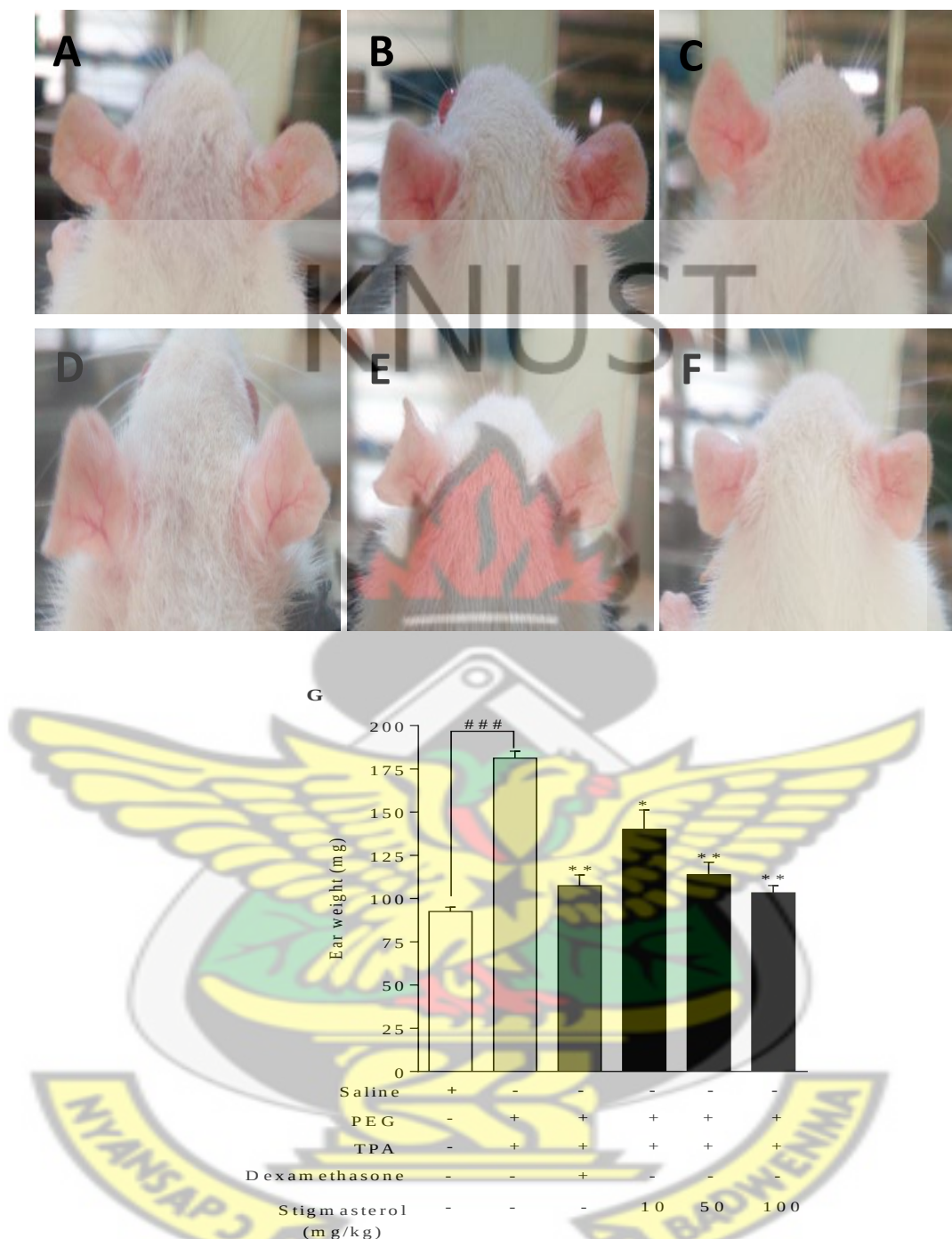


Fig. 3.3.3 Effect of stigmasterol on TPA-induced ear inflammation and oedema. Wistar rats of either sex (100 – 120 g) received either normal saline 5 ml/kg (naïve group) (A), polyethylene glycol 5ml/kg (PEG) (B), dexamethasone 3 mg/kg (C) or stigmasterol 10, 50 and 100 mg/kg (D –F). Rats received a topical application of 20 µg of TPA dissolved in acetone on each ear (inner and outer surfaces) 30 min after each daily treatment. Naïve rats were challenged with acetone. 5 h after the last TPA or acetone challenge, rats were sacrificed and both ears excised and

weighed. The total weights were used as a measure of TPA-induced oedema. Data is expressed as mean ear weight (g) ($n = 8$) $\pm$ SEM (G) $**P < 0.01$ and $*P < 0.05$ as compared to PEG-treated, TPA challenged group. $###P < 0.0001$ as compared to saline-treated, unchallenged (naïve) group using One-way ANOVA followed by Dunnet's *post hoc* test.

3.3.4.2.2 Effect of stigmasterol on neutrophil count

Blood neutrophil count was determined to be $2.25 \pm 0.17 \times 10^3$ cells/ μ l in saline-treated naïve control rats (Fig. 3.3.4). TPA challenge induced severe neutrophilia in the PEG-treated control rats with neutrophil cell count of $60.80 \pm 6.54 \times 10^3$ cells/ μ l (Fig. 3.3.4). This represented a significant 60-fold increase in the count obtained in naïve rats. Stigmasterol at all doses used inhibited this TPA-induced neutrophilia. Treatment with stigmasterol at 10, 50 and 100 mg/kg recorded $15.12 \pm 1.77 \times 10^3$ cells/ μ l, $14.47 \pm 1.07 \times 10^3$ cells/ μ l and $4.00 \pm 0.77 \times 10^3$ cells/ μ l (Fig. 3.3.4) representing 75.13 $\pm$ 2.90%, 76.20 $\pm$ 1.75% and 93.41 $\pm$ 1.27% inhibition of neutrophil count relative to PEG-treated TPA challenged control. Similarly, dexamethasone exhibited significant inhibition of neutrophilia with a mean neutrophil count of $12.33 \pm 1.21 \times 10^3$ cells/ μ l (Fig. 3.3.4) representing 79.73 $\pm$ 1.99% inhibition of TPA-induced neutrophilia.

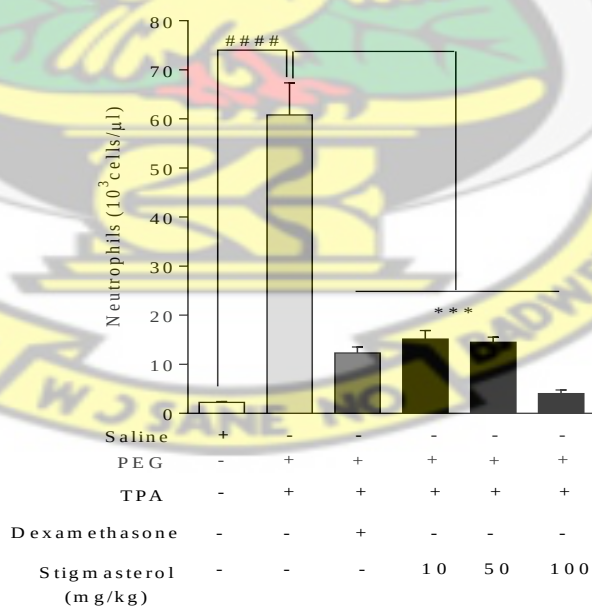


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Fig. 3.3.4 Effect of stigmasterol on TPA-induced neutrophilia. Wistar rats of either sex (100 – 120 g) received either normal saline 5 ml/kg (naïve group), polyethylene glycol 5 ml/kg (PEG), dexamethasone 3 mg/kg or stigmasterol 10, 50 and 100 mg/kg. Rats received a topical application of 20 µg of TPA dissolved in acetone on each ear (inner and outer surfaces) 30 min after each daily treatment. Naïve rats were challenged with acetone. Five (5) hours after the last TPA or acetone challenge, rats were sacrificed, blood collected and neutrophil counts determined. Data is expressed as mean neutrophil count (10^3 cells/µl) $\pm$ SEM ***P<0.001 as compared to PEG-treated, TPA challenged group. ####P<0.0001 as compared to saline-treated, unchallenged (naïve) group using One-way ANOVA followed by Dunnet's *post hoc* test.

3.3.4.2.3 Effect of stigmasterol on serum TNF α concentration

TPA challenge induced significant increase in serum levels of TNF α in the PEG-treated TPA-challenged control. Mean TNF α concentration in serum collected 5 h after the last TPA challenge was 162.90 ± 6.33 pg/ml, a 6-fold increase above naïve mean levels of 27.40 ± 5.49 pg/ml (Fig. 3.3.5). Both stigmasterol and dexamethasone treatments suppressed TPA-induced increase in serum TNF α . Treatment with 10, 50 and 100 mg/kg of stigmasterol reduced mean serum TNF α levels to 79.95 ± 10.00 pg/ml, 82.92 ± 10.98 pg/ml, 60.21 ± 12.02 pg/ml (Fig. 3.3.5) indicating $50.92 \pm 6.14\%$, $49.10 \pm 6.74\%$ and $63.04 \pm 7.38\%$ inhibitions respectively compared to PEG-treated TPA challenged control. Serum TNF α concentration of 63.00 ± 10.53 pg/ml was obtained in the dexamethasone-treated mice (Fig. 3.3.5) indicating a $61.14 \pm 6.47\%$ inhibition compared to PEG-treated control group.

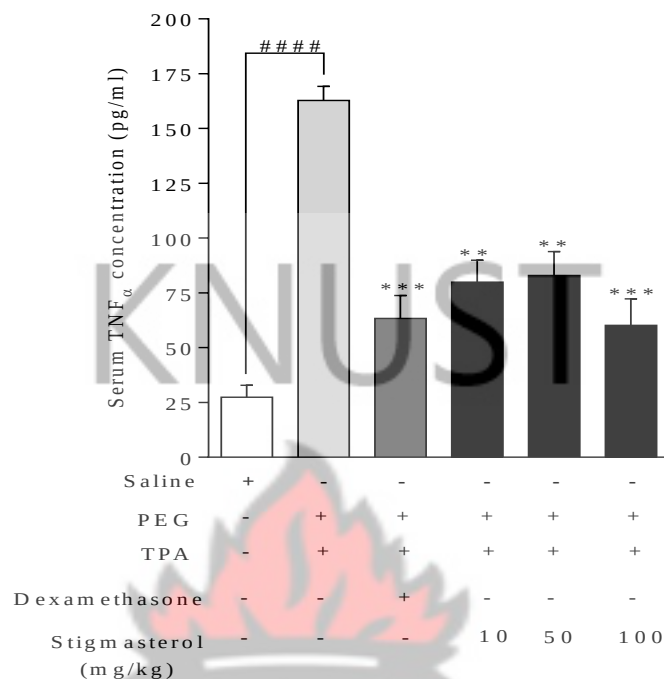


Fig 3.3.5 Effect of stigmasterol on TPA-induced increase in TNF α serum concentration.

Wistar rats of either sex (100 – 120 g) received either normal saline 5 ml/kg (naïve group), polyethylene glycol 5ml/kg (PEG), dexamethasone 3 mg/kg or stigmasterol 10, 50 and 100 mg/kg. Rats received a topical application of 20 μ g of TPA dissolved in acetone on each ear (inner and outer surfaces) 30 min after each daily treatment. Naïve rats were challenged with acetone. Five (5) hours after the last TPA or acetone challenge, rats were sacrificed, blood collected and serum separated by centrifuging at 1000 rpm for 15 min. Serum concentration of tumor necrosis factor alpha (TNF α) was quantified with sandwich ELISA. Data is expressed as TNF α concentration (pg/ml) $\pm$ SEM (n = 5) ***P<0.001, **P<0.01 as compared PEG-treated, TPA challenged group. ####P<0.001 as compared to saline-treated, unchallenged (naïve) group using One-way ANOVA followed by Dunnet's *post hoc* test.

3.3.4.2.4 Effect of stigmasterol on skin histopathology

Skin sections from the saline-treated naïve control animals showed normal skin structure and cell distribution with no signs of hyperplasia of epidermal layer. No sign of edema was observed in the dermis (Fig. 3.3.6A). TPA induced histological changes consistent with severe inflammation. Skin sections from PEG-treated TPA-challenged rats showed significant distortions in skin architecture. Epidermal hyperplasia with

proliferation of epidermal keratinocytes and thickening of dermis contributed to an overall thickened skin section. The dermis was densely populated with clusters of polymorphonuclear leukocytes and lymphocyte infiltrates (Fig. 3.3.6B). Moderate cellular infiltrates and suppressed thickening compared to PEG-treated TPA-challenged rat ear skin sections were observed in stigmasterol 10 – 100 mg/kg (Fig 3.3.6D – F) and dexamethasone treated rats (Fig 3.3.6C). Indices of inflammatory damage such as skin thickness, epidermal hyperplasia and inflammatory cell infiltration of dermis were quantified. Skin thickness of PEG-treated TPA-challenged rats measured a mean of $995.00 \pm 57.49 \mu\text{m}$ corresponding to a 173.35% increase in mean thickness in rat ear skin of the naïve animals which measured $364.00 \pm 17.30 \mu\text{m}$ (Fig 3.3.6G). Mean thickness in rat ear skin of $647.60 \pm 15.04 \mu\text{m}$, $573.11 \pm 25.29 \mu\text{m}$ and $529.40 \pm 18.78 \mu\text{m}$ respectively were obtained with stigmasterol 10, 50 and 100 mg/kg thereby inhibiting the TPA-induced skin thickness by $34.91 \pm 1.51\%$, $42.41 \pm 2.54\%$ and $46.80 \pm 1.89\%$ respectively. Dexamethasone reduced the skin thickness of PEG-treated TPA-challenged rats to $566.70 \pm 27.47 \mu\text{m}$ giving a $43.04 \pm 2.76\%$ inhibition of skin thickness (Fig 3.3.6G). A similar trend was observed for the epidermal hyperplasia assessment. The average thickness of the epidermis, a measure of epidermal hyperplasia was found to be $24.28 \pm 3.47 \mu\text{m}$ in naïve rats (Fig 3.3.6H). Topical TPA challenge induced a 351.81% increase in thickness of the epidermal layer in the PEG-treated control group with a mean thickness of $109.70 \pm 9.10 \mu\text{m}$. Stigmasterol at 10, 50 and 100 mg/kg caused significant suppressive effects with average thickness of the epidermis of $46.20 \pm 5.85 \mu\text{m}$, $48.91 \pm 7.02 \mu\text{m}$ and $43.04 \pm 4.30 \mu\text{m}$ causing inhibitions of epidermal layer thickening by $57.89 \pm 5.34\%$, $55.41 \pm 6.40\%$ and $60.77 \pm 3.92\%$ respectively. Similarly, in the dexamethasone-treated rats, mean epidermis thickness of $35.47 \pm 4.67 \mu\text{m}$ representing $67.67 \pm 4.26\%$ inhibition compared to PEG-treated control group was obtained (Fig 3.3.6H). Quantification of cell distribution in dermal regions of naïve mice, recorded a mean cell (polymorphonuclear leukocytes and lymphocytes) count of 24.84 ± 2.19 per 0.2 mm^2 of dermis (Fig. 3.3.6I). Skin sections of TPA-induced PEG-treated rats recorded a mean inflammatory cell population of $112.40 \pm 8.80 \text{ cells/mm}^2$ (Fig 3.3.6I) representing a 352.50% increase in the count obtained for the naïve control. Stigmasterol 10, 50 and 100 mg/kg treatment gave cell

counts of 67.53 ± 6.91 cells/mm², 63.71 ± 5.61 cells/mm² and 43.33 ± 4.52 cells/mm² (Fig 3.3.6I) thereby reducing TPA-induced cell infiltration by $39.92 \pm 6.15\%$, $43.32 \pm 4.99\%$ and $61.45 \pm 4.02\%$ respectively. In the dexamethasone-treated rats recorded mean cell counts of 38.64 ± 4.74 cells/mm² (Fig 3.3.6I) representing an inhibition of $65.62 \pm 4.22\%$ was obtained.

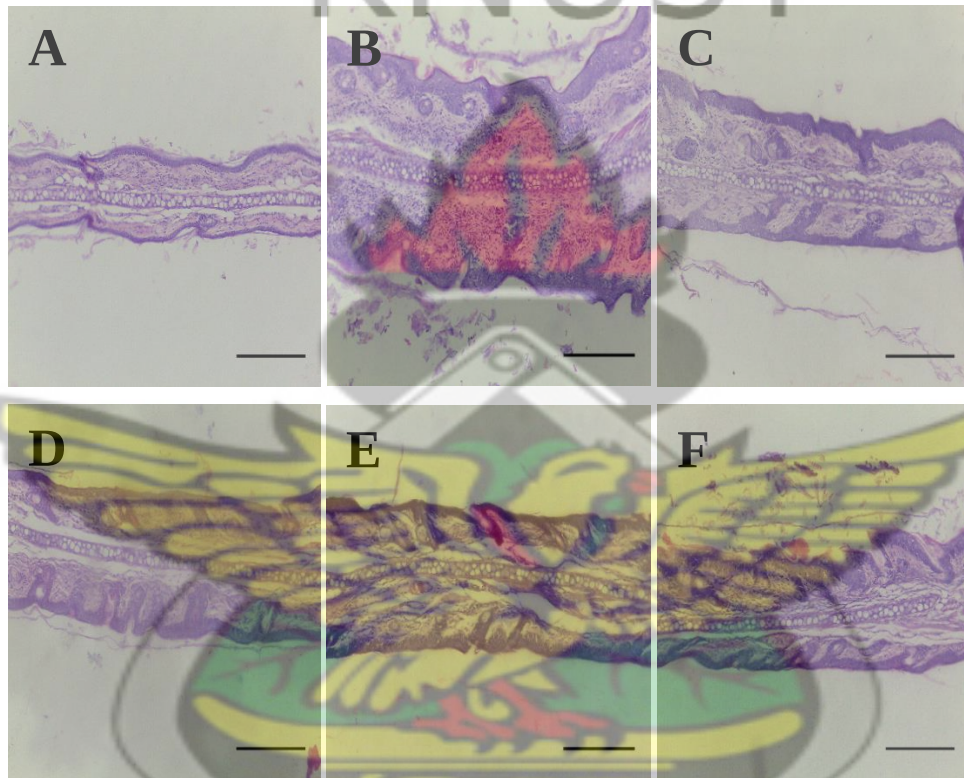


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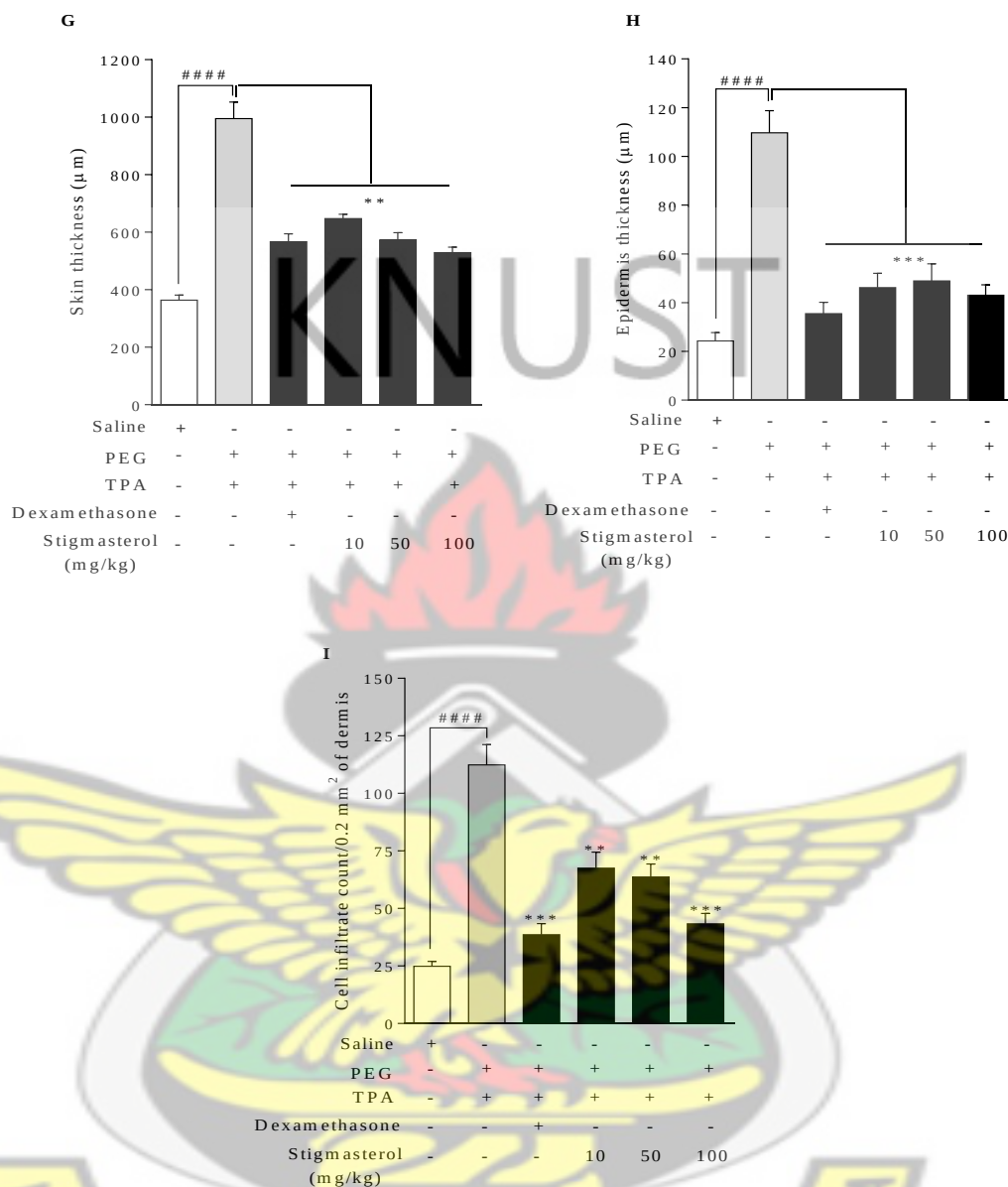


Fig 3.3.6 Effect of stigmasterol rat ear skin histology. Wistar rats of either sex (100 – 120 g) received either normal saline 5 ml/kg (naïve group) (A), polyethylene glycol 5ml/kg (PEG) (B), dexamethasone 3 mg/kg (C) or stigmasterol 10, 50 and 100 mg/kg (D – F). Rats received a topical application of 20 µg of TPA dissolved in acetone on each ear (inner and outer surfaces) 30 min after each daily treatment. Naïve rats were challenged with acetone. Five (5) hours after the last challenge, rats were sacrificed, both ears excised, weighed and stored in 10% buffered formalin. 3 µm skin sections were stained with H and E stain and observed for histopathological changes. Parameters of skin damage were assessed as described in the methods section. Data is expressed as mean skin thickness (µm) (n = 12) ± SEM (G), mean epidermis thickness (µm) (n

= 12) $\pm$ SEM (H) and mean cell infiltrate per field (n = 12) $\pm$ SEM (I). ***P < 0.001, ** P < 0.01 as compared to PEG-treated, TPA challenged group. ####P < 0.0001 as compared to saline-treated, unchallenged (naive) group using One-way ANOVA followed by Dunnet's *post hoc* test. Micron bar represents 300 μ m.

3.3.5 DISCUSSION

The effects of stigmasterol on selected allergic cutaneous responses were investigated in this section. The study focused on Compound 48/80-induced pruritus as well as TPA-induced irritant dermatitis which are both non-IgE dependent mechanisms.

It was demonstrated that scratching behavior induced by compound 48/80 in ICR mice was abated by stigmasterol. Pruritus is a major symptom of dermatological disease which has proven difficult to treat over the years (Cunha and Delfini Filho, 2012). Incessant scratching further worsens disease prognosis and affects both physical and psychological well-being (Yosipovitch and Bernhard, 2013). Release of histamine and its subsequent stimulation of histaminic-H₁ receptors is widely reported to be one of the major mechanisms of dermatological itch (Davidson and Giesler, 2010). Earlier studies by Dunford *et al.* (2006) have also implicated histamine- H₂ and H₄ . receptors in the itch response. Other pruritogens such as serotonin, capsaicin, tryptase and cannabinoids have all been reported to play important roles *via* a variety of receptors (Davidson and Giesler, 2010). Perhaps this explains the limited efficacy of antihistamine agents in pruritus. Compound 48/80 is a potent mast cell degranulator widely used in animal models to screen potential antipruritic agents. It acts by directly activating G proteins, stimulating protein tyrosine phosphorylation and triggering a rise in intracellular calcium (Seifert, 2015). Interestingly, Inagaki *et al.* (2002) demonstrated that compound 48/80-induced pruritus in mast cell-deficient mice. While the involvement of mast cells is irrefutable, this finding alongside the reported ineffectiveness of mast cell stabilizers such as cromolyn sodium confirms the possible involvement of other pathways. Consistent with literature, skin histology of the PEG-treated, C48/80-injected control mice showed an increase in mast cell numbers and widespread degranulation at injected site when compared to the saline-treated naïve control. Mast cell proliferation and

degranulation have been shown to be critical to the mediation of itch responses in animal studies (Yamashita *et al.*, 2005; Jin *et al.*, 2014). Both processes were shown to be inhibited by stigmasterol. Topical application of TPA induces skin inflammatory responses consistent with irritant contact dermatitis. In this process, the induction of pro-inflammatory cytokines and chemo-attractants, reactive oxygen species, recruitment of polymorphonuclear leukocytes into skin tissues and skin histopathology similar to irritant dermatitis is believed to be initiated by activation of protein kinase C, PKC (Passos *et al.*, 2013). TPA skin inflammation model is therefore useful in the screening of potential agents in allergic skin diseases (Rauh *et al.*, 2011). In this study, systemic intraperitoneal stigmasterol suppressed key local and systemic features of TPA-induced contact dermatitis. Stigmasterol at all doses used reduced ear weights, a measure of oedema, indicating significant inhibition of inflammatory processes. This is consistent with findings in an earlier study by Gomez *et al.* (1999), in which one of several topically applied phytosterol isolates from *Achillea ageratum* that inhibited TPA-induced skin oedema was found to be stigmasterol. In this section, it is reported also, that systemic administration of stigmasterol not only reversed oedema but suppressed other key features of the skin inflammatory response. One significant finding was the reduction in serum levels of TNF_α by stigmasterol. A strong correlation between TPA-induced or clinical contact dermatitis and pro-inflammatory cytokine activity has been reported widely (Otuki *et al.*, 2005). TNF_α which is both an ‘activator’ and product of skin cells is crucial in the pathophysiology of allergic skin disease. Its role in activating chemokines such as IL-8 and regulated on activation, normal T cell expressed and secreted RANTES also called CCL5 in the early stages of contact dermatitis contributes significantly to infiltration of skin tissue by inflammatory cells. It is therefore not surprising that interventions that control both skin tissue and serum TNF_α levels have been shown to improve features of TPA-induced dermatitis. (Lee *et al.*, 2009). Features such as increase in skin thickness, epidermal hyperplasia and dramatic transmigration of polymorphonuclear leukocytes and lymphocytes which were observed in naïve control rats were largely suppressed in the stigmasterol-treated rats. Migration of inflammatory cells into skin tissue is a key factor in skin damage in contact dermatitis. Release of chemokines by cells of injured skin encourage the infiltration and

trafficking of neutrophils and other inflammatory cells into tissue. Activities of such cells further aggravate the skin injury. Indeed, agents which have shown inhibition of both polymorphonuclear leukocyte activation and chemo-attraction into damaged skin tissue have offered significant improvement in allergic skin diseases (Otuki *et al.*, 2011). Following PKC activation and release of chemokines such as cytokine-induced neutrophil chemoattractant and macrophage inflammatory protein 2, MIP-2 in murine plasma, TPA induces severe systemic neutrophilia and intra-epidermal accumulation of neutrophils (Cataisson *et al.*, 2006). Both features of TPA-induced skin inflammation were significantly suppressed upon stigmasterol treatment. Perhaps this inhibition of neutrophil transmigration, in addition to reduced serum levels of $\text{TNF}\alpha$ could account for, albeit partly, the improvement in skin histopathology observed in stigmasterol-treated rats.

3.3.6 CONCLUSION

Stigmasterol ameliorated scratching behaviour induced by compound 48/80 possibly due to inhibition of skin mast cell degranulation and proliferation at injected site. Stigmasterol significantly inhibited TPA-induced skin damage by suppression of neutrophil accumulation in blood, infiltration of leukocytes into skin tissue and reduced serum levels of $\text{TNF}\alpha$. Stigmasterol therefore holds great potential in allergic skin disease therapy.

3.4 EFFECT OF STIGMASTEROL ON ALLERGEN-INDUCED AIRWAY INFLAMMATION

3.4.1 INTRODUCTION

Asthma is a chronic pulmonary disorder associated with airway hyper-responsiveness (AHR), inflammation and airway obstruction. The pathophysiology of asthma is characterized by severe inflammatory cell activation and accumulation, airway muscle hypertrophy, sub-mucosal fibrosis and excessive mucus production resulting in permanent airway remodeling (Maslan and Mims *et al.*, 2014). In allergic asthma, immunoglobulin E (IgE) type of antibodies are produced when cognate antigens also called allergens sensitize patients on first exposure. These antibodies remain in circulation in the blood or become attached to mast cells of the nasal or bronchial tissues and basophils. Subsequent re-exposure to the same antigen results in cross-linking of bound immunoglobulin E (IgE) to surface receptors (Galli *et al.*, 2002). At the molecular level, this antigen-antibody reaction in the early phase causes degranulation of the lung mast cells with the release of mediators such as histamine, 5-hydroxytryptamine, prostaglandins, the cysteinyl leukotrienes (LTB₄, LTC₄ and LTD₄) and cytokines such as the interleukins; IL-4, IL-5 and IL-13 (Gilfillan and Tkaczyk, 2006). Elias *et al.* (2003) report that these mediators of allergy sustain the late or delayed phase of asthma and they activate additional inflammatory cells such as eosinophils, basophils, leukocytes and alveolar macrophages to release more of the LTs and ILs. Recent advances in medicine notwithstanding, asthma is responsible for a yearly death toll of about 250, 000. This has ultimately imposed a global financial burden of about \$300 - \$1300 per patient (Pawankar *et al.*, 2011) with lower-to-middle income regions such as Sub-Saharan Africa recording the highest rate of asthma-related deaths (Zar *et al.*, 2001). In Ghana, a WHO report estimated an incident rate of 1.5/1000 per year (WHO, 2009). A review by Amoah *et al.*, (2012) however points to an increase in this trend.

New research and medications designed to tackle specific arms of the underlying pathophysiology have emerged (Xie and Merkel, 2015; Chung, 2016) in an attempt to address the current gap in knowledge and limitations in therapy respectively.

Stigmasterol, a naturally occurring steroid alcohol belongs to a larger class of plant compounds called phytosterols (Moreau *et al.*, 2002) which are widely distributed in foods of plant origin (Ryan *et al.*, 2007) and popular medicinal plants throughout the world (Zheng, 1994; Parra-Delgado *et al.*, 2004; Singh *et al.*, 2012). Phytosterols have been associated with emerging health benefits such as lipid lowering, anticancer and anti-inflammatory effects (Trautwein and Demonty, 2007). Several *in vivo* and *in vitro* studies have shown that the underlying anti-inflammatory mechanisms could account for the apparent lipid lowering and anti-atherogenic effects (Othman and Moghadasian, 2011). Demonstration of anti-arthritic and anti-inflammatory actions (Gabay *et al.*, 2010; Chen *et al.*, 2012) for stigmasterol suggests some immunomodulatory effects but there still exists a significant knowledge gap with regards to the extent and specifics of its role in immune and inflammatory disorders such as asthma.

The search for novel medications for asthma spans across synthetic molecules, molecular interventions and alternatives from natural sources. Particular interest in the latter has taken center stage, with some interesting findings already reported from both experimental and clinical investigations (Abidi *et al.*, 2014; Chen *et al.*, 2015; Subhashini *et al.*, 2016).

In this section, the potential benefits of stigmasterol in the treatment of asthma were investigated. It assesses the possible anti-inflammatory or immunomodulatory effects of stigmasterol in ovalbumin-induced asthma in guinea pig model of inflammation.

3.4.2 MATERIALS

3.4.2.1 Chemicals and reagents

Stigmasterol (95%), ovalbumin (OVA), polyethylene glycol, PEG and dexamethasone were obtained from Sigma Aldrich (St. Louis, USA). Guinea pig VCAM-1 and OVA sIgE ELISA quantification kits were purchased from MLBio Biotechnology Company Limited (Shanghai, China).

3.4.2.2 Animals

Guinea pigs (300 - 350 g) of either sex were obtained from Noguchi Memorial Institute for Medical Research, Legon - Ghana. Animals were kept under standard temperature and humidity (temperature $25 \pm 2^{\circ}\text{C}$ with a 12 h light-dark cycle) at the Animal House facility of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST and allowed access to commercial chow and distilled water ad libitum. All protocols used in this study were approved by the Faculty of Pharmacy Ethics Committee and animal handling was done in compliance with Animal Welfare Regulations (USDA 1985; US Code, 42 USC § 289d) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002).

3.4.3 METHODS

3.4.3.1 Ovalbumin-induced asthma; sensitization and challenge

Five groups ($n = 5$) of guinea-pigs (300 - 350 g) were sensitized by intraperitoneal injection of 100 μl OVA-solution (2 mg ovalbumin emulsified in 10 mg aluminium hydroxide (AlOH_3) dissolved in 10 ml normal saline (0.9% w/v NaCl) at the start of the experiment. A booster dose of 100 μl (1 mg ovalbumin dissolved in saline) was administered intraperitoneally on day 14. From day 21 to day 30, sensitized guinea pigs were challenged with aerosolized ovalbumin (1% w/v dissolved in phosphate buffered saline, PBS) daily for 10 min. Naïve animals ($n = 5$) were sham-sensitized with 100 μl normal saline and challenged with PBS only. Before each challenge, polyethylene glycol, PEG 50% w/v (10 ml/kg, *p.o.*), stigmasterol (10, 50, 100 mg/kg, *i.p.*) or dexamethasone (3 mg/kg, *p.o.*) respectively was administered to naïve and test groups 30 min after *i.p* or 1 h after oral administration. Guinea pigs were subjected to the following tests:

3.4.3.1.1 Haematology and Serum analysis

Animals were sacrificed with an overdose of ether, 24 h after the last OVA exposure, and immediately bled by dissection of the jugular vein. Blood was collected into EDTA tubes for differential blood cell count with automated analyzer (Sysmex KX-21N, Sysmex America Inc., Illinois, USA) and into sterile capillary tubes for serum analysis

respectively. Blood collected into sterile capillary tubes was allowed to clot and centrifuged for 15 min at 1000 rpm. Aliquots of serum were collected into Eppendorf tubes and stored at -70°C. Serum levels of vascular cell adhesion molecule-1 (VCAM-1) and ovalbumin-specific Immunoglobulin E (OVA sIgE) were quantified using enzyme-linked immunosorbent assay (ELISA) kits according to instructions of the manufacturers.

3.4.3.1.2 Broncho-alveolar lavage fluid (BALF) collection and analysis

Immediately after blood collection the trachea was carefully exposed avoiding contamination of luminal contents with blood and damage to lung tissue. The trachea was cannulated and broncho-alveolar fluid was collected by aspiration. Lung tissues were flushed with 3x 5 ml portions of PBS and aspirated after gentle massage. Recovered fluid was centrifuged at 1000 rpm for 10 min at 4°C. The supernatant was collected, protein concentration determined with the Bradford method, stored at -70°C and when needed subjected to analysis for the following oxidative stress markers:

a) Malondialdehyde (MDA)

MDA was measured as a product of lipid peroxidation by the method of Heath and Parker (1968). Briefly, 1 ml BALF was added to a 3 ml mixture of trichloroacetic acid, TCA (20%) and thiobarbituric acid, TBA (0.5%), heated at 95°C for 30 min and immediately cooled and centrifuged at 5,000 rpm for 10 min. Aliquots (200 µl) of supernatant was pipetted into 96-well plates in triplicate and absorbance read at 532 nm and 600 nm respectively with a Synergy H1 Hybrid Reader spectrophotometer (BioTek Technologies, Winooski, VT, USA) to correct for nonspecific absorbance. MDA concentration (nmol/ mg protein) was calculated with its molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ with the equation;

$$\text{nmol MDA per mg protein} = \left[\frac{\text{Absorbance}_{532 \text{ nm}} - \text{Absorbance}_{600 \text{ nm}}}{1.56 \times 10^5 \times \text{total protein}} \right] \times 10^6$$

b) Reduced glutathione (GSH)

GSH levels were determined by a method earlier described by Ellman (1959). Briefly, 100 µl BALF aliquot was mixed with 2.4 ml EDTA (0.02 M) at 4 °C for 10 min. To this 2 ml distilled water and 500 µl TCA (50%) were added and centrifuged at 3000 rpm for 5 min. To 1 ml of the supernatant, 50 µl 5, 5' – dithio-bis-2-nitro benzoic acid, DTNB (10 mM) and 2 ml Tris buffer (0.4 M, pH 8.9) were added. The absorbance was read within 5 min of DTNB addition at 412 nm against a blank (reagents only) with a Synergy H1 Hybrid Reader spectrophotometer (BioTek Technologies, Winooski, VT, USA). The final sulphhydryl concentration was interpolated from a standard curve with the equation: $y = 0.0004x + 0.0026$, where x is the absorbance at 412 nm.

c) Superoxide dismutase (SOD)

SOD activity was estimated with the modified method of Misra and Fridovich (1972). Briefly, 500 µl of BALF was treated with 150 µl ice-cold chloroform and 750 µl ethanol (96% v/v), vortexed for 1 min, and then centrifuged at 2000 rpm for 20 min at 25°C. To 500 µl portion of the supernatant, 500 µl EDTA (0.6 mM) and 1 ml carbonate bicarbonate buffer (0.1 M, pH 10.2) were added. The reaction was initiated by the addition of 50 µl adrenaline (1.3 mM). Absorbance was measured with a Synergy H1 Hybrid Reader spectrophotometer (BioTek Technologies, Winooski, VT, USA) at 480 nm against a blank. Activity of SOD, measured as the quantity of the enzyme required to inhibit the auto-oxidation of adrenaline was calculated using the equation:

$$\% \text{ inhibition} = \left[\frac{\text{Absorbance}_{(test)} - \text{Absorbance}_{(blank)}}{\text{Absorbance}_{test}} \right] \times 100$$

SOD level was expressed in units per mg protein, where 1 unit of enzyme activity is the quantity of enzyme required to prevent the auto-oxidation of adrenaline at 25°C and calculated with the equation:

$$\text{Units of SOD activity/mg protein} = \left[\frac{\% \text{ inhibition}}{50 \times \text{weight of protein}} \right] \times 100$$

d) Catalase (CAT)

The method described by Sinha (1972) with slight modifications was used. Briefly, to 100 µl aliquot of BALF, 1 ml phosphate buffer (0.01 M, pH 7.0) and 400 µl H₂O₂ (1.18 M) were added and the mixture incubated at room temp for 5 min. The reaction was halted by adding 2 ml of a 3:1 mixture of glacial acetic acid and dichromate (5%). Absorbance was measured at 620 nm with a Synergy H1 Hybrid Reader spectrophotometer (BioTek Technologies, Winooski, VT, USA). One unit of catalase activity defined as the amount of enzyme that degrades 1 mmol H₂O₂ per min at 25°C and pH 7.0, was expressed in terms of its molar extinction coefficient, 39.4 M⁻¹ cm⁻¹.

$$mUnit\ CAT/mg\ protein = \left[\frac{Absorbance_{620\ nm}}{3.94 \times weight\ of\ protein} \right] \times 1000$$

3.4.3.1.3 Histology

Lung tissues were carefully removed, 24 h after the last OVA exposure, and fixed in 10% formalin. Tissues were serially dehydrated in increasing concentrations of ethanol, cleared in xylene in a TP 1020 Tissue processor (Leica Biosystems, Wetzlar, Germany) and embedded in paraffin using a Leica EG 1160 Embedding machine (Leica Biosystems, Wetzlar, Germany). Transverse sections of 3 µm were cut with a Leica RM 2125 Microtome (Leica Biosystems, Wetzlar, Germany), deparaffinized and hydrated to distilled water. Tissue sections were stained appropriately for either airway inflammatory cell infiltration or collagen deposition and observed under light microscope (Leica DM2500 M). Quantitative analyses were performed with Image J analysis tool (version 1.50i, National Institutes of Health, USA).

a) Airway inflammatory cell infiltration

Sections were stained with hematoxylin and eosin (H & E) stain. A method previously described by Zare *et al.* (2008) with modifications was used to assess the degree of airway inflammatory cell infiltration with a brief scoring system as follows; 0, no cell; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2 - 4 cell layers deep; 4, a ring of cells > 4 cell layers deep in the peribronchiolar and perivascular regions.

Alveolar cell infiltration was assessed as follows; 0, no infiltrate or widening septa; 1, few infiltrates with widening septa; 2; obvious infiltrates with widening septa; 3, filled alveolar air spaces with thickened septa. Scores for peribronchiolar, perivascular and alveolar cell infiltration were summed into an 11-point composite score.

b) Assessment of collagen deposition

Sections were stained with Masson's trichrome stain. Remodeling was assessed by measuring the total length of the basement membrane of selected bronchioles from each treatment and the respective peribronchiolar fibrotic region (stained blue). The degree of fibrosis was quantified as the mean area of collagen deposition per unit length of the basement membrane. For each guinea pig, 5 random sections from the left lower lung were selected. Average sized bronchioles (5 – 7) from each section were analyzed and the average scores for each group calculated.

3.4.3.2 Statistical analysis

All results are presented as mean $\pm$ SEM. Data analysis was done using One-way analysis of variance (ANOVA). Multiple comparisons between treatment groups were performed using Dunnet's *post hoc* test. All statistical analyses were done using GraphPad for Windows version 6 (GraphPad Prism Software, San Diego, USA).

3.4.4 RESULTS

3.4.4.1 Haematology and Serum Analysis

3.4.4.1.1 Effect of stigmasterol on inflammatory cell count in blood

Ovalbumin challenge of previously OVA-sensitized guinea pigs was characterized by significant increase in blood eosinophils, lymphocytes and monocytes relative to the naïve control animals (Fig. 3.4.1A - C). The saline-treated OVA-sensitized and challenged groups recorded respectively 7, 5 and 3 fold increases in mean number of eosinophils, lymphocytes and monocytes compared to their respective naïve control groups and these were significantly reduced by dexamethasone (Fig. 3.4.1A - C). Stigmasterol administered at 10, 50 and 100 mg/kg significantly reduced the cell proliferative effect induced by ovalbumin challenge from 0.25 ± 0.01 to 0.05 ± 0.01 ,

0.03 $\pm$ 0.02 and 0.02 $\pm$ 0.01 respectively for eosinophils (Fig. 3.4.1A). Similarly, elevated blood lymphocyte counts of 2.12 $\pm$ 0.09 were significantly reduced by stigmasterol treatment to 1.05 $\pm$ 0.05, 0.86 $\pm$ 0.03 and 0.84 $\pm$ 0.04 respectively (Fig. 3.4.1B). For the monocytes, while significantly reduced numbers were counted from a control of 0.58 $\pm$ 0.03 to 0.19 $\pm$ 0.02 and 0.16 $\pm$ 0.01 respectively for 50 and 100 mg/kg stigmasterol treatment the value of 0.56 $\pm$ 0.03 obtained for the 10 mg/kg treatment was not significant (Fig. 3.4.1C).



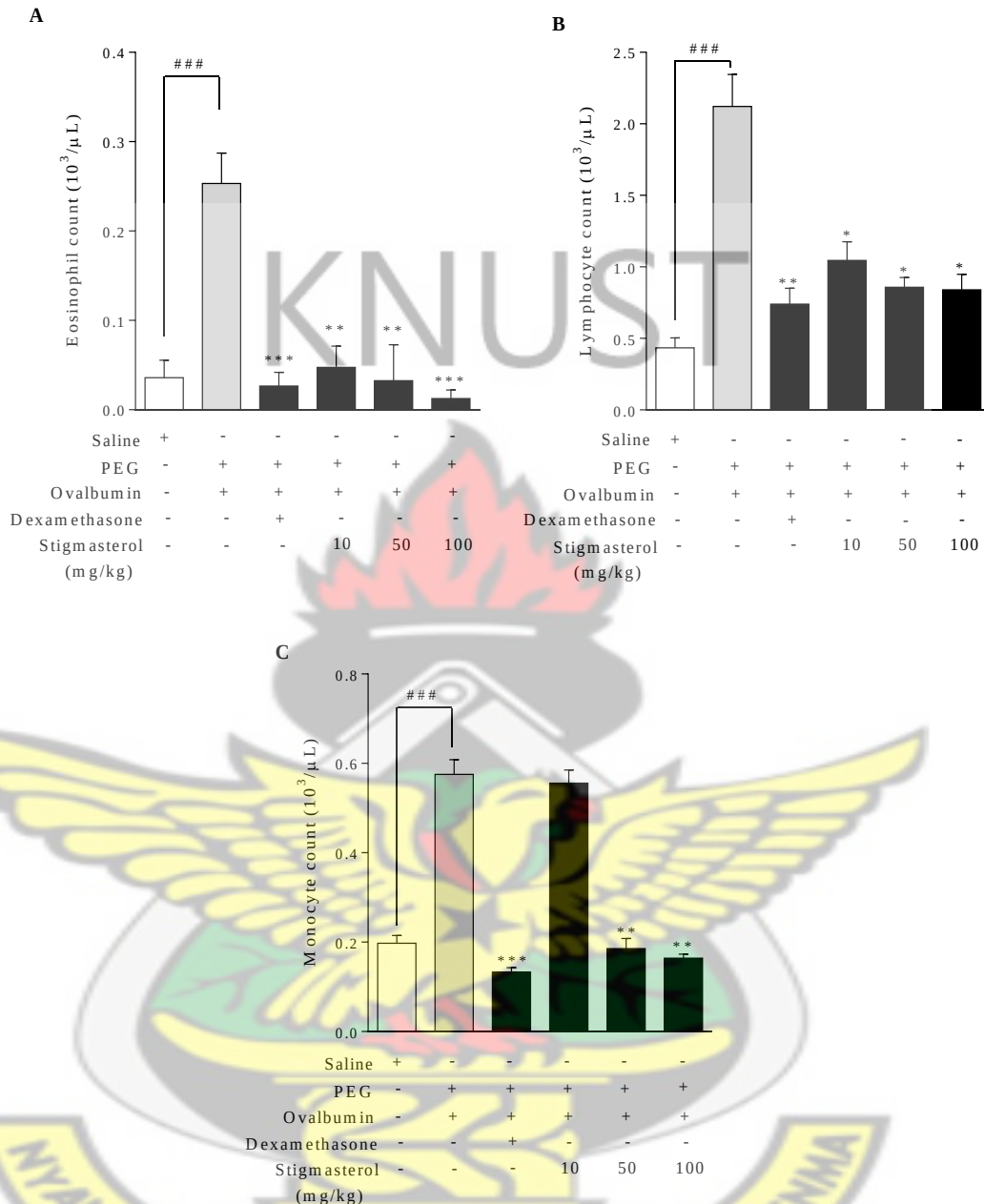


Fig 3.4.1 Effect of stigmasterol on inflammatory cell count in blood. Guinea pigs (300 – 350 g) received either normal saline 5 ml/kg, polyethylene glycol (PEG) 5 ml/kg, dexamethasone 3 mg/kg or stigmasterol 10, 50 and 100 mg/kg 1 h prior to each ovalbumin challenge. Naïve controls received normal saline only. Animals were sacrificed 24 h after the last challenge and blood was collected for counts of eosinophil (A), lymphocyte (B) and monocyte (C). Data is expressed as cell count ($10^3/\mu\text{L}$) $\pm$ SEM (n = 5) ***P<0.001, **P<0.01, *P<0.5 as compared to PEG-treated control; ###P<0.001 as compared to naïve control using One-way ANOVA followed by Dunnet's *post hoc* test.

3.4.4.1.2 Effect of stigmasterol on serum vascular cell adhesion molecule-1 (VCAM-1) levels

Serum analysis showed a significantly increased mean concentration of soluble vascular cell adhesion molecule-1 (VCAM-1) in saline-treated OVA-sensitized and challenged group to 205.20 ± 25.82 pg/ml from a mean value of 10.58 ± 3.18 pg/ml for the naïve control group (Fig. 3.4.2). As expected dexamethasone (3 mg/kg) significantly reduced the increased mean expression of VCAM-1 to 50.97 ± 7.52 pg/ml. Stigmasterol 50 and 100 mg/kg demonstrated inhibitory effects by significantly reducing the mean expression of VCAM-1 to 118 ± 12.54 pg/ml and 90.75 ± 7.12 pg/ml respectively when compared to the saline-treated OVA-sensitized and challenged control group. No significant inhibition was however observed with stigmasterol administered at 10 mg/kg with a concentration of 209.40 ± 13.11 pg/ml (Fig. 3.4.2).

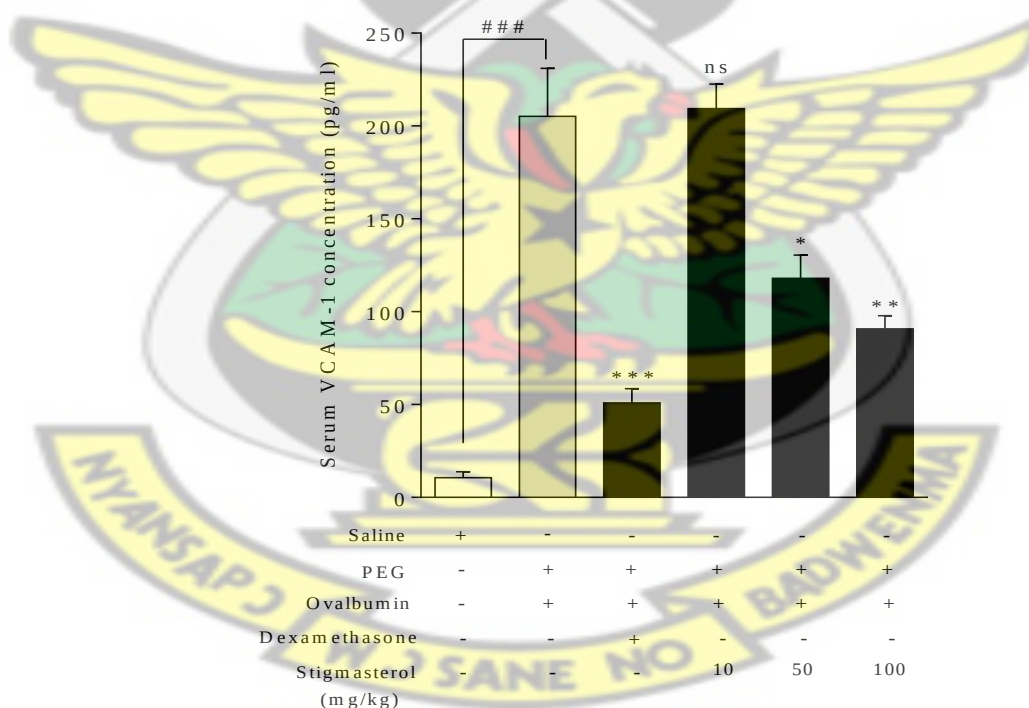


Fig 3.4.2 Effect of stigmasterol on serum vascular cell adhesion molecule-1(VCAM-1). Guinea pigs (300 – 350 g) received either normal saline 5 ml/kg, polyethylene glycol (PEG) 5 ml/kg, dexamethasone 3 mg/kg or stigmasterol 10, 50 and 100 mg/kg 1 h prior to each ovalbumin challenge. Naïve controls received normal saline only. 24 h after the last challenge,

animals were bled by dissection of jugular vein. Blood collected was allowed to clot and centrifuged at 1000 rpm for 15 min. Serum vascular cell adhesion molecule-1, VCAM-1 levels were quantified with sandwich ELISA. Data is expressed as VCAM-1 concentration (pg/ml) $\pm$ SEM (n = 5) ***P<0.001, **P<0.01, *P<0.5 as compared to PEG-treated control; ###P<0.001 as compared to naive control using One-way ANOVA followed by Dunnet's *post hoc* test.

3.4.4.1.3 Effect of stigmasterol on serum OVA specific Immunoglobulin E (OVA sIgE) levels

The mean OVA sIgE was significantly increased to 81.75 ± 7.5 ng/ml in the saline-treated OVA-sensitized and challenged group compared to the naïve control animals with values below detectable levels (Fig. 3.4.3). OVA sIgE levels in dexamethasone-treated animals were significantly reduced to 26.10 ± 2.08 ng/ml as compared to the saline-treated OVA-sensitized and challenged group (Fig. 3.4.3). Stigmasterol significantly reduced ovalbumin-elicited serum levels of OVA specific Immunoglobulin E to 39.83 ± 1.71 ng/ml, 42.79 ± 4.59 ng/ml and 34.02 ± 1.65 ng/ml respectively at 10, 50 and 100 mg/kg relative to the saline-treated OVA-sensitized and challenged control group (Fig. 3.4.3).

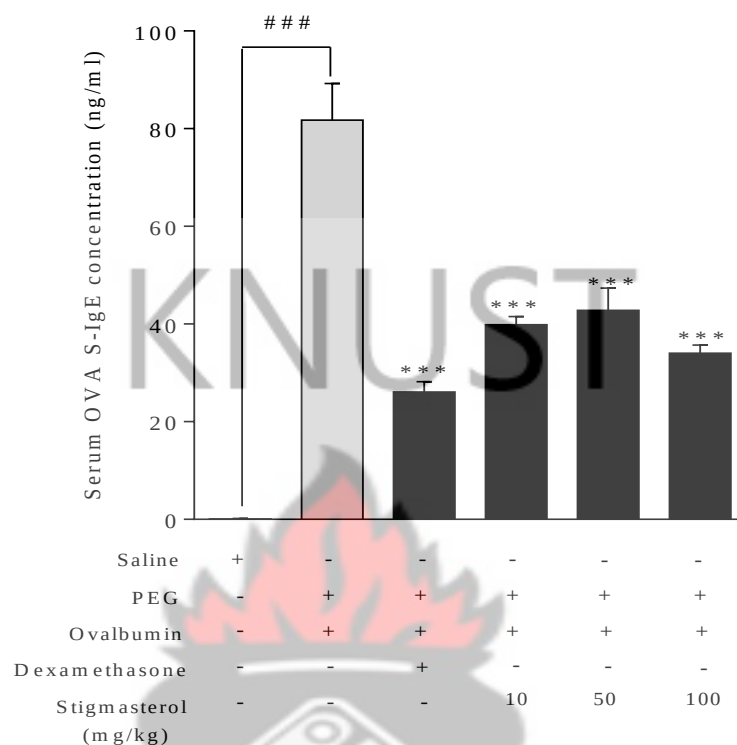


Fig 3.4.3 Effect of stigmasterol on serum OVA specific Immunoglobulin E (OVA sIgE).

Guinea pigs (300 – 350 g) received either normal saline 5 ml/kg, polyethylene glycol (PEG) 5 ml/kg, dexamethasone 3 mg/kg or stigmasterol 10, 50 and 100 mg/kg 1 h prior to each ovalbumin challenge. Naïve controls received normal saline only. 24 h after the last challenge, animals were bled by dissection of jugular vein. Blood collected was allowed to clot and centrifuged at 1000 rpm for 15 min. Serum OVA specific Immunoglobulin E, OVA sIgE levels were quantified with sandwich ELISA. Data is expressed as OVA sIgE concentration (ng/ml) $\pm$ SEM (n = 5) ***P<0.001 as compared to PEG-treated control; ###P<0.001 as compared to naïve control using One-way ANOVA followed by Dunnet's *post hoc* test.

3.4.4.2 Broncho-alveolar lavage fluid (BALF) analysis

3.4.4.2.1 Effect of stigmasterol on BALF oxidative stress markers

Analysis of supernatant showed an antioxidant profile consistent with severe inflammation. Levels of malondialdehyde (MDA) a direct product of lipid peroxidation was significantly elevated to 56.39 ± 5.15 nmol/mg protein in the saline-treated OVA-sensitized and challenged animals relative to 9.30 ± 1.01 nmol/mg protein in the naïve

animals (Fig. 3.4.4A). This evident oxidative stress induced by ovalbumin challenge was significantly mitigated by both dexamethasone and stigmasterol. Administered at 3 mg/kg dexamethasone suppressed the elevated MDA level to 28.36 ± 0.65 nmol/mg protein while stigmasterol at 10, 50 and 100 mg/kg significantly suppressed same to 34.98 ± 4.72 , 20.32 ± 2.08 , and 19.28 ± 0.52 nmol/mg protein respectively (Fig. 3.4.4A). Analysis of BALF from saline-treated OVA-sensitized and challenged group showed significant depletion of the markers reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) with dexamethasone significantly increasing their activity (Fig 3.4.4B-D). The mean GSH levels of 742.40 ± 26.23 μ mol/mg protein in naïve guinea pigs was a significantly reduced to 304.60 ± 4.04 μ mol/mg protein in the saline-treated OVA-sensitized and challenged animals. Dexamethasone as expected elevated it to 541.70 ± 29.87 μ mol/mg protein (Fig. 3.4.4B). Stigmasterol 50 and 100 mg/kg significantly maintained GSH levels at 434.20 ± 30.19 and 667.40 ± 15.14 μ mol/mg protein respectively while the mean value of 372.10 ± 9.65 μ mol/mg protein recorded in the 10 mg/kg stigmasterol-treated animals was however not statistically different from saline-treated ovalbumin-sensitized and challenged control (Fig. 3.4.4B). Superoxide dismutase level was significantly reduced to $9.78 \pm 0.28 \times 10^2$ U/mg protein in the saline-treated OVA-sensitized and challenged animals relative to $20.02 \pm 0.21 \times 10^2$ U/mg protein in the naïve animals (Fig. 3.4.4C). When treated with dexamethasone a significantly elevated SOD activity of $19.02 \pm 0.34 \times 10^2$ U/mg protein was attained relative to the saline-treated OVA-sensitized and challenged animals while stigmasterol at 10, 50 and 100 mg/kg significantly elevated mean SOD to 16.99 ± 0.70 , 18.74 ± 0.05 and $18.89 \pm 0.27 \times 10^2$ U/mg protein respectively (Fig. 3.4.4C). A similar observation was made for CAT activity (Fig. 3.4.4D). On OVA sensitization and challenge, saline-treated animals presented with 2.79 ± 0.45 mU/mg protein which was significantly different from 6.13 ± 0.46 mU/mg protein in the naïve animals. Dexamethasone significantly elevated CAT activity to 6.08 ± 0.26 mU/mg protein while the test drug stigmasterol at 10, 50 and 100 mg/kg significantly elevated CAT levels to 4.38 ± 0.28 , 5.77 ± 0.32 , and 5.35 ± 0.46 mU/mg protein respectively (Fig. 5.4D).

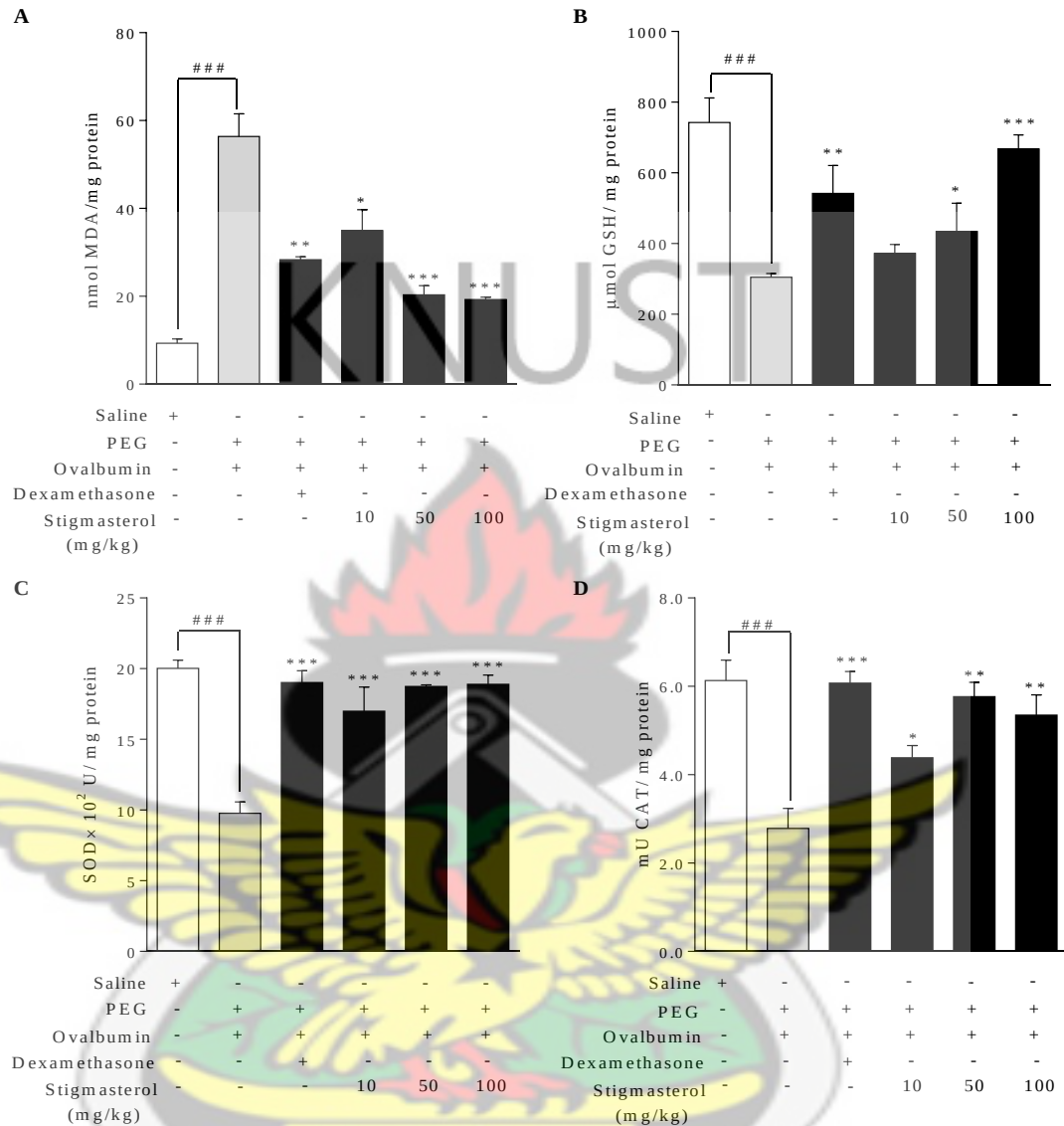


Fig. 3.4.4 Effect of stigmasterol on BALF oxidative stress markers. Guinea pigs (300 – 350 g) received either normal saline 5 ml/kg, polyethylene glycol (PEG) 5 ml/kg, dexamethasone 3 mg/kg or stigmasterol 10, 50 and 100 mg/kg 1 h prior to each ovalbumin challenge. Naïve controls received normal saline only. Bronchoalveolar fluid was collected by aspiration 24 h after the last ovalbumin challenge and centrifuged for 1000 rpm for 10 min. The supernatant was analyzed quantitatively for level of malondialdehyde (A), reduced glutathione (B), superoxide dismutase (C) and catalase (D). Data is expressed as mean concentration/mg protein $\pm$ SEM (n = 5) ***P<0.001, **P<0.01 and *P<0.05 as compared to PEG-treated group and ###P<0.001 as compared to naïve group using One-way ANOVA followed by Dunnet's *post hoc* test.

3.4.4.3 Histology

3.4.4.3.1 Effect of stigmasterol on inflammatory cell infiltration

Lung architecture in naïve animals was consistent with normal guinea pig lung structure. Alveolar spaces were clear with little or no accumulation of cells around the bronchioles (Fig. 3.4.5A). Ovalbumin challenge in the previously sensitized animals induced severe and extensive infiltration of lymphocytes, eosinophils and monocytes, forming thick cuffs of cells around bronchioles, blood vessels and alveolar septa in saline-treated OVA-sensitized and challenged group (Fig. 3.4.5B). Treatment with dexamethasone 3 mg/kg (Fig. 3.4.5C) as well as stigmasterol 10 - 100 mg/kg (Fig. 3.4.5D-F) reversed these features and presented less cellular congestion and alveolar septa thickening. These effects were quantified into a composite cell infiltration score by a method earlier described. A cell infiltration score of 9.95 ± 0.40 was recorded for saline-treated ovalbumin-sensitized and challenged group representing a significant increase when compared to 0.55 ± 0.16 for the naïve group (Fig. 3.4.5G). Compared to the untreated asthma group, dexamethasone exhibited a significantly reduced cell infiltration score of 3.75 ± 0.39 . Stigmasterol presented a significantly different dose-dependent reduction of ovalbumin-induced cell infiltration when compared to normal saline-treated ovalbumin-sensitized and challenged group, with scores of 5.86 ± 0.64 , 4.47 ± 0.40 and 3.43 ± 0.37 respectively at the doses of 10, 50 and 100 mg/kg (Fig. 3.4.5G).

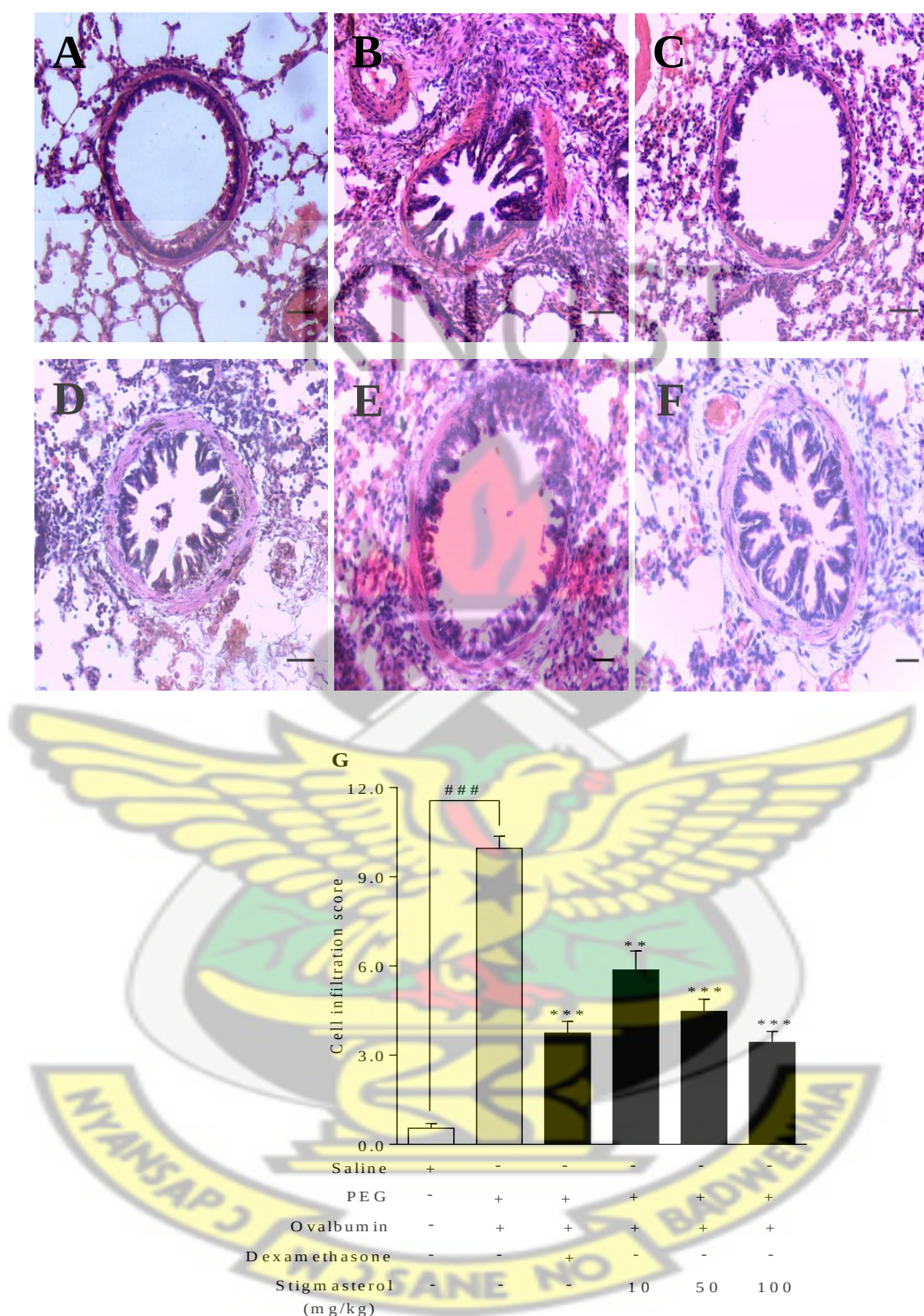


Fig. 3.4.5 Effect of stigmasterol on inflammatory cell infiltration. Guinea pigs (300 – 350 g) received either normal saline 5 ml/kg (A), polyethylene (PEG) 5 ml/kg (B), dexamethasone 3 mg/kg (C) or stigmasterol 10, 50 and 100 mg/kg (D – F) 1 h prior to each ovalbumin challenge. Naïve controls received normal saline only. H and E-stained lung sections (3 µm) were assessed

for degree of cell infiltration using a score described by Zare *et al.* (2008) with slight modifications. Data is expressed as mean cell infiltration score $\pm$ SEM (n = 5) (G) ***P<0.001 as compared to PEG-treated group and ###P<0.001 as compared to naïve group using One-way ANOVA followed by Dunnet's *post hoc* test. Micron bar represents 50 μ m.

3.4.4.3.2 Effect of stigmasterol on collagen deposition

Sub-epithelial collagen deposition (blue stain) was significantly pronounced in saline-treated OVA-sensitized and challenged group mostly in the perivascular and peribronchiolar regions (Fig. 5.6B), a feature absent in the lung tissue of the naïve animals (Fig. 3.4.6A). Sub-epithelial collagen deposition was significantly reduced in dexamethasone-treated guinea pigs (Fig. 3.4.6C). Similar reductions were noted with the stigmasterol-treated groups when compared to the saline-treated OVA-sensitized and challenged group (Fig. 3.4.6D-F). Morphometric analyses confirmed this observation. Quantitative measurements showed a collagen deposition index (stained area/per unit basement membrane length) of $1.02 \pm 0.16 \mu\text{m}^2/\mu\text{m}$ in saline-treated OVA-sensitized and challenged group relative to $0.01 \pm 0.01 \mu\text{m}^2/\mu\text{m}$ in the naïve control group (Fig. 3.4.6G). Dexamethasone was significantly effective in reducing the fibrotic region to $0.12 \pm 0.03 \mu\text{m}^2/\mu\text{m}$. Stigmasterol at the doses of 10, 50 and 100 mg/kg recorded indices of $0.33 \pm 0.05 \mu\text{m}^2/\mu\text{m}$, $0.36 \pm 0.05 \mu\text{m}^2/\mu\text{m}$ and $0.12 \pm 0.06 \mu\text{m}^2/\mu\text{m}$ respectively that were significant relative to the saline-treated OVA-sensitized and challenged group (Fig. 3.4.6G).

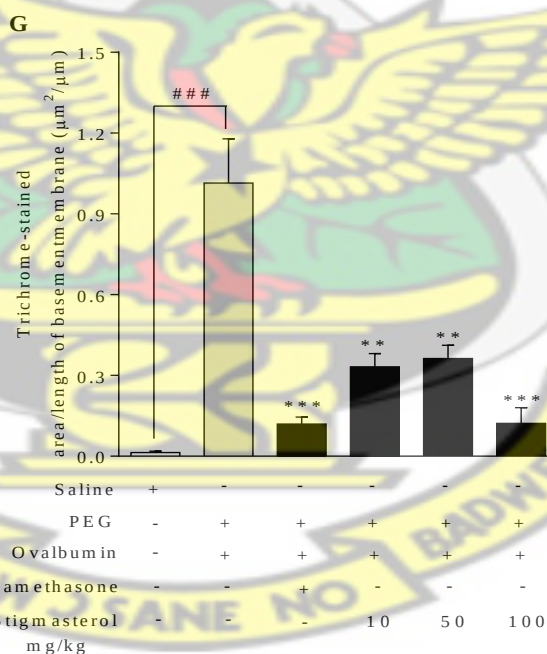
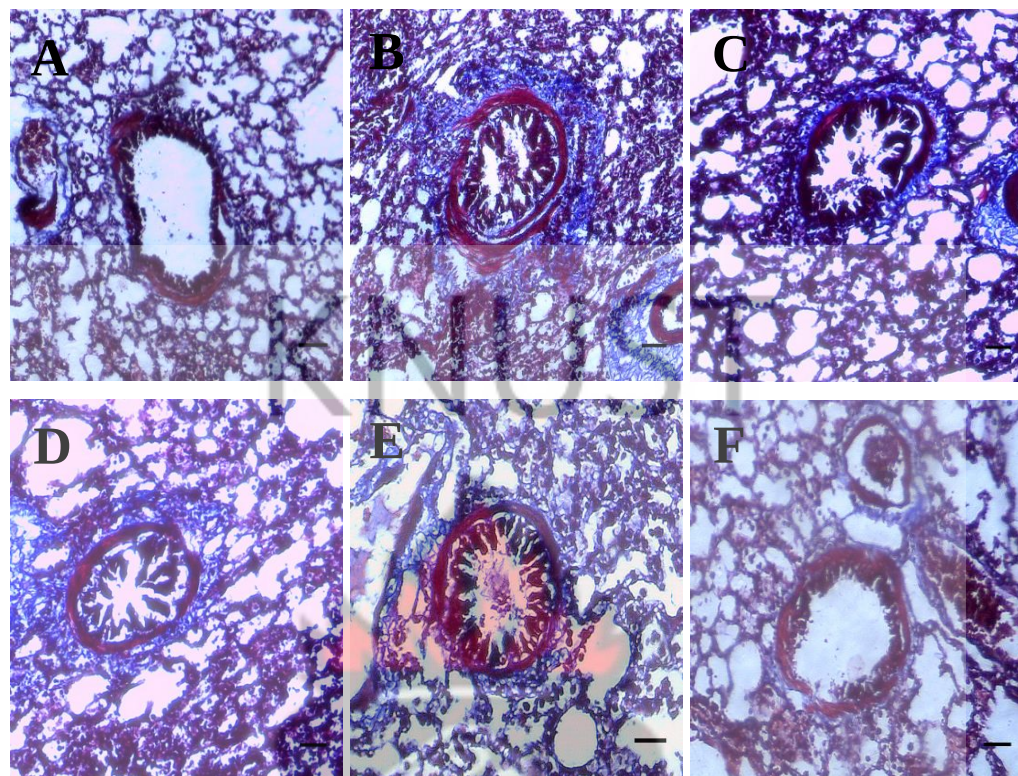


Fig. 3.4.6 Effect of stigmasterol on inflammatory collagen deposition. Guinea pigs (300 – 350 g) received either normal saline 5 ml/kg (A), polyethylene glycol (PEG) 5 ml/kg (B), dexamethasone 3 mg/kg (C) or stigmasterol 10, 50 and 100 mg/kg (D – F) 1 h prior to each ovalbumin challenge. Naïve controls received normal saline only. Lung sections (3 μ m) were

stained with Masson's trichrome solution to assess collagen deposition. Morphometric analysis was performed to quantify the extent of collagen deposition. Data is expressed as mean collagen deposition index $\pm$ SEM (n = 5) (G) ***P<0.001, **P<0.01 as compared to PEG-treated group and ###P<0.001 as compared to naïve group using One-way ANOVA followed by Dunnet's *post hoc* test. Micron bar 50 μ m.

3.4.5 DISCUSSION

This section explored the effects of stigmasterol on chronic airway inflammation induced by aerosolized ovalbumin and investigated its potential inhibitory effect on inflammatory features triggered by repeated challenge with ovalbumin in previously sensitized guinea pigs noting the possible mechanisms involved in the inhibition.

Cell infiltration into lung tissues and alveolar fluids, elevation of inflammatory cells in blood and changes to lung histology are features largely consistent with asthma (Pynn *et al.*, 2012). The ovalbumin-induced asthma model has been employed extensively and established to have characteristics that represent human asthma (Corry and Kheradmand, 2009). In this model, ovalbumin serves as the source of allergen. After sensitization and aerosol challenge, a Type 1 immune response is triggered. A Th2-skewed response which is characterized by Th2 cytokines such as IL-4, IL-5 and IL-13 tend to mediate production of ovalbumin specific IgE by B cells and recruitment of eosinophils, mast cells and other inflammatory cells. Pro-inflammatory cytokine participation mainly by IL-1, IL-6 and tumor necrosis factor alpha (TNF α) further intensify the inflammatory response (Wills-Karp, 2004). Consequently, there is a direct tissue injury and high cellular activity which invariably initiates production of reactive oxygen species (ROS) resulting in damage to macromolecular structures (Dworski, 2000). The extent of oxidative damage can be measured by levels of MDA, a product of lipid peroxidation and respective levels of the body's enzyme (SOD, catalase) and non-enzyme (GSH) antioxidant factors (Wood *et al.*, 2000; Ahmad *et al.*, 2012). This model is thus appreciably predictive in the investigation of potential anti-asthmatic agents.

In this study it was demonstrated that stigmasterol inhibited early phase immune responses to allergen exposure as shown by inhibition of IgE response. Analysis of serum collected from guinea pigs 24 h after the last ovalbumin exposure showed that the elevated levels of ovalbumin specific immunoglobulin E (OVA sIgE) in PEG-treated control group was significantly reduced in stigmasterol-treated guinea pigs. Elevated blood inflammatory cell count induced by OVA sensitization and challenge in the saline-treated animals was also suppressed by stigmasterol. Similar to dexamethasone, stigmasterol could significantly control eosinophil, lymphocyte and monocyte proliferation. In asthma, the proliferation of blood borne inflammatory cells and their subsequent migration into airway tissue drives epithelial tissue damage caused by chemical and inflammatory mediator release, leading to severe inflammation. Indeed, previous studies have established a direct link between Th2 cell control and a good asthma prognosis (Wynn, 2015).

Prior to tissue invasion, inflammatory cell movement and subsequent adherence to endothelial cells are mediated by several adhesion molecules. VCAM-1, identified as a major adhesion molecule in this process is shed from cytokine activated endothelial cells to promote subsequent leukocyte attachment (Hamzaoui *et al.*, 2001). Soluble or circulating forms of VCAM-1 were up-regulated in the saline-treated asthmatic control animals. Significantly reduced VCAM-1 levels in the stigmasterol-treated animals at 50 and 100 mg/kg were recorded, suggesting that stigmasterol may have an overall suppressive role on cell-mediated lung tissue damage and eventual remodeling.

Significant correlation between asthma severity and either systemic or airway oxidative stress has been established (Ahmad *et al.*, 2012). Cellular and tissue damage associated with asthma leads to massive production of reactive oxygen species (Zuo *et al.*, 2013). Reduction in β -adrenergic function in the lungs, increased sensitivity of airway smooth muscle, tracheal smooth muscle contraction and mucus production have been attributed to the role of ROS (Bowler, 2004). It is therefore not surprising that several studies have established a positive relationship between lung function and antioxidant intake. Levels of oxidative stress markers in breath condensates (Corradi *et al.*, 2003) and bronchoalveolar fluids (Schock *et al.*, 2003) have been used to predict asthma severity in both animal and clinical studies. From analysis of bronchoalveolar lavage fluid

(BALF), it was observed that treatment with stigmasterol inhibited oxidative stress evidenced in the preservation of the lung tissue antioxidant capacity. Markers such as reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) in BALF were significantly higher in the stigmasterol-treated animals compared to PEG-treated control animals. PEG-treated asthmatic control animals relative to the stigmasterol-treated animals presented with significantly higher levels of MDA, a product of lipid peroxidation and a positive indicator of oxidative stress. Results from antioxidant studies showed, that potentially damaging processes such as lipid peroxidation and superoxide anion-mediated free radical generation were abated. This apparent antioxidant effect of stigmasterol is consistent with some previous studies reporting antioxidant activity of stigmasterol in both *in vitro* and *in vivo* assays (Yoshida and Niki, 2003; Ghosh *et al.*, 2011).

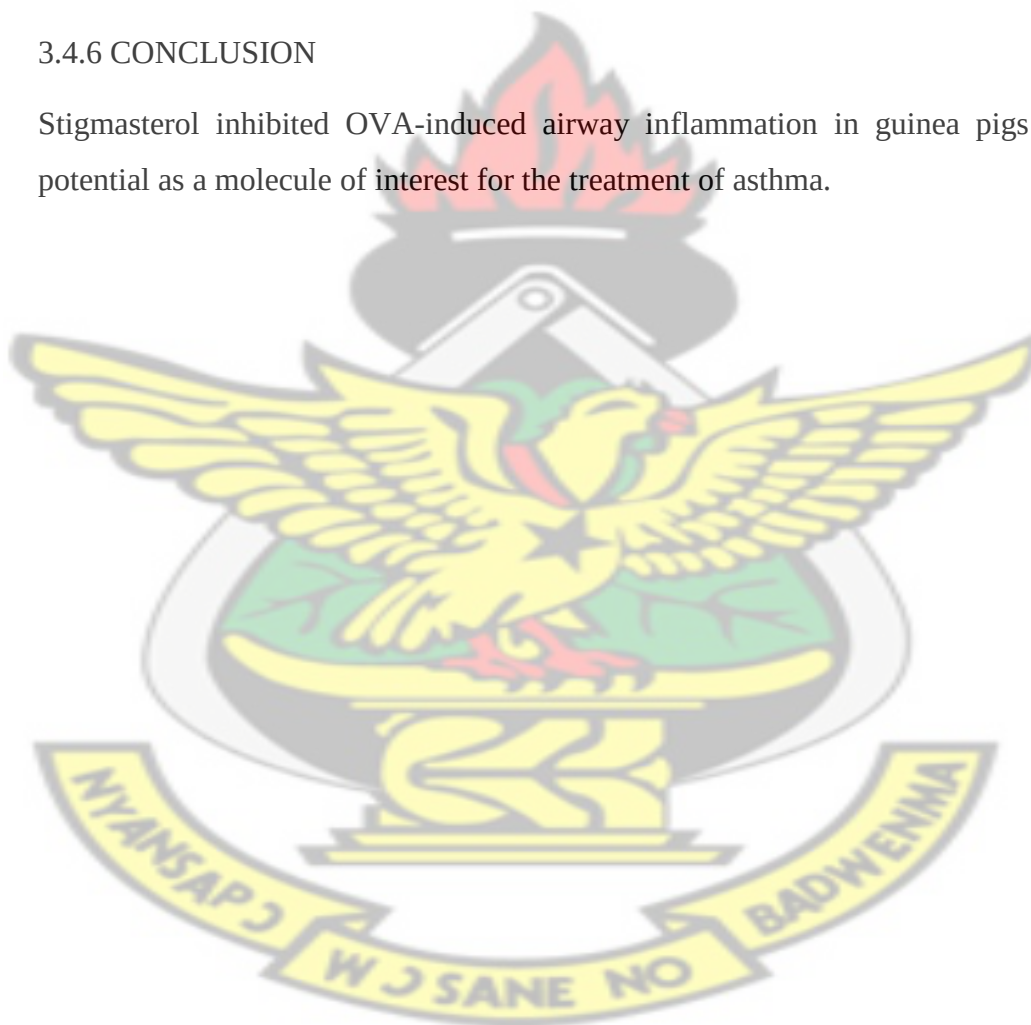
Hematoxylin and Eosin (H&E) staining revealed excessive infiltration of inflammatory cells, mostly eosinophils and lymphocytes in ovalbumin challenged groups compared to untreated naïve animals. Lung sections of saline-treated OVA-sensitized and challenged animals showed high cellularity especially in peribronchiolar and perivascular regions. Dexamethasone and stigmasterol lowered cell accumulation in the peribronchiolar, perivascular and alveolar regions, obtaining lower cell infiltration scores compared to the saline-treated OVA-sensitized and challenged asthmatic control guinea pigs. Stigmasterol treatment was associated with less congestion, sparsely distributed inflammatory cells in the alveolar region and reduced thickening of alveolar septa. Persistent uncontrolled airway inflammation and cell infiltration leads to a cycle of tissue damage and repair eventually causing permanent damage to lung tissue referred to as lung remodeling. It is associated with airway smooth muscle thickening, epithelial and goblet cell hyperplasia, basement membrane thickening and collagen deposition (Hirota and Martin, 2013). In this study, collagen deposition as an index for lung remodeling and tissue fibrosis was assessed using the Masson's trichrome stain. As expected, sections from the saline-treated OVA-sensitized and challenged asthmatic control animals showed extensive areas of collagen-positive staining especially around the bronchioles and blood vessel but significantly reduced in sections from stigmasterol-

treated animals. A collagen deposition index showed all three doses of stigmasterol effective in suppressing lung remodeling.

Taken together, the data demonstrates for the first time that stigmasterol suppresses airway inflammation and remodeling by inhibiting allergen-induced immunoglobulin E-mediated responses and also abolishes VCAM-1-aided cellular migration into lung tissues. Again, the results show that stigmasterol controls oxidative stress and preserves lung tissue anti-oxidant capacity and this mechanism is a key factor responsible for its anti-inflammatory action.

3.4.6 CONCLUSION

Stigmasterol inhibited OVA-induced airway inflammation in guinea pigs and has potential as a molecule of interest for the treatment of asthma.



3.5 IMMUNOTOXICITY EVALUATION OF STIGMASTEROL

3.5.1 INTRODUCTION

The immune system is a complex collection of physical and chemical barriers, a variety of circulating and resident immune cells and humoral factors. These are involved in general and specific defense mechanisms against host invasion by exogenous substances (Takahama, 2006; Modlin, 2012). The functions of this sophisticated system can however be affected by a range of biological, physical or chemical agents referred to as immunotoxicants (Luebke *et al.*, 2006). These include agents that induce abnormal immunologic responses such as hypersensitivity reactions or adverse drug reactions, suppress immune system functions or induce autoimmunity (Dietert, 2009). The term immunotoxicity therefore describes a permanent or reversible adverse change in the structure or function of the immune system. Immunotoxicity may therefore manifest as histopathological changes in lymphoid tissues, abnormalities in levels of cellular and humoral factors, distortions in 'self' and 'non-self' recognition by the immune system and disorders in normal innate and adaptive responses (Germolec *et al.*, 2017).

Immunosuppression is a common form of immunotoxicity. It is a state of impaired immune function often associated with marked reductions in circulating leukocytes, specific cellular and humoral mediated functions, depletion of lymphocyte populations resident in, or atrophy of, lymphoid organs (Delves and Roitt, 2000; Gaspar *et al.*, 2002). Susceptibility to infection, slow recovery from disease, tumorigenesis and low infant survival have all been linked to an impaired immune system function (Schacke *et al.*, 2002). Immunosuppressants can therefore be regarded as immunotoxicants. Immunosuppression may be due to primary causes such as genetic mutations (Lehman, 2015). It may also be secondary to infections (Douek *et al.*, 2002), antimicrobial use (Labro, 2005) or immunosuppressive drugs. In the clinical setting, immunosuppression may be a desired outcome in the prevention of graft rejection but may also be a side effect of treatment. Glucocorticoids, the most potent anti-inflammatory compounds, are associated with marked and often harmful effects on the immune system especially at high doses and with chronic use. Inhibition of gene transcription factors such as nuclear factor κ B (NF- κ B) and activator protein-1 (AP-1), subsequent inhibition of

proinflammatory cytokines and chemokines expression, disruption of inflammatory cell production and maturation, apoptosis of lymphocytes and atrophy of lymphoid organs are among documented effects (Bosscher *et al.*, 2002; Ashwell *et al.*, 2000; Dietert *et al.*, 2003; Löwenberg *et al.*, 2005). Glucocorticoids therefore present an interesting clinical dilemma since these effects are invariably linked to the mechanism of their anti-inflammatory actions. Therefore, while interaction between agents and immune system to achieve therapeutic goals is desired, potential harmful effects on the ‘health’ of the body’s defense system should be considered (Descotes, 2005). Immunotoxic assessment of potential immunomodulatory or immunoactive compounds is therefore critical.

Phytosterols have been reported to have anti-inflammatory and anticancer properties *via* modulation of aspects of the immune system (Trautwein and Demonty, 2007). Some earlier studies showed stigmasterol suppressed levels of proinflammatory cytokine expression both *in vivo* and *in vitro* with possible interaction with some transcription factors (Gabay *et al.*, 2010; Chen *et al.*, 2012). In the previous sections of this study, a wide range of anti-inflammatory and suppressive effects on immune responses have been observed. This suggests an obvious interaction between stigmasterol and key cellular and humoral components of the immune system.

In this section, the potential immunotoxic effect of stigmasterol, possibly arising from these interactions is assessed. The effects of stigmasterol on lymphoid organ histology and cell populations, levels of circulating leukocytes and T cell function are evaluated.

3.5.2 MATERIALS

3.5.2.1 Chemicals and reagents

Stigmasterol (95%), 1-chloro-2,4-dinitrochlorobenzene (DNCB), polyethylene glycol, PEG and dexamethasone were obtained from Sigma Aldrich (St. Louis, USA).

3.5.2.2 Animals

Wistar rats were obtained from Centre for Research into Plant Medicine (CRPM), Mampong-Akuapem, Ghana. Animals were kept at the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST Animal House facility

under standard temperature and humidity (temperature 25 ± 2 °C with a 12 h light-dark cycle). All animals had access to commercial chow and distilled water *ad libitum*. All protocols used in this study were approved by the Faculty of Pharmacy Ethics Committee and animal handling was done in compliance with Animal Welfare Regulations (USDA 1985; US Code, 42 USC § 289d) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002).

3.5.3 METHODS

3.5.3.1 Immunosuppression and immunotoxicity

Wistar rats (150 – 200 g) were randomized into seven (7) groups (n = 10) and treated daily for eight days intraperitoneally as follows;

Group I: vehicle control - polyethylene glycol, PEG (5 ml/kg)

Group II – IV: dexamethasone (1, 3 and 6 mg/kg) respectively

Group V – VII: stigmasterol (10, 50 and 100 mg/kg) respectively

Rats were randomly selected 24 h after the last treatment from each treatment group for the DNCB challenge test. Haematology and organ damage assessment was performed on the remaining surviving animals. The assessment of all parameters of immunotoxicity were performed as follows;

3.5.3.1.1 Body weight changes

The weight of each rat was recorded daily before feeding and treatment. Percentage increase in body weight was calculated using the following equation.

$$\% \text{ increase in body weight} = \left[\frac{W - W_0}{W_0} \right] \times 100$$

Where W is the daily weight of rat and W_0 is the weight of rat on day zero.

A time course curve was generated for percentage increases in body weight over the 8-day period and the total percentage increase was computed as the area under the curve (AUC).

3.5.3.1.2 Mortality

On the 8th day, record of all deaths were taken and percentage mortality for each treatment group calculated with the following equation:

$$\% \text{ Mortality} = \left[\frac{N_d}{N_0} \right] \times 100$$

Where N_d is the number of deaths recorded per each treatment group on day 8 and N_0 is the total number of animals in group on Day 0.

3.5.3.1.3 Effect on T cell-mediated immunity (DNCB challenge)

Five rats were randomly selected from each treatment group. The lower backs of rats were shaved and skin thickness measured with a digital vernier caliper. A thin layer of 0.2% w/v of 1-chloro-2,4-dinitrobenzene (DNCB) solution was applied on the lower backs of each rat and allowed to dry. Skin thickness of each rat was again assessed 24 h and 48 h after topical DNCB challenge. Percentage increase in skin thickness, a measure of the intensity of the T cell immune response, was calculated using the following equation:

$$\% \text{ increase in skin thickness} = \left[\frac{T_t - T_0}{T_0} \right] \times 100$$

Where T_t is the skin thickness either 24 h or 48 h after topical DCNB challenge and T_0 is the skin thickness before DNCB challenge.

3.5.3.1.4 White blood cell distribution

Rats were sacrificed 24 h after the last treatment by cervical dislocation. Following jugular vein dissection, blood was collected in EDTA tubes for total and differential white blood cell count with an automated analyzer (Sysmex KX-21N, Sysmex America Inc., Illinois, USA).

3.5.3.1.5 Thymus, spleen and adrenal glands weight assessment

Rats were sacrificed 24 h after the last treatment. The thymus gland, spleen and adrenal glands were harvested. Organs were trimmed of fatty tissue, dried by blotting on tissue paper and weighed. The relative organ weights were calculated using the formula:

$$\text{Relative organ weight} = \left[\frac{\text{Weight of organ}}{\text{Total body weight at sacrifice}} \right]$$

3.5.3.1.6 Spleen cellularity

Portions of harvested spleens were cut, weighed and grounded between two sterile glass slides. Fragments of fibrous materials were removed and ground spleen was suspended in 2 ml PBS. This suspension was forced through 20 gauge needles to form single cell suspensions which were kept on ice (5°C) and later analyzed for total WBC and lymphocyte count with an automated analyzer (Sysmex KX-21N, Sysmex America Inc., Illinois, USA). Total cellularity (either total WBC count or total lymphocyte count) was determined with the formula:

Total spleen cellularity

$$= \left[\frac{\text{Total weight of spleen}}{\text{Weight of spleen portion}} \right] \times \text{cell count for suspension}$$

Spleen cellularity index (spleen WBC or lymphocyte count per 0.5 g spleen tissue) was determined with the formula:

Spleen cellularity index

$$= \left[\frac{0.5 \text{ g}}{\text{Total weight of spleen}} \right] \times \text{Total spleen cellularity}$$

3.5.3.1.7 Histopathology

Spleen, thymus and adrenal glands tissue were collected from sacrificed rats and stored in 10% buffered formalin and embedded in paraffin. Three (3) µm sections were cut, deparaffinized, dehydrated and stained with haematoxylin and eosin (H & E) for

histopathological assessment. The tissue sections were observed under light microscope (Leica DM2500 M, Leica Microsystems, Wetzlar, Germany). Four random spleen sections from each treatment group were analyzed for area and distribution of white pulp regions relative to red pulp regions (cortex-medulla ratio). Four random thymus sections from each treatment group were assessed for reduced cellularity for both cortex and medulla regions, decreased cortex-medulla ratio, adipocyte infiltration and apoptosis and scored as 0- absent, 1- mild, 2- moderate, 3- severe. A total 12-point composite score was employed to assess thymus histopathology. Adrenal glands were scored for degree of fat infiltration, reduced cortex-medulla ratio, fat regeneration, and presence of apoptotic bodies. These features were scored individually as 0- absent, 1- mild, 2- moderate, 3- severe. Morphometric analyses were performed with Image J analysis tool (version 1.50i, National Institutes of Health, USA).

3.5.3.2 Statistical Analysis

Data is presented as mean $\pm$ standard error of mean (SEM). Data analysis was done using One-way analysis of variance (ANOVA). Multiple comparisons between treatment groups were performed using Dunnet's *post hoc* test. All statistical analyses were done using GraphPad for Windows version 6 (GraphPad Prism Software, San Diego, USA).

3.5.4 RESULTS

3.5.4.1 Effect of stigmasterol body weight

Daily body weight determinations of all rats over the 8-day period were presented as time course curves (Fig. 3.5.1A and C). The corresponding total percentage body weight gain within the same period was quantified as the area under the time course curve (AUC) (Fig. 3.5.1B and D). PEG-treated rats showed a gradual rise in body weight which continued till day 8 with a mean weight gain on day 8 of $19.87 \pm 1.58\%$ and a total weight gain (cumulative weight gain over 8-day study period) of 97.07 ± 9.47 (Fig. 3.5.1A and B). Stigmasterol at 10 mg/kg showed no significant effect on the pattern of mean weight gain and total weight gain compared to PEG-treated control group. Rats in 10 mg/kg stigmasterol group recorded a mean weight gain of $14.74 \pm 1.45\%$ and a

total weight gain of 65.51 ± 7.68 . 50 and 100 mg/kg stigmasterol recorded $14.42 \pm 1.13\%$ and $14.18 \pm 2.23\%$ mean weight gain taken on day 8 and total weight gain of 56.43 ± 9.94 and 58.29 ± 12.36 respectively (Fig. 3.5.1C and D). This represented significant decreases in the overall body weight gain compared to PEG-treated control rats. Dexamethasone treatment at all doses caused significant weight loss which began from day 0. Daily weight determinations showed a gradual but persistent loss in body weight which continued up to day 8 (Fig. 3.5.1A). Dexamethasone at 1, 3 and 6 mg/kg for recorded mean body weight losses of $9.38 \pm 2.62\%$, $14.13 \pm 1.22\%$ and $16.05 \pm 1.17\%$ respectively. The total body weight loss over the study period recorded for 1, 3 and 6 mg/kg dexamethasone was 42.48 ± 10.81 , 62.51 ± 4.17 and 46.74 ± 8.35 respectively (Fig. 3.5.1B).

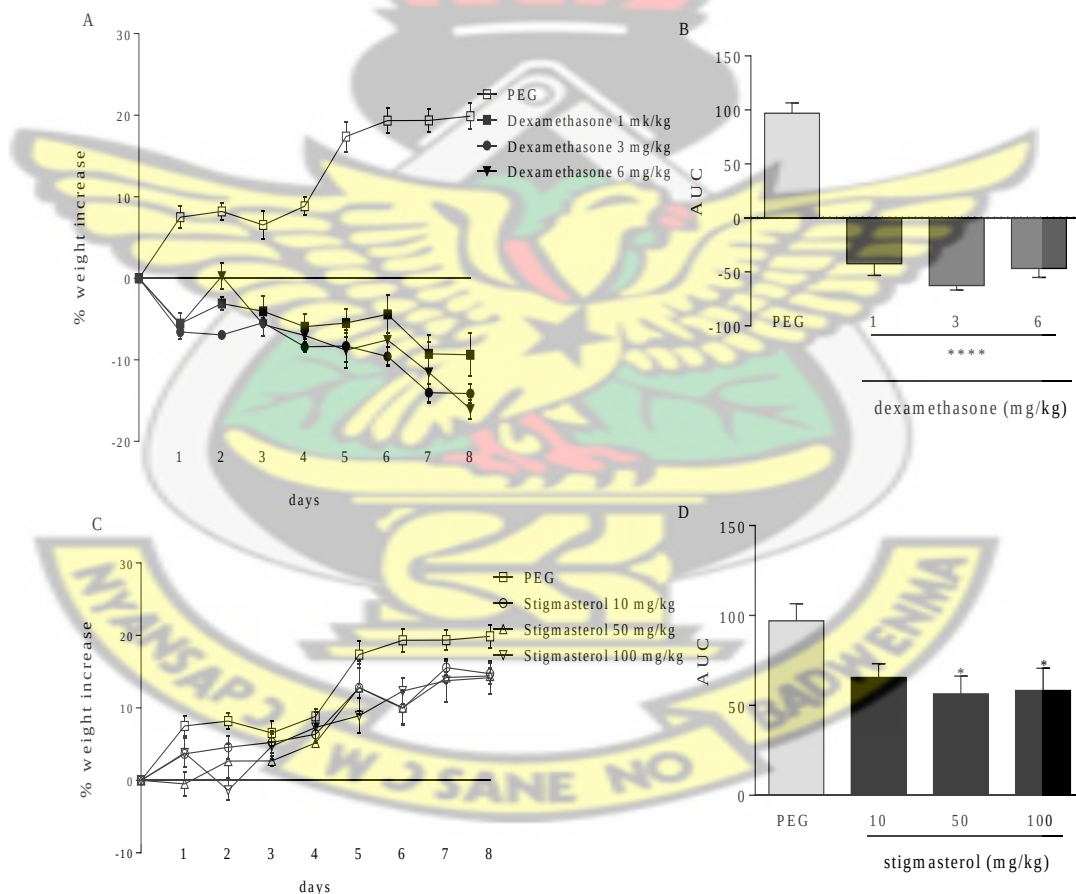


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Fig. 3.5.1 Effect of stigmasterol on body weight changes. Wistar rats (150 – 200 g) received either polyethylene glycol 5 ml/kg (PEG), dexamethasone (1 – 6 mg/kg) or stigmasterol (10 – 100 mg/kg). Weights of rats were taken daily for 8 days. Data is presented as time course curves of (A and C) and the total percentage increase in body weight is presented as mean area under the curve (AUC) $\pm$ S.E.M (n = 10) (B and D) ****P<0.0001 and *P<0.05 as compared to PEG-treated control group using One-way ANOVA followed by Dunnet's *post hoc* test.

3.5.4.2 Effect of stigmasterol on mortality

Consecutive, daily treatment with stigmasterol (10 – 100 mg/kg *i.p.*) for 8 days yielded no mortality. No visible signs of toxicity was observed compared to PEG-treated control rats. A similar treatment with dexamethasone 3 mg/kg and 6 mg/kg recorded 20% and 30% mortality respectively. All deaths recorded for both doses occurred on the 8th day of drug administration. Dexamethasone at 1 mg/kg however recorded no deaths (Table 3.5.1).

Table 3.5.1: Effect of stigmasterol on mortality of Wistar rats.

Treatment (mg/kg) (<i>i.p.</i>)	No. Per Group	No. of Deaths	% Mortality (After 8 days)
Polyethylene glycol (50% w/v)	5 10	0	0
Dexamethasone	1 10	0	0
	3 10	2	20
	6 10	3	30
Stigmasterol	10 10	0	0
	50 10	0	0
	100 10	0	0

Wistar rats (150 – 200 g) were randomly placed in the following treatment groups; polyethylene glycol (50% w/v), dexamethasone (1 – 6 mg/kg) and stigmasterol (10 – 100 mg/kg). Treatments

were done daily for 8 days and rats were observed for mortality for the duration of the experiment. Number of deaths were recorded and percentage mortality for each treatment group was calculated.

3.5.4.3 Effect of stigmasterol on T cell-mediated immune response

Topical application of DNCB induced a delayed type, T-cell mediated response observed as skin thickening which developed at 24 h and persisted till 48 h after challenge. A $393.30 \pm 27.32\%$ increase in skin thickness was recorded in PEG-treated control rats (Fig. 3.5.2A). Stigmasterol treatment at all three doses showed no significant inhibition of T-cell response. Stigmasterol 10, 50 and 100 mg/kg-treated rats recorded increases in skin thickness of $402.41 \pm 33.11\%$, $468.70 \pm 13.13\%$, $404.63 \pm 32.63\%$ at 24 h (Fig. 3.5.2A). This represented no significant difference compared to responses recorded in PEG-treated control rats. Dexamethasone however showed marked suppression of DNCB-induced, delayed type hypersensitivity response compared to PEG-treated control group. Rats treated with dexamethasone at 1, 3 and 6 mg/kg recorded reduced increases in skin thickness responses of $112.21 \pm 23.49\%$, $96.04 \pm 47.33\%$, $57.81 \pm 29.86\%$ 24 h post-challenge (Fig. 3.5.2A). The suppression offered by 1, 3 and 6 mg/kg after 24 h represents $71.48 \pm 5.97\%$, $75.58 \pm 12.03\%$, $85.30 \pm 7.59\%$ compared to PEG-treated control group (Fig. 3.5.2A).

A similar trend was observed for skin thickness recorded at 48 h. PEG-treated control group recorded a mean skin thickness of $361.20 \pm 10.28\%$ 48 h after challenge (Fig. 3.5.2B). No significant inhibition of skin thickness was recorded in stigmasterol-treated rats at all doses. Stigmasterol 10, 50 and 100 mg/kg-treated rats recorded increases in skin thickness of $369.21 \pm 29.16\%$, $370.13 \pm 33.49\%$, $345.72 \pm 39.04\%$ at 48 h (Fig. 3.5.2B). Dexamethasone 1, 3 and 6 mg/kg-treated rats recorded mean increases in skin thickness of $108.00 \pm 17.14\%$, $109.32 \pm 35.74\%$, $57.03 \pm 23.23\%$ representing $70.11 \pm 4.75\%$, $69.74 \pm 9.89\%$ and $84.21 \pm 6.43\%$ inhibition of skin response respectively.

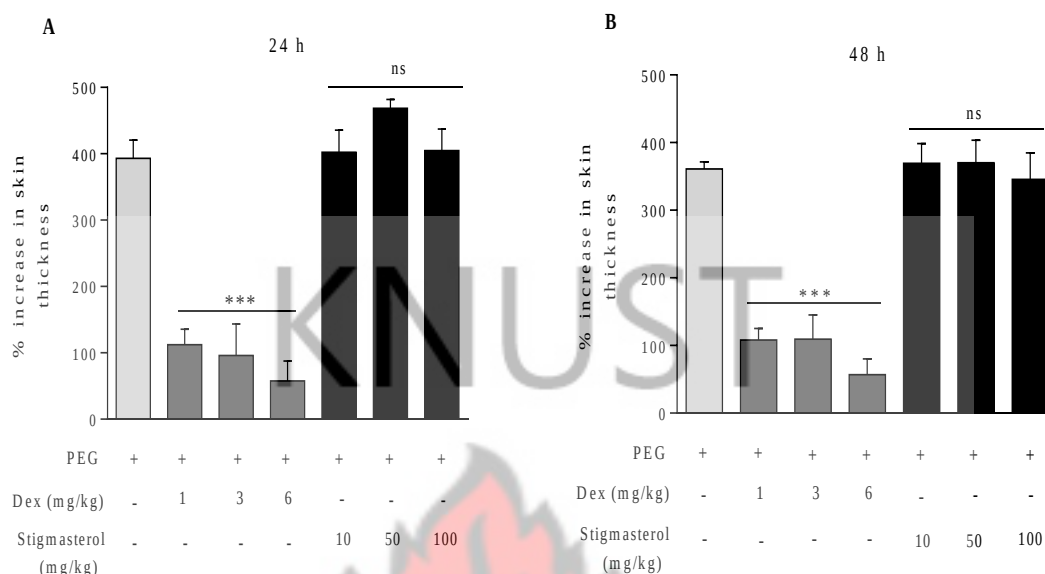


Fig. 3.5.2 Effect of stigmasterol on DNCB-induced, T-cell mediated response. Wistar rats (150 – 200 g) received intraperitoneal treatment of either polyethylene glycol (PEG), dexamethasone (1 – 6 mg/kg) or stigmasterol (10 – 100 mg/kg) daily for 8 days. 24 h after the last treatment, each rat received a topical challenge with 0.2% w/v of 1-chloro-2,4-dinitrochlorobenzene at their lower backs. Skin thickness was recorded at 24 h and 48 h after challenge. Data is represented as mean percentage increase in skin thickness after 24 h (A) and 48 h (B) $\pm$ S.E.M ***P < 0.001 and ns (not significant) as compared to PEG-treated control using One-way ANOVA followed by Dunnet's *post hoc* test.

3.5.4.4 Effect of stigmasterol on WBC distribution

A total white blood cell count of $9.78 \pm 0.95 \times 10^3$ cells/ μ L was recorded in PEG-treated control rats (Fig. 3.5.3A). Marked decreases in total white cell counts were however observed in dexamethasone-treated rats. Dexamethasone 1, 3 and 6 mg/kg treatments recorded white blood cell counts of $6.15 \pm 0.38 \times 10^3$ cells/ μ L, $5.42 \pm 0.47 \times 10^3$ cells/ μ L and $5.54 \pm 0.24 \times 10^3$ cells/ μ L respectively, and this was quantified as $37.13 \pm 3.85\%$, $44.63 \pm 4.76\%$ and $43.40 \pm 2.45\%$ inhibition of total WBC counts as compared to PEG-treated control group (Fig. 3.5.3A). A mean total count of $8.40 \pm 0.99 \times 10^3$ cells/ μ L, $8.94 \pm 0.62 \times 10^3$ cells/ μ L and $8.53 \pm 0.43 \times 10^3$ cells/ μ L was also determined in stigmasterol 10, 50 and 100 mg/kg treated rats respectively (Fig. 3.5.3A). This

represented no significant change compared to levels determined in PEG-treated control rats.

A similar trend was observed in differential white blood cell counts for lymphocytes, eosinophils and basophils. The mean lymphocyte count in PEG-treated control rats was $6.95 \pm 1.15 \times 10^3$ cells/ μ L. Significantly low counts of $3.43 \pm 0.37 \times 10^3$ cells/ μ L, $2.76 \pm 0.38 \times 10^3$ cells/ μ L, $1.80 \pm 0.28 \times 10^3$ cells/ μ L representing $50.60 \pm 5.26\%$, $60.28 \pm 5.49\%$ and $74.05 \pm 4.05\%$ reductions compared to PEG-treated group, were determined for dexamethasone 1, 3 and 6 mg/kg respectively (Fig. 3.5.3B). Stigmasterol 10, 50 and 100 mg/kg-treated rats recorded mean lymphocyte counts of $6.05 \pm 1.17 \times 10^3$ cells/ μ L, $6.38 \pm 0.53 \times 10^3$ cells/ μ L, $5.96 \pm 0.58 \times 10^3$ cells/ μ L respectively (Fig. 3.5.3B). No statistical difference was determined compared to PEG-treated control rats.

A mean eosinophil count of $0.16 \pm 0.02 \times 10^3$ cells/ μ L was determined in PEG-treated control rats. Significant reductions in eosinophil levels was observed in dexamethasone-treated rats. Eosinophil counts determined in dexamethasone 1, 3 and 6 mg/kg-treated rats were $0.03 \pm 0.01 \times 10^3$ cells/ μ L, $0.02 \pm 0.01 \times 10^3$ cells/ μ L and $0.04 \pm 0.01 \times 10^3$ cells/ μ L representing $80.31 \pm 5.26\%$, $85.95 \pm 5.43\%$, $73.64 \pm 4.10\%$ inhibition compared to PEG-treated group counts (Fig. 3.5.3C). Treatment with stigmasterol 10, 50 and 100 mg/kg recorded $0.18 \pm 0.03 \times 10^3$ cells/ μ L, $0.16 \pm 0.03 \times 10^3$ cells/ μ L and $0.17 \pm 0.02 \times 10^3$ cells/ μ L representing no significant inhibition compared to eosinophil count in PEG-treated control rats (Fig. 3.5.3C).

A mean basophil count of $0.12 \pm 0.02 \times 10^3$ cells/ μ L was recorded in PEG-treated control group (Fig. 3.5.3D). Dexamethasone 6 mg/kg treatment recorded a reduced count of $0.04 \pm 0.01 \times 10^3$ cells/ μ L representing an inhibition of $62.3 \pm 27.67\%$ compared to PEG-treated rats. Dexamethasone at 1 and 3 mg/kg recorded basophil counts of $0.09 \pm 0.01 \times 10^3$ cells/ μ L and $0.09 \pm 0.02 \times 10^3$ cells/ μ L (Fig. 3.5.3D). These reductions were however statistically insignificant. Treatment with stigmasterol 10, 50 and 100 mg/kg recorded $0.11 \pm 0.01 \times 10^3$ cells/ μ L, $0.09 \pm 0.01 \times 10^3$ cells/ μ L and $0.11 \pm 0.01 \times 10^3$ cells/ μ L. No statistical difference was determined compared to PEG-treated control rats.

Both stigmasterol and dexamethasone treatments did not show any significant inhibition in monocyte and neutrophil counts. Counts of $0.06 \pm 0.01 \times 10^3$ cells/ μ L and $2.53 \pm 0.44 \times 10^3$ cells/ μ L levels in monocytes (Fig. 3.5.3E) and neutrophils (Fig. 3.5.3F) respectively were determined in PEG-treated control rats. Treatment with dexamethasone 1, 3 and 6 mg/kg recorded monocyte counts of $0.05 \pm 0.01 \times 10^3$ cells/ μ L, $0.06 \pm 0.01 \times 10^3$ cells/ μ L and $0.05 \pm 0.01 \times 10^3$ cells/ μ L (Fig. 3.5.3E) and neutrophil counts of $2.27 \pm 0.22 \times 10^3$ cells/ μ L, $2.46 \pm 0.13 \times 10^3$ cells/ μ L and $3.50 \pm 0.39 \times 10^3$ cells/ μ L (Fig. 3.5.3F). Following stigmasterol 10, 50 and 100 mg/kg treatments, cell counts showed $0.06 \pm 0.01 \times 10^3$ cells/ μ L, $0.07 \pm 0.01 \times 10^3$ cells/ μ L and $0.06 \pm 0.01 \times 10^3$ cells/ μ L in monocyte levels (Fig. 3.5.3E) and $1.93 \pm 0.44 \times 10^3$ cells/ μ L, $2.23 \pm 0.14 \times 10^3$ cells/ μ L, $2.40 \pm 0.42 \times 10^3$ cells/ μ L levels in neutrophils (Fig. 3.5.3F) respectively.



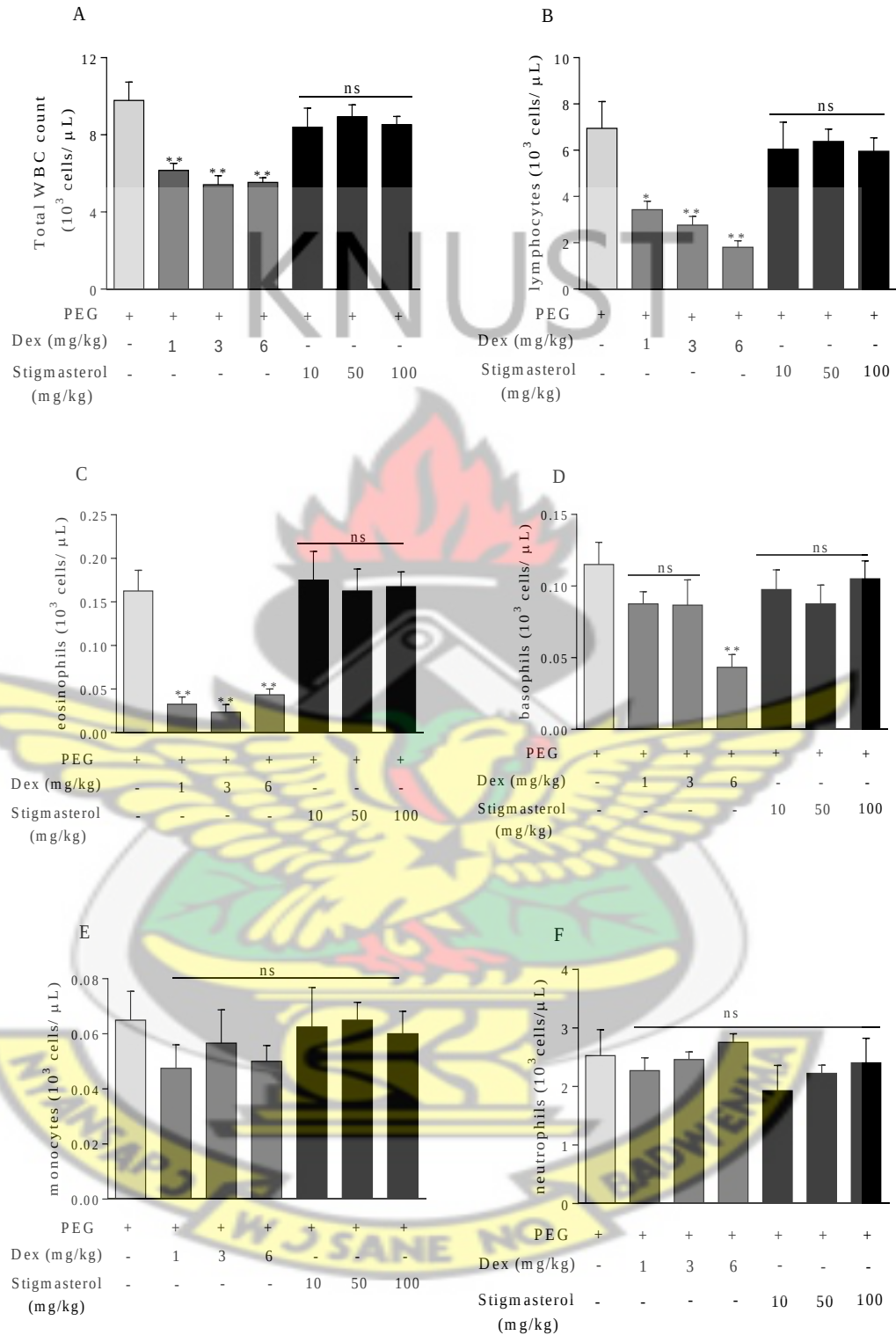


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Fig. 3.5.3 Effect of stigmasterol on total and differential WBC count. Wistar rats (150 – 200 g) received intraperitoneal treatment of either polyethylene glycol, 5 ml/kg (PEG), dexamethasone (1 – 6 mg/kg) or stigmasterol (10 – 100 mg/kg) daily for 8 days. 24 h after the last treatment blood was collected by dissection of the jugular vein for total and differential white blood cell count. Data is represented as mean total WBC count (A), lymphocyte (B), eosinophils (C), basophils (D), monocytes (E) and neutrophils (F) $\pm$ S.E.M ***P <0.01 and ns (not significant) as compared to PEG-treated control using One-way ANOVA followed by Dunnet's *post hoc* test.

3.5.4.5 Effect of stigmasterol on thymus, spleen and adrenal gland weights

A mean thymus weight index of 0.16 ± 0.02 was recorded for PEG-treated control rats. Dexamethasone at 1, 3 and 6 mg/kg treatment reduced the thymus weight index to 0.08 ± 0.01 , 0.08 ± 0.01 and 0.05 ± 0.01 (Fig. 3.5.4A) representing $49.94 \pm 3.67\%$, $53.11 \pm 3.03\%$ and $68.69 \pm 6.39\%$ inhibition of thymus weights compared to PEG-treated group. Treatment with 10, 50 and 100 mg/kg of stigmasterol recorded thymus weight indices of 0.13 ± 0.01 , 0.16 ± 0.02 , 0.16 ± 0.01 (Fig. 3.5.4A). This represented no significant reduction in thymus weights compared to PEG-treated control rats.

PEG-treated control rats recorded a mean spleen weight index of 0.50 ± 0.05 (Fig. 3.5.4B). Dexamethasone at 1, 3 and 6 mg/kg treatment significantly inhibited spleen weight index by $37.55 \pm 5.38\%$ (0.32 ± 0.03), $39.38 \pm 6.43\%$ (0.31 ± 0.03) and $67.31 \pm 6.50\%$ (0.17 ± 0.03) (Fig. 3.5.4B). Treatment with 10, 50 and 100 mg/kg of stigmasterol recorded thymus weight indices of 0.50 ± 0.05 , 0.46 ± 0.03 , 0.52 ± 0.02 (Fig. 3.5.4B). This represented no significant reduction in thymus weights compared to PEG-treated control rats.

A mean adrenal gland weight (both left and right adrenal glands) index of 0.041 ± 0.004 was recorded for PEG-treated control rats (Fig. 3.5.4C). Treatment with 1 mg/kg dexamethasone recorded no significant reduction in adrenal gland weight index (0.038 ± 0.001). Treatment with 3 and 6 mg/kg of dexamethasone however recorded significant reductions in adrenal gland weight, recording indices of 0.027 ± 0.002 and $0.021 \pm$

0.003 which represented $35.06 \pm 3.79\%$ and $49.40 \pm 7.36\%$ reductions respectively (Fig. 3.5.4C).

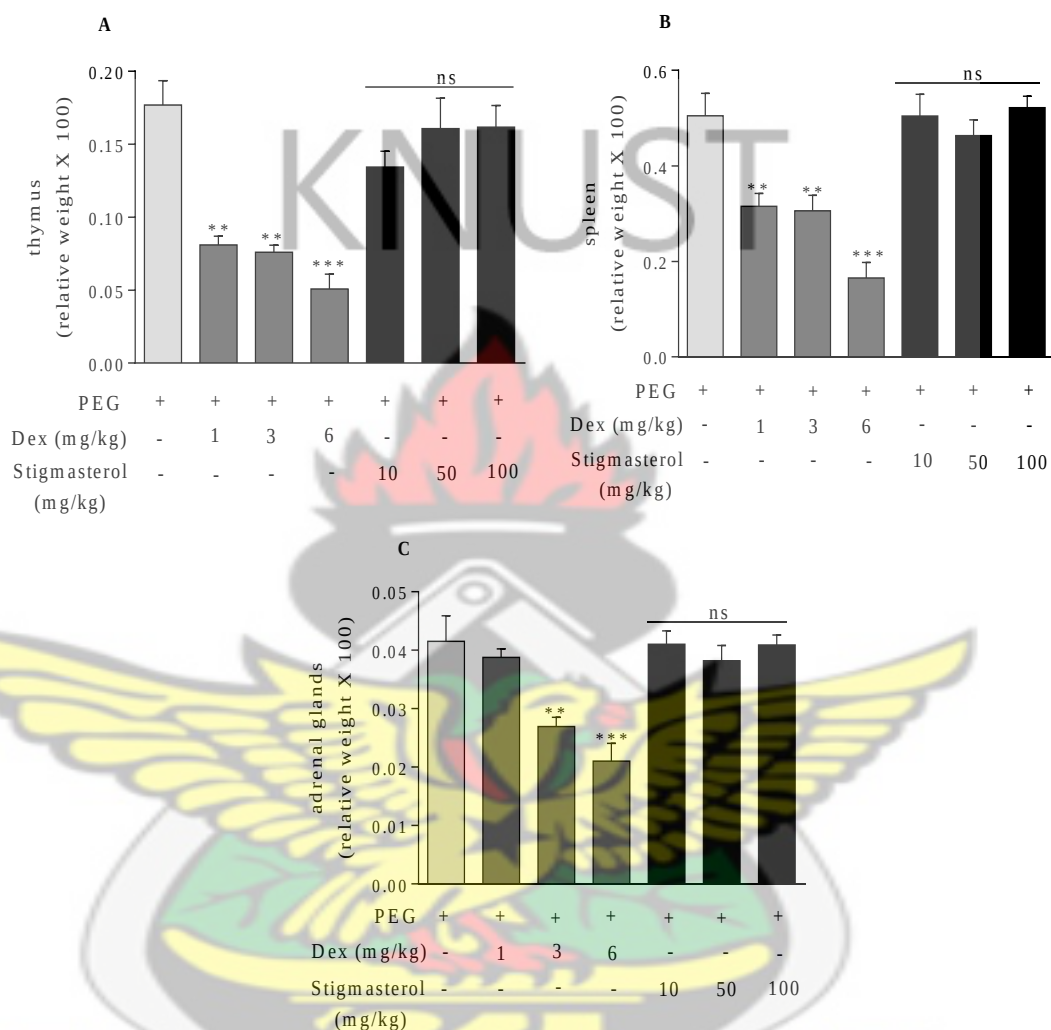


Fig. 3.5.4 Effect of stigmasterol on thymus, spleen and adrenal gland weights. Wistar rats (150 – 200 g) received intraperitoneal treatment of either polyethylene glycol, 5 ml/kg (PEG), dexamethasone (1 – 6 mg/kg) or stigmasterol (10 – 100 mg/kg) daily for 8 days. Twenty four (24) hours after the last treatment, rats were sacrificed by cervical dislocation and the thymus, spleen and adrenal glands harvested. Organs were trimmed of fatty tissue, blotted dry and weighed. Data is expressed as mean relative weight $\times 100$ of thymus gland (A), spleen (B), adrenal glands (C) $\pm$ SEM ***P < 0.001, **P < 0.01 and ns (not significant) as compared to PEG-treated control using One-way ANOVA followed by Dunnet's *post hoc* test.

3.5.4.6 Effect of stigmasterol on spleen cellularity

Leukocyte count of spleen cell suspensions showed a white blood cell count and lymphocyte count of $49.13 \pm 3.81 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen (Fig. 3.5.5A) and $39.53 \pm 3.17 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen (Fig. 3.5.5B) respectively in PEG-treated control rats. Dexamethasone at all three doses showed a marked inhibition of the cellularity of spleen tissues. Significant reductions in both total white blood cell counts and lymphocyte counts were recorded following dexamethasone treatment. Dexamethasone 1, 3 and 6 mg/kg reduced total WBC counts to $25.76 \pm 3.15 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen, $20.41 \pm 2.88 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen and $16.01 \pm 3.33 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen corresponding to $47.58 \pm 6.41\%$, $58.46 \pm 5.87\%$ and $67.41 \pm 6.78\%$ inhibition respectively (Fig. 3.5.5A). Again reduced lymphocyte counts of $7.35 \pm 1.08 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen, $5.10 \pm 0.57 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen and $7.19 \pm 1.82 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen representing $81.42 \pm 2.73\%$, $87.09 \pm 1.45\%$ and $81.80 \pm 4.60\%$ inhibition compared to PEG-treated control group were recorded for 1, 3 and 6 mg/kg of dexamethasone respectively (Fig. 3.5.5B). Treatment of rats with stigmasterol 10, 50 and 100 mg/kg recorded white blood cell counts of $50.76 \pm 4.65 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen, $46.82 \pm 2.70 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen and $43.94 \pm 5.52 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen (Fig. 3.5.5A) and lymphocyte counts of $7.35 \pm 1.08 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen, $5.10 \pm 0.57 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen and $7.19 \pm 1.82 \times 10^3 \mu\text{L}^{-1}$ / 0.5 g spleen (Fig. 3.5.5B) respectively. These represented no significant inhibition of spleen total white blood cell and lymphocyte counts compared to PEG-treated control rats.

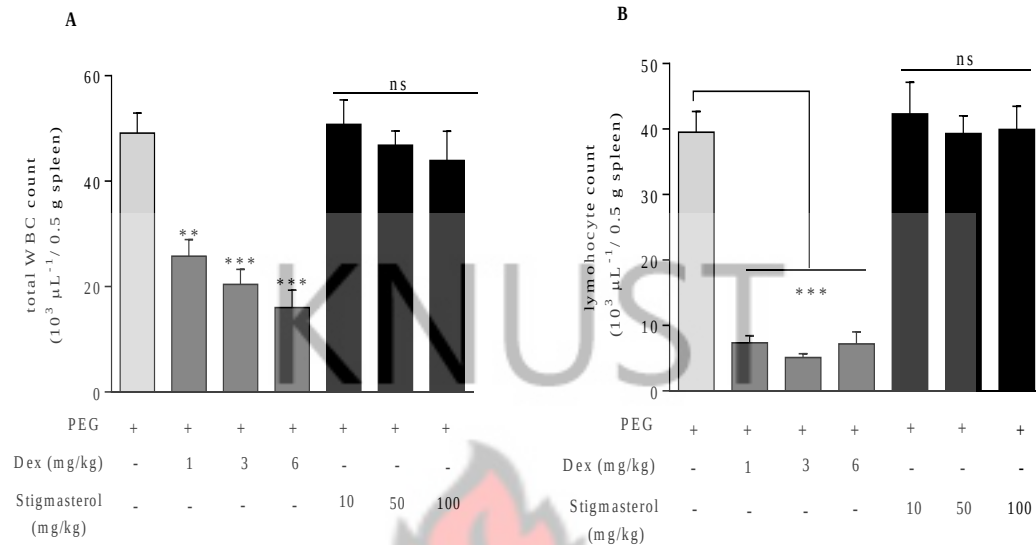


Fig. 3.5.5 Effect of stigmasterol on spleen cellularity. Wistar rats (150 – 200 g) received intraperitoneal treatment of either polyethylene glycol, 5 ml/kg (PEG), dexamethasone (1 – 6 mg/kg) or stigmasterol (10 – 100 mg/kg) daily for 8 days. Twenty four (24) hours after the last treatment, rats were sacrificed by cervical dislocation. Spleens were harvested and single cell suspensions were prepared from weighed portions of spleen. Spleen cell suspensions were analyzed for total WBC and lymphocyte counts. Data is represented as mean total WBC counts $\times 10^3$ cells/ μ L/ 0.5 g spleen (A) and mean lymphocyte $\times 10^3$ cells/ μ L/ 0.5 g spleen (B) $\pm$ SEM ***P < 0.001, **P < 0.01 and ns (not significant) as compared to PEG-treated control using One-way ANOVA followed by Dunnet's *post hoc* test.

3.5.4.7 Histopathology

3.5.4.7.1 Effect of stigmasterol on spleen

Spleen sections of PEG-treated control rats showed features consistent with normal spleen architecture. Both white and red pulp regions were distinct with no observable change in cellularity. PALS, follicular and marginal zones of the white pulp region were distinguishable (Fig. 3.5.6A). These features were consistent with stigmasterol-treated rats. Spleen sections showed no significant changes in spleen architecture (Fig. 3.5.6D–F). White pulp regions were however, markedly reduced or indistinguishable from red pulp regions in dexamethasone-treated rats. (Fig. 3.5.6 B and C). The PALS, follicular

and marginal zones of the white pulp were diffuse with significant reduction in cellularity. Incidents of reduced cellularity of both red and white pulp regions and presence of apoptotic cells were observed especially in the 3 and 6 mg/kg dexamethasone-treated rats (Fig. 3.5.6 B and C). The cortex-medulla ratio was determined as a measure of the size and distribution of the lymphocyte-populated white pulp regions (Fig. 3.5.6G). Spleen sections of PEG-treated rats recorded a cortex-medulla ratio of 0.31 ± 0.02 . This was significantly increased in dexamethasone-treated rats. Dexamethasone at 1, 3 and 6 mg/kg treatment recorded cortex-medulla ratios of 1.82 ± 0.33 , 2.74 ± 0.50 , 3.27 ± 0.56 representing $81.90 \pm 3.03\%$, $87.88 \pm 2.29\%$ and $90.03 \pm 1.46\%$ increases respectively. Stigmasterol 10, 50 and 100 mg/kg-treated rats recorded spleen cortex-medulla ratios of 0.41 ± 0.06 , 0.35 ± 0.06 and 0.29 ± 0.09 . These were considered statistically insignificant compared with PEG-treated control rats (Fig 3.5.6G).



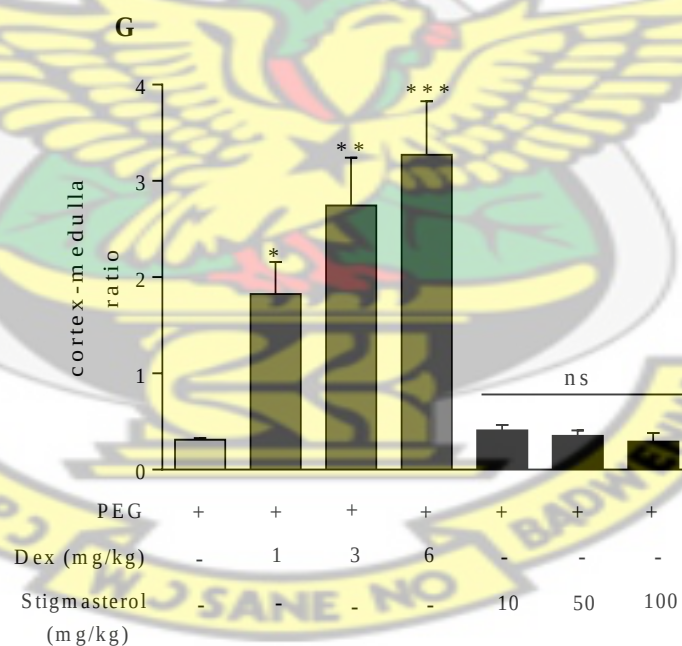
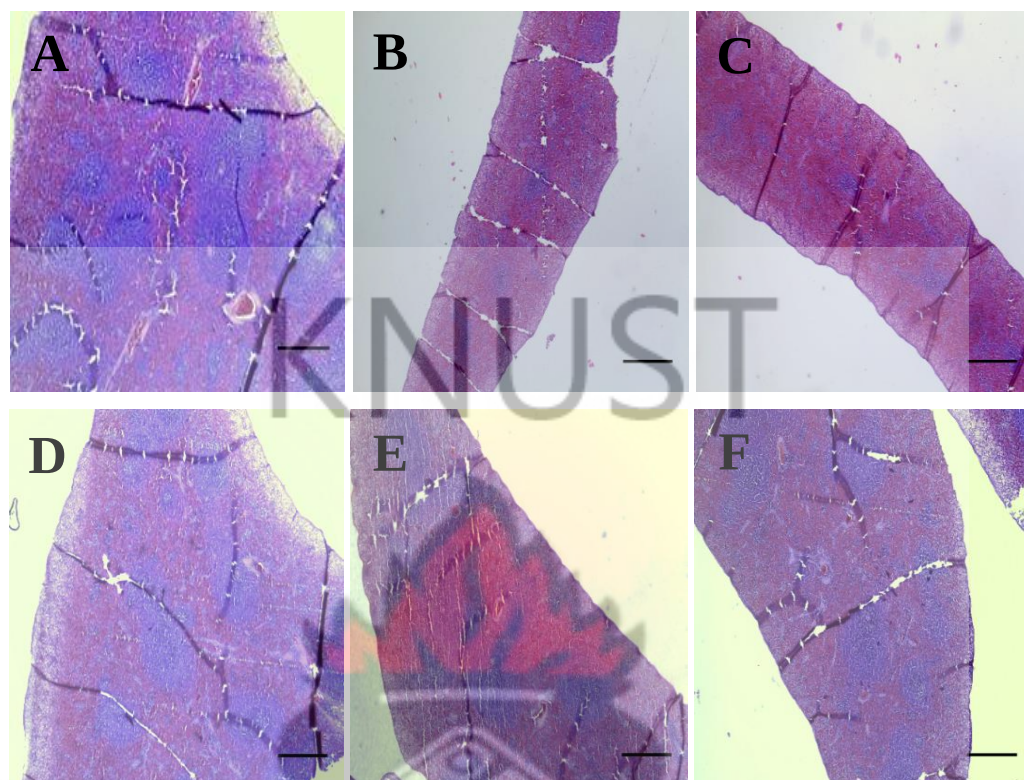


Fig. 3.5.6 Effect of stigmasterol on spleen histopathology. Wistar rats (150 – 200 g) received either polyethylene glycol 5 ml/kg (PEG), dexamethasone (1 – 6 mg/kg) or stigmasterol (10 – 100 mg/kg) daily for 8 days. 24 h after the last treatment, rats were sacrificed by cervical dislocation. 3 μ m sections of harvested spleens were stained with H & E stain for histological

examination. Micrographs represent spleen sections from PEG (A), dexamethasone 3 and 6 mg/kg (B and C) and stigmasterol (10 – 100 mg/kg) (D – F). Cortex-medulla ratios of spleen sections were determined with morphometric analysis (G). Data is represented as mean cortex-medulla ratio $\pm$ SEM ***P <0.001, **P <0.01, *P <0.05 and ns (not significant) as compared to PEG-treated control using One-way ANOVA followed by Dunnet's *post hoc* test. Micron bar represents 1 mm.

3.5.4.7.2 Effect of stigmasterol on thymus

Thymus sections from PEG-treated control rats showed normal lymphocyte density and distribution for both the dark staining cortex and paler staining medulla regions. Both regions were clearly distinct with no signs of reduced cellularity or apoptotic bodies (Fig. 3.5.7A). Significantly atrophied thymus, with reduced cortex and widespread incidents of apoptotic cortical lymphocytes were observed in dexamethasone-treated rats at all doses. Severe changes in thymus architecture were observed in 3 and 6 mg/kg dexamethasone-treated rats. Cortical regions were either completely lost or indistinguishable from medulla regions. Lymphocyte densities were dramatically reduced. Widened interlobular septa, areas of regeneration and infiltration of adipocytes were apparent (Fig. 3.5.7 B and C). These features were however absent in stigmasterol-treated rats. Minimal adipocyte infiltrations were however observed in some sections. The cortex and light staining medulla regions were distinguishable, with no significant reduction in the cellularity of both regions (Fig. 3.5.7 D – F). When quantified, dexamethasone at 1, 3 and 6 mg/kg recorded an overall histopathology score of 6.33 ± 0.33 , 9.67 ± 1.45 , and 10.00 ± 1.20 representing significant increases above the mean histopathology score of 1.67 ± 0.33 for PEG-treated rats. The thymus histopathology scores of 3.33 ± 0.88 , 2.67 ± 0.33 and 2.67 ± 0.67 determined for stigmasterol 10, 50 and 100 mg/kg-treated rats respectively, were not statistically different from the mean score for PEG-treated control rats (Fig. 3.5.7G).

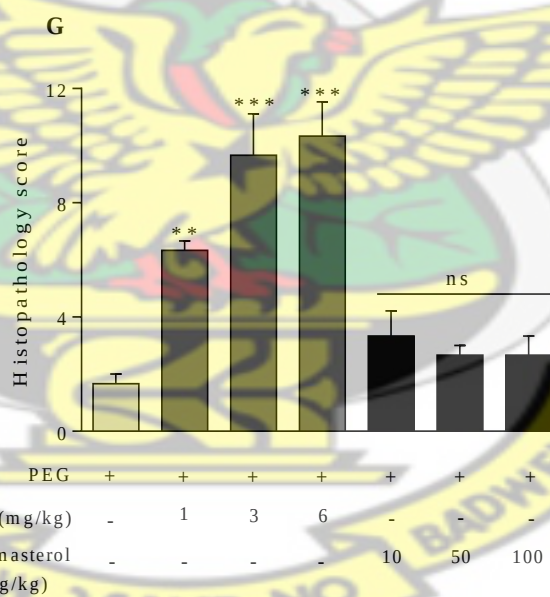
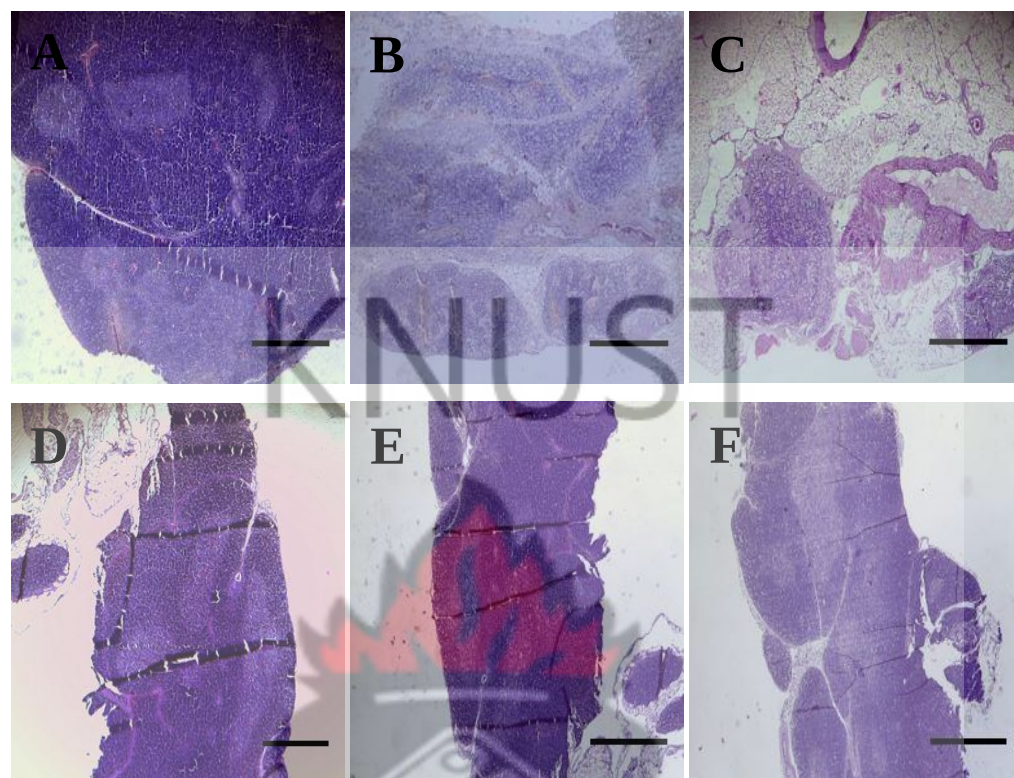


Fig. 3.5.7 Effect of stigmasterol on thymus histopathology. Wistar rats (150 – 200 g) received either polyethylene glycol, 5 ml/kg (PEG), dexamethasone (1 – 6 mg/kg) or stigmasterol (10 – 100 mg/kg) daily for 8 days. 24 h after the last treatment, rats were sacrificed by cervical dislocation. 3 μ m sections of harvested thymus were stained with H & E stain for histological

examination. Micrographs represent thymus sections from PEG (A), dexamethasone 3 and 6 mg/kg (B and C) and stigmasterol (10 – 100 mg/kg) (D – F). Data is represented as mean histopathology score $\pm$ SEM (G) ***P <0.001, **P <0.01 and ns (not significant) as compared to PEG-treated control using One-way ANOVA followed by Dunnet's *post hoc* test. Micron bar represents 1 mm.

3.5.4.7.3 Effect of stigmasterol on adrenal glands

Adrenal gland sections of PEG-treated control rats showed normal histology with distinct cortex and medulla (Fig. 3.5.8A). Adrenal glands from dexamethasone-treated rats showed significant signs of atrophy. Fat infiltration, regions of regeneration and vascular congestion, and reduced cortical: medulla ratios were observed at all doses of dexamethasone. Presence of apoptotic bodies were also observed in dexamethasone 3 and 6 mg/kg- treated rats (Fig. 3.5.8B and C). Stigmasterol-treated rats showed minimal signs of atrophy, with distinguishable cortex and medulla regions. Moderate fat infiltrations and slight reductions in cortical regions were however observed in 100 mg/kg stigmasterol treated rats (Fig. 3.5.8D – F). When the observed changes were quantified, dexamethasone at 1, 3 and 6 mg/kg recorded an overall histopathology score of 6.25 ± 0.48 , 7.00 ± 0.70 and 8.00 ± 0.41 , representing significant increases above the score of 1.00 ± 0.41 for PEG-treated control rats. The histopathology scores of 1.25 ± 0.25 , 1.75 ± 0.63 and 2.00 ± 0.41 determined for stigmasterol 10, 50 and 100 mg/kg-treated rats respectively, were not statistically different from the mean score for PEG-treated control rats (Fig. 3.5.8G)

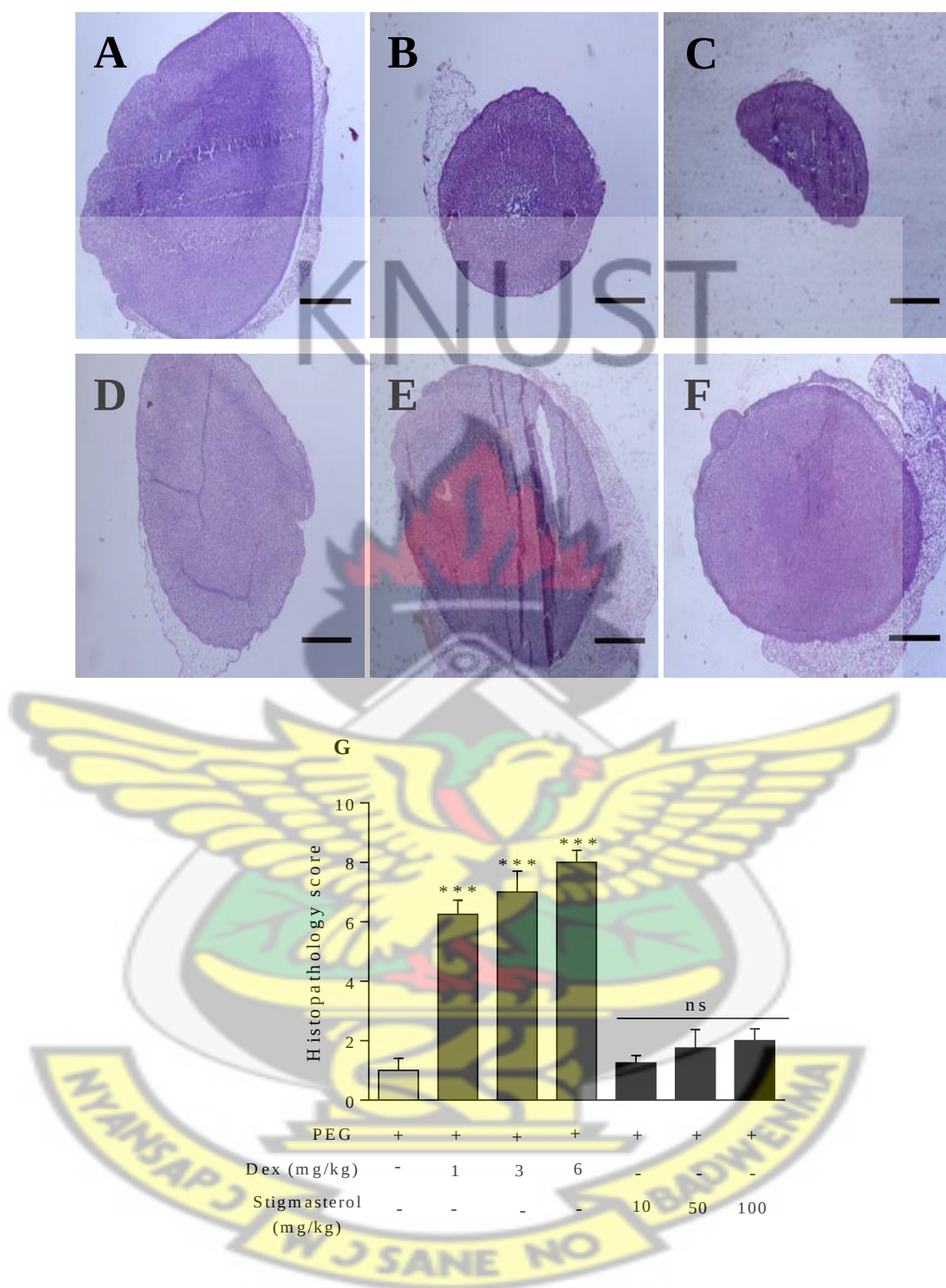


Fig. 3.5.8 Effect of stigmasterol on adrenal gland histopathology. Wistar rats received either polyethylene glycol, 5 ml/kg (PEG), dexamethasone (1 – 6 mg/kg) or stigmasterol (10 – 100 mg/kg) daily for 8 days. 24 h after the last treatment, rats were sacrificed by cervical dislocation. 3 μ m sections of harvested adrenal gland (right) were stained with H & E stain for histological examination. Micrographs represent adrenal gland sections from PEG (A), dexamethasone 3

and 6 mg/kg (B and C) and stigmasterol (10 – 100 mg/kg) (D – F). Data is represented as mean histopathology score $\pm$ SEM (G) ***P <0.001 and ns (not significant) as compared to PEG-treated control using One-way ANOVA followed by Dunnet's *post hoc* test. Micron bar represents 1 mm.

3.5.5 DISCUSSION

The optimum function of the immune system is critical to host survival (Parkin and Cohen, 2001). Components of the immune system such myeloid tissue, primary and secondary lymphoid organs as well as specific cellular and humoral functions have been shown to be either reversibly or permanently compromised by xenobiotics (Gao *et al.*, 2008; Kurtin, 2012). These agents range from environmental pollutants and organic solvents to therapeutic agents (Germolec *et al.*, 2017). Immunomodulatory drugs such as alkylating agents, corticosteroids and cyclosporins exert their effects by interacting with components of the immune system to modify immune function and responses. The deleterious effects on the immune system by such agents are well documented (Lallana and Fadul, 2011). The apparent risk to the immune system therefore makes the immunotoxicity assessment of potentially immune-active compounds necessary.

In previous sections, stigmasterol has been shown to modulate innate immune responses, immunoglobulin response to antigen, expression of cytokines and adhesion molecules and levels of circulating leukocytes in various inflammatory states. This section assesses if this blunting of varying immune responses by stigmasterol is accompanied by damage to key tissues of the immune system.

Stigmasterol showed no obvious signs of general toxicity or toxic effects in the parameters studied. No mortality, abnormal behaviour or significant weight loss was recorded among stigmasterol-treated rats throughout the study period.

Primary and secondary lymphoid tissues are responsible for the generation and maturation of B and T lymphocytes and initiation of antigen specific immune responses. Generally, a successful mounting of a cellular and humoral response is indicative of a healthy immune system. The DNCB challenge is used as a measure of the state of

cellular immunity. DNCB induces T cell-mediated, delayed type hypersensitivity (DTH) observed as skin inflammation (Shabbir *et al.*, 2016). Stigmasterol-treated rats showed no significant change in skin inflammation compared to PEG-treated control rats signifying an intact T lymphocyte function. Again, the severe depletion of circulating leukocytes observed in dexamethasone-treated rats was not present in stigmasterol-treated rats. Previous studies have established that dexamethasone markedly reduces total counts of peripheral leukocytes and promotes redistribution of leukocyte subpopulations in rats. Apoptosis of lymphocytes, eosinophils and basophils with corresponding promotion of neutrophil survival accounts mainly for these effects (Meagher *et al.*, 1996; Ohkaru *et al.*, 2010). Results from dexamethasone-treated rats in this study were consistent with these findings. In contrast, stigmasterol treatment showed no alteration in peripheral leukocyte cell count or distribution compared to control determinations. In addition, the severe reduction in lymphoid organ weights and spleen cellularity induced by dexamethasone treatment was absent in stigmasterol-treated rats at all doses. Changes in lymphoid organ weights and cellularity are highly predictive of immune system toxicity (Mitra *et al.*, 2013). Glucocorticoids are known to reduce, as part of its anti-inflammatory action, the size and cellularity of myeloid and lymphoid tissue via direct apoptosis of resident lymphocytes (Goya *et al.*, 2003). This presents clinically as a muted cellular response, leaving hosts in an immunocompromised state. Glucocorticoids are also documented to have suppressive effect on the Hypothalamus-Pituitary-Adrenal (HPA) axis (Tasker and Herman, 2011). This often manifests as a fall circulating cortisol levels and atrophy of adrenal glands even with low doses. Considering the bi-directional relationship between the HPA axis and immune system the quality of stress and immune responses are affected (Campbell *et al.*, 2012; Bellavance and Rivest, 2014). Treatment with effective dose range 10 – 100 mg/kg of stigmasterol as determined in previous experiments in this study, showed no change in lymphoid tissue size and cellularity. This was further supported by histopathological assessment of thymus and spleen sections. Stigmasterol-treated rats showed normal spleen and thymus architecture. Spleen white pulp regions and cortical thymocyte regions appeared unchanged, suggesting a largely preserved tissue lymphocyte population. No signs for atrophy was observed in stigmasterol-treated rats.

Further studies will however be required to assess the full extent of stigmasterol on the HPA axis.

3.5.6 CONCLUSION

Overall, stigmasterol showed no immunotoxic effects at the doses investigated. In addition to a preserved T cell response, no changes were recorded in lymphoid tissue size and cellularity. Histological assessment revealed no significant alteration in spleen and thymus architecture.



CHAPTER FOUR

GENERAL DISCUSSION

Immunomodulation describes processes by which immune responses are modified to achieve a particular therapeutic aim (Gea-Banacloche, 2006). In response to the increasing prevalence of immune system disorders and limitations of current therapy, research is now focused on screening alternative molecules against old and emerging therapeutic targets. Stigmasterol is a steroid alcohol belonging to the phytosterol class of compounds. Though phytosterols are known mainly for their cholesterol lowering properties (Ostlund, 2002; Thornton, 2011), many immune system modulatory effects have been attributed to phytosterol mixtures as well as individual compounds within the chemical class (Othman and Moghdasian, 2011). Against this backdrop, the study sought to investigate the potential anti-allergic and immune modulating properties of stigmasterol. The approach of the study was to establish the scope and some specific mechanisms involved in the actions of stigmasterol. The influence of stigmasterol on innate and adaptive immune responses as well as selected animal models of allergy was investigated.

The innate immune system represents a formidable first line of the hosts' defense against invasion by foreign bodies (Modlin, 2012). Deficiencies in the barrier, cellular and humoral components of this system is associated with significant risk of infection. Clinical and experimental evidence however has linked hyperactivity of the innate immune system with several disease states (Samarghitean *et al.*, 2007). Current immune modulatory interventions are focused on mechanisms of phagocytosis, pathogen recognition, antigen presentation, cell migration and cytokine signaling in the therapy of cancer, sepsis as well as autoimmune disorders. In this study, stigmasterol proved effective in suppressing innate immune responses induced by intraperitoneal injection of lipopolysaccharide. Pyrexia, multiple organ damage and mortality were significantly controlled in stigmasterol-treated mice. Analysis of blood and peritoneal fluid showed that the acute neutrophilia associated with Toll-like receptor activation was significantly suppressed by stigmasterol. Neutrophil accumulation and subsequent infiltration into tissues, release of chemoattractants for other inflammatory cells and generation of

reactive oxygen species are central in LPS-associated multiple organ damage and eventual death (Hsu and Wen, 2002; Czaikoski *et al.*, 2016). Perhaps the suppression of neutrophil accumulation by stigmasterol could account, in part for the limited organ damage and improved survival observed in stigmasterol-treated mice. Previous studies have also demonstrated the *in-vivo* antioxidant capacity of stigmasterol (Panda *et al.*, 2009; Ghosh *et al.*, 2014) and its inhibitory effect on prostaglandin and thromboxane expression following LPS stimulation of whole blood (Saadawi *et al.*, 2012). These reported effects support the findings of this study.

Rat mesentery tissues pretreated with stigmasterol showed reduced incidents of both immunogenic and non-immunogenic mast cell degranulation *in vitro*. Allergen and secretagogue induced mast cell degranulation represent distinct mechanisms of activation and degranulation with significant clinical relevance (Chinen and Shearer, 2008b; Blunk *et al.*, 2004). The former follows the classic allergic response in sensitized subjects. Immunoglobulin E-mediated activation of mast cells is central to Type I hypersensitivity reactions and diseases such as atopic dermatitis (Liu *et al.*, 2011). Targeting IgE interactions with the high affinity FcεRI or intracellular events leading to mast cell degranulation has shown promise in ameliorating allergic diseases (Holgate, 2014). Thus, inhibition of allergen induced, IgE-mediated mast cell degranulation could partly account for the suppression of active cutaneous anaphylaxis observed in stigmasterol-treated mice. The antigen-antibody reaction which follows cutaneous bovine serum albumin challenge in previously sensitized mice is largely driven by mast cells. Re-exposure to the allergen leads to cross-linking of bound IgE molecules, activation and degranulation of mast cells, release of vasoactive mediators, increases in vascular permeability and extravasation of Evans blue dye (Evans *et al.*, 2014). Pathways involved in secretagogue-induced degranulation are however not IgE-dependent. Mrgprb2 receptor in mice and MRGPRX2 in humans have been implicated in anaphylactoid reactions (McNeill *et al.*, 2015). Interestingly, the data showed significant inhibition of mast cell mesentery degranulation, as well as improved survival from systemic anaphylaxis both induced by compound 48/80 on stigmasterol treatment. Compound 48/80 is known to activate G proteins, diacylglycerol (DAG)/ protein kinase C (PKC) intracellular signaling pathways and triggering of intracellular calcium

mobilization (Seifert, 2015). These events occur without prior interaction between IgE and FcεRI of the mast cell. The observed effectiveness of stigmasterol in both immunogenic and non-immunogenic degranulation suggests, at least, that the mechanism of the mast cell stabilizing effect offered by stigmasterol does not involve or is not limited to IgE-mediated pathways. Bunluepuech *et al.* (2013) determined, that a mixture of stigmasterol and β sitosterol was found to have promising anti-allergic properties. They demonstrated that the mixture inhibited β -hexosaminidase release from RBL-2H3 cells. The findings in this study provides further evidence of the specific effects and potential mechanism of action of stigmasterol, further buttressing its anti-allergic potential.

The reported anti-inflammatory effects of stigmasterol in literature, as well as its inhibitory effect on innate responses and mast cell-mediated effects observed in this study, suggest that it is a worthy candidate for drug development for immune disorders with allergic and inflammatory pathophysiology. Therefore, the possible anti-allergic effects of stigmasterol as well as its modulatory effects on key pathways of allergic responses were further assessed in selected animal models of allergic diseases. The study focused on mechanisms of cutaneous allergic responses and allergen-induced airway inflammation such as inflammatory cell involvement, role of cytokines and molecules critical in the respective pathophysiology and pathological changes in tissues of interest.

Allergic skin diseases describe an array of cutaneous allergic manifestations characterized by pruritus, erythema, urticaria and angioedema (Fonacier *et al.*, 2010). Current therapy includes topical and systemic antihistamines, corticosteroids and immunosuppressive agents (Heller, 2012; Aslam *et al.*, 2014). Modulation of gene transcription, cytokine involvement, cell migration and trafficking and intracellular inflammatory signaling pathways have been the focus of recent efforts (Wollenberg *et al.*, 2014; Dai *et al.*, 2017). In this present study, stigmasterol significantly suppressed skin inflammatory responses induced by topical 12-O-tetradecanoylphorbol-13-acetate (TPA). Stigmasterol, administered intraperitoneally, suppressed ear skin oedema. This signifies a marked control of TPA-induced skin inflammation. This was supported by morphometric evaluation of ear skin sections. Skin thickness and hyperplasia of

epidermal layer were also markedly suppressed in stigmasterol-treated rats. The latter is especially key since resident keratinocytes in the epidermis are major drivers of the inflammatory response *via* cytokine release and chemoattraction. The data obtained showed two possible mechanisms of action of stigmasterol. Stigmasterol controlled neutrophil accumulation and infiltration into skin tissue, a process which is central to the pathophysiology of irritant dermatitis. In addition to this, TNF α expression, which promotes the inflammatory process was significantly reduced by stigmasterol. These two effects could account for the overall inhibitory action observed. Furthermore, the inhibition of compound 48/80-induced scratching behaviour in mice pretreated with stigmasterol supports its potential as an agent for allergic skin diseases. Pruritus is a common, often intractable symptom of allergic skin disorders. Its control improves healing of epithelial barrier and improves quality of life of patients (Blauvelt *et al.*, 2003; Heller, 2012). A microscopic study of skin sections provided further evidence of the effectiveness of stigmasterol. Skin sections of stigmasterol-treated mice were associated with reduced mast cell proliferation and degranulation, both of which have been linked extensively with pruritic responses. Mast cell proliferation and release of histamine, with or without degranulation is known to mediate itch responses *via* stimulation of mainly H₁ and other histamine receptors (Jin *et al.*, 2014). However, the report that compound 48/80-induced scratching behaviour in mast cell-deficient mice suggests the possible involvement of other pathways (Inagaki *et al.*, 2002). While this points to the complex nature of itch responses, the possible effect of stigmasterol on these alternative pathways is not covered under the scope of this study.

Asthma represents a typical allergic disorder. It is triggered by exposure to allergens by hypersensitive individuals, mediated by a TH2 skewed cytokine profile and IgE production, and involves significant roles by mast cells and eosinophils (Agrawal and Shao, 2010). The allergic reaction and ensuing inflammation are both critical to acute and chronic manifestations of allergic asthma. Assessing the effects of stigmasterol in models of allergen-induced asthma was therefore considered suitable in further understanding the modulatory activity of the steroid alcohol on diseases with underlying ‘allergy-based’ mechanisms. In this study, challenge of previously sensitized guinea pigs with aerosolized ovalbumin, yielded severe airway inflammation characterized by

marked proliferation of inflammatory cells in blood and subsequent infiltration into lung tissue, oxidative stress and marked changes to lung tissue architecture. Here, stigmasterol significantly suppressed the adaptive immune response to ovalbumin. Immunoglobulin E produced in response to ovalbumin sensitization and challenge was impaired in stigmasterol-treated mice. Interventions in the generation and perpetuation of an adaptive response such as modulation of antigen presentation, blockade of serum IgE and prevention of its receptor binding and modulation of TH2 cell mediated inflammatory responses have shown encouraging experimental and clinical outcomes (Gaurav and Agrawal, 2013; Pelaia *et al.*, 2017). Considering the crucial role of IgE in the pathophysiology of atopic asthma, the reduced levels in stigmasterol-treated guinea pigs could account for the improved features observed. Additionally, the mast cell stabilizing effect offered by stigmasterol against ovalbumin-induced degranulation may further support this assertion. The mast cell crucially interacts with circulating IgE to release proinflammatory mediators which drive allergic asthma. Chemokines released from mast cell also play critical roles in ‘inviting’ other inflammatory cells to contribute to the inflammatory milieu (Robinson *et al.*, 2004). Inhibition of immunoglobulin E response and prevention of mast cell degranulation would represent a significant blunting of the adaptive response and could account for the muted inflammatory cell proliferation in blood and the overall mechanism of action for stigmasterol in reversing ovalbumin-induced airway inflammation. The results also suggest that other factors may contribute to the overall mechanism of action of stigmasterol. While the above mechanisms are prominent in the early phase of asthma pathogenesis, stigmasterol exerted modulatory effect on later events as well. A key finding from this study was the inhibition of vascular cell adhesion molecule-1 (VCAM-1) expression in stigmasterol-treated guinea pigs. Infiltration of circulating eosinophils and other inflammatory cells into lung tissue precedes inflammatory cell-elicited oxidative damage, lung injury and inflammation of airway smooth muscles (Possa *et al.*, 2013). This migration is mediated by adhesion molecules such as selectins, ICAM-1 and VCAM-1 (Hamzaoui *et al.*, 2001). Unsurprisingly, reduction in circulating inflammatory cells and VCAM-1 levels following stigmasterol administration corresponded with suppressed levels of oxidative stress markers in broncho-alveolar lavage fluid and significantly controlled features of

lung damage such as peribronchiolar accumulation of inflammatory cells, airway smooth muscle hypertrophy and collagen deposition.

Immunotoxicity evaluation of stigmasterol showed no significant damage to the immune system. At the effective doses used throughout this study, no significant change was observed in circulating leukocytes and lymphoid organs histopathology and cellularity. This is contrary to effects seen for other immunomodulatory agents. For instance, glucocorticoids typically blunt immune responses by a variety of mechanisms including apoptosis of lymphocytes resident in lymphoid tissues and atrophy of these immune response-initiating tissues (Goya *et al.*, 2003). Their immunotoxicity is therefore heavily linked to their desired anti-inflammatory effect and also accounts for the severe immunosuppression associated with glucocorticoid treatment. Data from this study however suggests that the anti-inflammatory effects and suppression of immune responses exerted by stigmasterol is not associated with direct damage to lymphoid tissues.



CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

The study provided significant evidence of the immunomodulatory and anti-allergic effect of stigmasterol in animal models. Stigmasterol was demonstrated to have suppressive effects on innate immune responses and IgE-mediated adaptive responses. Immunotoxicity studies showed, that the suppression of immune responses exerted by stigmasterol is not associated with damage to lymphoid tissues of the immune system. Specific effects of stigmasterol determined by the study are as follows:

- Inhibition of both allergen- and inflammogen-induced inflammatory cell recruitment in blood and target organs.
- Suppression of endotoxin-driven febrile responses and multiple organ damage
- Inhibition of proinflammatory cytokine (TNF α) and vascular cell adhesion molecule (VCAM-1) expression in inflammatory reactions.
- Inhibition of mast cell degranulation by IgE-dependent and IgE-independent mechanisms
- Suppression of inflammation-generated oxidative stress.
- Blunting of allergen-induced immunoglobulin E production and resulting adaptive response.
- No significant change in circulating inflammatory cell count, T cell-mediated immune response, lymphoid tissue size, cellularity or histopathology, by stigmasterol at effective dose range used in the study.

5.2 RECOMMENDATIONS/ OUTLOOK

Further studies could be performed to further evaluate the immunomodulatory potential of stigmasterol as well as to fill knowledge gaps in the present study. Some suggestions include:

- Investigating the possible involvement of the glucocorticoid receptor (GR) in the mechanism of action of stigmasterol through receptor binding studies and/or use of selective GR antagonist RU-486.
- Structure activity relationship studies with stigmasterol derivatives.
- Detailed molecular mechanisms involved in the mechanism of action of stigmasterol using cell-line and protein identification-based approaches.
- Phase I clinical trials to assess its effect in humans for conditions such as allergy and asthma.



REFERENCES

- Abidi A, Gupta S, Agarwal M, Bhalla HL, Saluja M (2014). Evaluation of Efficacy of Curcumin as an Add-on therapy in Patients of Bronchial Asthma. *Journal of Clinical Diagnostic Research* 8(8):19-24.
- Acharya KR, Ackerman SJ (2014). Eosinophil Granule Proteins: Form and Function. *The Journal of Biological Chemistry* 289:17406-17415.
- Adorini L, Penna G, Giarratana N, Roncari A, Amuchastegui S, Daniel KC, Uskokovic M (2004). Dendritic cells as key targets for immunomodulation by Vitamin D receptor ligands. *The Journal of Steroid Biochemistry and Molecular Biology* 89-90(1-5):437-41.
- Agrawal DK, Shao Z (2010). Pathogenesis of Allergic Airway Inflammation. *Current Allergy and Asthma Reports* 10(1): 39–48.
- Ahamed BMK, Krishna V, Gowdru HB, Rajanaika H, Kumaraswamy HM, Rajshekarappa S, Dandin CJ, Mahadevan KM (2007). Isolation of Bactericidal Constituents from the Stem Bark Extract of *Grewia tiliaefolia* Vahl. *Research Journal of Medicinal Plant* 1(3): 72-82.
- Ahmad A, Shameem M, Husain Q (2012). Relation of oxidant-antioxidant imbalance with disease progression in patients with asthma. *Annals of Thoracic Medicine* 7(4):226-232.
- Akondy RS, Fitch M, Edupuganti S, Yang S, Kissick HT, Li KW, Youngblood BA, Abdelsamed HA, McGuire DJ, Cohen KW, Alexe G, Nagar S, McCausland MM, Gupta S, Tata P, Haining WN, McElrath MJ, Zhang D, Hu B, Greenleaf WJ, Goronzy JJ, Mulligan MJ, Hellerstein M, Ahmed R (2017). *Nature* 552(7685):362–367.
- Alexopoulou L, Holt AC, Medzhitov R, Flavell RA (2001). Recognition of double stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413:732–8.
- Al-Herz W, Bousfiha A, Casanova JL, Chatila T, Conley ME, Cunningham-Rundles C, Etzioni A, Franco JL, Gaspar HB, Holland SM, Klein C, Nonoyama S, Ochs HD, Oksenhendler E, Picard C, Puck JM, Sullivan K, Tang ML (2014). Primary

immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. *Frontiers in Immunology* 5:162.

Ali H, Dixit S, Ali D, Alqahtani SM, Alkahtani S, Alarifi S (2015). Isolation and evaluation of anticancer efficacy of stigmasterol in a mouse model of DMBA-induced skin carcinoma. *Drug Design, Development and Therapy* 28(9):2793–2800.

Amano MT, Camara NOS (2013). The immunomodulatory role of carbon monoxide during transplantation. *Medical Gas Research* 3:1.

Amin K (2012). The role of mast cells in allergic inflammation. *Respiratory Medicine* 106(1):9-14.

Amin K, Janson C, Boman G, Venge P (2005). The extracellular deposition of mast cell products is increased in hypertrophic airways smooth muscles in allergic asthma but not in nonallergic asthma. *Allergy* 60(10):1241-7.

Amoah AS, Forson AG, Boakye DA (2012). A review of epidemiological studies of asthma in Ghana. *Ghana Medical Journal* 46(2 Suppl): 23-28.

Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytnuik L, Raharjo E, Kubes P (2003). Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. *Journal of Clinical Investigation*. 111:1011–1020.

Andoniou CE, van Dommelen SL, Voigt V, Andrews DM, Brizard G, Asselin-Paturel C, Delale T, Stacey KJ, Trinchieri G, Degli-Esposti MA (2005). Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity. *Nature Immunology* 6(10): 1011- 1019.

Anochie PI (2013). Mechanisms of fever in humans. *International Journal of Microbiology and Immunology Research*. 2(5):37-43.

Arock M, Schneider E, Boissan M, Tricottet V, Dy M (2002). Differentiation of human basophils: an overview of recent advances and pending questions. *Journal of Leukocyte Biology* 71: 557–564.

Ashwell JD, Lu FW, Vacchio MS (2000). Glucocorticoids in T cell development and function. *Annual Review of Immunology* 218:309-345

Aslam I, Sandoval LF, Feldman SR (2014). What's new in the topical treatment of allergic skin diseases *Current Opinion in Allergy and Clinical Immunology* 14(5):436-50.

Bae MJ, Shin HS, Chai OH, Han JG, Shon DH (2013). Inhibitory effect of unicellular green algae (*Chlorella vulgaris*) water extract on allergic immune response. *Journal of the Science of Food and Agriculture* 93(12): 3133–3136.

Baker BS (2006). The role of microorganisms in atopic dermatitis. *Clinical & Experimental Immunology* 144(1): 1–9.

Balaji G, Chalamaiah M, Hanumanna P, Vamsikrishna B, Jagadeesh Kumar D, Venu babu V. (2014). Mast cell stabilizing and anti-anaphylactic activity of aqueous extract of green tea (*Camellia sinensis*). *International Journal of Veterinary Science and Medicine* 2:89–94.

Baliga CS, Paul ME, Chine J, Shearer WT (2008). HIV infection and the acquired immunodeficiency syndrome. In: Rich RR, editor. *Clinical immunology. Principles and practice*. 3th ed. Philadelphia: Elsevier Saunders pp 553-60.

Balk RA (2000). Severe sepsis and septic shock. Definitions, epidemiology, and clinical manifestations. *Critical Care Clinics* 16:179–192.

Bansil R, Turner BS (2017). The biology of mucus: Composition, synthesis and organization. *Advance Drug Delivery Reviews* doi: 10.1016/j.addr.2017.09.023.

Bascones-Martinez A, Mattila R, Gomez-Font R, Meurman JH (2014). Immunomodulatory drugs: Oral and systemic adverse effects. *Medicina Oral, Patologia Oral Y Cirugia Bucal* 19(1): 24–31.

Belkaid Y, Hand T (2014). Role of the Microbiota in Immunity and inflammation *Cell* 157(1): 121–141.

Bellavance MA, Rivest S (2014). The HPA - Immune Axis and the Immunomodulatory Actions of Glucocorticoids in the Brain. *Frontiers in Immunology* 5:136.

Black S, Kushner I, Samols D (2004). C-reactive protein. *Journal Biological Chemistry* 279:48487-48490.

Blauvelt A, Hwang ST, Udey MC (2003). Allergic and immunologic diseases of the skin. *Journal of Allergy and Clinical Immunology* 111(2):560-570.

Blunk JA, Schmelz M, Zeck S, Skov P, Likar R, Koppert W (2004) Opioid-induced mast cell activation and vascular responses is not mediated by mu-opioid receptors: an in vivo microdialysis study in human skin. *Anesthesia and Analgesia* 98(2): 364-70.

Boden SR, Wesley Burks A (2011). Anaphylaxis: a history with emphasis on food allergy. *Immunological Reviews* 242: 247-257.

Boes M (2002). Role of natural and immune IgM antibodies in immune responses. *Molecular Immunology* 37:1141-9.

Boix E, Carreras E, Nikolovski Z, Cuchillo CM, Nogués MV (2001). Identification and characterization of human eosinophil cationic protein by an epitope-specific antibody. *Journal of Leukocyte Biology* 69:1027–1035.

Boneberger S, Rupec RA, Ruzicka T (2010). Complementary therapy for atopic dermatitis and other allergic skin diseases: facts and controversies. *Clinical Dermatology* 28(1):57-61.

Bonilla FA, Bernstein IL, Khan DA, Ballas ZK, Chinen J, Frank MM, Kobrynski LJ, Levinson AI, Mazer B, Nelson RP Jr, Orange JS, Routes JM, Shearer WT, Sorensen RU, American Academy of Allergy, Asthma and Immunology, American College of Allergy, Asthma and Immunology, Joint Council of Allergy, Asthma and Immunology (2005). Practice parameter for the diagnosis and management of primary immunodeficiency. *Annals of Allergy, Asthma and Immunology* 94(5 Suppl 1): S1-63.

Bordon Y (2017). Allergy: Pollutants drive atopic dermatitis. *Nature Reviews Immunology* 17(1):1.

- Borregaard N. (2010). Neutrophils, from marrow to microbes. *Immunity* 33:657–670.
- Bosscher K, Vanden Berghe W, Haegeman G (2002). Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: negative interference of activated glucocorticoid receptor with transcription factors. *Journal of Neuroimmunology* 109(1): 16–22.
- Bouic PJ (2001). The role of phytosterols and phytosterolins in immune modulation: a review of the past 10 years. *Current Opinion in Clinical Nutrition and Metabolic Care* 4(6): 471-5.
- Bouvier-Navé P, Husselstein T, Benveniste P (1998). Two Families of Sterol Methyltransferases are Involved in the First and the Second Methylation Steps of Plant Sterol Biosynthesis. *European Journal of Biochemistry* 256(1): 88-96.
- Bowcock AM, Krueger JG (2005). Getting under the skin: The immunogenetics of psoriasis. *Nature Reviews Immunology* 5(9):699-711.
- Bowler RP (2004). Oxidative Stress in the pathogenesis of Asthma. *Current Allergy and Asthma Reports* 4(2):116-22.
- Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, Maitland M, Norgard MV, Plevy SE, Smale ST, Brennan PJ, Bloom BR, Godowski PJ, Modlin RL (1999). Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science* 285:732–736.
- Brogden KA (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology* 3:238-250.
- Broide DH (2009). Immunomodulation of Allergic Disease. *Annual Review of Medicine* 60:279–291.
- Brubaker SW, Bonham KS, Zanoni I, Kagan JC (2015). Innate Immune Pattern Recognition: A Cell Biological Perspective. *Annual Reviews of Immunology* 33:257-290.
- Budinger L, Hertl M (2000). Immunologic mechanisms in hypersensitivity reactions to metal ions: an overview. *Allergy* 55(2): 108–115.

Buffie CG, Pamer EG (2013). Microbiota-mediated colonization resistance against intestinal pathogens. *Nature Reviews Immunology* 13:790–801.

Bunluepuech K, Wattanapiromsakul C, Madaka F, Tewtrakul S (2013). Anti-HIV-1 integrase and anti-allergic activities of *Bauhinia strychnifolia* Songklanakarin. *Journal of Science and Technology* 35(6):659-664.

Calpe-Berdiel L, Escola-Gil JC, Benitez S, Bancells C, González-Sastre F, Palomer X, Blanco-Vaca F (2007). Dietary phytosterols modulate T-helper immune response but do not induce apparent anti-inflammatory effects in a mouse model of acute, aseptic inflammation. *Life Sciences* 80(21):1951–1956.

Campbell LS, Chevalier M, Levy RA, Rhodes A (2012). Hypothalamic–Pituitary–Adrenal Axis Suppression Related to Topical Glucocorticoid Therapy in a Child with Psoriatic Exfoliative Erythroderma. *Pediatric Dermatology* 29(1):101–104.

Cash HL, Whitham CV, Behrendt CL, Hooper LV (2006). Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* 313:1126–1130.

Cataisson C, Pearson AJ, Tsien MZ, Mascia F, Gao JL, Pastore S, Yuspa SH (2006). CXCR2 ligands and G-CSF mediate PKC α -induced intraepidermal inflammation. *The Journal of Clinical. Investigation* 116:2757–2766.

Chang JT, Wherry EJ, Goldrath AW (2014). Molecular regulation of effector and memory T cell differentiation. *Nature Immunology*. 15(12):1104–1115.

Chapel H, Lucas M, Lee M, Bjorkander J, Webster D, Grimbacher B, Fieschi C, Thon V, Abedi MR, Hammarstrom L (2008). Common variable immunodeficiency disorders: division into distinct clinical phenotypes. *Blood* 112: 277–86.

Chen G, Tang J, Ni Z, Chen Q, Li Z, Yang W, Din J, Luo X, Wang X (2015). Antiasthmatic effects of resveratrol in ovalbumin-induced asthma model mice involved in the upregulation of PTEN. *Biological and Pharmaceutical Bulletin* 38(4):507-513.

Chen K, Cerutti A (2011). The function and regulation of immunoglobulin D. *Current Opinion in Immunology* 23(3):345-352.

Chen WP, Yu C, Hu PF, Bao JP, Tang JL, Wu LD (2012). Stigmasterol blocks cartilage degradation in rabbit model of osteoarthritis. *Acta Biochimica Polonica* 59(4):537–541.

Chilvers MA, O'Callaghan C (2000). Local mucociliary defence mechanisms. *Paediatric Respiratory Reviews* 1(1):27-34.

Chinen J, Shearer WT (2008). Advances in basic and clinical immunology in 2007. *Journal of Allergy Clinical Immunology* 122:36–41.

Chinen J, Shearer WT (2008). Secondary immunodeficiencies, including HIV infection. *Journal of Allergy and Clinical Immunology* 121(2 Suppl): S388-92.

Chung A, Rollman E, Johansson S, Kent SJ, Stratov I (2008). The utility of ADCC responses in HIV infection. *Current HIV Research* 6: 515-519.

Chung KF (2016). Asthma phenotyping: a necessity for improved therapeutic precision and new targeted therapies. *Journal of Internal Medicine* 279(2): 192-204.

Conley ME, Dobbs AK, Farmer DM, Kilic S, Paris K, Grigoriadou S, Coustan-Smith E, Howard V, Campana D (2009). Primary B cell immunodeficiencies: comparisons and contrasts. *Annual Review of Immunology* 27:199-227.

Cork MJ, Danby SG, Vasilopoulos Y, Hadgraft J, Lane ME, Moustafa M, Guy RH, Macgowan AL, Tazi-Ahnini R, Ward SJ (2009). Epidermal barrier dysfunction in atopic dermatitis. *Journal of Investigative Dermatology* 129:1892-1908.

Corradi M, Folesani G, Andreoli R, Manini P, Bodini A, Piacentini G, Carraro S, Zanconato S, Baraldi E (2003). Aldehydes and glutathione in exhaled breath condensate of children with asthma exacerbation”, *American Journal of Respiratory Critical Care Medicine* 167(3): 395-399.

Corry DB, Kheradmand F (2009). Toward a comprehensive understanding of allergic lung disease. *Transactions of the American Clinical and Climatological Association* 120: 33-48.

Coulter EM, Jenkinson C, Farrell J, Lavergne SN, Pease C, White A, Aleksic M, Basketter D, Williams DP, King C, Pirmohamed M, Park BK, Naisbitt DJ (2010). Measurement of CD4+ and CD8+ T-lymphocyte cytokine secretion and gene

expression changes in p-phenylenediamine allergic patients and tolerant individuals. *Journal of Investigative Dermatology* 130(1):161–174.

Cuenca J, Martín-Sanz P, Álvarez-Barrientos AM, Boscá L, Goren N (2006). Infiltration of Inflammatory Cells Plays an Important Role in Matrix Metalloproteinase Expression and Activation in the Heart during Sepsis. *American Journal Pathology* 169(5):1567–1576.

Cunha PR, Delfini Filho O (2012). Pruritus: still a challenge. *Anais Brasileiros de Dermatologia* 87(5):735-41.

Cunningham-Rundles C (2002). Hematologic complications of primary immune deficiencies *Blood Reviews* 16(1):61-4.

Cusick MF, Libbey JE, Fujinami RS (2012). Molecular Mimicry as a Mechanism of Autoimmune Disease. *Clinical Review of Allergy and Immunology* 42(1): 102–111.

Czaikoski PG, Mota JM, Nascimento DC, Sônego F, Castanheira FV, Melo PH, Scortegagna GT, Silva RL, Barroso-Sousa R, Souto FO, Pazin-Filho A, Figueiredo F, Alves-Filho JC, Cunha FQ (2016). Neutrophil Extracellular Traps Induce Organ Damage during Experimental and Clinical Sepsis. *PLoS One* 5:11(2): e0148142.

Dahlin JS, Hallgren J (2015). Mast cell progenitors: Origin, development and migration to tissues. *Molecular Immunology* 63(1):9-17.

Dai J, Choo MK, Park JM, Fisher DE (2017). Topical ROR Inverse Agonists Suppress Inflammation in Mouse Models of Atopic Dermatitis and Acute Irritant Dermatitis. *Journal of Investigative Dermatology*. 137(12):2523-2531.

Davidson S, Giesler GJ (2010). The multiple pathways of itch and their interactions with pain. *Trends in Neurosciences* 33(12):550-558.

De Smet K, Contreras R (2005). Human antimicrobial peptides: defensins, cathelicidins and histatins. *Biotechnology Letters* 27:1337-1347.

Delamarre L, Pack M, Chang H, Mellman I, Trombetta ES (2005). Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* 307: 1630–1634.

Delves PJ, Roitt IM (2000). The immune system. Second of two parts. *The New England Journal of Medicine* 343(2): 108-117.

Demonty I, Ras RT, van der Knaap HC, Duchateau GS, Meijer L, Zock PL, Geleijnse JM, Trautwein EA (2009) Continuous Dose-Response Relationship of the LDL-Cholesterol–Lowering Effect of Phytosterol Intake. *Journal of Nutrition* 139(2):271-84.

Descotes J (2005). Immunotoxicology: role in the safety assessment of drugs. *Drug Safety* 28(2):127-36.

Diacovich L, Gorvel JP (2010). Bacterial manipulation of innate immunity to promote infection. *Reviews Microbiology* 8(2):117-128.

Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303:1529–31.

Dietert RR (2009). Developmental immunotoxicology: focus on health risks. *Chemical Research in Toxicology* 22(1):17-23.

Dietert RR, Ji-Eun Lee JE, Olsen J, Kevin Fitch K, Marsh JA (2003). Developmental immunotoxicity of dexamethasone: comparison of fetal versus adult exposures. *Toxicology* 194(1-2): 163–176.

Dinarelli CA (2004). Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. *Journal Endotoxin Research* 10(4):201-22.

Divkovic M, Pease CK, Gerberick GF, Basketter DA (2005). Hapten protein binding: from theory to practical application in the in vitro prediction of skin sensitization. *Contact Dermatitis* 53(4):189–200.

Dokmeci E, Herrick CA (2008). The immune system and atopic dermatitis. *Seminars in Cutaneous Medicine and Surgery* 27(2):138-43.

Douek DC, Brenchley JM, Betts MR, Ambrozak DR, Hill BJ, Okamoto Y, Casazza JP, Kuruppu J, Kuntsman K, Wolinsky S, Grossman Z, Dybul M, Oxenius A, Price DA., Connors M, Koup RA (2002). HIV preferentially infects HIV-specific CD4⁺ T cells. *Nature* 417(6884): 95-98.

Doyle SL, O'Neill LA (2006). Toll-like receptors: from the discovery of NF κ B to new insights into transcriptional regulations in innate immunity. *Biochemical Pharmacology* 72:1102–1113.

Dunford PJ, Williams KN, Desai PJ, Karlsson L, McQueen D, Thurmond RL (2006). Histamine H₄ receptor antagonists are superior to traditional antihistamines in the attenuation of experimental pruritus. *Journal of Allergy and Clinical Immunology*. 119 (1).

Dunkelberger JR, Song WC (2010). Complement and its role in innate and adaptive immune responses. *Cell Research* 20:34–50.

Dworski R (2000). Oxidant stress in asthma. *Thorax* 55(2):51–53.

Edilu A, Adane L, Woyessa D (2015). *In vitro* antibacterial activities of compounds isolated from roots of *Caylusea abyssinica*. *Annals of Clinical Microbiology and Antimicrobials* 14:15.

Egawa G, Weninger W (2015). Immunology. Pathogenesis of atopic dermatitis: A short review *Cogent Biology* 1: 1103459.

Ehrlich P (1878). Beiträge zur Theorie und Praxis der histologischen Färbung. (Leipzig University.).

Elias JA, Lee CG, Zheng MB, Homer RJ, Zhu Z (2003). New insights into pathogenesis of asthma. *Journal of Clinical Investigation* 111(3): 291-297.

Elias PM (2005). Stratum corneum defensive functions: an integrated view. *Journal of Investigative Dermatology* 125:183–200.

Elias PM (2007). The skin barrier as an innate immune element. *Seminars in Immunopathology* 29:3–14.

Elias PM, Choi EH (2005). Interactions among stratum corneum defensive functions. *Experimental Dermatology* 14: 719–726.

Ellman GL (1959). Tissue sulfhydryl groups. *Archives of Biochemistry Biophysics* 82(1):70-77.

Epelman S, Lavine KJ, Randolph GJ (2014). Origin and Functions of Tissue Macrophages *Immunity* 41(1):21-35.

Erle DJ, Sheppard D (2014). The cell biology of asthma. *Journal of Cell Biology* 205(5): 621–631.

Esmon CT, Xu J, Lupu F (2011). Innate Immunity and Coagulation. *Journal of Thrombosis and Haemostasis* 9(Suppl 1): 182–188.

Evans H, Killoran KE, Mitre E (2014). Measuring Local Anaphylaxis in Mice. *Journal of Visualized Experiments* (92): e52005.

Falcone FH, Haas H, Gibbs BF (2000). The human basophil: a new appreciation of its role in immune responses. *Blood* 96: 4028-4038.

Falcone FH, Zillikens D, BF Gibbs BF (2006). The 21st century renaissance of the basophil? Current insights into its role in allergic responses and innate immunity. *Experimental Dermatology* 15: 855–864.

Faurschou M, Borregaard N (2003). Neutrophil granules and secretory vesicles in inflammation. *Microbes and Infection* 5:1317–1327.

Finn DF, Walsh JJ (2013). Twenty-first century mast cell stabilizers. *British Journal of Pharmacology* 170(1):23–37.

Finn OJ (2012). Immuno-oncology: understanding the function and dysfunction of the immune system in cancer. *Annals of Oncology*. Suppl 8:6-9. doi: 10.1093/annonc/mds256.

Fonacier LS, Dreskin SC, Leung DYM (2010). Allergic skin diseases. *Journal of Allergy and Clinical Immunology* 125:138-149.

Forrest S, Dunn K, Elliott K, Fitzpatrick E, Fullerton J, McCarthy M, Brown J, Hill D, Williamson R (1999). Identifying genes predisposing to atopic eczema. *Journal of Allergy and Clinical Immunology* 104(5): 1066-1070.

França TGD, Ishikawa LLW, Zorzella-Pezavento SFG, Chiuso-Minicucci F, da Cunha MLRS, Sartori A (2009). Impact of malnutrition on immunity and infection. *Journal of Venomous Animals and Toxins including Tropical Diseases* 15(3):374-390.

French LE, Prins C (2013). Erythema multiforme, Stevens-Johnson syndrome and toxic epidermal necrolysis. In: Bologna JL, Jorizzo JL, Schaffer JV (eds) *Dermatology* 3(3):319–334.

Gabay C, Kushner I (1999). Acute-phase proteins and other systemic responses to inflammation. *New England Journal of Medicine* 340:448-454.

Gabay O, Sanchez C, Salvat C, Chev F, Breton M, Nourissat G, Wolf C, Jacques C, Berenbaum F (2010). Stigmasterol: a phytosterol with potential anti-osteoarthritic properties. *Osteoarthritis Cartilage* 18:106–116.

Galli SJ, Kalesnikoff J, Grimbaldston MA, Piliponsky AM, Williams CM, Tsai M (2005). Mast cells as ‘tunable’ effector and immunoregulatory cells: Recent advances. *Annual Review of Immunology* 23: 749-786.

Galli SJ, Wedemeyer J, Tsai M (2002). Analyzing the roles of mast cells and basophils in host defense and other biological responses. *International Journal of Hematology* 75(4): 363-369.

Gao S, Wang Y, Zhang P, Dong Y, Li B (2008). Subacute Oral Exposure to Dibromoacetic Acid Induced Immunotoxicity and Apoptosis in the Spleen and Thymus of the mice. *Toxicological Sciences* 105(2), 331–341.

García MD, Sáenz MT, Gómez MA, Fernández MA (1999). Topical Anti-inflammatory Activity of Phytosterols Isolated from *Eryngium foetidum* on Chronic and Acute Inflammation Models. *Phytotherapy Research*. 13(1)78–80.

Gaspar HB, Sharifi R, Gilmour KC, Thrasher A.J (2002). X-linked lymphoproliferative disease: clinical, diagnosis and molecular prospective. *British. Journal of Haematology*. 119(3): 585-595.

Gasque P (2004). Complement: a unique innate immune sensor for danger signals. *Molecular Immunology* 41(11):1089-1098.

Gaurav R, Agrawal DK (2013). Clinical View on the Importance of Dendritic Cells in Asthma *Expert Review of Clinical Immunology* 9(10): 899–919.

Gay NJ, Gangloff M (2007). Structure and function of Toll receptors and their ligands. *Annual Review of Biochemistry* 76:141–165.

Gea-Banacloche JC (2006) Immunomodulation. In: Runge M.S., Patterson C. (eds) *Principles of Molecular Medicine*. Humana Press pp. 893-904.

Gea-Banacloche JC (2006). Immunomodulation. In: Runge M.S., Patterson C. (eds) *Principles of Molecular Medicine*. Humana Press pp. 893 - 904. doi:10.1007/978-1-59259-963-9_92

Gerberick F, Aleksic M, Basketter D, Casati S, Karlberg AT, Kern P, Kimber I, Lepoittevin JP, Natsch A, Ovigne JM, Rovida C, Sakaguchi H, Schultz T (2008). Chemical reactivity measurement and the predictive identification of skin sensitizers. The report and recommendations of ECVAM Workshop 64. *Alternatives to Laboratory Animals* 36(2):215–242.

Germolec D, Luebke R, Rooney A, Shipkowski K, Vandebriel R, van Loveren H (2017). Immunotoxicology: A brief history, current status and strategies for future immunotoxicity assessment. *Current Opinion in Toxicology* 5:55-59.

Ghably J, Saleh H, Vyas H, Peiris E, Misra N, Krishnaswamy G (2015). Paul Ehrlich's mastzellen: a historical perspective of relevant developments in mast cell biology. *Methods in Molecular Biology* 1220:3-10. doi: 10.1007/978-1-4939-1568-2_1.

Ghosh T, Ghosh S, Maity TK (2014). Antihyperglycemic activity of stigmasterol isolated from *Bacopa monnieri* Linn. aerial parts against alloxan-induced diabetic rats. *International Journal of Natural Products Research* 4(2): 40-46.

Ghosh T, Maity TK, Singh J (2011). Evaluation of antitumor activity of stigmasterol, a constituent isolated from *Bacopa monnieri* Linn aerial parts against Ehrlich Ascites Carcinoma in mice. *Oriental Pharmacy and Experimental Medicine* 11(1): 41-49.

Ghosh TR, Ghosh S, Maity TK, Antihyperglycemic activity of stigmasterol isolated from *Bacopa monnieri* Linn. Aerial parts against alloxan-induced diabetic rats. *International Journal Natural Product Research* 4(2):40–46.

Gilfillan AM, Tkaczyk C (2006). Integrated signalling pathways for mast cell activation”, *Nature Reviews Immunology* 6(3): 218-30.

Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jéhanho M, Viala J, Tedin K, Taha MK, Labigne A, Zähringer U, Coyle AJ, DiStefano PS, Bertin J, Sansonetti PJ, Philpott DJ. (2003). NOD1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300:1584–7.

Githinji CG, Mbugua PM, Kanui TI, Kariuki DK (2012). Analgesic and anti-inflammatory activities of 9-Hexacosene and Stigmasterol isolated from *Mondia whytei*. *Phytopharmacology* 2(1) 212-223.

Gomez MA, Saenz MT, Garcia-Gimenez MD, Fernandez MA (1999). Study of the topical anti-inflammatory activity of \A\ on chronic and acute inflammation models 54(11):937-941.

Gong J, Chen SS (2003). Polyphenolic antioxidants inhibit peptide presentation by antigen-presenting cells. *International Immunopharmacology* (13-14):1841-52.

Goraca A, Huk-Kolega H, Kleniewskal P, Piechota-Polańczyk A, Skibska B (2013). Effects of lipoic acid on spleen oxidative stress after LPS administration. *Pharmacological Reports* 65:179-186.

Gough SCL, Simmonds MJ (2007). The HLA Region and Autoimmune Disease: Associations and Mechanisms of Action. *Current Genomics* 8(7): 453–465.

Goya RG, Cónsole GM, Spinelli OM, Carino MH, Riccillo F, Corrons FJ (2003). Glucocorticoid-induced apoptosis in lymphoid organs is associated with a delayed increase in circulating deoxyribonucleic acid. *Apoptosis* 8(2):171-7.

Grimbacher B, Warnatz K, Yong PFK, Korganow AS, Peter HH (2016). The crossroads of autoimmunity and immunodeficiency: Lessons from polygenic traits and monogenic defects. *Journal of Allergy and Clinical Immunology* 137(1):3-17.

Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, Lopez-Botet M (2004). Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* 104: 3664–3671.

Hankin CS, Cox L (2014). Allergy immunotherapy: what is the evidence for cost saving? *Current Opinions in Allergy and Clinical Immunology* 14(4):363-70

Hamzaoui A, Ammar J, El Mekki F, Borgi O, Ghrairi H, Brahim MB, Hamzaoui K (2001). Elevation of serum soluble E-selectin and VCAM-1 in severe asthma. *Mediators of Inflammation* 10(6): 339–342.

Harborne JB (1998). Phytochemical Methods. A Guide to Modern Techniques of Plant Analysis. *Blackwell Publishing Limited* doi:10.1046/j.1365-3059.1999.00318. x.

Hardy RR, Hayakawa K (2001). B cell development pathways. *Annual Review of Immunology* 19:595–621.

Harvima IT, Levi-Schaffer F, Draber P, Friedman S, Polakovicova I, Gibbs BF, Blank U, Nilsson G, Maurer M. (2014). Molecular targets on mast cells and basophils for novel therapies. *Journal of Allergy and Clinical Immunology* 134(3):530-44.

Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A (2001). The innate immune response to bacterial flagellin is mediated by Toll- like receptor 5. *Nature* 410:1099–103.

Heath RL, Packer L (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* 125(1) 189-98.

Heller AJ (2012). Allergic Skin Disease. *Facial Plastic Surgery Clinics of North America* 20:31–42.

Heneka MT, Golenbock DT, Latz E (2015). Innate immunity in Alzheimer's disease. *Nature Immunology* 16:229–236.

Hirota N, Martin JG (2013). Mechanisms of Airway Remodeling. *Chest* 144(3):1026-32.

Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ (2000). Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *The Journal of Immunology* 165:618–622.

Hoet S, Pieters L, Muccioli GG, Habib-Jiwan JL, Opperdoes FR, Quetin-Leclercq J (2007) Antitrypanosomal Activity of Triterpenoids and Sterols from the Leaves of *Strychnos spinosa* and Related Compounds. *Journal of Natural Products* 70:1360-1363.

Hogarth PM, Pietersz GA (2012). Fc receptor-targeted therapies for the treatment of inflammation, cancer and beyond. *Nat Reviews Drug Discovery* 11(4):311–331.

Holgate ST (2014). New strategies with anti-IgE in allergic diseases. *World Allergy Organization Journal* 7(1):17.

Holgate ST, Davies DE (2009). Rethinking the Pathogenesis of Asthma. *Immunity* 31(3):362-367. Malo JL, Lemiere C, Gautrin D, Labrecque M (2004). Occupational asthma. *Current Opinions in Pulmonary Medicine* 10(1):57–61.

Holgate ST, Polosa R (2008). Treatment strategies for allergy and asthma. *Nature Reviews Immunology* 8(3):218-30.

Holland SM. Chronic granulomatous disease (2010). *Clinical Review of Allergy Immunology* 38(1):3-10.

Holmes MC, Zhang P, Nelson S, Summer WR, Bagby GJ (2002). Neutrophil modulation of the pulmonary chemokine response to lipopolysaccharide *Shock* 18:555–560.

Hopkin J (2009). Immune and genetic aspects of asthma, allergy and parasitic worm infections: evolutionary links. *Parasite Immunology* 31(5):267-73.

Hsu HY, Wen MH (2002). Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *The Journal of Biological Chemistry* 277(25):22131-9.

Huang JG, Zhou LJ, Xu HH, Li WO (2009). Insecticidal and Cytotoxic Activities of Extracts of *Cacalia tangutica* and Its Two Active Ingredients against *Musca domestica* and *Aedes albopictus* *Journal of Economic Entomology* 102(4):1444-1447.

Hughes S, Kelly P (2006). Interactions of malnutrition and immune impairment, with specific reference to immunity against parasites. *Parasite Immunology* 28(11): 577–588.

Hume DA, Ross IL, Himes SR, Sasmono RT, Wells CA, Ravasi T (2002). The mononuclear phagocyte system revisited. *Journal of Leukocyte Biology* 72: 621–627.

Iikura M, Miyamasu M, Yamaguchi M, Kawasaki H, Matsushima K, Kitaura M, Morita Y, Yoshie O, Yamamoto K, Hirai K (2001). Chemokine receptors in human basophils: inducible expression of functional CXCR4. *Journal of Leukocyte Biology* 70(1):113-20.

Imokawa, G (2002). Ceramides as natural moisturizing factors and their efficacy in dry skin. In: Leyden JJ, Rawlings AV, eds. Skin Moisturisation. *Cosmetic Science and Technology Series* 25:267–302.

Inagaki N, Igeta K, Kim JF, Nagao M, Shiraishi N, Nakamura N, Nagai H (2002). Involvement of unique mechanisms in the induction of scratching behaviour in BALB/c mice by compound 48/80. *European Journal of Pharmacology* 448(2–3):175–183.

Invernizzi P, Gershwin ME (2009). The Genetics of human autoimmune disease. *Journal of Autoimmunity* 33:303-308

Irvine AD, McLean WI, Leung DY (2011). Filaggrin mutations associated with skin and allergic diseases. *New England Journal of Medicine* 365(14): 1315–1327.

Issazadeh-Navikas S, Teimer R, Bockermann R (2012). Influence of Dietary Components on Regulatory T Cells. *Molecular Medicine* 18(1): 95–110.

Iwasaki A, Medzhitov R (2004). Toll-like receptor control of the adaptive immune responses. *Nature Immunology* 5(10):987-95.

Jantan I, Ahmad W, Bukhari SNA (2015). Plant-derived immunomodulators: an insight on their preclinical evaluation and clinical trials. *Frontiers in Plant Science* 6: 655.

Jayasekera JP, Moseman EA, Carroll MC (2007). Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. *Journal of Virology* 81:3487–3494.

Je IG, Kim HH, Park PH, Kwon TK, Seo SY, Shin TY, Sang-Hyun Kim SH (2014). SG-HQ2 inhibits mast cell-mediated allergic inflammation through suppression of histamine release and pro-inflammatory cytokines. *Experimental Biology and Medicine* 240(5):631-638.

Jenerowicz D, Silny W, Danczak-Pazdrowska A, Polanska A, Osmola-Mankowska A, Olek-Hrab K (2012). Environmental factors and allergic diseases. *Annals of Agricultural and Environmental Medicine* 19:475–481.

Jeong HJ, Lee SA, Moon PD, Na HJ, Park RK, Um JY, Kim HM, Hong SH (2006). Alginic acid has anti-anaphylactic effects and inhibits inflammatory cytokine expression via suppression of nuclear factor-kappa B activation. *Clinical and Experimental Allergy* 36(6): 785–794.

Jie Q, Kodithuwakku ND, Yuan X, He G, Chen M, Xu S, Wu Y (2015). Anti-allergic and anti-inflammatory properties of a potent histamine H1 receptor antagonist desloratadine citrate disodium injection, and its anti-inflammatory mechanism on EA.hy926 endothelial cells. *European Journal of Pharmacology* 754:(1–10).

Jin X, Zhao W, Kirabo A, Park SO, Ho WT, Sayeski PP, Zhao ZJ (2014). Elevated levels of mast cells are involved in pruritus associated with polycythemia vera in JAK2V617F transgenic mice. *Journal of Immunology* 193(2):477-484.

Joh EH, Jeong JJ, Kim DH (2104). Inhibitory effect of echinocystic acid on 12-O-tetradecanoylphorbol-13-acetate-induced dermatitis in mice. *Archives of Pharmacal Research* 37(2):225-31.

Jörg S, Grohme DA, Erzler M, Binsfeld M, Haghikia A, Müller DN, Linker RA, Kleinewiet M (2016). Environmental factors in autoimmune diseases and their role in multiple sclerosis. *Cellular and Molecular Life Sciences* 73(24): 4611–4622.

Judge AD, Zhang X, Fujii H, Surh CD, Sprent J (2002). Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8(+) T cells. *The Journal of Experimental Medicine*. 196:935–46.

Kaech SM, Cui W (2012). Transcriptional control of effector and memory CD8+ T cell differentiation. *Nature Reviews Immunology* 12(11):749–761.

Kaminogawa S, Nanno M (2004). Modulation of Immune Functions by Foods. *Evidence Based Complementary and Alternative Medicine* 1(3): 241–250.

Kang YH, Tan LA, Carroll MV, Gentle ME, Sim RB (2009). Target pattern recognition by complement proteins of the classical and alternative pathways. *Advances in Experimental Medicine Biology* 653:117–28.

Kaplan AP, Giménez-Arnau AM, Saini SS (2017). Mechanisms of action that contribute to efficacy of omalizumab in chronic spontaneous urticaria. *Allergy* 72(4):519-533.

Kapur R, Einarsdottir HK, Vidarsson G (2014) IgG-effector functions: “The Good, The Bad and The Ugly” *Immunology Letters* 160(2): 139-144.

Kay AB (2001). Allergy and Allergic Diseases. Second of Two Parts. *The New England Journal of Medicine* 344(2):109-13.

Kazatchkine MD, Kaveri SV (2001). Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. *The New England Journal of Medicine* 345(10):747-55

Kemp SF, Lockey RF (2002). Anaphylaxis: A review of causes and mechanisms. *Journal of Allergy and Clinical Immunology* 110(3):341-8.

Khalil DN, Smith EL, Brentjens RJ, Wolchok JD (2016). The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nature Reviews Clinical Oncology* 13(5): 273–290.

Kim SJ, Jeong HJ, Yi BJ, Kang TH, An NH, Lee EH, Yang DC, Kim HM, Hong SH., Um JY (2007). Transgenic *Panax ginseng* inhibits the production of TNF-alpha, IL-6,

and IL-8 as well as COX-2 expression in human mast cells. *American Journal of Clinical Medicine*. 35:329–339.

Kim WY, Hong SB (2016). Sepsis and Acute Respiratory Distress Syndrome: Recent Update. *Tuberculosis and Respiratory Diseases (Seoul)*. 79(2):53-7.

Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H (2000). Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. *Clinical and Experimental Allergy* 30(4): 501–508.

Kingeter LM, Lin X (2012). C-type lectin receptor-induced NF- κ B activation in innate immune and inflammatory responses. *Cellular and Molecular Immunology* 9(2):105-112.

Klein C, Welte K (2010). Genetic insights into congenital neutropenia. *Clinical Review of Allergy and Immunology* 38(1):68-74.

Klein L, Hinterberger M, Wirnsberger G, Kyewski B (2009). Antigen presentation in the thymus for positive selection and central tolerance induction. *Nature Reviews Immunology* 9(12):833-844.

Kobayashi SD, Voyich JM, Burlak C, DeLeo FR (2005). Neutrophils in the innate immune response *Archivum Immunologiae et Therapia Experimentalis* 53:505–517.

Kou X, Qi S, Dai W, Luo L, Yin Z (2011). Arctigenin inhibits lipopolysaccharide-induced iNOS expression in RAW264.7 cells through suppressing JAK-STAT signal pathway *International Immunopharmacology* 11:1095–1102.

Kraft S, Kinet JP (2007). New developments in Fc ϵ RI regulation, function and inhibition. *Nature Reviews Immunology* 7:365–378.

Krystel-Whittemore M, Dileepan KN and Wood JG (2016) Mast Cell: A Multi-Functional Master Cell. *Frontiers in Immunology* 6:620.

Kuijpers TW, Baars PA, Dantin C, van den Burg M, van Lier RA, Roosnek E (2008). Human NK cells can control CMV infection in the absence of T cells. *Blood* 112: 914–915.

- Kumar D, Arya V, Kaur R, Bhat ZA, Gupta VK, Kumar V (2012). A review of immunomodulators in the Indian traditional health care system. *Journal of Microbiology, Immunology and Infection* 45(3):165-84
- Kumar V, Sharma A (2010). Neutrophils: Cinderella of innate immune system. *International Immunopharmacology* 10(11):1325-34.
- Kun L, Li L (2016). Therapeutic Strategies towards Allergic Diseases. *International Journal of Allergy Medications* 2:011.
- Kuna P, Jurkiewicz D, Czarnecka-Operacz MM, Pawliczak R, Woroń J, Moniuszko M, Emeryk A (2016). The role and choice criteria of antihistamines in allergy management – expert opinion. *Postepy Dermatologii Alergologii* 33(6): 397–410.
- Kurtin S (2012). Myeloid Toxicity of Cancer Treatment. *Journal of the Advance Practitioner in Oncology* 3(4): 209–224.
- Labro MT (2005). Influence of antibacterial drugs on the immune system. In: Nijkamp FP, Parnham MJ (Eds.), *Principles of Immunopharmacology*, 2: 407-439.
- Lacy P, Abdel-Latif D, Steward M, Musat-Marcu S, Man SF, Moqbel R (2003). Divergence of mechanisms regulating respiratory burst in blood and sputum eosinophils and neutrophils from atopic subjects. *The Journal of Immunology* 170:2670–2679.
- Lallana EC, Fadul CE (2011). Toxicities of Immunosuppressive Treatment of Autoimmune Neurologic Diseases. *Current Neuropharmacology* 9(3): 468–477.
- Lanier LL (2005). NK cell recognition. *Annual Review of Immunology* 23: 225–274.
- Latiff AH, Kerr MA (2007). The clinical significance of immunoglobulin A deficiency. *Annals of Clinical Biochemistry* 44: 131–139.
- Lee DY, Choo BK, Yoon T, Cheon MS, Lee HW, Lee AY, Kim HK (2009) Anti-inflammatory effects of *Asparagus cochinchinensis* extract in acute and chronic cutaneous inflammation. *Journal of Ethnopharmacology* 121:28–34.

Lehman HK (2015). Autoimmunity and Immune Dysregulation in Primary Immune Deficiency Disorders. *Current Allergy and Asthma Reports* 15(9): 53.

Lerner A, Jeremias P, Matthias T (2015). The World Incidence and Prevalence of Autoimmune Diseases is Increasing *International Journal of Celiac Disease* 3(4):151-155.

Li J, Huston G, Swain SL (2003). IL-7 promotes the transition of CD4 effectors to persistent memory cells. *The Journal Experimental Medicine* 198(12):1807–15.

Liang B, Gardner D, Griswold D, Song XR (2007). Protection against Lipopolysaccharide-induced Death by An Anti-Interleukin-6 Monoclonal Antibody. *American Journal of Immunology* 3(1):4-9.

Lieberman P, Nicklas RA, Oppenheimer J, Kemp SF, Lang DM, Bernstein DI, Bernstein JA, Burks AW, Feldweg AM, Fink JN, Greenberger PA, Golden DB, James JM, Kemp SF, Ledford DK, Lieberman P, Sheffer AL, Bernstein DI, Blessing-Moore J, Cox L, Khan DA, Lang D, Nicklas RA, Oppenheimer J, Portnoy JM, Randolph C, Schuller DE, Spector SL, Tilles S, Wallace D (2010). The diagnosis and management of anaphylaxis practice parameter: 2010 update. *Journal of Allergy and Clinical Immunology* 126(3):477- 80.

Lindblom A, Heyman M, Gustafsson I, Norbeck O, Kaldensjö T, Vernby A, Henter JJ, Tolfvenstam T, Broliden K (2008). Parvovirus B19 infection in children with acute lymphoblastic leukemia is associated with cytopenia resulting in prolonged interruptions of chemotherapy. *Clinical Infectious Diseases* 46(4): 528-36.

Lipper GM, Arndt KA, Dover JS (2000). Recent therapeutic advances in dermatology. *JAMA* 283(2):175-177.

Liu FT, Goodarzi H, Chen HY (2011). IgE, mast cells, and eosinophils in atopic dermatitis. *Clinical Reviews in Allergy and Immunology* 41(3):298-310.

Lorenz D, Wiesner B, Zipper J, Winkler A, Krause E, Beyermann M, Lindau M, Bienert MJ (1998). Mechanism of peptide-induced mast cell degranulation. Translocation and patch-clamp studies. *The Journal of General Physiology* 112(5): 577-91.

Lowenberg M, Tuynman J, Bilderbeek J, Gaber T, Buttgereit F, van Deventer S, Peppelenbosch M, Hommes D. Rapid immunosuppressive effects of glucocorticoids mediated through Lck and Fyn. *Blood* 106(5): 1703-1710.

Lowes DA, Webster NR, Murphy MP, Galley HF (2013). Antioxidants that protect mitochondria reduce interleukin-6 and oxidative stress, improve mitochondrial function, and reduce biochemical markers of organ dysfunction in a rat model of acute sepsis. *British Journal of Anaesthesia* 110(3):472-480.

Luckheeram RV, Zhou R, Verma AD, Xia B (2012). CD4⁺T Cells: Differentiation and Functions. *Clinical and Developmental Immunology*. doi: 10.1155/2012/925135.

Luebke RW, Holsapple MP, Ladics GS, Luster MI, Selgrade MJ, Smialowicz RJ, Woolhiser MR, Germolec DR (2006). Immunotoxicogenomics: The potential of genomics technology in the immunotoxicity risk assessment process. *Toxicological Sciences* 94(1): 22–27.

Ma HD, Deng YR, Tian Z, Lian ZX (2013). Traditional Chinese medicine and immune regulation. *Clinical Review of Allergy and Immunology* 44(3):229-41.

MacLeod MKL, Kappler JW, Marrack P (2010). Memory CD4 T cells: generation, reactivation and re-assignment *Immunology* 130(1): 10–15.

Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440:237–41.

Maslan J, Mims JW (2014). What is asthma? Pathophysiology, demographics, and health care costs. *Otolaryngologic Clinics of North America* 47(1): 13-22.

Mazmanian SK, Round JL, Kasper DL (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 453:620–625.

Mbugi EV, Chilongola JO (2010). Allergic Disorders in Africa and Africans: Is It Primarily a Priority? *World Allergy Organization Journal*. 3(5): 175–181.

McCann SE, Freudenheim JL, Marshall JR, Graham S (2003). Risk of human ovarian cancer is related to dietary intake of selected nutrients, phytochemicals and food groups. *Journal of Nutrition* 133: 1937–1942.

McCusker C, Warrington R (2011). Primary immunodeficiency. *Allergy, Asthma and Clinical Immunology* 7(Suppl 1): S11.

McNeil BD, Pundir P, Meeker S, Han L, Undem BJ, Kulka M, Dong X (2015). Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature* 519(7542): 237–41.

Meagher LC, Cousin JM, Seckl JR, Haslett C. (1996). Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *Journal of Immunology* 156: 4422–44428.

Medeiros R, Otuki MF, Avellar MC, Calixto JB (2007). Mechanisms underlying the inhibitory actions of the pentacyclic triterpene alpha-amyrin in the mouse skin inflammation induced by phorbol ester 12-O-tetradecanoylphorbol-13-acetate. *European Journal of Pharmacology* 559: 227–235.

Melo CM, Carvalho KM, Neves JC, Morais TC, Rao VS, Santos FA, Brito GA, Chaves MH (2010). Alpha, beta-amyrin, a natural triterpenoid, ameliorates L-arginine-induced acute pancreatitis in rats. *World Journal of Gastroenterology* 16:4272– 4280.

Metcalfe DD, Peavy RD, Gilfillan AM (2009) Mechanisms of mast cell signaling in anaphylaxis. *Journal of Allergy and Clinical Immunology* 124(4): 639–648.

Metz M, Maurer M (2009). Innate immunity and allergy in the skin. *Current Opinion in Immunology* 21:687–693.

Metz M, Siebenhaar F, Maurer M (2008). Mast cell functions in the innate skin immune system. *Immunobiology* 213:251-260.

Misra HP, Fridovich I (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *The Journal of Biological Chemistry* 247(10):3170-3175.

Mitra S, Keswani T, Ghosh N, Goswami S, Datta A, Das S, Maity S, Bhattacharyya A (2013). Copper induced immunotoxicity promote differential apoptotic pathways in spleen and thymus. *Toxicology* 306:74–84.

Miyara M, Sakaguchi S (2007). Natural regulatory T cells: mechanisms of suppression. *Trends in Molecular Medicine* 13(3):108-16.

Modlin RL (2012). Innate Immunity: Ignored for Decades, but Not Forgotten. *Journal of Investigative Dermatology* 132:882–886.

Moore KJ, Freeman MW (2008). Targeting Innate Immunity for CV Benefit. *Drug Discovery Today: Therapeutic Strategies* 5(1): 15–23.

Moreau RA, Whitaker BD, Hicks KB (2002). Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Progress in Lipid Research* 41(6): 457-500.

Muniz VS, Weller PF, Neves JS (2012). Eosinophil crystalloid granules: structure, function, and beyond. *Journal of Leukocyte Biology* 92(2):281-288.

Mygind N (2014). Allergic rhinitis. *Chemical Immunology Allergy* 100: 62–68.

Nashed B, Yeganeh B, HayGlass KT, Moghadasian MH (2005). Antiatherogenic effects of dietary plant sterols are associated with inhibition of proinflammatory cytokine production in Apo E-KO mice. *Journal of Nutrition* 135:2438–2444.

Nathan C (2006) Neutrophils and immunity: challenges and opportunities. *Nature Reviews. Immunology* 6:173–182.

Navarro A, De las Heras B, Villar A (2001). Anti-inflammatory and immunomodulating properties of a sterol fraction from *Sideritis foetens* CLEM. *Biological and Pharmaceutical Bulletin* 24(5):470–473.

Nes WD, Venkatramesh M (1999). Enzymology of Phytosterol Transformations. *Critical Reviews in Biochemistry and Molecular Biology* 34(2): 81–93.

Nessler N, Launey Y, Aninat C, Morel F, Mallédant Y, Seguin P (2012). Clinical review: The liver in sepsis. *Critical Care*. 16(5):235.

Nissinen MJ, Gylling H, Miettinen TA (2006). Effects of plant stanol esters supplied in a fat free milieu by pastilles on cholesterol metabolism in colectomized human subjects. *Nutrition, Metabolism and Cardiovascular Diseases* 16:426-435.

Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamas V, Piraino J, Huttner K, Gallo RL (2001). Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 414:454-457.

Nong X, Yang YJ, Yang GY, Chen FZ, Tang M, Wang G (2017) Toxicity of Stigmasterol Isolated from Crofton Weed, *Eupatorium adenophorum* Spreng. Against a Rabbit Ear Mite, *Psoroptes cuniculi*. *Pakistan Journal of Zoology* 49(4): 1197-1200.

Normen L, Frohlich JJ, Trautwein EA (2004). Role of Plant Sterols in Cholesterol Lowering. In: Dutta PC, ed. *Plant Sterols: Analytical, Nutritional, and Safety Aspects as Functional Food*. New York: Marcel Dekker 243-315.

Notarangelo LD (2010). Primary immunodeficiencies. *Journal of Allergy and Clinical Immunology* 125: S182-94.

Nualkaew S, Padee P, Talubmook C (2015). Hypoglycemic activity in diabetic rats of stigmasterol and sitosterol-3-O- β -D-glucopyranoside isolated from *Pseuderanthemum palatiferum* (Nees) Radlk. leaf extract. *Journal of Medicinal Plants Research* 9(20):629-63.

Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM (2015). The generation of antibody-secreting plasma cells. *Nature Reviews Immunology* 15:160-171.

Nweze NE (2012). *In vitro* anti-trypanosomal activity of *Morinda lucida* leaves. *African Journal of Biotechnology* 11(7): 1812-1817

Ogawa Y, Grant JA (2007). Mediators of anaphylaxis. *Immunology and Allergy Clinics of North America* 27:249–260.

Ohkaru Y, Arai N, Ohno H, Sato S, Sakakibara Y, Suzuki H, Aritoshi S, Akimoto S, Ban T, Tanihata J, Tachiyashiki K, Imaizumi K (2010). Acute and Subacute Effects of Dexamethasone on the Number of White Blood Cells in Rats. *Journal of Health Science* 56(2):215-220.

Opelz G, Dohler B (2009). Influence of immunosuppressive regimens on graft survival and secondary outcomes after kidney transplantation. *Transplantation* 87:795-802.

Ostlund RE (2002). Phytosterols in human nutrition. *Annual Review Nutrition* 22:533–549.

Othman RA, Moghadasian MH (2011). Beyond cholesterol-lowering effects of plant sterols: Clinical and experimental evidence of anti-inflammatory properties. *Nutrition Reviews* 69(7): 371-82.

Otuki MF, Schwob O, Prudente AS, Zaltsman I, Shai Meretski S, Segal J, Yedgar S, Rosenblat G (2011). Attenuation of experimental TPA-induced dermatitis by acetylenic acetogenins is associated with inhibition of PLA2 activity. *European Journal of Pharmacology* 672:175–179.

Otuki MF, Vieira-Lima F, Malheiros A, Yunes RA, Calixto JB (2005). Topical anti-inflammatory effects of the ether extract from *Protium kleinii* and alpha-amyrin pentacyclic triterpene. *European Journal of Pharmacology* 507:253–259.

Panda S, Jafri M, Kar A, Meheta BK (2009). Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from *Butea monosperma*. *Fitoterapia* 80(2): 123-6.

Pan-Hammarström Q, Hammarström L (2008). Antibody deficiency diseases. *The European Journal of Immunology* 38(2):327-333.

Park SJ, Lee HA, Kim JW, Lee BS, Kim EJ (2012). *Platycodon grandiflorus* alleviates DNCB-induced atopy-like dermatitis in NC/Nga mice. *Indian Journal of Pharmacology* 44(4):469-74.

Parkin J, Cohen B (2001). An overview of the immune system. *Lancet* 357(9270):1777-89.

Parra-Delgado H, Ruiz GG, Camacho AN, Martínez-Vázquez M (2004). Anti-inflammatory activity of some extracts and isolates from *Leonotis nepetaefolia* on TPA-induced Edema. *Journal of the Mexican Chemical Society* 48: 293-295.

Pasare C, Medzhitov R (2004) Toll-like receptors: linking innate and adaptive immunity. *Microbes and Infection* 6:1382–1387.

Passos GF, Medeiros R, Marcon R, Nascimento AF, Calixto JB, Pianowski LF (2013). The role of PKC/ERK1/2 signaling in the anti-inflammatory effect of tetracyclic triterpene euphol on TPA-induced skin inflammation in mice. *European Journal of Pharmacology* 698(1-3):413-20.

Pawankar R (2014). Allergic diseases and asthma: a global public health concern and a call to action. *World Allergy Organization Journal* 7:55.

Pawankar R, Canonica GW, Holgate ST, Lockey RF (2011). The World Allergy Organization (WAO) White Book on Allergy. World Allergy Organization: 1-216.

Peiser L, Mukhopadhyay S, Gordon S (2002). Scavenger receptors in innate immunity. *Current Opinions in Immunology* 14:123-128.

Pelaia G, Canonica GW, Matucci A, Paolini R, Triggiani M, Paggiaro P (2017). Targeted therapy in severe asthma today: focus on immunoglobulin E. *Drug Design, Development and Therapy* 11:1979-1987.

Peres MTL, Delle Monache F, Pizzolatti MG, Santos ARS, Beirith A, Calixto JB, Yunes RA (1998). Analgesic Compounds of *Croton urucurana* Baillon. Pharmaco-chemical Criteria Used in their Isolation. *Phytotherapy Research* 12:209–211.

Peters-Golden M, Gleason MM, Togias A (2006). Cysteinyl leukotrienes: multifunctional mediators in allergic rhinitis. *Clinical and Experimental Allergy* 36(6): 689–703.

Piironen V, Lindsay DG, Miettinen TA, Toivo J, Lampi AM (2000). Plant Sterols: Biosynthesis, Biological Function and Their Importance to Human Nutrition. *Journal of the Science of Food and Agriculture* 80: 939-966.

Ponce RA, Gelzleichter T, Haggerty HG, Heidel S, Holdren MS, Lebrech H, Mellon RD, Pallardy M (2014). Immunomodulation and lymphoma in humans. *Journal of Immunotoxicology* 1:1-12.

Possa SS, Leick EA, Prado CM, Martins MA, Tibério IFLC (2013). Eosinophilic Inflammation in Allergic Asthma. *Frontiers in Pharmacology* 4: 46.

Pynn MC, C. A. Thornton CA, and G. A Davies GA (2012). Asthma Pathogenesis. *Pulmao RJ* 21(2): 11-17.

Pynn MC, Thornton CA, Davies GA (2012). Asthma Pathogenesis. *Pulmao RJ* 21(2):11-17.

Quinton LJ, Jones MR, Robson BE, Mizgerd JP (2009). Mechanisms of the Hepatic Acute-Phase Response during Bacterial Pneumonia. *Infection and Immunity* 77(6): 2417–2426.

Ramos PS, Shedlock AM, Langefeld CD (2015). Genetics of autoimmune diseases: insights from population genetics. *Journal of Human Genetics* 60:657–664.

Rao K, Brown M (2008). Mast cells: multifaceted immune cells with diverse roles in health and disease. *Annals of the New York Academy Sciences* 1143: 83-104.

Rauh LK, Horinouchi CD, Loddi AM, Pietrovski EF, Neris R, Souza-Fonseca-Guimarães F, Buchi DF, Biavatti MW, Otuki MF, Cabrini DA (2011). Effectiveness of *Vernonia scorpioides* ethanolic extract against skin inflammatory processes. *Journal of Ethnopharmacology* 138:390– 397.

Ray A, Khare A, Krishnamoorthy N, Qi Z, Ray P (2010). Regulatory T cells in many flavors control asthma. *Mucosal Immunology* 3(3): 216-229.

Reust CE (2013). Evaluation of primary immunodeficiency disease in children. *American Academy of Family Physicians* 87(11):773-8.

Rice AL, Sacco L, Hyder A, Black RE (2000). Malnutrition as an underlying cause of childhood deaths associated with infectious diseases in developing countries. *Bulletin of the World Health Organization* 78(10):1207-21.

Ripoll E, Merino A, Grinyó JM, Torras J (2013). New Approaches for the Treatment of Lupus Nephritis in the 21st Century. *Immunotherapy*. 5(10):1089-1101.

Rivera J, Gilfillan AM (2006). Molecular regulation of mast cell activation. *Journal of Allergy and Clinical Immunology* 117(6):1214-25.

Riyazzudin M, Sharma M, Kaur G, Chaudhry N (2016). A review on Therapeutic Potential of plant derived natural compounds. *Bio-Chemiae Acta* 1(1):30-34.

Robinson DS (2004). The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? *Journal of Allergy and Clinical Immunology* 114(1):58-65.

Rodgers E (2011). Nutritionally acquired immune deficiency syndromes (NAIDS): common but often not diagnosed early. *Pacific Health Dialog* 17(1):149-53.

Rosas LV, Cordeiro MSC, Campos FR, Nascimento SKR, Januário AH, França SC, Nomizo A, Toldo MPA, Albuquerque S, Pereira PS (2007). *In vitro* evaluation of the cytotoxic and trypanocidal activities of *Ampelozizyphus amazonicus* (Rhamnaceae). *Brazilian Journal of Medical and Biological Research* 40(5): 663-670.

Rosenwasser LJ (2011) Mechanisms of IgE inflammation. *Current Allergy and Asthma Reports* 11:178–183.

Ross LE, van Wagtendonk WJ, Wulzen R (1949). Proceedings of the Society for Experimental Biology and Medicine, 71, 281–83, 1949.

Rukwied R, Lischetzki G., McGlone F., Heyer G., Schmelz M (2000). Mast cell mediators other than histamine induce pruritus in atopic dermatitis patients: a dermal microdialysis study. *British Journal of Dermatology* 142:1114–1120.

Ryan E, Galvin K, O'Connor TP, Maguire AR, O'Brien NM (2007). Phytosterol, squalene, tocopherol content and fatty acid profile of selected seeds, grains, and legumes. *Plant Foods for Human Nutrition* 62: 85–91.

Saadawi BS, Jalil J, Jasamai M, Jantan I (2012). Inhibitory Effects of Acetylmeleodorinol, Chrysin and Polycarpol from *Mitrella kentii* on Prostaglandin E2 and Thromboxane B2 Production and Platelet Activating Factor Receptor. *Molecules* 17:4824-4835.

Saag KG, Teng GG, Patkar NM, Anuntiyo J, Finney C, Curtis JR, Paulus HE, Mudano A, Pisu M, Elkins-Melton M, Outman R, Allison JJ, Suarez Almazor M, Bridges SL Jr, Chatham WW, Hochberg M, MacLean C, Mikuls T, Moreland LW, O'Dell J, Turkiewicz AM, Furst DE; American College of Rheumatology (2008). Recommendations for the use of nonbiologic and biologic disease-modifying antirheumatic drugs in rheumatoid arthritis. *Arthritis and Rheumatology* 59(6):762-84.

Saint-Mezard P, Krasteva M, Chavagnac C, Bosset S, Akiba H, Kehren J, Kanitakis J, Kaiserlian D, Nicolas JF, Berard F (2003). Afferent and efferent phases of allergic contact dermatitis (ACD) can be induced after a single skin contact with haptens: evidence using a mouse model of primary ACD. *Journal of Investigative Dermatology* 120(4):641–647.

Saint-Mezard P, Rosieres A, Krasteva M, Berard F, Dubois B, Kaiserlian D, Nicolas JF (2004). Allergic contact dermatitis. *European Journal Dermatology* 14(5): 284-95.

Samarghitean C, Valiaho J, Vihinen M (2007). IDR knowledge base for primary immunodeficiencies. *Immunome Research* 3:6.

Sansonetti PJ (2004). War and peace at mucosal surfaces. *Nature Reviews Immunology* 4: 953–964.

Santos ARS, Niero R, Filho VC, Yunes RA, Pizzolatti MG, Delle Monache F, João B. Calixto JB (1995). Antinociceptive Properties of Steroids Isolated from *Phyllanthus corcovadensis* in Mice. *Planta Medica* 61(4):329-332.

Saxena R, Sharma A, Bharti M, Rathore M (2012). Immunomodulator A New Horizon: An overview. *Journal of Pharmacy Research* 5(4):2306-2310.

Schacke H, Docke WD, Asadullah K (2002) Mechanisms involved in the side effects of glucocorticoids. *Pharmacology & Therapeutics* 96(1): 23– 43.

Schmitt EG, Williams CB (2013). Generation and function of induced regulatory T cells. *Frontiers in Immunology* 4:152.

Schock BC, Young IS, Brown V, Fitch PS, Shields MD, Ennis M (2003). Antioxidants and oxidative stress in BAL fluid of atopic asthmatic children. *Pediatric Research* 53(3):375-81.

Schroeder HW, Cavacini L (2010) Structure and function of immunoglobulins. *Journal of Allergy and Clinical Immunology* 125: S41-52.

Seifert R (2015). How do basic secretagogues activate mast cells? *Naunyn-Schmiedeberg's Archives of Pharmacology* 388(3):279-81.

Serbina NV, Jia T, Hohl TM, Pamer EG (2008). Monocyte-mediated defense against microbial pathogens. *Annual Reviews of Immunology* 26:421–452.

Shabbir A, Arshad HA, Shahzad M, Shamsi S, Ashraf MI (2016). Immunomodulatory activity of mefenamic acid in mice models of cell-mediated and humoral immunity. *Indian Journal of Pharmacology* 48(2): 172–178.

Shafizadeh M, Rajaba A, Imran Khan M, Ostadhadi S, Rastegar H, Dehpour (2014). Anti-pruritic activity of pioglitazone on serotonin-induced scratching in mice: Possible involvement of PPAR-gamma receptor and nitric oxide. *European Journal of Pharmacology* 744:103-107.

Shamri R, Xenakis JJ, Spencer LA (2011). Eosinophils in innate immunity: an evolving story. *Cell and Tissue Research* 343(1):57–83.

Shanthakumar B, Sathish M, Suresh AJ (2013). *In Vitro* Anti-Oxidant Activity of Extracts and Stigmasterol from Leaves of *Clerodendrum inerme* Linn. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* 4(4): 1411-1418.

Shapiro N, Howell MD, Bates DW, Angus DC, Ngo L, Talmor D (2006). The association of sepsis syndrome and organ dysfunction with mortality in emergency

department patients with suspected infection. *Annals of Emergency Medicine* 48(5):583-90.

Shelley O, Murphy T, Paterson H, Mannick JA, Lederer JA (2003). Interaction between the innate and adaptive immune systems is required to survive sepsis and control inflammation after injury. *Shock* 20:123–129.

Siegmund B, Lear-Kaul KC, Faggioni R, Fantuzzi G (2002). Leptin deficiency, not obesity, protects mice from Con A-induced hepatitis. *European. Journal of Immunology* 32:552–560.

Silva MT (2010). When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *Journal of Leukocyte Biology* 87(1):93-106.

Simons FER (2008). Anaphylaxis. *Journal of Allergy and Clinical Immunology* 121 (Suppl. 2): S402–S407

Simons KJ, Simons FER (2010). Epinephrine and its use in anaphylaxis: current issues. *Current Opinion in Allergy and Clinical Immunology* 10(4):354–361.

Simons, FER. (2010). Anaphylaxis. *Journal of Allergy and Clinical Immunology* 125(2 Suppl 2): S161-81.

Sin B, Togias A (2011). Pathophysiology of Allergic and Nonallergic Rhinitis. *Proceedings of the American Thoracic Society* 8(1):106-114.

Singh N, Verma P, Pandey BR, Bhalla M (2012). Therapeutic Potential of *Ocimum sanctum* in Prevention and Treatment of Cancer and Exposure to Radiation: An Overview. *International Journal of Pharmaceutical Sciences and Drug Research* 4(2): 97-104.

Sinha AK (1972). Colorimetric assay of catalase. *Analytical Biochemistry* 47(2):389-94.

Skibska B, Józefowicz-Okonkwo G, Goraca A (2006). Protective effects of early administration of alpha-lipoic acid against lipopolysaccharide-induced plasma lipid peroxidation *Pharmacological Reports* 58:399-404.

Skoner DP (2001). Allergic rhinitis: Definition, epidemiology, pathophysiology, detection, and diagnosis. *Journal of Allergy and Clinical Immunology* 108(1 Suppl): S2-8.

Sneller MC (2001). Common variable immunodeficiency. *The American Journal of the Medical Sciences* 321: 42-48.

Sperandio B, Fischera N, Sansonettia PJ (2015). Mucosal physical and chemical innate barriers: Lessons from microbial evasion strategies. *Seminars in Immunology* 27(2):111-118.

Stahn C, Buttgereit F (2008). Genomic and non-genomic effects of glucocorticoids. *Nature Clinical Practice Rheumatology* 4:525-33.

Steele TA (2002). Chemotherapy-induced immunosuppression and reconstitution of immune function. *Leukemia Research* 26:411–414.

Subhashini, Chauhan PS, Singh R (2016). Ovalbumin-induced allergic inflammation lead to structural alterations in mouse model and protective effects of intranasal curcumin: A comparative study”, *Allergologia et Immunopathologia* 44(3):246-56.

Sugimoto Y, Umakoshi K, Nojiri N, Kamei C (1998). Effects of histamine H1 receptor antagonists on compound 48/80-induced scratching behaviour in mice. *European Journal Pharmacology* 351:1-5.

Sun JC, Lanier LL (2009) Natural killer cells remember: An evolutionary bridge between innate and adaptive immunity? *European Journal of Immunology* 39: 2059–2064.

Takahama Y (2006). Journey through the thymus: stromal guides for T-cell development and selection. *Nature Reviews Immunology* 6(2):127–135.

Takeda K, Kaisho T, Akira S (2003). Toll-like receptors. *Annual Review of Immunology* 21:335–376.

Takeuchi O, Akira S (2010). Pattern recognition receptors and inflammation. *Cell* 140(6):805-20.

Tanaka M, Miyake Y (2007). Apoptotic cell clearance and autoimmune disorder. *Current Medicinal Chemistry* 14(27):2892-7.

Tasker JG, Herman JP (2011). Mechanisms of rapid glucocorticoid feedback inhibition of the hypothalamic-pituitary-adrenal axis. *Stress* 14(4):398-406

Thornton SJ, Wong IT, Neumann R, Kozlowski P, Wasan KM (2011). Dietary supplementation with phytosterol and ascorbic acid reduces body mass accumulation and alters food transit time in a diet-induced obesity mouse model. *Lipids in Health and Disease* 10:107.

Thurmond RL, Kazerouni K, Chaplan SR, Greenspan AJ (2015). Antihistamines and itch. *Handbook of Experimental Pharmacology* 226:257-90.

To T, Stanojevic S, Moores G, Gershon AS, Bateman ED, Cruz AA, Boulet LP (2012). Global asthma prevalence in adults: findings from the cross-sectional world health survey. *BMC Public Health* 12:204.

Togias A (2003). H1-receptors: localization and role in airway physiology and in immune functions. *Journal of Allergy and Clinical Immunology* 112(4 Suppl): S60–S68.

Trautwein EA, Demonty I (2007). Phytosterols: natural compounds with established and emerging health benefits. *Oilseeds fats Crops Lipids* 14: 259-266.

Tsicopoulos A, Sénéchal S, de Nadai P, Ralainirina N, Tonnel AB (2003). Chemokines in cutaneous allergic reactions: the SCID model as a powerful tool to evaluate their interest as therapeutical targets. *Revue française d'allergologie et d'immunologie clinique* 43:206–210.

Tuchinda P, Gaspari AA (2010). Langerhans cells in allergic contact dermatitis. *Giornale Italiano di Dermatologia e Venereologia* 145(6):747-62.

Underhill DM, Ozinsky A (2002). Toll-like receptors: key mediators of microbe detection. *Current Opinions in Immunology* 14:103-110.

Unsworth DJ (2008). Complement deficiency and disease. *Journal of Clinical Pathology* 61:1013-7.

Vane J.R (2000). The Mechanism of Action of Anti-Inflammatory Drugs. *Advances in Eicosanoid Research* 31:1-23.

Vega MA, Corbí AL (2006). Human macrophage activation: Too many functions and phenotypes for a single cell type *Immunologia* 25(4): 248-272.

Vo TS, Kim SK (2013). Down-regulation of histamine-induced endothelial cell activation as potential anti-atherosclerotic activity of peptides from *Spirulina maxima*.

European Journal of Pharmaceutical Sciences 50: 198–207.

von Bernuth H, Picard C, Jin Z, Pankla R, Xiao H, Ku CL, Chrabieh M, Mustapha IB, Ghandil P, Camcioglu Y, Vasconcelos J, Sirvent N, Guedes M, Vitor AB, Herrero-Mata MJ, Aróstegui JJ, Rodrigo C, Alsina L, Ruiz-Ortiz E, Juan M, Fortuny C, Yagüe J, Antón J, Pascal M, Chang HH, Janniere L, Rose Y, Garty BZ, Chapel H, Issekutz A, Maródi L, Rodriguez-Gallego C, Banchereau J, Abel L, Li X, Chaussabel D, Puel A, Casanova JL (2008). Pyogenic bacterial infections in humans with MyD88 deficiency. *Science* 321:691-6.

Wallace DV, Dykewicz MS, Bernstein DI, Blessing-Moore J, Cox L, Khan DA, Lang DM, Nicklas RA, Oppenheimer J, Portnoy JM, Randolph CC, Schuller D, Spector SL, Tilles SA; Joint Task Force on Practice; American Academy of Allergy; Asthma & Immunology; American College of Allergy; Asthma and Immunology; Joint Council of Allergy, Asthma and Immunology. The diagnosis and management of rhinitis: An updated practice parameter. *Journal of Allergy and Clinical Immunology* 122(2 Suppl): S1-84.

Walport MJ, Davies KA, Botto M (1998). C1q and systemic lupus erythematosus. *Immunobiology* 199: 265–85.

Wang B, Esche C, Mamelak A, Freed I, Watanabe H, Sauder DN (2003). Cytokine knockouts in contact hypersensitivity research. *Cytokine and Growth Factor Reviews* 14(5):381–389.

Wang D, Ma Y, Wang J, Liu X, Fang M (2013). Natural Killer cells in Innate Defense against Infective Pathogens. *Journal of Clinical and Cellular Immunology* S13:006. doi:10.4172/2155-9899.S13-006.

Ward PP, Conneely OM (2004). Lactoferrin: role in iron homeostasis and host defense against microbial infection. *Biometals* 17:203–208.

Warrington R., Watson W., Kim HL, Antonetti FR (2011). An introduction to immunology and immunopathology. *Allergy, Asthma & Clinical Immunology* 7(Suppl 1): S1.doi:10.1186/1710-1492-7-S1-S1.

Wickelgren I (2006). Targeting the Tolls. *Science*. 312:184–187.

Wille JJ, Kydonieus A (2003). Palmitoleic acid isomer (C16:1 Δ 6) in human skin sebum is effective against gram-positive bacteria. *Skin Pharmacology Applied Skin Physiology* 16:176–187.

Wills-Karp M (2004). Interleukin-13 in asthma pathogenesis. *Immunological Reviews* 202: 175–190.

Winter WE, Hardt NS, Fuhrman S (2000). Immunoglobulin E Importance in Parasitic Infections and Hypersensitivity Responses. *Archives of Pathology Laboratory Medicine* 124(9):1382-5.

Wollenberg A, Seba A, Antal AS (2014). Immunological and molecular targets of atopic dermatitis treatment. *British Journal of Dermatology* 170 Suppl 1:7-11.

Wood LG, Fitzgerald DA, Gibson PC, Garg ML (2000). Lipid peroxidation as determined by plasma isoprostanes is related to disease severity in mild asthma *Lipids* 35:967.

Woof JM, Kerr MA (2006). The function of immunoglobulin A in immunity. *The Journal of Pathology* 208(2): 270–82.

Woof JM, Kerr MA, Moro I, Mestecky JR (2004). Mucosal immunoglobulins. In: Mestecky JR, Bienenstock J, Lamm ME, Mayer L, McGhee JR, Strober W, eds. *Mucosal Immunology* (Chapter 9). 3rd edn. San Diego: Academic Press Inc. 153–81.

World Health Organization. Chronic Respiratory Diseases and Arthritis Team WHO/WAO Meeting on the Prevention of Allergy and Allergic Asthma (2002: Geneva, Switzerland). (2003) Prevention of allergy and allergic asthma WHO/NMH/MNC/CRA/03.2.

World Health Organization. Country Profile of Environmental Burden of Disease: Ghana. In: WHO, ed. *Public Health and the Environment* (2009). Geneva: World Health Organization.

Wynn TA (2015). Type 2 cytokines: mechanisms and therapeutic strategies”. *Nature Reviews Immunology* 15: 271-282.

Xie Y, Merkel OM (2015). Pulmonary Delivery of sRNA via Polymeric Vectors as Therapies of Asthma. *Arch Pharm Chemistry in Life Sciences* 348(10): 681–688.

Xu X, Bittman R, Duportail G, Heissler D, Vilcheze C, London E (2001). Effect of the Structure of Natural Sterols and Sphingolipids on the Formation of Ordered Sphingolipid/Sterol Domains (Rafts) Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebroside, and ceramide. *The Journal of Biological Chemistry* 276(36):33540–33546.

Yamamoto K (2004). Mechanisms of Autoimmunity. *Japan Medical Association Journal* 47(9):403–406.

Yamashita H, Michibata Y, Mizukami H, Ogihara Y, Morita A, Nose M. (2005). Dermal mast cells play a central role in the incidence of scratching behaviour in mice induced by multiple application of the hapten, 2,4,6-trinitrochlorobenzene. *Experimental Dermatology* 14:438–444.

Yang B1, Li J, Liu X, Ma L, Deng L, Liu J, Liu Z, Ji Q (2013). Herbal Formula-3 inhibits food allergy in rats by stabilizing mast cells through modulating calcium mobilization. *International Immunopharmacology* 17:576–584.

Yang Y, Yin C, Pandey A, Abbot D, Sasseti C, Kelliher MA (2007). NOD2 pathway activation by MDP or Mycobacterium tuberculosis infection involves the stable polyubiquitination of Rip2. *The Journal of Biological Chemistry* 282:36223–9.

Yi JS, Cox MA, Zajac AJ (2010) Immunology. T-cell exhaustion: characteristics, causes and conversion 129(4): 474–481.

Yin H, Li X, Gong Q, Jin X, Gu H, Yuan B, Zhang B, Zheng F, Gong F, Zhu J (2010). Heme oxygenase-1 upregulation improves lipopolysaccharide-induced acute lung injury involving suppression of macrophage migration inhibitory factor. *Molecular Immunology* 47(15): 2443–2449.

Yoshida Y, Niki E (2003). Antioxidant effects of phytosterol and its components. *Journal of Nutritional Science and Vitaminology* 49(4): 277–280.

Yosipovitch G, Bernhard JD (2013). Chronic pruritus. *New England Journal of Medicine* 368:1625–1634.

You Q, Holt M, Yin H, Li G, Hu CJ, Ju C (2013). Role of hepatic resident and infiltrating macrophages in liver repair after acute injury. *Biochemical. Pharmacology* 86: 836–843.

Yuk JE, Woo JS, Yun CY, Lee JS, Kim JH, Song GY, Yang EJ, Hur IK, Kim IS (2007). Effects of lactose-beta sitosterol and beta-sitosterol on ovalbumin-induced lung inflammation in actively sensitized mice. *International Immunopharmacology* 7(12):1517–1527.

Zar HJ, Ehrlich RI, Workman L, Weinberg EG (2007). The changing prevalence of asthma, allergic rhinitis and atopic eczema in African adolescents from 1995 to 2002. *Pediatric Allergy and Immunology* 18:560–565.

Zar HJ, Stickells D, Toerien A, Wilson D, Klein M, Bateman ED (2001). Changes in fatal and near-fatal asthma in an urban area of South Africa from 1980-1997. *European Respiratory Journal* 18(1):33-37.

Zare A, Farzaneh P, Pourpak Z, Zahedi F, Moin M, Shahabi S, Hassan ZM (2008). Purified aged garlic extract modulates allergic airway inflammation in BALB/c mice. *Iranian Journal of Allergy, Asthma and Immunology*. 7(3): 133-141.

Zasloff M (2002). Antimicrobial peptides of multicellular organism. *Nature* 415:389–95.

Zhang N, Bevan M (2011). CD8(+) T cells: foot soldiers of the immune system. *Immunity* 35(2):161-8.

Zheng GQ (1994). Cytotoxic terpenoids and flavonoids from *Artemisia annua*. *Planta Medica* 60(1): 54-57.

Zheng Y, Zhu D (2016). Molecular hydrogen therapy ameliorates organ damage induced by sepsis, *Oxidative Medicine and Cellular Longevity* doi: 10.1155/2016/5806057.

Zhou JC, Zhu Y, Chen Z, He S, Zheng S, Mo J, Liu X, Gong C, Hou B, Yang H (2015). Association of IgE-mediated allergen sensitivity and promoter polymorphisms of chemokine (C–C motif) ligand 5 gene in Han Chinese patients with allergic skin diseases. *Genes and Genomics* 37(5):451-458.

Zhu J, Paul WE (2010). Heterogeneity and plasticity of T helper cells. *Cell Research* 20(1):4–12.

Zuo L, Otenbaker NP, Rose BA, Salisbury KS (2013). Molecular mechanisms of reactive oxygen species-related pulmonary inflammation and asthma. *Molecular Immunology* 56(2): 57–63.

APPENDIX

1. DRUG PREPARATION AND ADMINISTRATION

50 g of polyethylene glycol (grade 4000) was dissolved in 100 ml distilled water and stirred continuously to form a clear 50 %w/v PEG solution. Stigmasterol was dissolved in 50 %w/v and given *via* intraperitoneal route for all *in vivo* treatments.

2. BUFFERS AND REAGENTS

2.1 BUFFERS FOR ANTIOXIDANT ASSAY

2.1.1 TRIS-HCL Buffer (pH 8.9)

Solution A: Dissolve 21.03 g Tris (hydroxymethyl) aminomethane in 1000 ml of distilled water

Solution B: Add 10 ml of 1 M HCL to distilled water, adjust to 100 ml

Mix 50 ml of solution A and 17.6 ml of solution B.

2.1.2 Carbonate bicarbonate Buffer (pH 10.2)

Dissolve 1.05 g of sodium bicarbonate and 9.275 g of sodium bicarbonate (anhydrous) in distilled water. Add distilled water to the 1 L mark.

2.2 TOLUIDINE BLUE

Stock Solution:

1 g of Toluidine Blue O was dissolved in 10 ml of 70 % alcohol and mixed gently.

1% Sodium Chloride:

0.5 g of sodium chloride was dissolved in 50 ml of distilled water. Solution was prepared freshly.

Working Solution:

5 ml stock Toluidine blue solution was dissolved in 45 ml of 1 % Sodium chloride. pH was adjusted to < 4 with glacial acetic acid. Working solution was prepared freshly and discarded after use.

2.3 10 % PHOSPHATE BUFFERED FORMALIN

0.8 g of sodium phosphate monobasic and 1.3 g of sodium phosphate dibasic was dissolved in 20 ml of 40 % formaldehyde. It was made up to 180 ml with distilled water and pH adjusted to 7.2.

3. ELISA PROTOCOL

1. Prepare serial dilutions of standard (VCAM-1, OVA sIgE or TNF α) with standard diluent.
2. Add sample: Set blank wells separately (Do not add sample and HRP-Conjugate reagent to blank comparison wells. All other steps should be performed same as testing sample wells). Add 40 μ l sample diluent to the testing sample wells, then add 10 μ l testing sample (sample final dilution is 5-fold). Gently mix.
3. Incubate: After closing plate with closure plate membrane, incubate for 30 min at 37°C.
4. Wash solution: Dilute wash 30-fold (or 20-fold) with distilled water and reserve.
5. Washing: Remove closure plate membrane and discard liquid. Dry plate by swing. Add washing buffer to every well, still for 30 s then drain. Repeat 5 times and dry by patting on absorbent material.
6. Add enzyme: Add 50 μ l of HRP-Conjugate reagent to each well, except blank well.
7. Incubate: Repeat step 3.
8. Washing: Repeat step 5.
9. Color: Add 50 μ l Chromogen Solution A and 50 μ l Chromogen Solution B to each well. Keep away from light for 10 min at 37°C

10. Stop the reaction: Add 50µl Stop Solution to each well. Stop the reaction (the blue color changes to yellow).

11. Assay: Take blank well as zero. Read absorbance at 450 nm within 15 min after adding Stop Solution.

12. Calculate: Plot standard curve [optical density, OD, (y axis) against concentration, (x axis)]. Determine the straight line regression equation of the standard curve with the standard density and the OD value. With the sample OD value in the equation, calculate the sample concentration, multiplied by the dilution factor, the result is the actual sample concentration.

4. PUBLICATIONS FROM STUDY

- Antwi AO, Obiri DD, Osafo N, Essel LB, Forkuo AD, Atobiga C. (2018) Stigmasterol Alleviates Cutaneous Allergic Responses in Rodents. *BioMed Research International*. 10.1155/2018/3984068.
- Antwi AO, Obiri DD, Osafo N, Forkuo AD, Essel LB. (2017) Stigmasterol Inhibits Lipopolysaccharide-induced Innate Immune Responses in Murine Models. *International Immunopharmacology* 53:105-113.
- Antwi AO, Obiri DD, Osafo N. (2017) Stigmasterol Modulates Allergic Airway Inflammation in Guinea Pig Model of Ovalbumin-Induced Asthma. *Mediators of Inflammation*. 10.1155/2017/2953930.