TITLE

EVALUATION OF THE ANTI-INFLAMMATORY AND ANALGESIC ACTIVITIES OF *Schumanniophyton magnificum* LEAVES

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This work is dedicated to God Almighty.

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ABSTRACT

This study was aimed at evaluating the possible anti-inflammatory and analgesic activities of methanol extract of the leaves of *S. magnificum*. The extractive yield obtained from the methanol crude extract was 07.02 %. The phytochemical composition showed the presence of bioactive compounds such as tannins, phenols, alkaloids, terpenoids, saponins and flavonoids. The acute toxicity test showed no lethality or behavioural changes at 5000 mg/kg b.w. The egg albumin-induced acute inflammation showed significant ($p < 0.05$) decrease in paw volume of rats treated with the extract and ibuprofen compared with group 1 treated with 2ml/kg of 3% Tween-80. A significant ($p < 0.05$) decrease in paw diameter was observed in extract treated groups compared with group 2 (induced but not treated) after 10 days of treatment in formaldehyde-induced sub-chronic inflammation. There were significant ($p < 0.05$) inhibitory activities of phospholipase A_2 and membrane haemolysis subjected to hypotonic stress by the extract, partitions and fractions, when compared with that of standard drugs, prednisolone and indomethacin. The extract significantly ($p < 0.05$) inhibited platelet aggregation compared with indomethacin. The extract exhibited significant ($p < 0.05$) free radical-scavenging activities in all concentrations in a manner comparable with ascorbic acid. However, low ferric reducing capacity was observed in the extract when compared with gallic acid. The extract exhibited a significant ($p < 0.05$) and dose-dependent inhibition of vascular permeability induced by acetic acid in rats compared with indomethacin. The extract caused a significant ($p < 0.05$) reduction in *in vivo* leukocyte migration induced by agar when compared with indomethacin. There was significant ($p < 0.05$) decrease in malondialdehyde concentration in both extract and standard drug groups compared with group 2 treated with 2ml/kg of 3% Tween-80. There was a significant ($p < 0.05$) increase in Hb, RBC, WBC and PCV and a significant ($p < 0.05$) decrease in platelet count in all extract treated groups as well as the standard drug groups when compared with group 2. Extract treated groups significantly ($p < 0.05$) decreased AST, ALT and ALP activities compared with group 2. The extract treated groups significantly ($p < 0.05$) decreased cholesterol, TAG and LDL concentrations and significantly ($p < 0.05$) increased HDL concentration when compared with group 2. The extract produced a significant ($p < 0.05$) dose-dependent reduction in the number of abdominal constriction induced by intraperitoneal injection of acetic acid in mice when compared with aspirin. A significant ($p < 0.05$) and dose-dependent elevation of the post-treatment reaction time and pain reaction time to thermal pain was evident in the extract treated animals comparable to that produced by 10 mg/kg of pentazocine. The extract at 400 mg/kg showed higher activity in most determinations. In the FTIR spectroscopic studies, the most active fraction (fraction 1) revealed the presence of these functional groups: amines, phenols, carboxylic acids, alcohols, alkenes, alkanes, esters and nitrites. The GCMS result of fraction 1 confirmed the presence of 10 compounds with Hexadecanoic acid, 2-methyl-, methyl ester (24.906%) identified as the major peak. The result of this study revealed that *S. magnificum* leaves possess anti-inflammatory, antioxidant and analgesic activities, as well as the potential to restore the concentrations of other biochemical parameters generated during inflammation and oxidative stress. This result could be attributed to the abundant phytoconstituents present in the plant.

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AA	Arachidonic acid
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AP	Alternative pathway
AST	Aspartate aminotransferase
ATP	Adenosine Triphosphate
b.w.	Body weight
BHA	Butylatedhydroxyanisole
BHT	Butylatedhydroxytoluene
BK	Bradykinin
CNS	Central nervous system
COX	Cyclooxygenase
CP	Classical pathway
CRP	C reactive protein
DAMPs	Damage-associated molecular patterns
DH	Dorsal horn
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDTA	Ethylene diamino tetracetic acid
FXII	Factor XII (Hageman factor)
GCMS	Gas Chromatography-Mass Spectroscopy
GCSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSH	Glutathione
Hb	Haemoglobin
HL-60	Human leukemia-60
HRBCs	Human red blood cells

IL	Interleukin
LCAT	Lecithin cholesterol acyltransferase
LOX	Lipoxygenase
LP	Lectin pathway
LT	Leukotrienes
MDA	Malondialdehyde
MHC	Major histocompactibility complex
NF-κB	Nuclear factor kappa B
NSAIDs	Non-steroidal anti-inflammatory drugs
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PAMPs	Pathogen-associated molecular patterns
PG	Prostaglandin
PK	Plasma kallikrein
PLA_2	Phospholipase A_2
PMNs	Polymorphonuclear leukocyte
PNS	Peripheral nervous system
PRRs	Pattern-recognition receptors
RNS	Reactive nitrogen specie
ROS	Reactive oxygen specie
TFPI	Tissue Factor Pathway Inhibitor
THcell	T helper cell
TLC	Thin layer chromatography
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor alpha

CHAPTER ONE

INTRODUCTION

Medicinal herbs are the local heritage with global importance. The search for novel natural products with biological properties is an ongoing exercise. Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Cosa *et al*., 2006). Botanicals and herbal preparations for medicinal usage contain various types of bioactive compounds that can be used to treat chronic as well as infectious diseases (Duraipandiyan *et al*., 2006). Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, man turned to ethnopharmacognosy. Researchers found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with less adverse effect. However, clinical trials are necessary to demonstrate the effectiveness of these bioactive compounds to verify their traditional claims. Clinical trials directed towards understanding the pharmacokinetics, bioavailability, efficacy, safety and drug interactions of newly developed bioactive compounds and their formulations (extracts) require careful evaluation (Sasidharan *et al*., 2011). The World Health Organisation (WHO) has shown great interest in plant-derived medicines which have been described in the folklore medicines of many countries (Mukherjee, 2002). These plants are used in diverse forms, including infusion, essential oils or extracts. Both plants and vegetables vary considerably in their nutrient contents and are good sources of vitamins, essential amino acids, proteins as well as minerals and antioxidants. The use of plants in the management and treatment of diseases started with life. In more recent years, with considerable research, it has been found that many plants do indeed have medicinal values. Some important plant-derived active molecules used to date in conventional medicine include, among others, aspirin from salicin, isolated from leaves and bark of willow, quinine from the bark of *Cinchona sp*., artemisinin from *Artemisia annua* (Wang and Zheg, 2001). In Nigeria, *Garcina kola* is used in the treatment of asthma, *Carica papaya* is used as a remedy for hypertension, *Ocimum basilicum*, a cure for typhoid fever, and *Cola nitida*, for treatment of pile (FAO, 1996). There has been a tremendous growth in the search for alternative therapies and the therapeutic use of natural products, especially those derived from plants. Plant sources have provided inspiration in the development of an impressive number of synthetic drugs. These resources provide a host of novel chemical compounds to yield therapeutic agents, which have been optimized on the basis of their biological activities.

Inflammation is a complex pathophysiologic response of vascularised tissue to injury arising from various stimuli including thermal, chemical or physical damage, ischemia, infectious agents, antigen-antibody interactions and other biologic processes (Clark, 2002). The term inflammation refers to the events that occur in the tissues in response to an invading pathogen or due to the presence of a noxious substance. It is usually associated with pain as a secondary process resulting from the release of analgesic mediators. Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage (Kumar and Elavarasi, 2016). Pain and inflammation are the major health problems commonly treated with traditional remedies mainly using medicinal plants because of their low cost, accessibility, and eco-friendly advantages. Both inflammation and pain involve a complex array of biochemical processes such as enzyme activation, inflammatory mediator release and extravasation of fluid, cell migration, and tissue damage and repair (Simon *et al*., 2000; Liu and Hong, 2002; Medzhitov, 2008). There are various medicines for controlling and suppressing inflammatory crisis; steroids, non-steroid anti-inflammatory drugs, and immunosuppressant are the practical examples of these medications which are associated with adverse effects. The quest for the discovery of new anti inflammatory and antioxidants drugs was born out of the increasing side effects associated with the use of Nonsteroidal anti-inflammatory drugs (NSAIDs) which include gastrointestinal upset, gastric ulcer, bleeding, and liver damage. This is due to their nonselective inhibition of both constitutive (COX-1) and inducible (COX-2) isoforms of the cyclooxygenase enzymes (Tapiero *et al*., 2002). Because of this, the search for safe and effective newer agents is growing. As one of research areas, screening medicinal plants with claimed analgesic and anti-inflammatory effect may create the opportunity of discovering new compounds with more safety and efficacy (Geremew *et al*., 2015).

1.1 *Shumanniophyton magnificum*

1.1.1 Ecological and Some Pharmacological Properties of *Shumanniophyton magnificum*

Shumanniophyton magnificum is a species of the *Rubiaceae* family. It is a flowering plant that is locally called “mgba mmiri” in Igbo language of Nigeria. *Shumanniophyton magnificum* is widely used in African ethnomedicine in the treatment of various diseases, in particular, fever and malaria. A decoction of the stem bark is used in Cameroon in the treatment of dysentery (Tane *et al*., 1990), and as a lotion after circumcision (Irvine, 1961). In Congo, the stem bark is used as antiseptic for the treatment of blennorrhoea, syphilitic

cankers or ulcers. The bark infusion is used as a purgative or vermifuge in order to relieve stomach ache (Dounias, 2000). The juice from the stem bark is used as snake bite remedies in Nigeria and the protective effects of the extract against snake venom have been demonstrated (Akunyili and Akubue, 1986; Houghton *et al*., 1992). This plant is also well-known as snake repellent (Okogun *et al*., 1983). *Shumanniophyton magnificum* extracts are active against *Plasmodium falciparum* (Bickii *et al*., 2007). Rohitukine (5,7-dihydroxy-2-methyl-8-[4-(3-hydroxy-1-methyl)-piperidinyl]-4*H*-1-benzopyran-4-one) is a chromone alkaloid isolated from the bark of *Schumanniophyton magnificum* (Rubiaceae) (Houghton, 1988) and *Schumanniophyton problematicum* (Rubiaceae) (Houghton and Woldemariam, 1993) showed anti-inflammatory and immunomodulatory activities (Naik *et al*., 1988). It also showed moderate cytotoxicity against human HL-60 promyelocytic leukemia and HCT-116 colon cancer cells (Ismail *et al*., 2009). In traditional medicine, the alcoholic extract from root of *Schumanniophyton problematicum* is used for treatment of agitated psychotic patients. In fact, the root extract of the plant showed remarkable neuropsychopharmacological activities (Amadi *et al*., 1991). In addition to the above mentioned compound several complex chromone alkaloids, such as schumanniophytine, have also been isolated from *Schumanniophyton* species.

Figure 1: *Schumanniophyton magnificum* plant

1.2 An Overview of Inflammation

Inflammation is a protective strategy evolved in higher organisms in response to detrimental insults such as microbial infection, tissue injury and other noxious conditions (Ahmed, 2011). It is an essential immune response by the host that enables the removal of harmful stimuli as well as the healing of damaged tissue and then restoring tissue homeostasis (Benly, 2015). The inflammatory process involves a complex biological cascade of molecular and cellular

signals that alter physiological responses (Libby, 2007; Enechi and Nwodo, 2015). The agents that cause inflammations are infective agents such as bacteria, viruses, and their toxins, fungi, parasites; immunological agents: cell–mediated and antigen-antibody reactions; physical agents: heat, cold, radiation, mechanical trauma; chemical agents: organic and inorganic poisons; inert material: foreign bodies (Harsh, 2002; Anupama *et al.*, 2008; Oyedapo *et al*., 2008). Mankind has known the classical symptoms of inflammation for hundreds of years, which include redness, pain, swelling, heat and loss of function (Medzhitov, 2008; Ekwueme *et al*., 2011). The redness is caused by increased blood volume in the inflamed part; pain is as a result of increased pressure on nerve ending which is induced by certain chemical mediators of inflammation; swelling comes as a result of increased blood flow and the presence of other substances which exude from the blood vessels into the surrounding tissues; heat is due to increased flow of blood through the inflamed region and loss of function is often attributed to pain that inhibits motility or severe swelling that prevents movement in the area (Ferrero-Miliani *et al*., 2007; Markiewski and Lambris, 2007). There are also various components to an inflammatory reaction such as oedema formation, leukocyte infiltration and granuloma formation that can contribute to the associated symptoms and tissue injury (Trivedi *et al*., 2017).

The primary functions of inflammation are to rapidly destroy or isolate the underlying source of the disturbance, remove damaged tissue, and then restore tissue homeostasis (Medzhitov, 2008, Soehnlein and Lindbon, 2010). Inflammatory responses are essential for the maintenance of normal tissue homeostasis. As a protective strategy for the host, one of the main aims of inflammation is to reinstate cellular homeostasis in response to any damaging condition. The mechanism underlying the initiation of inflammation is therefore tightly connected to the physiological state of homeostasis. And so, inflammation is considered as an ‘adaptive response’ to any harmful effect threatening the integrity of the cellular homeostasis. It is quite plausible to understand that such an adaptive response operates at the expense of normal cellular functions (Medzhitov, 2010). As a result, the longer this response persists in the host the more damaging the consequences (Italiani and Boraschi, 2014). Prolonged inflammations are often associated with severe detrimental side effects on health, if inflammation is not controlled and the body continually fights this battle, symptoms of chronic inflammation can show itself as arthritis (Ekwueme *et al*., 2015), colitis, chronic fatigue, sinusitis, cataracts, chronic pain, hair loss, heart disease, stroke, Alzheimer’s and other ailments and conditions. The immune system is often involved with inflammatory disorders, demonstrated in both allergic reactions and some myopathies, with many immune

system disorders resulting in abnormal inflammation. Non- immune diseases with etiological origins in inflammatory processes are thought to include cancer, atherosclerosis and ischemic heart disease (Trivedi *et al*., 2010).

In response to a proper stimulant such as microbial infection, foreign invaders or any irritant (external or internal), inflammation is usually initiated within minutes in any host with a functional innate immune system. As innate immune system is the major contributor to inflammation, immune cells such as macrophages, dendritic cells, mast cells, neutrophils and lymphocytes play important roles in inflammatory responses (Akira *et al*., 2006). Apart from immune cells, non-immune cells such as epithelial cells, endothelial cells and fibroblasts also contribute to inflammatory processes. Based on the nature of stimulants, inflammatory pathways vary significantly and so are their target tissues (Ahmed, 2011). In the case of bacterial infection, the immune cells through specific receptors immediately sense pathogens. Activation of pathogen-specific receptors induces the production of inflammatory mediators such as inflammatory cytokines [e.g. tumour necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6)] and chemokines. These mediators rapidly accelerate the progression of inflammation through the modification of vascular endothelial permeability as well as the recruitment of neutrophils and excess plasma (containing antibodies and complement factors) into the site of infection (Rosser and Mauri, 2015). At the same time, the invading pathogens are targeted and destroyed by the immune cells. The duration of inflammatory responses vary depending on the extent of damage caused by the infection. Inflammation is controlled by a balance of pro-inflammatory and anti-inflammatory signals, resulting in the development of an immune response followed by temporarily pre-resolution factors that lead to inflammation switching off, and injured tissues returning to normal physiology (Rajakariar *et al*., 2008).

1.2.1 Role of Inflammation

As part of the immune response, inflammation plays an important role in defending the body against pathogens such as viruses, bacteria, fungi, and other parasites (Sowjanya *et al*., 2017). Other roles or functions include:

i. Inflammation is the first step in the healing or repair process after some physical or chemical injury or stress. It is a normal bodily function and it is a healthy action when it is controlled. If there is no inflammation, even a small cut on the finger could lead to death.

ii. Inflammation prevents the spread of damaged cells to other areas of the body that could cause secondary problems. A local infection, for example, can be contained due to the inflammatory response, instead of causing a body-wide infection.

iii. Inflammation rids the body of damaged and dead cells.

1.2.2 Drawbacks of Inflammation

Inflammatory responses may be harmful, such as in an anaphylactic shock. Inflammation of the peritoneum leads to fibrous bands that cause intestinal obstruction. Pericardial inflammation results in the formation of dense pericardium that impairs the functioning of the heart (Chishti, 1991).

1.3 Phases of Inflammation

Inflammatory responses can be classified into two main phases namely: Acute and chronic phases.

1.3.1 Acute Phase

Acute inflammation known to be the first line of defence against injury occurs within minutes or at most hours after tissue injury, and may be characterized by the classical symptoms of redness, heat, and oedema. It is a short term process (Ahmed, 2011; Sowjanya *et al*., 2017). It is characterized by the exudation of fluids and plasma proteins and the migration of leukocytes, most importantly, neutrophils into the injured area which are induced by the actions of the various inflammatory mediators (Anosike *et al*., 2012b). Acute inflammation encompasses the immediate and early responses to an injurious agent and is quickly resolved. This acute inflammatory response is useful to the defence mechanism aimed at killing of bacteria, virus and parasites while still facilitating wound repairs (Oishi and Manabe, 2015). Once the disturbance is removed, there is strong selective pressure for the inflammatory response to end so that tissue repair can restore functionality (Noah *et al*., 2012).

1.3.2 Chronic Phase

Chronic inflammation is of a more prolonged duration and characterized by the presence of lymphocytes and macrophages, resulting in fibrosis and tissue necrosis (Sowjanya *et al*., 2017). Inflammation becomes chronic if the agent causing the inflammation persists for a prolonged period of time. Chronic inflammation can result from a viral or microbial infection, environmental antigen (e.g. pollen), autoimmune reaction, or persistent activation

of inflammatory molecules. The beginning of chronic inflammation is often characterized by the replacement of neutrophils with macrophages and other immune cells, such as T cells (Medzhitov, 2008). During a chronic inflammatory state, granulomas are typically formed in a final attempt to wall off the host from pathogens. Chronic inflammation is primarily mediated by monocytes and long-lived macrophages (Scott *et al*., 2004); monocytes mature into macrophages once they leave the bloodstream and enter tissues. Macrophages engulf and digest microorganisms and senescent cells (Lippy, 2007). They release several different chemical mediators, including IL-1, TNF-α, and prostaglandins, that perpetuate the pro-inflammatory response. At later stages, other cells, including lymphocytes, invade the affected tissues: T lymphocytes kill virus-infected cells and B lymphocytes produce antibodies that specifically target the invading microorganisms for destruction (Scott *et al*., 2004).

Macrophages and other leukocytes release ROS and proteases that destroy the source of inflammation; however, damage to the body's own tissues often results in chronic inflammation. In chronic inflammation, damaged tissues are repaired through replacement with cells of the same type or with fibrous connective tissue. Another important characteristic of chronic inflammation is local angiogenesis — the development of new blood vessels (Bian *et al*., 2004). In some instances, the body is unable to repair tissue damage, and the inflammatory cascade continues. Chronic inflammation is abnormal and does not benefit the body, it is involved in a number of disease states such as asthma, Crohn's disease, rheumatoid arthritis (Ekwueme *et al*., 2015), polymyalgia rheumatica, tendonitis, bursitis, laryngitis, gingivitis, gastritis, celiac disease, diverticulitis, and inflammatory bowel disease. Additionally, a number of chronic diseases have inflammatory components, such as atherosclerosis (Blake and Ridker, 2002), obesity (Horton, 2009), diabetes mellitus, stroke (Hotamisligil, 2006), cancer and perhaps even Alzheimer's disease. The biochemical mechanisms underlying several of these diseases are unknown, and the role of inflammation in disease pathogenesis is under investigation.

1.4 Mechanism of Inflammation

Inflammation consists of a tightly regulated cascade of immunological, physiological, and behavioural processes that are orchestrated by soluble immune signalling molecules called cytokines (Ashley *et al*., 2012). The inflammatory process is a combination of many pathways like a synthesis of prostaglandin, interleukin or other chemo toxin, adhesive protein receptor action, platelet-activating factors (Medzhitov, 2008). The first step of the

inflammatory cascade involves recognition of infection or damage by germ-line encoded receptors called pattern-recognition receptors (PRRs) such as transmembrane Toll-like receptors (TLRs) and intracellular nucleotide binding domain and leucine-rich-repeat containing receptors (NOD-like receptors or NLRs) (Lange *et al*., 2001, Proell *et al*., 2008; Ahmed, 2011). This is typically achieved by the detection of pathogen-associated molecular patterns (PAMPs), which are specifically directed toward general motifs of molecules expressed by pathogens that are essential for pathogen survival. Damage-associated molecular patterns (DAMPs) are endogenous molecules that signal damage or necrosis and are also recognized by the innate immune system. Once recognition of ligands occurs, TLRs activate common signaling pathways that culminate in the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). This transcription factor is found in virtually all cell types and remains in an inactivated state bound to an inhibitor protein, IκB (Ashley *et al*., 2012).Upon transduction of the signal, NF-κB is released from IκB and translocates to the nucleus, where transcription is upregulated through binding to target genes (Ahmed, 2011).

Transcription and translation of genes lead to the third stage of the inflammatory cascade, precisely proinflammatory cytokines, such as interleukin-1-beta (IL- 1β), IL-6, tumor necrosis factor-alpha (TNF-α), and others. In conjunction with chemokines (attractants) and various co-stimulatory molecules, these soluble proteins facilitate the recruitment of effector cells, such as monocytes and neutrophils, to the site of disturbance. Neutrophils create a cytotoxic environment by releasing noxious chemicals from cytoplasmic granules (a process called degranulation). Rapid release of these chemicals requires consumption of both glucose and oxygen, known as the respiratory burst. Toxic chemicals released include highly reactive oxygen and nitrogen species (ROS and RNS, respectively) and various proteinases (Wight *et al*., 2010; Ashley *et al*., 2012). These substances are destructive to both pathogens and hosts and essentially induce phagocytosis or liquefaction of surrounding tissue to stave off microbial metastasis (Nathan, 2002). Macrophages and dendritic cells also participate in phagocytosis of antigen (Ashley *et al*., 2012). Studies have demonstrated the ability of platelets to regulate most the effector functions of neutrophils and macrophages such as production of reactive oxygen species (ROS), the secretion of neutrophil granule content, phagocytocis, or the formation of neutrophil extracellular traps (Gros *et al*., 2014). The net effect of these interactions culminates in the stereotypical cardinal signs of local inflammation: heat, swelling, redness, pain, and loss of function (Ashley *et al*., 2012; Hemalata and Shiva, 2015).

The last phase of inflammation is its resolution, which is critical for limiting collateral damage to the host (Serhan and Savill, 2005). After the first few hours of inflammation, a coordinated programme of resolution is set into motion by tissue-resident and recruited macrophages. During acute inflammation, these cells produce proinflammatory prostaglandins and leukotrienes, but rapidly switch to lipoxins, which block further neutrophil recruitment and instead favour enhanced infiltration of monocytes important for wound healing (Serhan *et al*., 2007; Huang and Glass, 2010).

1.5 Cellular Components of Inflammation

The cells that are involved in the mediation of inflammation include neutrophils, lymphocytes, basophils, eosinophils, mast cells, tissue macrophages and platelets. From the blood, platelets and leucocytes move into the inflammatory area but the neutrophils, mast cells and tissue macrophages are normally present in tissues (Rang *et al.*, 2008).

a. Lymphoctes

They are the major cells of the specific immune system involved in humoral and cell-mediated immune responses. They are also involved in the initiation and maintenance of inflammation. These cells can further be stimulated to maintain inflammation through the action of adaptive cascade through lymphocytes: T cells, B cells, and antibodies (Rajakariar *et al*., 2008).

b. Neutrophils

Neutrophils are the most abundant white blood cells in human circulation; they are also called neutrophilic granulocytes or polymorphonuclear leukocytes (PMNs) (Mocsai, 2013). These are characteristic of inflammation in the early stages - they are the first cells to appear in an infected area, and any section of recently inflamed (within a couple of days or so) tissue viewed under a microscope will appear packed with them. They perform many important functions, including phagocytosis and the release of extracellular chemical messengers. Neutrophils only live for a couple of days in these interstitial areas, so if the inflammation persists for a longer duration then they are gradually replaced by longer lived monocytes (Wight *et al*., 2010). Previous studies have shown significant increases in levels of chemokines, granulocyte colony stimulating factor (GCSF) and granulocyte-macrophage CSF (GM-CSF) in animal models of acute inflammation and in patients with infections, (Shahbazian *et al.*, 2004; Sugimoto *et al.*, 2005; Cataisson *et al.*, 2006) suggesting that these chemokines and cytokines may potentially stimulate mobilization of leukocytes, especially

neutrophils from the bone marrow during inflammatory reactions. G-CSF was also demonstrated to play a critical role in the leukocytosis caused by bacterial infection (Gregory *et al.*, 2007).

c. Macrophages/Monocytes

These are large phagocytic leucocytes, which are able to travel outside of the circulatory system by moving across the cell membrane of capillary vessels and entering the areas between cells in pursuit of invading pathogens. These usually, act after neutrophils and are the most efficient of the phagocytes. They can eat substantial numbers of bacteria or other cells (Braga *et al*., 2015). The binding of bacterial molecules to receptors on the surface of a macrophage triggers it to engulf and destroy the bacteria through the generation of a "respiratory burst". Pathogens also stimulate the macrophage to produce chemokines, which summon other cells to the site of infection. These release Tumor Necrosis Factor alpha (TNF-α), IL-1 in response to activation of toll-like receptors (Braga *et al*., 2015; Gensel and Zhang, 2015).

d. Basophils

Basophils are the very rare type of granulocyte, they mature in the bone marrow and are then released into circulation. Basophils are increased during inflammation responses. They synthesize histamine from histidine due to the actions of histidine decarboxylase (Raap *et al*., 2015) and also respond to a range of chemokines and cytokines that facilitate their migration (Gibbs, 2008).

e. Eosinophils

They are innate immune cells that originate from the bone marrow. They depend on cytokines for development, survival, expansion and terminal differentiation (Wong *et al*., 2014). Eosinophils are important in host defence against tumor and allergic inflammation. Khoury *et al*. (2014) reported roles of eosinophils in more homeostatic functions, including tissue repair and remodelling and maintenance of plasma cells in the bone marrow and of alternatively activated macrophages in adipose tissue. Eosinophils secrete chemo attractants for the recruitment of T cells, macrophages and dendritic cells to tissue sites (Wong *et al*., 2014).

f. Mast cells

They are a type of innate immune cell that reside in the connective tissue and in the mucous membranes, and are intimately associated with pathogen defense and wound healing, and are

often also associated with allergy and anaphylaxis. When activated by stretch receptors, mast cells rapidly release characteristic granules, rich in histamine and heparin, along with various hormonal mediators and chemokines or chemotactic cytokines into the environment (Gibbs, 2008). Histamine dilates blood vessels, causing the characteristic signs of inflammation, and also recruits neutrophils and macrophages. This is especially important in cases of trauma. Generally, mast cells make molecules that help repair traumatic injuries, including those involved with blood clotting (Markiewski and Lambris, 2007).

1.6 Mediators of Inflammation

Inflammation is a physiologic response to a variety of stimuli such as infections and tissue injury. Mainly, inflammatory mediators orchestrate the process. These are soluble molecules, released from cells or from matrix proteins. They act on the target inflammatory cells through specific receptors (Richard *et al.,* 2000; Parkin and Cohen, 2001; Arunachalam *et al*., 2009). Inflammatory mediators are grouped into two:

a. Cell derived mediators

b. Plasma protein derived mediators.

1.6.1 Cell Derived Mediators

Circulating platelets, basophils, PMNs, endothelial cells monocyte/macrophages, tissue mast cells and the injured tissue itself are all potential cellular sources of vasoactive mediators. In general, these mediators are derived from metabolism of phospholipids and arachidonic acid (e.g., prostaglandins, thromboxanes, leukotrienes, lipoxins, platelet activating factor [PAF]), preformed and stored in cytoplasmic granules (e.g., histamine, serotonin, lysosomal hydrolases) or derived from altered production of normal regulators of vascular function (e.g., nitric oxide and neurokinins).

a. Prostaglandins

Prostaglandins (PGs) are group of arachidonic acid-derived molecules that mediate allergic reactions. The biologic effects of prostaglandins generated during mast cell-dependent reactions in tissues include modulation of smooth muscle contractility, vascular permeability, sensations of pain, and platelet aggregation and degranulation (Kalinski, 2012). PG's and thromboxane A_2 termed prostanoids are formed when arachidonic acid is released from plasma membrane phospholipds by phospholipase A_2 and metabolized by PG G/H synthase or cyclooxygenase (COX) an enzyme associated with the endoplasmic reticulum of mast cells (Zarghi and Arfaei, 2011). Similar to 5-lipoxygenase, cyclooxygenase catalyzes the formation of relatively unstable intermediates PG_2 and PGH_2. These intermediates may then

be converted non-enzymatically or enzymatically by specific isomerase/peroxidase or synthase enzymes to yield the primary prostaglandins PGD_2, PGE_2, PGF_2, PGI_2 and TXA_2 (fig. 2) (Schwartz and Austen, 1984; Zarghi and Arfaei, 2011; Stolfi *et al*., 2013). The most abundant cyclooxygenase product generated by the immunologic activation of human lung mast cells is PGD_2. PGD_2 is generated by human mast cells after they are activated through the IgE receptor or by calcium ionophore (Lewis *et al*., 1981).

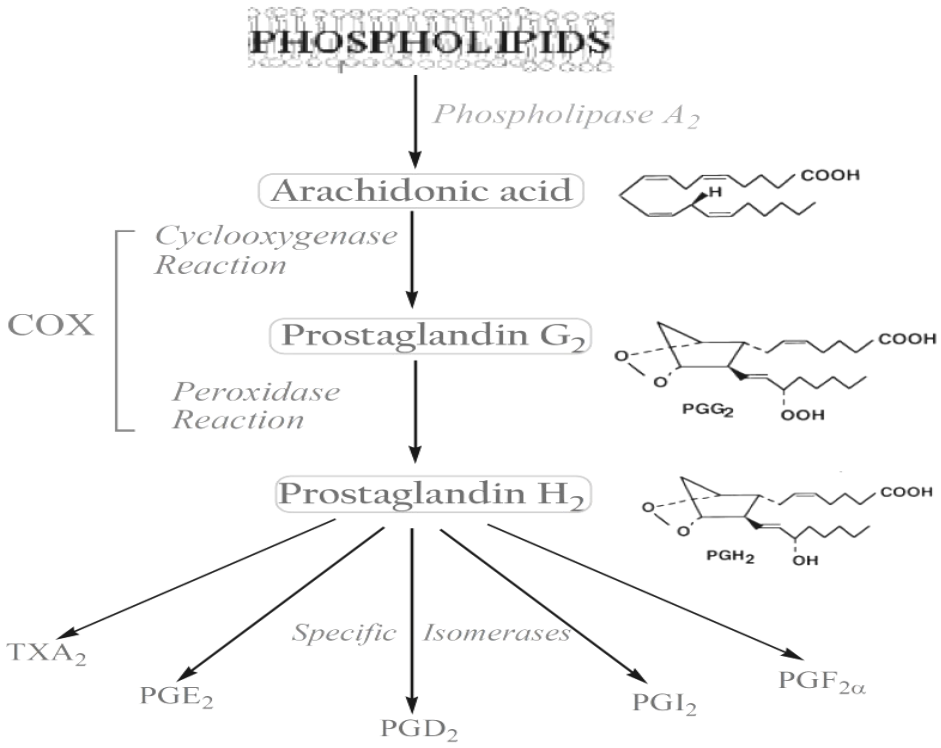


Figure 2: Biosynthesis of Prostanoids.
Source: Zarghi and Arfaei. (2011)

b. Histamine

Histamine (β-Imidazolylethylamine) is a biogenic amine synthesized in the golgi apparatus of mast cells and basophils by decarboxylation of the amino acid histidine and is then stored in secretory granules in complexes with heparin, protein, or both (Benly, 2015). In sensitized individuals histamine release occurs with the binding of specific allergens to IgE antibodies on mast cells and basophils, initiating a Ca^{2+}-dependent degranulation reaction (Zampeli and Tiligada, 2009). Many effects produced by histamine during inflammation include vasodilation, a constrictor of smooth muscle, and a potent stimulant of vascular permeability, respiratory mucus, and gastric acid secretion. It exerts its effects on a variety of cell types including smooth muscle cells, neurons, glandular cells (endocrine and exocrine), blood cells, and cells of the immune system. In addition to its role in immediate hypersensitivity reactions, histamine can exert H_2-receptor- mediated anti-inflammatory activity including

inhibition of human neutrophil lysosomal enzyme release, inhibition of IgE-mediated histamine release from peripheral leukocytes, and activation of suppressor T-lymphocytes (Benly, 2015).

c. Cytokines

Cytokines are small secreted proteins released by cells and are predominatly produced by helper T cells (Th) and macrophages. Cytokine is a general name, other names include monokine, chemokine, lymphokine and interleukin (Zhang and An, 2007). Cytokines such as interleukins (IL) -1, -2, -4, -5, -6 to 30, tumour necrosis factor (TNF-α), colony stimulating factors (CSF), interferon (IFN), chemokines (CKs) and growth factors (GF) influence the course of allergic inflammation by modulating the activity of inflammatory cells including macrophages, T cells, B cells, and eosinophils. IL-1 enhances the growth of T-helper cells and the growth and proliferation of B cells, whereas IL-2 causes proliferation of T lymphocytes and activation of B lymphocytes. IL-4 causes differentiation of B lymphocytes into IgE-secreting plasma cells and, along with TNF-α, upregulates both high- and low-affinity IgE receptor expression on antigen presenting cells. IL-5 causes activation of B lymphocytes and the differentiation and increased longevity of eosinophils, and IL-6 causes increased synthesis and secretion of immunoglobulins by B lymphocytes (Luster, 1998, Silva, 2005). Cytokines play a critical role in late-phase inflammatory events associated with allergic rhinitis and asthma, and that the mast cell is a source of many multifunctional cytokines (Bradding and Holgate, 1996). There are both pro-inflammatory (IL-Iβ, IL-6 and TNF-α) and anti-inflammatory cytokines (IL-4, IL-10 and IL-13). Pro-inflammatory cytokines up-regulate inflammatory reactions. Anti-inflammatory cytokines are generally T-cell derived cytokines and down-regulate inflammatory reactions. Among all the anti-inflammatory cytokines IL-10 has the ability to repress the expression of cytokine such TNF-α, IL-6 and IL-1 (Kubo and Motomura, 2012).

d. Leukotrienes

Leukotrienes (LTs) are derived from arachidonic acid, which is made available from cell membrane phospholipids by the action of phospholipase A_2 or by the sequential action of phospholipase C and diacylglycerol lipase (Montuschi, 2010). After being released from cell membranes by the action of phospholipase A_2, arachidonic acid is converted by 5-lipoxygenase to 5*S* hydroperoxy-6,8-*trans*-11,14-*cis*eicosatetraenoic acid. The same 5-lipoxygenase pathway catalyzes the conversion of 5*S*-hydroperoxy-6,8- *trans*-11,14-*cis*-eicosatetraenoic acid to LT_4 (LTA_4). LTA_4 can then be converted to LTB_4 or conjugated with

reduced glutathione to form LTC_4. In the later phases of the allergic reaction, after transport into the extracellular environment, LTC_4 can undergo further metabolism to LTD_4 and LTE_4 (Montuschi, 2010). Leukotriene (LT) C_4 and its products, LTD_4 and LTE_4, make up the biologic mixture previously known as the slow-reacting substance of anaphylaxis. Leukotrienes are generated by most cell types that participate in inflammatory reactions including mast cells, basophils, eosinophils, neutrophils, and monocytes. As chemical mediators of inflammation, they have biologic activity similar to that of histamine. Studies of the effects of H1- receptor antagonists on leukotriene release reveal that the mechanism may involve blocking the activity of receptor-coupled G proteins (Rihoux *et al*., 1997; Sharma and Mohammed, 2006).

e. Serotonin

Serotonin also known as 5-hydroxy tryptamine (5-HT) is a neurotransmitter and hormone that contributes to the regulation of various physiological functions by its actions in the central nervous system. It is a vasoactive mediator similar to mast cell histamine (Shajib and Khan, 2015). Serotonin can lead to both increases and decreases in pro-inflammatory cytokines as it dilates capillaries, increases vascular permeability and causes contraction of smooth muscle (Nau *et al*., 2013).

f. Platelet Activating Factors

Platelet activating factor (PAF) is an ether-linked phospholipid, designated as such because of its discovery as a basophil-derived mediator. PAF may be produced by several of the cells that participate in the inflammatory response including mast cells, macrophages, neutrophils, and eosinophils (Barnes, 1993). The synthesis of PAF occurs as a 2-step pathway in which phospholipase A_2 hydrolyzes a 1-O-alkyl-2-acyl-glyceryl- 3-phosphorylcholine to produce 1-O-alkyl-2-acylglyceryl- 3-phosphocholine (lyso-PAF), which is then converted by an acetyltransferase enzyme to PAF. The biologic activities of PAF include platelet activation, activation of neutrophils, and smooth muscle contraction (Garcia *et al*., 2010). PAF is rapidly inactivated in *vivo*; it is likely that it triggers a chain of inflammatory events. PAF stimulates the accumulation of eosinophils to endothelial surfaces, which may be the first step in the recruitment of eosinophils into tissues (Levi *et al*., 2004). Eosinophils also are a source of PAF and can attract additional eosinophils, which can potentiate the inflammatory reaction. PAF stimulates eosinophils to release basic proteins, leading to epithelial cell damage, and causes an increased expression of low-affinity IgE receptors on eosinophils and monocytes (Barnes, 1993; Garcia *et al*., 2010).

g. Nitric Oxide

Nitric oxide (NO) is a short-lived free radical that mediates many biological processes. It is produced from the amino acid L-arginine by nitric oxidase synthesis (NOS) (Liu *et al*., 2015). One of the functions of NO is to enhance the bactericidal and tumoricidal activities of activated macrophages (Singh *et al*., 2012); other functions include vasodilation (Egbuonu and Ezeanyika, 2013), blood pressure and immune regulation, neural signal transduction (Hosseini *et al*., 2012). Three NOS isoenzymes involved in nitic oxide synthesis *in vivo* are: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS and eNOS are constituively expressed and are responsible for the production of low NO in response to physiological stimuli while iNOS is induced by pro-inflammatory cytokines which activates macrophages and neutrophils leading to the production of high amount of NO (Kobayashi, 2010; Nworu and Akah, 2015). Excessive production of NO could however potentially lead to tissue damage and activation of pro-inflammatory mediators (Guzik *et al*., 2003; Ahmad *et al*., 2015).

h. Reactive Oxygen Species

Reactive oxygen species (ROS) are produced during functional activities and cellular metabolism. All reactive oxygen species are responsible for creating oxidative stress during inflammation by producing different cytotoxic cytokines through the phosphorylation of nuclear factor kappa-B (Bala and Haldar, 2013). Under certain stress conditions, oxygen becomes much more active and forms ROS, such as superoxide anion radical (O_2^-), hydroxyl radical (OH^-), peroxyl radical (ROO^-) and non radical species, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($_1O^2$) (Bala and Haldar, 2013). ROS have important roles in cell signaling, apoptosis, gene expression and ion transportation (Lu *et al*., 2010).

1.6.2 Plasma Protein Derived Mediators

Plasma protein derived mediators are mainly produced in the liver and present in circulation. They act as precursors that must be activated usually by a series of proteolytic cleavages. They include contact, complement and coagulation systems.

a. Contact System

The plasma contact system drives proinflammatory and procoagulant pathways and is composed of plasma proteases, substrates, and inhibitors produced and secreted by the liver (Renne, 2012). It consists of the serine proteases factor XII (FXII), FXI, plasma prekallikrein (PPK) zymogens, the non-enzymatic cofactor high molecular weight kininogen (HK), and C1

esterase inhibitor (C1INH) (Maas *et al*., 2011). FXII becomes activated when it comes into contact with negatively charged surfaces and undergoes a conformational change, which generates small amounts of activated FXII (FXIIa). FXIIa cleaves PPK to form plasma kallikrein (PK), which reciprocally activates FXII and generates a positive feedback loop of FXII activation. PK subsequently digests HK to release bradykinin (BK), a potent proinflammatory peptide and the end product of the kallikrein-kinin pathway. BK activates signaling pathways resulting in increased vascular permeability, vasodilation, and chemotaxis of neutrophils (Leeb-Lundberg *et al*., 2005; Muller and Renne, 2008). FXIIa also activates the intrinsic coagulation cascade via FXI cleavage to generate activated FXI (FXIa). FXIa initiates a series of Ca^{2+}-dependent sequential proteolytic cleavage events that lead to thrombin generation, fibrin formation, and production of a fibrin clot in plasma. Under physiological conditions, the contact system proteins are inactive zymogens circulating in plasma or are bound to various cardiovascular cells such as platelets, leukocytes, and endothelial cells via plasma membrane heparin and chondroitin sulfate–type proteoglycans. Additionally, intracellular cytoskeleton and mitochondrial proteins have been described to bind HK. FXII and HK can bind directly to surfaces of cardiovascular cells, while PK and FXI are anchored to cells indirectly through bimolecular complex formation with HK. PK also converts plasminogen to plasmin, linking the kallikrein-kinin system to fibrinolysis (Renne, 2000). Further, contact system–dependent inflammation and thrombosis can be activated by multiple different bacteria and parasites (Frick *et al*., 2007; Loof *et al*., 2014) which make the contact system an attractive therapeutic target for a variety of diseases.

b. Complement System

Complement system is composed of membrane-bound regulators and receptors as well as numerous plasma proteins that interact with various cells and mediators of immune system (Markiewski and Lambris, 2007). Complement system can be activated via four pathways: classical pathway (CP), lectin pathway (LP), alternative pathway (AP) and extrinsic protease pathway (Ricklin *et al*., 2010). Activation of the complement reaction sequence produces several active components that can be classified as chemical mediators of inflammation. Chemotactic activity derived from the complement system has been identified with C3a, C5a. Anaphylatoxin, a product of C3 or C5, enhances vascular permeability and causes contraction of smooth muscle. Complement components C1-C5 can be demonstrated to play a role in enhanced phagocytosis (Sarma and Ward, 2011). Complement components that are activated in plasma and body fluids are engaged in the regulation of all phases of acute inflammatory

reaction, including changes in vascular flow, the increase in vascular permeability, extravasation of leukocytes, and chemotaxis (Markiewski and Lambris, 2007).

c. Coagulation System

Inflammatory mechanisms shift the haemostatic balance to favour the activation of coagulation and, in the extremes, either disseminated intravascular coagulation or thrombosis.Inflammatory mediators can elevate platelet count, platelet reactivity, downregulate natural anticoagulant mechanisms, initiate the coagulation system, facilitate propagation of the coagulant response and impair fibrinolysis. Similarly, clotting can increase the inflammatory response both by releasing mediators from platelets and by activating cells, thereby promoting cell–cell interactions that increase the inflammatory responses (Esmon, 2005). After injury, thrombin is generated at the wound site. Thrombin then recruits platelets and generates fibrin. The activated platelets expressing P-selectin bind to circulating microparticles released from leucocytes or vascular cells (Day *et al*., 2005). Inflammatory mediators presumably increase both the number of microparticles through leucocyte activation and the concentration of tissue factor on the particle surface. Since the microparticles are generated from the cell surface and are devoid of an ATP generating system, they will have high concentrations of negatively charged phospholipid on the membrane surface. There are three major anticoagulant mechanisms that control the blood clotting process: tissue factor pathway inhibitor (TFPI), the heparin-antithrombin pathway and the protein C anticoagulant pathway. Deficiencies in the heparin-antithrombin or the protein C pathway in humans leads to increased risk of thrombosis, while the impact of deficiencies of the tissue factor pathway in humans is less clear (D'Angelo, 2015). One impact of inflammation on coagulation is to increase fibrinogen concentration. Fibrinogen, which is an acute phase reactant, is increased in inflammatory situations (Hantgan *et al*., 2001). Elevations in fibrinogen levels have been associated with an increase risk of thrombotic disease. Probably the best known contribution of inflammation to coagulation is the induction of tissue factor expression on the cell surface of leucocytes, particularly monocytes (Lindmark *et al*., 2000). Endotoxin, tumour necrosis factor-a (TNF-a) or CD40 ligand all induce tissue factor. If negatively charged phospholipids are exposed on activated monocytes, tissue factor gains major procoagulant activity. Inflammation also increases C reactive protein (CRP) concentrations in the blood. CRP has now been shown to facilitate monocyte–endothelial cell interactions (Han *et al*., 2004) and to promote plasminogen activator inhibitor-1 (PAI-1) and tissue factor (Devaraj *et al*., 2003) formation. Elevated CRP, probably a reflection of systemic inflammatory responses, is correlated with increased risk of

myocardial infarction (Ballantyne *et al*., 2004). In acute inflammatory situations, such as sepsis, antithrombin is consumed and/or inactivated. Antithrombin inhibitory activity decreases markedly during severe sepsis, often to less than 50% of normal levels (Opal, 2000). Decreased antithrombin concentrations result in roughly proportional decreases in the rates of inhibition of the target proteases. Thus, this decrease in antithrombin concentration results in delayed inhibition of coagulation enzymes that favour intravascular coagulation.

1.7 Anti-Inflammatory Agents

According to Chishti (1991), inflammatory response maybe harmful as seen in the case of pericardial inflammation which results in the formation of dense pericardium that impairs the functioning of the heart. There will be need for anti-inflammatory drugs when inflammatory response becomes inappropriate or sustained and the commonly used anti-inflammatory agents are non-steroidal anti-inflammatory drugs and corticosteroids.

1.7.1 Non-Steroidal Anti-Inflammatory Drugs

NSAIDs are the most frequently used medication for treating pain and inflammation. One mechanism by which NSAIDs exert anti-inflammatory effect is by inhibiting the cyclooxygenase (COX) pathway (Fig. 3) by which prostaglandins and thromboxanes are produced from arachidonic acid (AA) (Iwueke *et al*., 2006; Mizushima, 2010). Other mechanisms of action of NSAIDs include inhibition of neutrophil adherence, inhibition of neutrophil elastase activity, inhibition of neutrophil apoptosis, decreased degranulation and oxidant production (Wight *et al*., 2010), suppression of PLA_2 and inhibition of glutathione S-transferase (Summ and Evers, 2013). The reduction in prostaglandin production limits the increase in vascular permeability and neutrophil chemotaxis in the inflammatory response (Vane, 1971). COX has two major isoforms: COX-1 and COX-2. COX-1 is stimulated by growth factor and hormones. It is expressed constitutively in many tissues and PGs produced by COX-1 mediate “housekeeping” function such as: cytoprotection of gastric mucosa, regulation of renal blood flow and platelet aggregation (Monther *et al*., 2012). COX-2 is induced by stimuli such as pro-inflammatory cytokines, lipopolysaccharide, oncogenes, growth factors and hormones (Zarghi and Arfaei, 2011). Inhibitors of COX-1 bind at the active site and prevent AA from binding. Inhibition of COX-1 suppresses gastric prostaglandin systhesis and decreases thromboxane production causing ulcer and bleeding respectively (Musumba *et al*., 2009). However, to overcome the health challenges associated with the use of COX-1 inhibitors, COX-2 selective NSAIDs are recently used. COX-2 active

site is more bulky as a result of exchange of valine with bulky isoleucine at position of the active site of the enzyme; this allows access to additional side pocket, which is a prerequisite for COX-2 drug selectivity (Zarghi and Arfaei, 2011). According to Mizushima (2010), increasing selectivity for COX-2 also increases toxicity, since the anti-thrombotic prostacyclin is formed by COX-2 and inhibiting its synthesis precipitated heart hearts. COX-3 is a variant of COX-1 and is derived from the same gene by alternative splicing of the COX-1 gene. The only difference between the two is the retention of intron 1 of the COX-1 gene in COX-3 (Monther *et al*., 2012). Clinically, NSAIDs effectively treat many acute and chronic inflammatory reactions. NSAIDs such as aspirin inhibit both COX isoforms by covalent modification through acetylation of serine residue in the active site of the COX enzyme. Acetylation of COX-1 causes conformational change in the active site which disables it from oxidizing AA (Stolfi *et al*., 2013). NSAIDs indicate the symptomatic relief of rheumatoid arthritis, osteoarthritis, inflammatory arthropathies (e.g. ankylosing spondylitis, psoriatic arthritis, Reiter's syndrome), acute gout, metastatic bone pain, headache and migraine, postoperative pain, mild-to-moderate pain due to inflammation and tissue injury, pyrexia, renal colic. The two main adverse drug reactions (ADRs) associated with NSAIDs relate to gastrointestinal (Gl) effects and renal effects of the agents (Rang *et al.*, 2008).

1.7.2 Corticosteroids

Corticosteroids are synthetic analogues of natural steroid hormones produced by the adrenal cortex. Examples of corticosteroids include prednisone, prednisolone, hydrocortisone and dexamethasone. These potent anti-inflammatory agents exert various effects that result in reduction in the numbers and activity of immune system cells (Liu *et al*., 2013). They are regularly used in anti-inflammatory therapy. Corticosteroid inhibits PLA_2 thereby preventing the release of AA, and its subsequent conversion of eicosanoids (fig. 3) (Kubo and Motomura, 2012). Corticosteroid treatment causes a decrease in number of circulating lymphocytes because of either steroid induced lysis of lymphocytes (lympholysis) or alterations in lymphocyte-circulation patterns. Some species (eg., hamster, mouse, rat and rabbit) are particularly sensitive to corticosteroid-induced lympholysis. Corticosteroids also reduce both the phagocytic and killing ability of macrophages and neutrophils, and this effect may contribute to their anti-inflammatory action (Barnes, 2006). In addition, they reduce chemotaxis, so that fewer inflammatory cells are attached to the site of THcell activation. In presence of corticosteroids, expression of class II MHC molecules and IL-1 production by macrophages is dramatically reduced; such reduction would be expected to lead to corresponding reductions in THcell activation. Corticosteroids also stabilize the lysosomal

membrane, so that decreased levels of lysosomal enzymes are released at the site of inflammation (Norman *et al.,* 2004). Side effects of corticosteroids include respiratory depression, sedation, constipation, immunosuppression hallucination (Oray *et al*., 2016).

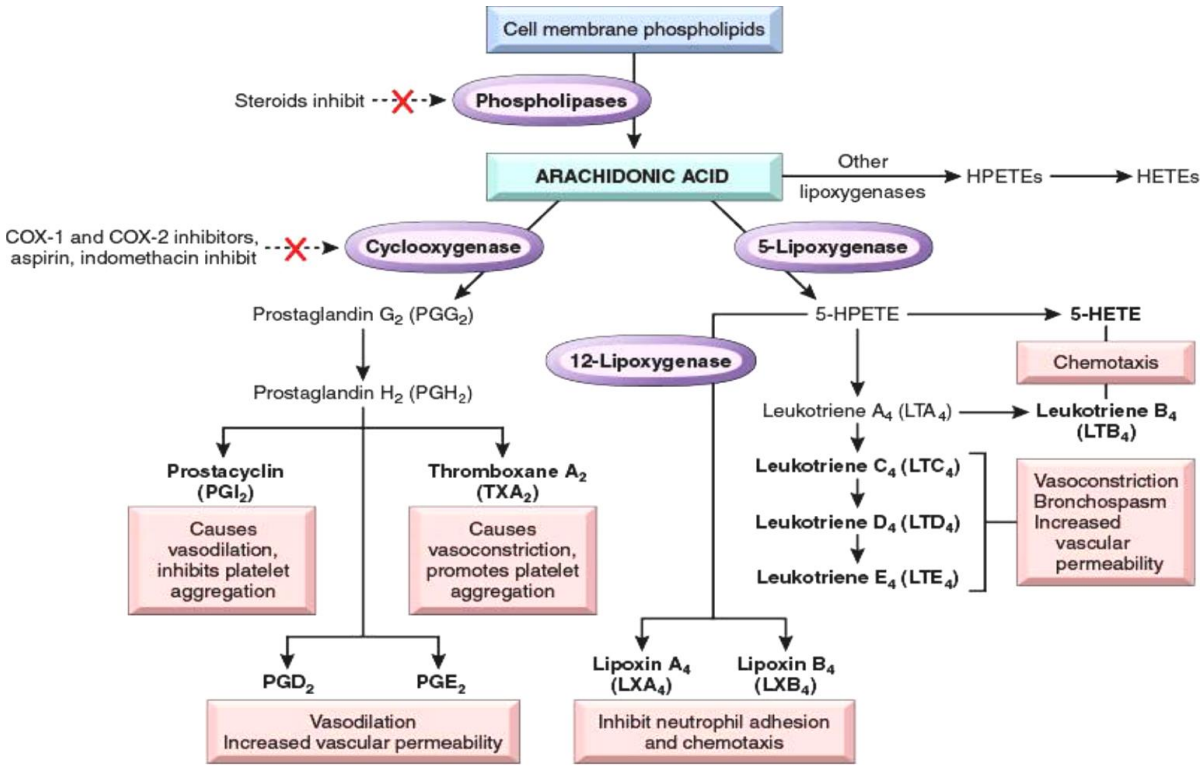


Figure 3: Arachidonic Acid Metabolites and Inflammation
Source: Robbins and Cotran, 2010.

1.7.3 Herbs

Many medicinal plants have been used in the treatment of many diseases due to their rich sources of bioactive components with curative properties (Newman *et al*., 2003; Duraipandiyan *et al*., 2006; Njoku *et al*., 2017). Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethnopharmacognosy. They found literally thousands of phytochemicals (from plants) as safe and broadly effective alternatives with less adverse effect (Naczk and Shahidi, 2006). The bioactive principles in these plant species have been linked to secondary metabolites such as phenolic compounds (curcumins, flavonoids and tannins), saponins, terpenoids and alkaloids (Iwalewa *et al*., 2007; Hodzic *et al*., 2009). The mechanisms of action of many phenolic compounds such as flavonoids, tannins and curcumins are thought to be via their free radical scavenging activities or the inhibition of pro inflammatory enzymes such as cyclo-oxygenases (COX) and lipoxygenases (LOX) in the inflammatory cascades (Sadik *et al*., 2003). Many beneficial biological activities such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity were reported (Sasidharan *et al*., 2011**)**.

Herbs such as *Tithonia diversifolia* and *Carum copticum* (Owoyele *et al*., 2003; Thangam and Dhanajayan, 2003) have been shown to possess anti-inflammatory and analgesic effects and such plants can be used as sole therapy in managing inflammatory conditions or as complementary therapy allowing patients to take smaller doses of conventional anti-inflammatory drugs, thereby minimizing the side effects of these drugs. Evidence exists that drugs derived from natural products can modulate various inflammatory mediators, the production and/or action of secondary messengers and expression of key pro-inflammatory molecules such as COX-2 and cytokines (TNFα and IL-Iβ) (Bellik *et al*., 2013). However, clinical trials are necessary to demonstrate the effectiveness of a bioactive compound to verify this traditional claim. Some herbs such as *Curcuma longa, Aegle marmelos, Cassia spp., Centaurea cyanus* and *Butea monosperma* have clinical evidence that they can significantly reduce inflammation (Doshi *et al*., 2011).

1.8 Oxidants and Antioxidants

1.8.1 Oxidants

Oxidants, also known as oxidizing agents, are chemical compounds that easily transfer oxygen or substances that gain electrons in a redox chemical reaction. Anti-oxidants help organisms deal with oxidative stress that is caused by free radical damage. Free radicals are chemical species, which contain one or more unpaired electrons which make them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability (Pietta, 2000; Shahin *et al*., 2008). Excess free radicals can result from tissue damage and hypoxia, overexposure to environmental factors (smoking, ultraviolet radiation, and pollutants), a lack of antioxidants, or destruction of free radical scavengers. When the production of damaging free radicals exceeds the capacity of the body's antioxidant defences to detoxify them, a condition known as oxidative stress occurs. The cellular injury caused by oxidative stress has been linked to over 200 clinical disorders (Pukalskas, 2008). Any free radical involving oxygen is referred to as reactive oxygen species (ROS) (McDermott, 2000). The most commonly formed ROS are superoxide anion radical ($O^{2\bullet-}$) and hydroxyl radical (•OH) (Wilson and Mogil, 2001). $O^{2\bullet-}$ is formed when one electron is added to an oxygen molecule, and is considered the least reactive type of ROS (Kohen and Nyska, 2002). Once $O^{2\bullet-}$ is produced, it triggers a rapid cascade of events that creates other free radicals, eventually terminating in the formation of H_2O. In humans, $O^{2\bullet-}$ is the most commonly produced free radical. Phagocytic cells such as macrophages and neutrophils are prominent sources of $O^{2\bullet-}$. In an inflammatory response, these cells generate free radicals that attack invading pathogens such as bacteria. Production of $O^{2\bullet-}$ by activated phagocytic cells in

response to inflammation is one of the most studied free radical-producing systems (Gutteridge and Mitchell, 1999). If oxygen attracts two hydrogen molecules, hydrogen peroxide (H_2O_2) is formed. Athough H_2O_2 is not technically considered an oxygen free radical, it is a member of the ROS family and may selectively participate in free radical generation (Kerr *et al*., 1996). The majority of the H_2O_2 is broken down to oxygen and water by the cellular enzyme catalase. In addition to catalase, the enzyme glutathione peroxidase is responsible for the breakdown of H_2O_2 and any peroxides that form on lipids within the body (Gutteridge and Mitchell, 1999). The hydroxyl radical (•OH) is the most reactive of the free radical molecules and damages cell membranes and lipoproteins by a process called lipid peroxidation.

1.8.2 Antioxidants

An antioxidant is defined as any substance or compound present at low concentrations that can prevent biomolecules from undergoing oxidative stress through free radical mediated reaction (Proestos *et al*., 2013). Antioxidants help organisms deal with oxidative stress, caused by free radical damage. Reactive oxygen species (ROS) formed *in vivo*, such as superoxide anion, hydroxyl radical and hydrogen peroxide, are highly reactive and potentially damaging transient chemical species. These are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signalling and immune function. ROS are regulated by endogenous superoxide dismutase, glutathione peroxidase and catalase but due to over-production of reactive species, induced by exposure to external oxidant substances or a failure in the defence mechanisms, damage to cell structures, DNA, lipids and proteins occur which increases risk of more than 30 different disease processes (Valko *et al.*, 2006). The most notorious among them being neurodegenerative conditions like Alzheimer's disease (AD) (Smith *et al*, 2000), mild cognitive impairment (MCI) (Guidi *et al*., 2006) and Parkinson's disease (PD) (Bolton *et al.*, 2000). All these diseases are associated with significant increase in the specific and persistent lipid peroxidation marker F2-isoprostane (Greco *et al.*, 2000).

The body interestingly possesses defence mechanisms against free radical-induced oxidative stress, which involve preventative mechanisms, repair mechanisms, physical defenses and antioxidant defenses. Enzyme antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). The non-enzyme antioxidants include ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids and flavonoids. All these act by one or more of the mechanisms (Table 1) like reducing activity, free radical-

scavenging, potential complexing of pro-oxidant metals and quenching of singlets oxygen (Lu *et al*., 2010). It is possible to reduce the risks of chronic diseases and prevent disease progression by either enhancing the body's natural anti-oxidant defences or by supplementing with proven dietary antioxidants (Stanner *et al.*, 2004). This is one of the reasons why discovery and synthesis of novel antioxidants is a major active area. Synthetic antioxidants like butylatedhydroxytoluene (BHT) and butylatedhydroxyanisole (BHA) commonly used in processed foods have side effects and are carcinogenic. In recent years, the use of natural antioxidants present in food and other biological materials has attracted considerable interest due to their presumed safety, nutritional and therapeutic value (Ajila *et al*., 2007). Anti-oxidants derived from fruits, vegetables, spices and cereals are very effective and have reduced interference with the body's ability to use free radicals constructively (Wolfe *et al.*, 2003). Natural antioxidants mainly come from plants in the form of phenolic compounds, flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols, ascorbic acid and carotenoids. The quest for natural antioxidants for dietary, cosmetic and pharmaceutical uses has become a major industrial and scientific research challenge over the last two decades. Efforts to gain extensive knowledge regarding the power of antioxidants from plants and to tap their potential are therefore on the increase.

Table 1: Mechanism of Action of Various Anti-oxidants in Different Disease Conditions

Compounds	**Pathology**	**Mechanism of action**
Glutathione	Cancer	Glutathione in the nucleus maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression
Catalase (CAT)	Cancer, diabetic retinopathy	Destroys hydrogen peroxide in high concentration by catalyzing its two-electron dismutation into oxygen and water
Glutathione Peroxide (GPx)	Neurodegenerative disease	Catalyse the reduction of hydroperoxide at the expense of GSH. In the process, hydrogen is reduced to water whereas hydroperoxides are reduced to alcohol
Superoxide Dismutase	Neurodegenerative disease	Catalyse the one-electron dismutation of superoxide into hydrogen peroxide oxygen
Phenolics	Cancer. diabetic retinopathy	Inhibit the oxidation of lipids, fats and proteins by donation of a phenolic hydrogen atom to free radical
Carotenoids	Cancer, diabetic retinopathy, chronic inflammation	Mainly act as physical quenches of reactive oxygen
Catechins	Neurodegenerative disease	Enhances activity of SOD and catalase
Alkaloids	Neurodegenerative disease, cancer, chronic inflammation	a. inhibition of topoisomerase I and II b. cytotoxicity against different tumor cell lines

Source: Ali *et al*., 2008

1.9 Pain

Pain is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey and Bogduk, 1994). It is a sensory modality which in many cases represents the only symptom for the diagnosis of several diseases. It often has a protective function (Almeida *et al*., 2001). The perception of a series of sensory events is required for the brain in order to detect pain and produce a response towards the threat. There are generally three main stages in the perception of pain. The first stage is pain sensitivity, followed by the second stage where the signals are transmitted from the periphery to the dorsal horn (DH), which is located in the spinal cord via the peripheral nervous system (PNS). Lastly, the third stage is to perform the transmission of the signals to the higher brain via the central nervous system (CNS) (Mun *et al*., 2018). Pain is a normal manifestation of everyday life and serves as a vital defensive function. However, uncontrolled pain can dramatically diminish quality of life and for this reason compounds that can control or eliminate pain have to be looked for through research. Trauma and stimulation of the peripheral or central nervous system can change immune responses. As a consequence, peptides and receptors are activated, followed by the translation of the signal to the intracellular environment (Lacerte and Shah, 2003). Hence, advances in the knowledge of the neuranatomy of conduction pathways, neuropharmacology, and pathophysiology of pain facilitate the development of research on new treatment modalities. Thus, effective analgesia for painful syndromes is still a great challenge.

1.9.1 Classification of Pain

Pain has constantly been described as a symptom. However, current advances in the understanding of neural mechanisms have confirmed that unrelieved pain may lead to changes in the nervous system and as such pain, particularly chronic pain, may be considered a disease in itself (Smith *et al.*, 2000). Pain is categorized according to its duration (acute and chronic pain) and types (nociceptive, neuropathic and inflammatory pain).

1.9.1.1 Pain – According to Duration

a. Acute Pain

Acute pain is of recent onset and (probably) limited duration, usually having an identified temporal and causal relationship to injury or disease. Acute pain generally comprises two phases. The first phase (lasting seconds) "alerts" the individual to potentially dangerous stimuli and the second, subchronic phase (lasting hours to days) may be regarded as a

"protective" mechanism characterized by "guarding" of the injured tissue as a means of promoting healing and recovery (Merskey and Bogduk, 1994). Intensity of acute pain is from mild to severe and it is used to describe conditions, such as post-operative pain, pain following trauma or procedural pain. Somatic acute pain arises from injury to skin, bone, joint, muscle, and connective tissue, and it is generally localized to the site of injury (Abdel-Salem and El-Batran, 2005).

b. Chronic Pain

Chronic pain is defined as "pain lasting for long periods of time. It persists beyond the point at which healing would be expected to be complete, or that occurring in disease processes in which healing does not take place, may be accompanied by severe psychological and social disturbance (DeLeo, 2006). Persistent pain is often regarded as a maladaptive response that confers no physiological advantage, such that the pain state itself has become the "disease", requiring treatment (Cousins, 2007). This type of pain includes the pain associated with various neuropathic and musculoskeletal disorders such as headaches, fibromyalgia, rheumatoid arthritis, and osteoarthritis. It is now known that in chronic pain, presynaptic receptors on sensory nerve terminals in the periphery contribute to increased excitability of sensory nerve endings (peripheral sensitization) (Agarwal *et al.*, 2007). The hyperexcitable sensory neuron bombards the spinal cord, leading to increased excitability and synaptic alterations in the dorsal horn. Such changes appear to be important in chronic inflammatory and neuropathic pain states (Agarwal *et al.*, 2007; Trevisani *et al.*, 2007).

1.9.1.2 Pain – According to Type

a. Nociceptive Pain

Nociception used interchangeably with nociperception is the response of our bodies' sensory nervous systems towards actual or potentially harmful stimuli. The sensory endings that are activated by such stimuli are known as nociceptors which are mainly responsible for the first stage of pain sensations (Mun *et al*., 2018). The nociceptive system extends from the receptors in the periphery to the spinal cord, brain stem, and to the cerebral cortex where pain sensation is perceived. This system is a key physiological function that prevents further tissue damage due to the body's autonomic withdrawal reflex (Baron and Treede, 2007; Suardiaz *et al.*, 2007). When tissue damage occurs despite the nociceptive defense system, inflammatory pain ensues. The body now changes focus from protecting against painful stimuli to protecting the injured tissue. The inflammatory response contributes to pain hypersensitivity

that serves to prevent contact or movement of the injured part until healing is complete, thus reducing further damage (Anseloni and Gold, 2008; Harvey and Dickenson, 2008).

b. Neuropathic Pain

Neuropathic pain is commonly described as a nerve injury or nerve impairment and serves no purpose in terms of defense system for the body, and the pain could be in the form of continuous sensation or episodic incidents (Mun *et al*., 2018). It is also a spontaneous pain and hypersensitivity to pain associated with damage to or pathologic changes in the peripheral nervous system as in painful diabetic peripheral neuropathy (DPN), acquired immunodeficiency syndrome (AIDS), polyneuropathy, post-herpetic neuralgia (PHN); or pain originating in the central nervous system (CNS), that which occurs with spinal cord injury, multiple sclerosis, and stroke (Baron and Treede, 2007; Garcia-Larrea and Magnin, 2008). At present, persistent inflammatory and neuropathic pains present great challenges to general practitioners and pain specialists alike, because the currently available drugs used to treat these conditions have significant limitations. Moreover, opioids that are effective for the relief of moderate to severe nociceptive pain are often considerably less effective for the relief of neuropathic pain, particularly when administered by systemic routes.

c. Inflammatory Pain

Inflammatory pain such as arthritis may be defined as hypersensitivity that arises in inflamed tissue following sensitization of peripheral nerve terminals. Inflammation is a natural biological response produced by the tissues within our body as a reaction to the harmful stimuli in order to eradicate the necrotic cells and initiate the tissue repairing process. Neutrophils are usually the first respondents of an inflammatory response and gather at the site of injury via the bloodstream, followed by the release of other chemical mediators (Yoo *et al*., 2011). Inflammation may lead to three major responses: hyperalgesia, allodynia and sympathetic maintained pain. An inflammation can also induce mast cell degranulation, which subsequently leads to the release of platelet activating factor (PAF) and stimulates the release of 5-HT from the circulating platelet. The cardinal signs of inflammation include the hot inflamed site due to increase in blood flow towards the region, redness, and swelling due to vascular permeability pain caused by the activation and sensitization of primary afferent neurons and lasting loss of function (Medzhitov, 2008; Ekwueme *et al*., 2011). The localized inflammatory response then induces the release of free arachidonic acid (AA) from the phospholipids, which are converted into prostaglandins (PG) via the cyclooxygenase (COX) pathways (Basbaum *et al*., 2009). There are some mediators produced at the site of injured

tissue, which include 5-HT, kinins, histamine, nerve growth factors (NGF), adenosine triphosphate (ATP), PG, glutamate, leukotrienes, nitric oxide (NO), NE and protons (Basbaum *et al*., 2009). During the process of inflammation, these chemical inflammatory mediators are produced from the necrotic tissues, and interact to activate the nociceptors within the inflamed area.

1.9.2 Basic Mechanisms of Action of Pain

Fundamentally, the basic pain mechanism undergoes three events—transduction, transmission and modulation when there is a presence of noxious stimuli (Mun *et al*., 2018). Transduction occurs along the nociceptive pathway following such order: (1) stimulus events are converted to chemical tissue events; (2) chemical tissue and synaptic cleft events are then changed into electrical events in the neurons; and (3) electrical events in the neurons are transduced as chemical events at the synapses. After the completion of transduction, the following mechanism would be transmission. It takes place by transmitting the electrical events along the neuronal pathways, while neurotransmitters in the synaptic cleft transmit information from a post-synaptic terminal of one cell to a pre-synaptic terminal of another. Meanwhile, the modulation event takes place at all level of nociceptive pathways through the primary afferent neuron, DH and higher brain centre by up- or down-regulation. All these lead to one end result, and the pathway of pain has been initiated and completed, thus allowing us to feel the painful sensation triggered by the stimulus (Basbaum *et al*., 2009).

1.9.3 Pain Management

The primary goal of pain management is to acutely provide comfort and safety; secondary goals are to prevent the immediate and long-term complications of pain. In general, physicians are more comfortable treating acute pain, which usually is caused by soft tissue damage, infection and/or inflammation among other causes. It is usually treated simultaneously with pharmaceuticals, commonly analgesics, or appropriate techniques for removing the cause and for controlling the pain sensation. The failure to treat acute pain properly may lead to chronic pain in some cases (Greco *et al.*, 2000).

a. Anesthesia

Anesthesia is the condition of having the feeling of pain and other sensations blocked by drugs that induce a lack of awareness. It may be a total or a minimal lack of awareness throughout the body (i.e. general anesthesia), or a lack of awareness in a part of the body (regional or local anesthesia).

b. Analgesia

Analgesia is an alteration of the sense of pain without loss of consciousness. The body possesses an endogenous analgesia system, which can be supplemented with painkillers or analgesic drugs to regulate nociception and pain. Analgesia may occur in the central nervous system or in peripheral nerves and nociceptors. The endogenous central analgesia system is mediated by three major components: the periaqueductal grey matter, the nucleus raphe and the nociception-inhibitory neurons within the dorsal horns of the spinal cord, which act to inhibit nociception-transmitting neurons also located in the spinal dorsal horn. The peripheral regulation consists of several different types of opioid receptors that are activated in response to the binding of the body's endorphins. These receptors, which exist in a variety of areas in the body, inhibit firing of neurons that would otherwise be stimulated to do so by nociceptors (Straube *et al.*, 2009).

Opioids are the mainstay for the treatment and prevention of pain. Opioids stimulate μ-opioid receptors, a subtype of G protein– coupled receptors that inhibit the release of inflammatory and excitatory mediators and interfere with the propagation of nerve signals, ultimately to dampen the excitability of nociceptors. They also activate N-methyl-d-aspartate (NMDA) receptors, which may lead to stimulation of nociceptors and development of tolerance. When administered in equianalgesic doses, the opioids have similar efficacy and similar non-cardiovascular adverse effects, including tolerance, constipation, suppression of cough reflexes, and respiratory depression (Chu, 2008). Pentazocine is an opioid analgesic, less potent than morphine. It acts by binding to opiate receptors in the central nervous system causing inhibition of ascending pain pathways thereby altering the perception of, and response to pain (Tade *et al*., 2009). Non-opioid analgesics which include most NSAIDs exert their analgesic properties principally by inhibiting the enzyme cyclo-oxygenase, resulting in a reduction in the synthesis of mediators of the acute inflammatory response, including prostaglandin synthesis in the spinal cord (Oyler *et al*., 2015). However, the choice of analgesic agent depends on a variety of patient factors, including the type, duration, and severity of pain; hemodynamic status; prior response or tolerance to therapy; presence of organ dysfunction and other comorbidities; potential for harmful drug interactions and adverse drug events; and the required onset and duration of pain relief. The route of administration of analgesia is an important consideration as well.

1.10 Isolation and Purification of Bioactive Compounds

Natural products, such as plant extracts, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity (Cosa *et al*., 2006; Naczk and Shahidi, 2006). An essential part in the investigation of plant is the identification of the biologically active compounds present in a plant leading to further biological and pharmacological studies (Momin *et al*., 2014; Farid *et al*., 2015). The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compounds, toxicological evaluation and clinical evaluation.

1.10.1 Extraction of Plant Material

Extraction is a process of obtaining one or more components from a solid material or withdrawal of a solute from a liquid to another liquid. Solid samples are usually cut into small pieces or ground into fine particles to facilitate solvent penetration. Stirring or shaking can be applied to increase the diffusion rate. Botanicals and herbal preparations for medicinal use contain various types of bioactive compounds and extraction is the first and most important step in the analysis of constituents present in botanicals and herbal preparations (Sasidharan *et al*., 2011). This is necessary to extract the desired chemical components from the plant materials for further separation and characterization. The basic steps include; pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample. Different solvents are available to extract the bioactive compound from natural products. Solvents used for the extraction of biomolecules from plants are chosen based on the polarity of the solute of interest. A solvent of similar polarity to the solute will properly dissolve the solute (Altemimi *et al*., 2017). The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate while that of lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are used. In some instances, extraction with hexane is used to remove chlorophyll (Cosa *et al*., 2006). The commonly used classical techniques are: soxhlet extraction, maceration and hydro distillation to obtain a crude extract which is then concentrated using a rotary evaporator (Azmir *et al*., 2013). The crude extracts are complex mixture of compounds, and are therefore not suitable for instant bioactivity screening. Thus, the crude extracts need to be fractionated prior to bioactivity screening. There are several techniques available for fractionation of crude extracts. This includes High Pressure Liquid Chromatography, column chromatography and flash chromatography. Fractionation is a procedure used to separate mixtures into fractions, which have compositions consistent with the gradient.

1.10.2 Column Chromatography

In column chromatography, the stationary phase (a solid adsorbent) is placed in a vertical glass column and the mobile phase (a liquid) is added at the top and flows down through the column (by either gravity or external pressure). Column chromatography is generally used as a purification technique to isolate desired compounds from a mixture (Kenkel, 2003). The crude extract to be purified by column chromatography is applied at the top of the column. The liquid solvent (the eluent) is passed through the column by gravity or by the application of air pressure. Equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column. Because the different components in the mixture have different interactions with the stationary and mobile phases, they will be carried along with the mobile phase to varying degrees and a separation will be achieved. The individual components, or elutants, are collected as the solvent drips from the bottom of the column (Harvey, 2000). Silica gel (SiO_2) and alumina (Al_2O_3) are the two adsorbents commonly used for column chromatography. These adsorbents are sold in different mesh sizes, indicated by a number on the bottle label. The polarity of the solvent which is passed through the column affects the relative rates at which compounds move through the column (Harvey, 2000). Polar solvents can compete more effectively with the polar molecules of a mixture for the polar sites on the adsorbent surface and will also solvate the polar constituents better. Consequently, a highly polar solvent will move even highly polar molecules rapidly through the column. If a solvent is too polar, movement becomes too rapid, and little or no separation of the components of a mixture will result. If a solvent is not polar enough, no compounds will elute from the column. Proper choice of an eluting solvent is thus crucial for the successful application of column chromatography as a separation technique. Often series of increasingly polar solvent systems are used to elute a column. A non-polar solvent is first used to elute the less-polar compounds. Once the less-polar compound is off the column, a more-polar solvent is added to the column to elute the more-polar compounds (Kenkel, 2003).

1.11 Identification of Bioactive Compounds

Plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities. There is need to isolate and identify these bioactive compounds from plant extracts which remain a big challenge, this is to ascertain which is responsible for its pharmacological activities. A number of different separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography and HPLC, should be used to obtain pure compounds (Huie, 2002; Sasidharan *et al*., 2011). The

pure compounds are then used for the determination of structure using methods such as FTIR, GCMS and NMR (Popova *et al*., 2009).

1.11.1 Thin Layer Chromatography (TLC)

Thin-layer chromatography (TLC) is an easy, cheap, rapid, and widely used method for the analysis and isolation of natural and synthetic products. Silica gel column chromatography and thin-layer chromatography (TLC) have been used for separation of bioactive molecules with some analytical tools (Zhang *et al*., 2005). Separation by TLC is effected by the application of a mixture or extract as a spot or thin line on to a sorbent that has been applied to a backing plate. The plate is then placed into a tank with sufficient suitable solvent just to wet the lower edge of the plate/sorbent but not adequate to wet the part of the plate where the spots were applied (origin). The solvent front then migrates up the plate through the sorbent by capillary action. A factor in quantifying migration of a compound on a particular sorbent and solvent system is the retention factor (R_f) value (Merck, 1980). This is defined as:

$$R_f = \frac{\text{distance traveled by the solute}}{\text{distance traveled by the solvent}}$$

TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound (Sasidharan *et al*., 2011). Additional tests involve the spraying of phytochemical screening reagents, which cause color changes according to the phytochemicals existing in a plants extract; or by viewing the plate under the UV light (Merck, 1980). It is also used to analyze the fractions obtained from column chromatography to determine if the fraction contains more than one component and if fractions can be combined without affecting their purity (Kenkel, 2003). This has also been used for confirmation of purity and identity of isolated compounds. TLC analysis also has short comings: low resolution, low sensitivity and the difficulty of detection of trace components, etc. (Zhang, *et al*., 2011).

1.11.2 Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR has played a vital role in pharmaceutical analysis in recent years (Thenmozhi *et al*., 2011; Murugesh and Vino, 2017). FTIR has proven to be a valuable tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plants extract (Eberhardt *et al*., 2007; Hazra *et al*., 2007**)**. In addition, FTIR spectra of pure compounds are usually so unique that they are like a molecular "fingerprint". For most common plant compounds, the spectrum of an unknown compound can be identified by comparison to a library of those of known compounds.

Fourier transform infrared (FTIR) spectroscopy analysis can identify chemical bonds by using an infrared spectrum that is absorbed by the material (Munajad *et al*., 2018). Samples for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of sample between two plates of sodium chloride. The drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) to and the compressed into a thin pellet which can be analyzed. Otherwise, solid samples can be dissolved in a solvent such as methylene chloride, and the solution then placed onto a single salt plate. The solvent is then evaporated off, leaving a thin film of the original material on the plate (Sasidharan *et al*., 2011).

1.11.3 Gas Chromatography Mass Spectroscopy (GCMS)

Gas Chromatography Mass Spectroscopy is a very compatible technique and the most commonly used technique for the identification and quantification purpose. The unknown organic compounds in a mixture can be determined by interpretation and also by matching the spectra with reference spectra (Ronald, 1997). It is most useful for the analysis of trace amounts of organically extractable, non-polar, volatile compounds and highly volatile compounds. Moreover, the use of GC-MS in the scan mode allows for non-targeted metabolic profiling and the discovery of novel compounds and metabolites (Krone *et al*., 2010). GC-MS with high specificity, high sensitivity, stability and small amount of sample characteristics, are unanimously accepted as the method for the analysis of volatile constituents. Moreover, the high selectivity of capillary columns enables separation of many volatile compounds simultaneously within very short time. GC-MS has limitations in the analysis of highly polar compounds due to their thermolability and low volatility (Yusuke *et al*., 2012).

1.12 Rationale for the Study

Inflammation and oxidative stress are now becoming major health challenges. Presently in developing countries, synthetic drugs are not only expensive but are also often with adulterations and side effects. *Schumanniophyton magnificum* is widely used in African ethnomedicine in the treatment of various diseases, in particular, malaria (Bickii *et al*., 2007), dysentery (Tane *et al*., 1990) and as snake repellent (Okogun *et al*., 1983). However, there is need to evaluate the anti-inflammatory and analgesic activities of *Schumanniophyton magnificum* leaves.

1.13 Aim and Objectives of the Study

1.13.1 Aim of the Study

The aim of this study was to evaluate the anti-inflammatory and analgesic activities of *Schumanniophyton magnificum* leaves and to determine the nature of the anti-inflammatory constituents responsible.

1.13.2 Specific Objectives of the Study

The aim of this study was achieved through the following specific objectives:

i. Determination of the phytochemical constituents of methanol leaf extract of *Schumanniophyton magnificum*
ii. Determination of the median lethal dose toxicity (LD_{50}) of the extract.
iii. Determination of the effect of methanol extract of the leaves of *S. magnificum* on egg albumin-induced oedema (acute inflammatory study) and formalin-induced oedema (sub-chronic inflammatory study)
iv. Assay for phospholipase A_2 activity of methanol crude extract, partitions and fractions of the leaves of *S. magnificum*
v. Determination of the effect of methanol extract of the leaves of *S. magnificum* on membrane stabilization of human red blood cell and anti-platelet aggregatory response
vi. Assay for some *in vitro* antioxidant activities of methanol extract of the leaves of *S. magnificum*
vii. Determination of the effect of methanol extract of the leaves of *S. magnificum* on acetic acid-induced vascular permeability and *in vivo* leukocyte mobilisation
viii. Determination of the effect of methanol extract of the leaves of *S. magnificum* on lipid peroxidation (MDA concentration), some haematological parameters, liver enzyme activities and lipid profile of rats with formalin-induced oedema
ix. Determination of the analgesic activities of methanol extract of the leaves of *S. magnificum* using acetic acid-induced writhing, hot plate analgesic studies and tail flick response in mice.
x. Fractionation and identification of the bioactive compounds in the most active fraction.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Material

The leaves of *Schumanniophyton magnificum* were used for this study. The fresh leaves were collected from Ukehe town in Igboetiti Local Government Area of Enugu State, Nigeria.

2.1.2 Animals, Blood Samples and Microorganism

Adult Wistar albino rats and Swiss albino mice were used for this study. All the animals used were obtained from the animal house of the Department of Zoology, University of Nigeria Nsukka. The animals were kept in well aerated steel cages in the animal house of Department of Biochemistry. The animals were acclimatized for 14 days under standard laboratory environment and maintained on a regular feed and water before use.

Blood samples used for *in vitro* studies were collected from healthy donors who were ascertained to be free from any drug treatment for at least two weeks before blood collection.

Bacillus cereus used as a source of phospholipase A_2 was obtained from the Department of Microbiology, University of Nigeria, Nsukka.

2.1.3 Equipment and Instruments

Centrifuge (Vickas Ltd, England), Electron microscope (Vickas Ltd, England), Haematocrit Centrifuge (Vickas Ltd, England), Haemoglobin graph (Jenway, UK), FTIR machine (Shimadzu FTIR– 8400s), Oven (Gallenkamp, England), GC-MS analyzer (GC-MS-QP 2010 Shimadzu, Japan), Pasteur pipette (Pyrex, England), Refrigerator Thermocool (England), Rotary evaporator Eyla N-1000 (Japan), Spectrophotometer (E312 Model) (Jenway, UK), Water bath (Gallenkamp, England), Weighing Balance (Vickas Ltd, England).

2.1.4 Chemicals and Reagents

The chemicals and reagents used were of analytical grade and were products of Sigma Aldrich, (USA), Qualikems (India), Merek Darmstadt (Germany), British Drug House (BDH) (England), May and Baker (England), Reagents used were commercial kits and products of Randox, (USA). Sterile distilled water was used in the preparation of the chemicals and/or reagents.

2.2 Methods

2.2.1 Collection of Plant Material

The fresh leaves were collected from Ukehe town in Igbo Etiti Local Government Area of Enugu State, Nigeria and identified by Mr. Alfred Ozioko of International Centre for Ethnomedicine and Drug Development (InterCEDD) Nsukka, Enugu State Nigeria where the voucher number Intercedd/837 was deposited. The leaves were dried under room temperature and pulverized to coarse powder using a milling machine.

2.2.2 Extraction of Plant Material

A weighed quantity (1974.92 g) of the ground leaves was extracted with methanol by cold maceration for 48 hours at room temperature (26-28^{O}C). The macerate was filtered using Whatman No. I filter paper and concentrated using a vacuum rotary evaporator 40-60^{O}C. The extract was stored in an air-tight plastic container in the refrigerator (4^{O}C).

The extractive yield was calculated using the relation:

$$\text{Yield (\%)} = \frac{\text{weight of extract (g)}}{\text{weight of plant material (g)}} \; x \; 100$$

2.2.3 Partitioning of the Crude Extract

Partitioning of the crude extract was achieved using n-hexane, dichloromethane, ethylacetate and aqueous methanol (90:10 v/v) according to their polarity as described by Rajbir *et al*. (2008). Crude extract (10 g) was dissolved in 250 ml of aqueous methanol (90:10) and poured into a separating funnel. n-hexane (250 ml) was poured and shaken vigourously and allowed to stand. Two layers were formed; upper and lower layers. The upper layer was collected and designated n-hexane partition. The lower layer was poured back to the funnel and re-washed with n-hexane. Dichloromethane was introduced into the aqueous portion (residue), and was treated same as n-hexane. However, the lower layer was the dichloromethane partition. After re-washing with dichloromethane, ethylacetate was introduced and treated likewise. After re-washing the last portion was the aqueous methanol partition (Fig. 4). In all, 30g of the crude extract was partitioned. The partitions were concentrated and used for qualitative phytochemical screening and PLA_2 activity. Phospholipase A_2 activity was carried out on the four partitions to show which partition was the most potent.

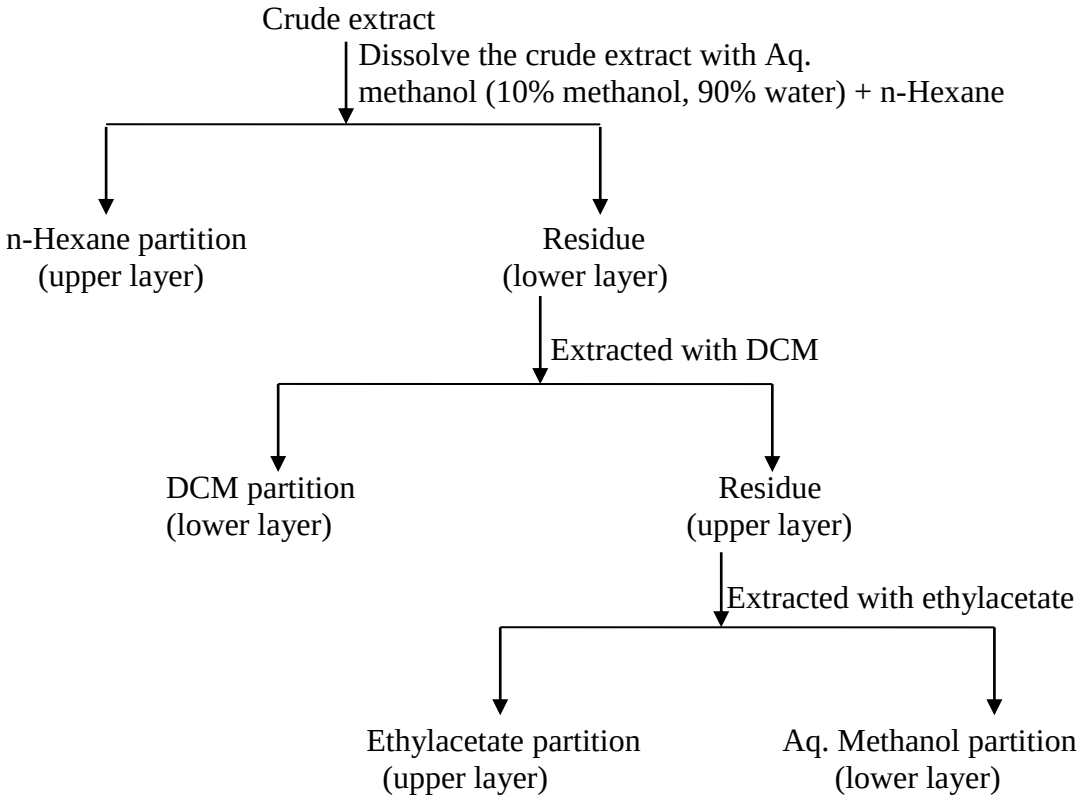


Figure 4: Partitioning scheme using immiscible solvents

2.2.4 Procedure for Thin Layer Chromatography of n-hexane Partition of Methanol Extract of *Schumanniophyton magnificum* Leaves

The most potent partition (n-hexane) was spotted on thin layer chromatographic plate pre-coated with silica-gel (Merck, silica gel 60 F_{254}). The plates were developed in chromatographic tanks with toluene:ethylacetate (80:20) as the solvent system which gave about 6-8 visible bands when viewed under ultraviolet (UV) light. The plate was allowed to stay for elution to be achieved. The n-hexane partition was taken to column chromatography for further purification.

2.2.5 Procedure for Column Chromatography of n-hexane Partition of Methanol Extract of *Schumanniophyton magnificum* Leaves

Silica gel (100 g) of mesh size 60-120 were mixed with 250 ml of eluent toluene:ethylacetate (80:20) and stirred for few minutes. The column was packed with the silica gel using a funnel. The sample was introduced by pouring on the wall of the column and subsequently eluted at about 5 ml volume interval. In all, 80 fractions of about 5 ml each were collected. The fractions were spotted on TLC plate and their R_f value calculated. The fractions with similar retention factor (R_f) value were pooled together. In all, four sub fractions were obtained.

2.2.6 Procedure for FTIR of Toluene:Ethylacetate (80:20) Fractions of n-hexane Partition of Methanol Extract of *Schumanniophyton magnificum* Leaves

The FTIR spectra of the fractions were carried out using FTIR-8400S spectrophotometer (Shidmazu model), available at the National Research Institute for Chemical Technology (NARICT) Laboratory Zaria. The fractions were used in a form of a thin film, held in between two potassium bromide discs. The liquid paste was dropped on each disc and they spread into a thin film. The disc was then mounted in the FTIR spectrometer in the range 1.20 × 1013–1.20 × 1014 Hz) within electromagnetic spectrum of infrared section. Absorption was written in terms of wave numbers (units cm^{-1}).

2.2.7 Procedure for GCMS of Toluene:Ethylacetate (80:20) Fractions of n-hexane Partition of Methanol Extract of *Schumanniophyton magnificum* Leaves

Chemical composition of active extracts and fractions was determined by GC/MS-QP-2010 plus Ultra (Shimadzu, Kyoto Japan) using a DB-5 MS fused silica capillary column (30 × 0.25 m internal diameter, film thickness 0.25 µm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Carrier gas was Helium gas, flow rate was 1.2 mL/min. Injector and MS transfer line temperature were set at 260^{o}C and 270^{o}C respectively. Oven temperature was initially maintained for 2 min, and then increased to 210^{o}C at a rate of 8^{o}C/min to 280^{o}C; hold time was 10 min. Samples were completely dissolved in absolute ethanol and 0.3 µL was injected through auto-sampler in the split mode, split ratio was 1:100. Relative percentage of each constituent was expressed as percentages by peak area normalization. Each component was identified based on its column retention time relative to computer-based matching of mass spectra with those of standards (NIST and Wiley libraries for GC-MS system).

2.2.8 Preparation of Reagents

i. Ferric chloride solution (5% (w/v))

A quantity, 5.0 g ferric chloride was dissolved in 100 ml of distilled water.

ii. Ammonium solution

Stock concentrated ammonium solution (187.5 ml) was diluted in 31.25 ml of distilled water and then made up to 500 ml with distilled water.

iii. Ethanol (45% (v/v))

A quantity, 45 ml of absolute ethanol was mixed with 55 ml of distilled water.

iv. Aluminium chloride solution

Aluminium chloride (0.5 g) was dissolved in 100 ml of distilled water.

v. Dilute sulphuric acid

A 10.9 ml aliquot of concentrated sulphuric acid was mixed with 5 ml of distilled water and made up to 100 ml.

vi. Lead sub acetate solution

A quantity, 45 ml of 15% lead acetate (i.e. 15.0 g of lead acetate in 100 ml of distilled water) was dissolved in 20 ml of absolute ethanol and made up to 100 ml with distilled water.

vii. Wagner's reagent

A known quantity, 2.0 g, iodine crystals and 3.0 g potassium iodide were dissolved in minimum amount of water and then made up to 100 ml with distilled water.

viii. Mayer's reagent

A known weight, 13.5 g, of mercuric chloride was dissolved in 50ml of distilled water. Also, 5.0 g of potassium iodide was dissolved in 20 ml of distilled water. The two solutions were mixed and the volume was made up to 100 ml with distilled water.

ix. Dragendorff's reagent

Bismuth carbonate (0.85 g) was dissolved in 100 ml of glacial acetic acid and 40 ml of distilled water to give solution A. Another solution designated solution B was prepared by dissolving 8.0 g of potassium iodide in 20 ml of distilled water. Both solutions were mixed to give a stock solution.

x. Hydrochloric acid (2% (v/v))

Measured volume, 2.0 ml concentrated hydrochloric acid was diluted with some distilled water and made up to 100 ml.

xi. Picric acid (1% (w/v))

Picric acid (1.0 g) was dissolved in 100 ml of distilled water.

xii. Preparation of normal saline

Normal saline was prepared by dissolving 0.9 g sodium chloride in distilled water and made up to 100 ml.

xiii. Preparation of ranitidine

A known quantity, 150 mg of ranitidine was dissolved in 10 ml of distilled water to give 15 mg/ml.

xiv. Preparation of 25% trichloroacetic acid (TCA)

A known weight, 25 g trichloroacetic acid was dissolved in distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder.

xv. Preparation of 1% thiobarbituric acid (TBA)

One (1 g) thiobarbituric acid was dissolved in distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder.

xvi. Preparation of 0.3% sodium hydroxide (NaOH)

Sodium hydroxide (0.3 g) was dissolved in a little amount of water and made up to 100 ml mark with distilled water in a measuring cylinder.

xvii. Preparation of 20% Sodium dodecyl sulphate (SDS)

A quantity, (20 g) sodium dodecyl sulphate was dissolved in some quantity of distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder with distilled water.

xviii. Preparation of 2% glacial acetic acid

A known quantity, 2 g of glacial acetic acid was dissolved in distilled water and made up to 100 ml with distilled water in a measuring cylinder.

xix. Preparation of phosphate buffer pH 7.4

Phosphate buffer was prepared by dissolving (5.67 g) anhydrous monopotassium phosphate in 1 litre of distilled water and the pH adjusted.

2.2.9 Qualitative Phytochemical Analyses of the Methanol Extract of *Schumanniophyton magnificum* Leaves

The phytochemical screening of the leaves of *Schumanniophyton magnificum* was carried out according to the methods of Harborne (1998) and Trease and Evans (2002).

Test for Alkaloids

A quantity, 0.2 g of the sample was boiled with 5 ml of 2% HCl on a steam bath. The mixture was filtered and 1ml aliquots of the filtrate were treated with 2 drops of the following reagents

(i) Dragendorff's reagent: An orange precipitate indicated the presence of alkaloids.

(ii) Mayer's reagent: A creamy-white precipitate indicated the presence of alkaloids.

(iii) Wagner's reagent: A reddish-brown precipitate indicated the presence of alkaloids.

(iv) Picric acid (1%): A yellow precipitate indicated the presence of alkaloids.

Test for Flavonoids

A quantity, 0.2 g of the sample was heated with 10 ml ethyl acetate in boiling water bath for 3 minutes. The mixture was filtered, and the filtrate was used for the following tests.

(i) Ammonium test: 4 ml of the filtrate was shaken with 1ml of dilute ammonium solution to obtain two layers. The layers were allowed to separate. A yellow precipitate observed in the ammonium layer indicated the presence of flavonoids.

(ii) Aluminium chloride test: 4ml of the filtrate was shaken with 1 ml of 1% aluminium chloride solution and observed for light yellow colouration that indicated the presence of flavonoids.

Test for Saponins

The sample (0.1 g) was boiled with 5ml of distilled water for 5 minutes. The mixture was filtered while still hot. The filtrate was used for the following tests.

(i) Emulsion test: A quantity, 1 ml of the filtrate was added to two drops of olive oil. The mixture was shaken and observed for the formation of emulsion.

(ii) Frothing test: A quantity, 1 ml of the filtrate was diluted with 4 ml of distilled water. The mixture was shaken vigorously and then observed on standing for a stable froth.

Test for Glycosides

A quantity, (2.0 g) of the sample was mixed with 30 ml of distilled water and 15 ml of dilute sulphuric acid respectively and heated in a boiling water bath for 5 minutes. The mixtures were filtered and the filtrates used for the following test.

(i) To 5 ml of each of the filtrate, 0.3 ml Fehling's solution mixtures of A and B was added until it turned alkaline (tested with litmus paper) and heated on a boiling water bath for 2 minutes. A brick-red precipitate indicated the presence of glycosides.

Test for Tannins

A known amount, 2 g of the sample was boiled with 5 ml of 45% ethanol for 5 minutes. The mixture was cooled and then filtered and the filtrate was treated with the following solutions.

(i) Lead sub acetate solution: To 1ml of the filtrate, 3 drops of lead sub acetate solution was added. A gelatinous precipitate indicated the presence of tannins.

(ii) Bromine water: To 1 ml of the filtrate was added 0.5 ml of bromine water and then observed for a pale brown precipitate.

(iii) Ferric chloride solution: a quantity, 1 ml of the filtrate was diluted with distilled water and then 2 drops of ferric chloride solution was added. A transient greenish to black colour showed the presence of tannins.

Test for Terpenoids and Steroids

A measured volume, 9 ml of ethanol was added to 1g of the sample and refluxed for a few minutes and filtered. The filtrate was concentrated to 2.5 ml on a boiling water bath, and 5 ml of hot water was added. The mixture was allowed to stand for 1hour, and the waxy matter filtered off. The filtrate was extracted with 2.5 ml of chloroform using a separating funnel. To 0.5 ml of the extract in a test tube was carefully added 1 ml of concentrated sulphuric acid to form a lower layer. A reddish-brown interface shows the presence of steroids. Another 0.5 ml aliquot of the extract was evaporated to dryness on a water bath and heated with 3 ml of concentrated sulphuric acid for 10 minutes on water. A grey colour showed the presence of terpenoids.

Test for Phenols

Distilled water (2 ml) was added to 0.1 g of methanol extract, followed by addition of sodium carbonate (0.5 ml) and Folin Ciocalteau's reagent (0.5 ml). Formation of green colour indicated the presence of phenols.

Test for Reducing Sugars

A quantity, 0.1 g of the sample was shaken vigorously with 5 ml of distilled water and filtered. To the filtrate was added equal volumes of Fehling's solutions A and B and shaken vigorously. A brick-red precipitate indicated the presence of reducing sugars.

Test for Carbohydrate

To 2 ml of test sample, 3 drops of Molisch reagent were added. The mixture was shaken and concentrated sulphuric acid was poured down into the inclined side of the test tube. An appearance of a purple ring at the junction of the two layers indicated the presence of carbohydrate.

Test for Protein

Presence of protein in extract was carried out by taking 2 ml (1 mg/ml) of extract and mixed with 1ml of 10% NaOH and 1ml of 1% $CuSO_4$. A violet colouration confirmed the presence of a peptide bond (protein).

2.2.10 Quantitative Phytochemical Analyses of Methanol Extract of *Schumanniophyton magnificum* Leaves

Quantitative Determination of Tannin Content

The method of Swain (1979) was used for the determination of the tannin content of the extract. A quantity, 0.2 g of finely ground sample was measured into a 50 ml beaker. About

20 ml of 50% methanol was added and covered with paraffin and placed in a water bath at 77-80^{o}C for 1 hr and stirred with a glass rod to prevent bumping. The extract was filtered using a double layer of Whitman No. 1 filter paper into a 50 ml volumetric flask then 20 ml distilled water, 2.5 ml Folin-Denis reagent and 10ml of 17% Na_2CO_3 were added and mixed properly. The mixture was made up to mark with distilled water and allowed to stand for 20 mins when a Mish-green colouration developed. Standard tannic acid solutions of range 0.10 mg were treated similarly as 1mI of sample above. The absorbances of the tannic acid standard solutions as well as samples were read after colour development at 760 nm. The tannin content was calculated using the formular:

$$\text{Tannin (mg/100 g)} = \frac{\text{Absorbance of sample x Average gradient x Dilution factor}}{\text{Weight of sample x 1000}}$$

Quantitative Determination of Flavonoid

This was determined according to the method of Harborne (1998). A quantity, 5 g of the sample was boiled in 50 ml of 2 MHCl solution for 30min under reflux. It was allowed to cool and then filtered through Whatman No. 1 filter paper. A measured volume of the filtrate was treated with equal volume of ethyl acetate starting with drops. The solution was filtered into a weighed crucible and heated to dryness in an oven at 60^{0}C. The dried crucible was weighed again and the difference in the weight gave the quantity of flavonoids present in the sample.

Quantitative Determination of Alkaloid

The quantitative determination of alkaloid was described by Harborne (1998). A known quantity, 5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 2 hours. This was filtered and the filtrate was concentrated down in a water bath to one – quarter (1/4) of the original volume. Concentrated ammonia was added drop-wise to the filtrate till a precipitate was formed. The precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue, the alkaloids, was dried and weighed.

Quantitative Determination of Saponin

The method used was that of Obadoni and Ochuko (2001). The samples were ground and 20 g of each were put into a conical flask and 100 cm^3 of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 550C. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90^{O}C. The concentrate was

transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n- butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight; the saponin content was calculated as mg/g.

Quantitative Determination of Total Phenol

Folin Ciocalteau reagent (1/10 dilution) and 1.5 ml of Na_2CO_3 2% (w/v) were added to the extract (0.5 g) and mixed. The blend was incubated in the dark at room temperature for 15 minutes. The absorbances of blue-coloured solution were measured at a wavelength of 765nm. The results were expressed in mg of gallic acid equivalent (GAE).

Preparation of calibration curve using gallic acid as standard

A quantity, 10 mg of standard gallic acid was accurately weighed and dissolved in 100 ml of distilled in a volumetric flask to make the stock solution. From the above stock solution prepared, different concentrations (0.5 – 2.5 ml) were pipetted out into 25 ml volumetric flasks. Then, 10 ml of distilled water and 1.5 ml of Folin Ciocalteu reagent were added to each of the above volumetric flasks. After 5 minutes, 4ml of 20% sodium carbonate solution were added and the volume was made up to 25 ml with distilled water. Absorbance was recorded after 30 minutes at a wavelength of 765 nm and a calibration curve of absorbance against concentration was plotted.

Quantitative Determination of Terpenoid

A quantity, (0.5 g) of extract was macerated with 50 ml of ethanol and filtered. To the filtrates (2.5 ml), 2.5 ml of 5% aqueous phosphomolybdic acid solution was added and 2.5 ml of concentrated H_2SO_4 was gradually added and mixed. The mixture was left to stand for 30 minutes and then made up to 12.5 ml with ethanol. The absorbance was read at a wavelength of 700 nm. The analysis was performed in triplicates.

2.2.11 Determination of Biological Activities of the Methanol Extract of *S. magnificum* Leaves

2.2.11.1 Acute Toxicity Test of the Methanol Extract of *S. magnificum* Leaves

The method of Lorke (1983) was used for the acute toxicity test of the methanol extract of *Schumanniophyton magnificum* leaves. Eighteen (18) albino mice were utilized in this study. The test involved two phases. In phase one, the animals were grouped into three (3) groups of three mice each and were given 10, 100 and 1000 mg/kg body weight of the extract

respectively and in the second phase, 1600, 2900 and 5000 mg/kg body weight of the extract were administered to the groups respectively. The administration of the extract was done orally using intubation tube. The animals were then observed for 24hrs for nervousness, dullness, in-coordination or death. LD_{50} was calculated as follows:

$LD_{50} = \sqrt{D_0} \times D_{100}$

Where D_0 = highest dose that gave no mortality

D_{100} = lowest dose that produced mortality

2.2.11.2 Anti-Inflammatory Studies

2.2.11.2.1 Determination of Effects of Methanol Extract of *S. magnificum* Leaves on Egg Albumin-induced Rat Paw Oedema

This was assessed in rats using the method of Winter *et al*. (1962) as modified by Ezekwesili and Nwodo (2000). Acute inflammation was determined using increase in the right hind paw volume induced by the sub-plantar injection of fresh egg albumin. Twenty (20) adult Wistar rats of either sex used were divided five groups of four rats each. The rats were fasted and deprived of water for 18 hours before the experiment, to ensure uniform hydration and to minimize variability in oedematous response. After the fasting and deprivation, the right hind paw volume of the rats were measured with mercury displacement at time zero (t = 0). Then, group 1 (control) received 2ml/kg b.w. of 3% Tween-80, group 2 received 100 mg/kg b.w. of Ibuprofen, groups 3–4 received 100, 200 and 400 mg/kg b.w. of methanol extract respectively intraperitoneally. Acute inflammation was induced one hour after the administration of test substances, by injecting 0.1ml of undiluted fresh egg albumin into the sub-plantar of the right hind paw of the rats. Paw volumes were then measured at 1, 2, 3, 4 and 5 hours after egg albumin injection. Oedema formation was assessed by finding the difference in the zero time paw volume and its volume at the different times after egg albumin injection. This was used to calculate average inflammation, % inflammation and % inhibition of inflammation using the following relation.

(i) Average inflammation = $(V_t - V_0)$

Where V_t = volume of oedema at time t (1, 2, 3, 4 and 5 hours)

V_0 = volume of oedema at time zero (initial time)

(ii) $$\% \text{ inflammation} = \frac{\text{Average inflammation of treated groups at Time t}}{\text{Average inflammation of control at Time t}} \times 100$$

$$\% \text{ inflammation} = \frac{(V_t - V_0) \text{ treated groups}}{(V_t - V_0) \text{ control}} \times 100$$

(iii) % inhibition of inflammation = $\frac{(V_t - V_0) \text{ control} - (V_t - V_0) \text{ treated groups}}{(V_t - V_0) \text{ control}} \times 100$

2.2.11.2.2 Determination of Effects of Methanol Extract of *S. magnificum* Leaves on Formalin-Induced Rat Paw Oedema

The effect of the extract on sub-chronic inflammation was assessed using arthritis induced by formaldehyde in rats as contained in the method of Chau (1989). A total of 28 adult wistar rats were divided into seven groups of 4 rats each. On day one, group 1 (control) received 2ml/kg b.w. of 3% Tween-80, group 2 received 3% Tween-80 2 ml/kg, group 3 received 1mg/kg b.w. of Dexamethazone, group 4 received 100 mg/kg b.w. of Aspirin, groups 5 – 7 received 100, 200 and 400 mg/kg b.w. of methanol extract respectively orally. Thirty minutes later, arthritis was induced by subplantar injection of 0.1 ml of 2% formaldehyde solution. On the 3rd day, groups 2 - 7 were injected with formaldehyde solution to sustain the inflammation. The increase in paw oedema was measured with Vernier caliper (Joseph *et al*., 2005). The paw thickness was measured before and once daily for 10 days after induction of inflammation by using vernier caliper. The difference in paw thickness after and before induction of inflammation was calculated and presented as mean increase in paw thickness (cm).

The ability of anti-inflammatory drug and extract to suppress paw inflammation was expressed as a percentage of inhibition of paw oedema (Duffy *et al*., 2001) and this percentage was calculated according to the following equation:

$$\% \text{ inhibition} = 1 - \left[\frac{b - a}{a}\right] \times 100$$

where b = mean increase in paw diameter of rats after induction of formalin

a = mean increase in paw diameter of rats before induction of formalin (baseline)

On the 10th day, blood samples of the rats were collected and used for biochemical studies.

2.2.11.3 Assay of Effect of Methanol Extract of the Leaves of *S. magnificum* on Phospholipase A_2 Activity

The effect of the extract on phospholipase A_2 activity was determined using the method of (Vane, 1971) as modified by Oyekachukwu *et al*. (2017). Fresh human blood samples were centrifuged at 3,000 rpm for 10 min and the supernatant (plasma) discarded. The red cells were washed three times with equal volume of normal saline, measured and reconstituted as a 40% (v/v) suspension with normal saline. This served as the substrate for phospholipase A_2. Bacterial enzyme preparation was obtained from *Bacillus cereus* strain culture. The organism

was cultured in MaCconkey agar for three days and the culture transferred into a test tube containing normal saline. This was centrifuged at 3,000 rpm for 10 min after which the bacterial cells settled at the bottom of the test-tube while the supernatant contained the enzyme preparation that was used for enzyme assay. Aliquots (0.1 ml) of re-suspended erythrocytes (substrate), normal saline (1.2 ml), 2 mM calcium chloride (0.2 ml), 1 ml varying concentrations of extract (0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml) in 3% tween 80 (dissolved in normal saline) and 0.2 ml of the enzyme preparation were incubated at 37°C for 1 hr. Control tubes contained 0.1 ml of the erythrocytes, 1.2 ml normal saline, 0.2 ml calcium chloride, 1 ml 3% tween 80 and 0.2 ml of the enzyme preparation. The blanks contained everything in the test except the enzyme. After incubation, the reaction mixture was centrifuged at 3,000 g for 10 min and the absorption of the supernatant read against the blank at 418 nm. Prednisolone (0.4 mg/ml), a known inhibitor of the enzyme was used as the standard control. The percentage inhibition of enzyme activity was calculated thus:

$$\% \text{ Enzyme activity} = \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

$$\% \text{ inhibition} = 100 - \% \text{ Enzyme activity}$$

2.2.11.4 Determination of Effect of Methanol Extract of the Leaves of *S. magnificum* on Membrane Stabilization of Human Red Blood Cell

The effect of the methanol extract of *S. magnificum* leaves on heamolysis of human red blood cell (HRBC) induced by hypotonic solution (distilled water) was evaluated using a modification of the method of Shinde *et al*. (1999). Blood samples (3 ml each) obtained from healthy volunteers were placed into EDTA bottles, centrifuged at 3,000 rpm for 10 minutes and washed three times with equal volumes of normal saline. For each, the blood volume was measured and reconstituted as a 40% (v/v) suspension with normal saline. Methanol extract was dissolved in distilled water (hypotonic solution) and normal saline (isotonic solution). The hypotonic solution (5 ml) containing graded concentration of extracts (0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml) was put into three sets of centrifuge tubes. The isotonic solution (5 ml) containing graded concentrations of extracts (0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml) was also put into triplicate (per dose) of the centrifuge tubes. Control tubes contained 5 ml of distilled and 5 ml of 0.2 and 0.4 mg/ml of Indomethacin respectively. Erythrocyte suspension (0.1 ml) was added to each of the tubes and mixed gently. The mixtures were incubated for 1 hour at room temperature, and afterwards, centrifuged at 1300 g for 3 minutes. The absorbance (OD) of the haemoglobin content of the supernatant was determined at a wavelength of 418 nm using

Jenway Spectrophotometer. The percentage haemolysis was calculated by assuming the haemolysis produced in the presence of distilled water as 100%. The percent inhibition of haemolysis of the extract was calculated thus:

$$\% \text{ Inhibition of Haemolysis} = 1 - \frac{OD_2 - OD_1}{OD_3 - OD_1} \times 100$$

Where OD_1 = absorbance of test sample in isotonic solution

OD_2 = absorbance of test sample in hypotonic solution

OD_3 = Absorbance of control sample in hypotonic solution.

2.2.11.5 Determination of Anti-Platelet Aggregatory Activity of the Methanol Extract of the Leaves of *S. magnificum*

This was carried out by the method of Born and Cross (1963). Fresh blood samples (3 ml each) were taken from healthy volunteers into plastic tubes containing 0.01 ml of 1% EDTA as an anticoagulant. The tubes were centrifuged at 3000 rpm for 10 minutes and the supernatants were collected, diluted twice with normal saline and then used as the platelet rich plasma (PRP). The PRP (0.2 ml), varying concentrations of the extract (0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml) as well as 0.2 and 0.4 mg/ml indomethacin in phosphate buffer were added into the cuvette. Aggregation was induced by the addition of 0.4 ml of 2 M $CaCl_2$ in normal saline into the cuvette. Immediately, the absorbance of each solution was measured at a wavelength of 520 nm at intervals of 30 seconds to 150 seconds. The tests were performed in triplicates.

2.2.11.6 Some *in vitro* Antioxidant Activities of Methanol Extract of the Leaves of *S. magnificum*

Determination of Nitric Oxide Radical Scavenging Activity of the Methanol Extract of the Leaves of *S. magnificum*

Nitric oxide radical scavenging activity of the extract was measured spectrophotometrically according to the method described by Sreejayan and Rao (1997). Sodium nitroprusside (2.0 ml of 10 nM) in phosphate buffer (pH 7.4) and phosphate buffer (5.0 ml) were mixed with 0.5 ml of different concentrations (15.63, 31.25, 62.50, 125, 250, 500 and 1000 μg/ml) of the extract and incubated at room temperature for 150 minutes. After the incubation period, Griess reagent (2 ml) was added to 2 ml of each of the incubate and incubated again at room temperature for 30 minutes. The absorbance of the pink chromophore formed by the diazotization of nitrite with α-napthlethylene diamine dihydrochloride was measured at

wavelength of 546 nm. Ascorbic acid was used as the positive control and the results were expressed as percentage inhibition of nitric oxide. All analyses were performed in triplicates.

$$\% \text{ inhibition} = 100 \times \frac{A_C - A_T}{A_C}$$

Where: A_C = Absorbance of the control

A_T = Absorbance of the test drug/ extract

Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity of the Methanol Extract of the Leaves of *S. magnificum*

Determination of DPPH radical-scavenging activity of the extract was carried out as described by Koleva *et al*. (2002). A volume, 1 ml of varying concentration (15.63, 31.25, 62.50, 125, 250, 500 and 1000 µg/ml) of the extract and ascorbic acid dissolved in methanol were mixed with 0.5 ml of 0.078 mM DPPH (in methanol) in a test tube. The absorbance of the residual DPPH was taken at a wavelength of 517 nm after 1 hour of incubation in the dark at room temperature. An aliquot, 0.5 ml of the 0.078 Mm DPPH solution plus 1.0 ml of methanol were used a negative control. All tests were performed in triplicate. Results were expressed as percentage of blank (100 %). The concentration required to cause a 50 % decrease (IC_{50}) in DPPH activity was also calculated.

The % DPPH scavenging effect of the antioxidant was calculated as follows:

$$\% \text{ DPPH scavenging effect} = 100 \times \frac{A_C - A_T}{A_C}$$

Where: A_C = Absorbance of the control

A_T = Absorbance of the test drug/ extract

Determination of Ferric Reducing Power Activity of the Methanol Extract of the Leaves of *S. magnificum*

Ferric reducing power capacity of the extract was determined by the method of Oyaizu (1986). Different concentrations (15.63, 31.25, 62.50, 125, 250, 500 and 1000 µg/ml) was mixed with 0.5 ml phosphate buffer (pH 6.6) and 0.5 ml 0.1 % potassium hexacyanoferrate, followed by incubation at 50 ^{0}C in water bath for 20 minutes. After incubation, 0.5 ml of 10 % TCA was added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml of distilled water and 0.1 ml 0.01 % $FeCl_3$ solution. The reaction mixture was left for 10 minutes at room temperature and the absorbance was measured at 700 nm against blank solution. All tests were performed in triplicate. A higher absorbance of the reaction mixture indicated greater reducing power. Gallic acid was used as a positive control.

2.2.11.7 Determination of the Effect of Methanol Extract of the Leaves of *S. magnificum* on Acetic Acid-Induced Vascular Permeability in Rats

The effect of the extract on acetic acid-induced vascular permeability in rats was assessed by the method of Whittle (1964). Twenty (20) adult Wistar rats of either sex (120 g – 200 g) divided into five groups of four rats each were used. The animals were fasted for 10 hours prior to the experiment. Then, group 1 (control) was administered 2 ml/kg b.w. of 3% Tween 80, group 2 (standard group) was administered 50 mg/kg b.w. of indomethacin, groups 3, 4 and 5 were administered 100, 200 and 400 mg/kg b.w. of methanol extract respectively. Three hours later, each animal was given a 0.5 ml intravenous injection of 1% Evans blue solution. Vascular permeability was induced thirty minutes afterwards, by (i. p) injection of 1 ml of 0.6% acetic acid. The animals were sacrificed 20 minutes later, and their peritoneum washed with 10 ml of normal saline. The recovered peritoneal fluid was centrifuged and the absorbance of the supernatant measured at 610 nm using a spectrophotometer. Percentage inhibition of vascular permeability was calculated as follows:

$$\% \text{ inhibition of vascular permeability} = 100 \times \frac{A_C - A_T}{A_C}$$

Where: A_C = Absorbance of the control

A_T = Absorbance of the test drug/ extract

2.2.11.8 Determination of the Effect of Methanol Extract of the Leaves of *S. magnificum* on *in vivo* Leukocyte Mobilisation in Rats

The effect of the methanol extract on *in vivo* leukocyte mobilization induced by an inflammatory stimulus was evaluated in albino rats using the method of Rebeiro *et al.* (1991). Twenty five (20) adult Wistar rats of either sex (120 g – 200 g) divided into five groups of four rats each were used for the test. Groups 3, 4 and 5 were administered varied doses of the extract (100, 200 and 400 mg/kg b.w.), while groups 1 (vehicle control) and 2 (treatment control) received 2 mg/kg b.w. of 3% Tween 80 and indomethacin (50 mg/kg b.w.) respectively. Three hours after oral administration of the extracts, Tween 80 or reference drug, each animal in the respective groups (n=4) received intraperitoneal injection (i. p) of 0.5 ml of 3% w/v agar suspension in normal saline. Four hours later, the animals were sacrificed and the peritoneal cavities washed with 5 ml of a 5% solution of EDTA in phosphate buffered saline (PBS). The peritoneal fluid was recovered and both total and differential leukocyte counts (TLC and DLC) were performed on the perfusates using a manual cell counter after staining with Wright's stain.

Estimation of Total Leukocyte Count (TLC): The perfusates in the tubes were drawn up to the 0.02ml calibration on the white blood cell capillary and then mixed with 0.38ml of leukocytes diluting fluid (3% acetic) and allowed to stand for 10 min for all the erythrocyte to lyse. Thereafter, the Neubauer counting chamber was filled with the diluted sample and the set-up examined microscopically in oil-immersion under 100 x magnification. The total number of white cells in the 4 large squares was counted at the end, the total leukocyte count (TLC) was calculated from the relation: TLC = n x d x v Where n = number of cells counted, d = the dilution factor, which in this case was 20 v = volume factor for counting chamber = 1/0.4 (i.e. 2.5) thus the relationship reduces to: TLC = n x 20 x 2.5. The percent inhibition of leukoctye migration was calculated using the formula: $\%\ \text{Leukocyte inhibition}\ (\%LI) = \left(1 - \left(\frac{T}{C}\right)\right) \times 100$ Where T and C represent the leukocyte count of the treated and control groups respectively.

Estimation of Differential Leukocyte Count (DLC): The Leishman staining technique was used. A drop of the fluid was placed on one end of the glass slide using an applicator. Another glass slide was used to make a smear of the fluid on the glass slide using the push wedge technique. The stain was made to cover the film and then left to stand for 2 min. Thereafter, distilled water, two times the quantity of stain used to flood the film was added; the setup was rocked gently for 2 min and then allowed to stand for 15 min before rinsing off the stain. The slide was left to dry and then examined on the microscope using the oil immersion objective lens of 100 x magnification. The cells were counted and differentiated on morphological basis using tally counter.

2.2.11.9 Determination of Malondialdehyde Concentration of Methanol Extract of the Leaves of *S. magnificum* on Rats with Formalin-Induced Oedema

Lipid peroxidation product was determined by measuring spectrophotometrically the concentration of the lipid peroxidation product, malondialdehyde (MDA) as described by Wallin *et al.* (1993).

Principle: Lipid degradation occurs forming such products as malondialdehyde (from fatty acids with three or more double bonds), ethane and pentane (from the ῳ-terminal carbons of 3 and 6 fatty acids, respectively). Malondialdehyde appears in the blood and urine and is used as an indicator of free radical damage. Malondialdehyde (MDA) is a sign of lipid peroxidation.

Method: A quantity, 0.1 ml of the serum was mixed with 0.9 ml of distilled water in a beaker. 0.5 ml of 25% trichloroacetic acid (TCA) and 0.5 ml of 1% thiobarbituric acid (TBA) in 0.3% NaOH were also added to the mixture. The mixture was boiled for 40 minutes in water-bath and then cooled in cold water. 0.1 ml of 20% sodium dodecyl sulfate (SDS) was

also added to the cooled solution and mixed properly. Then, the absorbance was read at wavelengths 532 nm and 600 nm against a blank.

2.2.11.10 Determination of the Effect of Methanol Extract of the Leaves of *S. magnificum* on Some Haematological Parameters of Rats with Formalin-Induced Oedema

Haematological parameters were determined using the method described by Dacie and Lewis (1991).

Determination of Packed Cell Volume (PCV)

Principle: When whole blood sample is subjected to a centrifugal force for maximum RBC packing, the space occupied by the RBCs is measured and expressed as percentage of the whole blood volume.

Method: Using microhaematocrit method, a well-mixed anticoagulated whole blood was allowed to enter capillary haematocrit tubes until they are approximately 2/3 filled with blood. Blood filling was done for each tube. One end of each tube was sealed with plastic seal and placed in the medial groves of the centrifuge, head exactly opposite each other, with the scaled end away from the centre of the centrifuge. All tubes were spun for five minutes at 1000 rpm. The tubes were removed as soon as the centrifuge had stopped spinning.

Calculation: PCV was obtained for each tube using microhaematocrit-reader by measuring the height of the RBC column and expressing this as a ratio of the height of the total blood column.

$$\text{PCV (\%)} = \frac{\text{Height of cell column}}{\text{Height of total blood column}} \times 100$$

Determination of Haemoglobin (Hb) Concentration

Principle: Whole blood is added to Drabkin's regent: a solution containing KCN and kite $(CN)_6$ KCN converts Hb-Fe^{2+} (ferrous) to Hb-Fe^{3+} (ferric) state to form methaemoglobin which then combines with KCN to form a stable pigment, cyanmethaemoglobin complex. The colour intensity of this mixture is measured in a spectrophotometer at a wavelength of 540 nm or using a yellow-green filter. The optical density (OD) of the solution is proportional to the haemoglobin concentration. All forms of Hb (Hb-C, Hb-O, etc) except Hb-S are measured with this cyanmet-method.

Method: Exactly 5.0 ml of drabkin's reagent was pipetted into two test tubes 1 and 2 and a well-mixed sample of EDTA-treated blood (0.02 ml) was pipetted into the tubes, rinsing the pipette five times with the reagent, until all the blood had been removed from the pipette. The

solutions were well mixed and allowed to stand at 25ºC for 10 minute in order to allow the formation of cyano-methaemoglobin. The mixtures were read in a spectrophotometer at a wavelength of 540 nm. The Drabkin's reagent in tube I was used to blank the machine (setting the percentage transmittance at 100%). The readings were recorded using a pre-calibrated chart and the actual Hb values in g/dl were calculated.

Determination of White Blood Cells (WBC) Count

Principle: When whole blood is mixed with weak acid solution, it dilutes the blood and haemolyses the RBCs, enabling the WBCs to be counted.

Method: Manual WBC counting method was used as follows:

Dilution of Blood:

(a) The blood specimen was mixed approximately for one minute; using the aspirator and WBC pipette, blood was drawn to the 0.5 mark in the pipette.

(b) Blood was removed from the outside of the pipette with clean gauze.

(c) Holding the pipette almost vertically, the tip was placed into the counting diluting fluid to draw it slowly. While gently rotating the pipette, to ensure proper mixing, the diluting fluid was aspirated until it reached the 11 mark.

(d) The pipette was placed in a horizontal position and firmly holding the index finger of either hand over the opening in the tip of the pipette, aspirator was detached from the other end of the pipette. This is 1:20 dilution

(e) Having now completed the dilution of blood, the counting chamber and cover glass were cleaned with a lint-free cloth.

Filling the counting chamber: Approximately 0.02 ml of well mixed EDTA-anticoagulated blood sample was added to 0.38 ml of dilute fluid, dispensed into a small container. One of the grids of the counting chamber was filled with re-mix of the diluted blood sample using a Pasteur pipette, taking care not to overfill the area. The filled area was left undisturbed for two minutes to allow time for the white blood cells to settle, after which the underside of the chamber was dried and placed on the microscope stage.

Counting the white blood cells: Using the x10 objective with the condenser iris closed sufficiently to give good contrast, the ruling of the chamber and white cells were focused until the cells appeared as small black dots. The cells in the four large squares of the chamber were then squarely counted.

Calculation:

a) The number of white cells per litre of blood was calculated as follows:

b) The total number of cells counted was divided by 2

c) The number obtained was then divided by 10

d) The result was then multiplied by 10^3 to give the white cell count.

Determination of Red Blood Cell (RBC) Count

Principle: When whole blood is diluted with an isotonic fluid, it prevents lysis and facilitates counting of the red cells. Some isotonic solutions in use include Hayem's solution, Gower's solution or 0.85% sodium chloride (NaCl) solutions.

Method: Using the Thoma (manual counting) method, anti-coagulated blood was drawn up to the 0.5 ml mark in the RBC count pipette and diluted to a 101 mark with RBC diluting fluid (1:200 dilution). Dilution was repeated with the replicate tube. The counting chamber was cleaned; both pipettes were shaken three times; counting chamber filled (first expelling the first 4 drops of the mixture), allowing approximately three minutes for the RBCS to settle, Red cells were counted using the counting steps as follows:

1. The filled counting chamber was carefully placed on the microscope stage.
2. Using low power (x10 objective) the large centre square was placed in the middle of the field of vision and the entire large square was carefully examined for even distribution of RBCs.
3. The high-dry objective was carefully changed, moving the counting chamber so that the small upper left corner square (this square is further sub-divided into 16 even smaller squares) is completely in the field of vision.
4. All the RBC were counted in the squares, also counting the cells on the two of the margins but excluding those lying on the other two sides

Calculation The RBCs (in mm^3) =cells counted × correction for volume × correction for dilution=RBCs counted in 5 small squares × 200 × 1.0 / 0.2 (or 50) = number of RBCs counted in five squares × 10^4.

Determination of Platelet Count

This was done using standard haematological techniques as described by Ochei and Kolhaktar (2008). Well mixed whole blood was diluted with 2 % mmonium oxalate in the ratio of 1:20 and allowed to stand for 30 minutes. The mixture was loaded into a charged counting chamber and the appropriate squares counted.

2.2.11.11 Assay of the Effect of Methanol Extract of the Leaves of *S. magnificum* on the Activities of Some Liver Enzymes of Rats with Formalin-Induced Oedema

Liver Function Test (ALT, AST and ALP) was carried out using the method of Reitman and Frankel (1957) and Klein *et al*. (1960).

Assay of Aspartate Aminotransferase Activity

Principle: Activity of AST or SGOT was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine. The colour intensity is measured against the blank at 546 nm.

Method: The blank and sample test tubes were set up in duplicates. A volume, 0.1ml of serum was pipetted into the sample tubes and 0.5ml of reagent 1 was pipette into both sample and blank tubes. The solutions were thoroughly mixed and incubated for exactly 30 minutes at 37 ^{0}C ml and pH 7.4. 0.5ml of Reagent 2 containing 2, 4-dinitrophenylhydrazine was added into all the test tubes followed by 0.1ml of sample into the blank tubes. The tubes were mixed thoroughly and incubated for exactly 20 minutes at 25 ^{0} C and 5.0ml of sodium hydroxide solution was then added to each tube and mixed. The absorbance was read against the blank, after 5 minutes, at 546 nm.

Assay of Alanine Aminotransferase Activity

Principle: Activity of ALT was measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine. The colour intensity is measured against the blank at 546nm.

Method: The blank and sample test tubes were set up in duplicates. 0.1ml of serum was pipetted into the sample tubes. To these were added 0.5ml buffer solution containing phosphate buffer, L-alanine and α-oxoglutarate. The mixtures were thoroughly mixed and incubated for exactly 30 minutes at 37 ^{0}C and pH 7.4. A volume, 0.5ml of reagent containing 2, 4-dinitrophenylhydrazine was later added to both tubes while 0.1ml of sample was added to sample blank tube. The tubes were mixed thoroughly and incubated for exactly 20 minutes at 25 ^{0} C. Five militres of sodium hydroxide solution was then added to each tube and mixed. The absorbance was read against the blank, after 5 minutes, at 546 nm.

Assay of Alkaline Phosphatase Activity

Principle: The principle of this method is based on the reaction involving serum alkaline phosphatase and a colourless substrate of phenolphthalein monophosphate, giving rise to phosphoric acid and phenolphthalein which at alkaline pH values, turn pink that can be determined spectrophotometrically.

P-nitrophenylphosphate + H_20 --------→ ALP ------→ PO_4^{2-} + P-nitrophenol (pink at pH=9.8)

Method: The blank and sample test tubes were set up in duplicates and 0.05ml of sample was pipetted into the sample test tubes. Distilled water (0.05ml) was pipetted into the blank tube. Three mililitres (3.0ml) of substrate was pipetted into each tube respectively, which was then mixed and the initial absorbance taken at 405nm. The stop watch was started and the absorbance of the sample and the blank read again three more times at one minute intervals.

Calculation: alkaline phosphatase activity was calculated as follows:

$$\text{Activity of ALP (in U/L)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \quad \text{x} \quad 100$$

2.2.11.12 Determination of the Effect of Methanol Extract of the Leaves of *S. magnificum* on Lipid Profile of Rats with Formalin-Induced Oedema

Determination of Cholesterol Concentration

The method of Abell *et al.* (1952) was followed.

Principle: Cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxidase and 4-aminoantipyrine in the presence of phenol and peroxidase

Test procedure: Three (3) test tubes were set up in a test tube rack and labelled blank, standard and sample respectively. To the blank, was added (10 µl) distilled H_2O, 10 µl standard specimen to the standard test tube and 10 µl sample (serum) to the sample test tube. To each of these test tubes was added 1000 µl of the cholesterol reagent. It was thoroughly mixed and incubated for 10minutes at room temperature (20-25^oC). The absorbance of the sample (A_{sample}) against the blank was read within 60 minutes at 500nm.

Determination of Triacylglycerol Concentration

The concentration of triacylglycerol (TAG) was determined according to the method of Otvos (1999).

Clinical significance:

Triacylglycerols measurements are used in the diagnosis and treatment of diseases involving lipid metabolism and various endocrine disorders example diabetes mellitus, nephrosis and liver obstruction.

Principle: The triacylglycerols are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

$$\text{Triacylglycerol} + H_2O \xrightarrow{\text{lipases}} \text{Glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

Glycerol-3-phosphate + O_2 $\xrightarrow{GPO}$ Dihydroxyacetone phosphate + H_2O_2

$2H_2O_2$ + 4-aminophenazone + 4 chlorophenol $\xrightarrow{POD}$ Quinoneimine + HCl + $4H_2O$

Method: A quantity, 0.1 ml of the sample was pipetted into a clean labelled tube and 1.0 ml of trichloroacetic acid (TCA) was added, mixed and then centrifuged at 300 rpm for 10 minutes. The supernatant was decanted and reserved for use. The procedure was carried out as follows:

Reagents (ml)	Blank	Standard	Sample
Distilled water	0.5	-	-
Standard solution	-	0.5	-
TCA	0.5	0.5	-
Supernatant	-	-	1.0
Reagent mixture	1.0	1.0	1.0

The mixtures were allowed to stand for 20 minutes at 25 ^{0}C and the absorbance of the sample and standard read against the blank at 540 nm.

Calculation: The concentration of triacylglycerol in serum was calculated as follows:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Standard concentration (mmol/l)}$$

Determination of High Density Lipoprotein Concentration

The concentration of high density lipoprotein (HDL) was determined according to the method of Kameswara *et al.* (1999).

Principle: LDL and VLDL (low and very low density lipoproteins) are precipitated from serum by the action of a polysaccharide in the presence of divalent cations. Then, high density lipoproteins (HDL) present in the supernatant is determined.

Procedure: The precipitant solution, 0.1ml, was added to 0.3ml of the sample and mixed thoroughly and allowed to stand for 15 min. This was centrifuge at 2,000 x g for 15 min. The cholesterol concentration in the supernatant was determined.

Determination of Low Density Lipoprotein Concentration

The concentration of low density lipoprotein (LDL) cholesterol was determined according to the method of Kameswara *et al.* (1999).

Principle: LDL-C can be determined as the difference between total cholesterol and the cholesterol content of the supernatant after precipitation of the LDL fraction by polyvinyl sulphate (PVS) in the presence of polyethyleneglycol monomethyl ether.

Procedure: The serum samples were kept at 2-8^{0}C. The precipitant solution (0.1ml) was added to 0.2ml of the serum sample and mixed thoroughly and allowed to stand for 15 min. This was centrifuged at 2,000 x g for 15 min. The cholesterol concentration in the supernatant was determined. The concentration of the serum total cholesterol, as described by Kameswara *et al.* (1999), was used.

Calculation:

LDL-C (mmol/L) = Total Cholesterol (mmol/L) – 1.5 x Supernatant Cholesterol (mmol/L).

2.2.11.13 Analgesic Activities of Methanol Extract of the Leaves of *S. magnificum* using Acetic Acid-Induced Writhing and Hot Plate Analgesic Studies.

2.2.11.13.1 Effect of Extract on Acetic Acid-Induced Writhing

The method described by Koster *et al.* (1959) and Taber *et al*. (1969) was used. Twenty mice were fasted for 6 hours and divided into five groups of four animals per group. Groups 3-5 received the extract intraperitoneally (100, 200 and 400 mg/kg b.w. respectively) while groups 1 (vehicle control) and 2 (treatment control) received 1 mg/kg b.w. of 3% Tween 80 and Aspirin (100 mg/kg b.w.) respectively. Thirty (30) minutes before administration of 1% acetic acid solution (0.1 ml/kg, i.p). The writhes (each of which is characterized by a wave of contraction of abdominal musculature followed by extension of the hind limbs) were counted 5 minutes after acetic acid injection for a period of 30 minutes. The percentage protection against writhing was taken as an index of analgesia (Shreedhara *et al*., 2009) and calculated using the following formula:

$$\% \text{ Analgesic Activity} = \frac{\text{Mean writhing count (control group} - \text{Treated group)}}{\text{Mean writhing count of control group}} \times 100.$$

2.2.11.13.2 Effect of Extract on Hot Plate Analgesia

Twenty rats were fasted for 6 hours and divided into five groups of four animals per group. Groups 3-5 received the extract intraperitoneally (100, 200 and 400 mg/kg b.w. respectively) while groups 1 (vehicle control) and 2 (treatment control) received 2 ml/kg b.w. of 3% Tween 80 and Pentazocine (10 mg/kg b.w.) respectively. Each rat was gently placed on the hot plate maintained at 55 ± 0.5/C and the times required by the rat to lick the paw or jump were taken as the response (Turner, 1965). The cutoff time or latency response was 15 seconds to avoid tissue damage (Neto *et al*., 2005). Percentage increase in reaction time or pain threshold inhibition was derived, using the formula (Dash *et al*., 2015).

$$\% \text{ Elongation} = \frac{\text{Latency (test)} - \text{Latency (control)}}{\text{Letency (test)}} \times 100.$$

2.2.11.13.3 Effect of Extract on Tail Flick Response in Mice

The experiment was carried out by measuring tail withdrawal time from hot water as described by Adzu *et al*. (2003). Twenty rats were fasted for 12 hours and divided into five groups of four animals per group. Groups 3-5 received the extract intraperitoneally (100, 200 and 400 mg/kg b.w. respectively) while groups 1 (vehicle control) and 2 (treatment control) received 2 ml/kg b.w. of 3% Tween 80 and Pentazocine (10 mg/kg b.w.) respectively. One hour post drug treatment, about 2 cm of the tail of each mouse was dipped into a water bath containing warm water maintained at a temperature of 50±1°C. The time taken for the mouse to flick the tail, known as the pain reaction time (PRT), was recorded for all the mice.

$$\text{Percentage increase in PRT} = \frac{\text{PRT in test/standard - PRT in control}}{\text{PRT in control}} \times 100$$

2.2.11.14 Statistical Analysis

Data obtained were analysed using one and two way of Analysis of Variance and subjected to LSD post hoc test for multiple comparisons. Results are presented as Mean ± Standard Deviation and differences between means are considered significant at 95% confidence interval.

CHAPTER THREE
RESULTS

3.1 Yield of Extract

The % yield was calculated using the relation:

$$\text{Yield (\%)} = \frac{\text{Weight of extract (g)}}{\text{Weight of plant material (g)}} \times 100$$

Weight of extract (g) = 139.47

Weight of plant material (g) = 1974.92

% extractive yield of crude extract = 7.06%

3.2 Qualitative Phytochemical Composition of Methanol Crude Extract, n-hexane, Dichloromethane, Ethylacetate and Aqueous Methanol Partitions of the Leaves of *S. magnificum*

The crude extract revealed secondary metabolites such as tannins and flavonoids in low concentration as seen in Table 2. Alkaloids, terpenoids, phenols and saponins were found to be in moderate concentration, while glycosides, steroids and reducing sugars were not detected. Carbohydrates were found to be in high concentration and protein in moderate concentration. n-hexane partition contained moderate concentration of alkaloids, terpenoids, glycosides and steroids; phenols, flavonoids, carbohydrates and reducing sugars were found to be in low concentration while saponins, tannins and protein were not detected. DCM partition contained high concentration of terpenoids and steroids; moderate concentration of alkaloids, phenols and tannins; low concentration of flavonoids, carbohydrates and glycosides while saponins, reducing sugars and protein were not detected. Ethylacetate partition contained high concentrations of phenols and tannins; moderate concentration flavonoids; low concentrations of alkaloids, carbohydrates, glycosides and reducing sugars while terpenoids, saponins, protein and steroids were not detected. Aq. methanol partition contained moderate concentration of tannins; low concentration of alkaloids, phenols, saponins, carbohydrates, glycosides and reducing sugars while terpenoids, tannins, protein and steroids were not detected.

3.3 Quantitative Phytochemical Composition of Methanol Extract of the Leaves of *S. magnificum*

Table 3 shows quantitative phytochemical composition of *S. magnificum* leaf ectract. Bioactive compounds such as saponins were found to be highest in concentration (5.000 ± 1.000 mg/100g) compared to other phytochemicals: terpenoids (1.720 ± 0.400 mg/100mg), tannins (0.860 ± 0.040 mg/100g), alkaloids (0.440 ± 0.040 mg/100g) and phenols (0.260 ± 0.040 mg/100g). Flavonoids were found to be lowest in concentration (0.050 ± 0.010 mg/100g).

Table 2: Qualitative phytochemical composition of methanol crude extract, n-hexane, dichloromethane, ethylacetate and aqueous methanol partitions of the leaves of *S. magnificum*

Phytochemicals	Methanol crude extract	N-hexane partition	Dichloromethane Partition	Ethylacetate partition	Aq. Methanol partition
Alkaloids	++	++	++	+	+
Terpeniods	++	++	+++	ND	ND
Phenols	++	+	++	+++	+
Saponins	++	ND	ND	ND	+
Flavonoids	+	+	+	++	++
Tannins	+	ND	++	+++	ND
Carbohydrates	+++	+	+	+	+
Protein	++	ND	ND	ND	ND
Glycosides	ND	++	+	+	+
Reducing Sugars	ND	+	ND	+	+
Steroids	ND	++	+++	ND	ND

Key: + Present in low conc.
++ Present in moderate conc.
+++ Present in highly conc.
ND Not detected

Table 3: Quantitative phytochemical composition of methanol extract of the leaves of *S. magnificum*

Phytochemical composition	**Quantity/Amount (mg/100g)**
Tannins	0.860 ± 0.040
Flavonoids	0.050 ± 0.010
Terpenoids	1.720 ± 0.400
Alkanoids	0.440 ± 0.040
Saponins	5.000 ± 1.000
Phenols	0.260 ± 0.040

Mean ± SD (n = 3)

3.4 Acute toxicity (LD_{50}) Test of Methanol Extract of the Leaves of *S. magnificum*

The acute toxicity test of the methanol extract of *S. magnificum* leaves showed no death up to 5000 mg/kg body weight within 24 hours of administration as shown in Table 4. This result shows that the extract is safe at doses below 5000 mg/kg body weight.

3.5 Effect of Methanol Extract of *S. magnificum* Leaves on Egg Albumin-Induced Rat Paw Oedema

As shown in Table 5, the paw volumes of the rats increased significantly 1 hour after egg albumin injection. Subsequently, there was significant ($p < 0.05$) decrease in paw oedema in rats treated with methanol extract of *S. magnificum* in both time and dose dependent manner compared with rats in the positive control (indomethacin) group. The reduction in paw volume (oedema) was highest at the 5^{th} hour in all extract doses while maximum percentage inhibition of 73.13 % was observed in group 5 administered 400 mg/kg b. w. of extract at the 5^{th} hour. Percentage inhibitory effect and decrease in paw volume of the extract were comparable with that of the standard anti-inflammatory drug, indomethacin.

3.6 Effect of Methanol Extract of *S. magnificum* Leaves on Formaldehyde-Induced Oedema

Table 6 shows that the daily oral administration of the extract for 10 days significantly ($p < 0.05$) suppressed the development of formaldehyde arthritis in a non-dose related manner when compared with that of group 2 (untreated control). The decrease in paw diameter was most observed in groups 5 and 7 administered 100 and 400 mg/kg b. w. of extract respectively on the 10^{th} day. A relative decrease was observed in groups 3 and 4 treated with 1mg/kg dexamethazone and 100 mg/kg aspirin respectively. However, group 6 administered with 200 mg/kg b. w. of extract exhibited the highest percentage inhibition at 100 % on day 10 while groups 5 and 7 had 90.00 % and 97.67 % inhibition on day 10.

Table 4: The median lethal dose of methanol extract of leaves of *S. magnificum*

Phase 1	Dose mg/kg body weight	Mortality
Group 1	10	0/3
Group 2	100	0/3
Group 3	1000	0/3
Phase 11		
Group 1	1600	0/3
Group 2	2900	0/3
Group 3	5000	0/3

Table 5: Effect of methanol extract of *S. magnificum* leaves on egg albumin-induced rat paw oedema

	Volume of mercury displaced (ml) and (%) inhibition of oedema					
Groups	**0hr**	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**5 hr**
1	0.37±0.02	0.47±0.06	0.60±0.06	0.63±0.05	0.65±0.07	0.67±0.07
2	0.24±0.03	0.27±0.05 (42.55)	0.31±0.02 (48.33)	0.23±0.03 (63.49)	0.24±0.02 (63.08)	0.17±0.03 (74.63)
3	0.37±0.02	0.35±0.02 (25.53)	0.40±0.04 (33.33)	0.40±0.02 (36.51)	0.32±0.04 (50.77)	0.30±0.04 (55.22)
4	0.36±0.04	0.32±0.04 (31.91)	0.39±0.02 (35.00)	0.38±0.02 (39.68)	0.30±0.04 (53.85)	0.25±0.05 (62.69)
5	0.35±0.02	0.29±0.04 (38.30)	0.35±0.02 (41.67)	0.32±0.05 (49.21)	0.22±0.02 (66.15)	0.18±0.02 (73.13)

Values presented as mean ± SD, n = 4, $p < 0.05$, % inhibition of oedema in parenthesis

Key:
Group 1=2 ml/kg of % Tween-80 + egg albumin
Group 2=Ibuprofen 100mg/kg + egg albumin
Group 3=100mg/kg of Extract + egg albumin
Group 4=200mg/kg of Extract + egg albumin
Group 5=400mg/kg of Extract + egg albumin

Table 6: Effect of methanol extract of *S. magnificum* leaves on formaldehyde-induced oedema

	Δ in paw diameter (cm) and (%) inhibition of oedema										
Grps	**Baseline**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**
1	0.45±0.10	0.45±0.10	0.45±0.10	0.45±0.10	0.45±0.10	0.45±0.10	0.41±0.10	0.40±0.08	0.38±0.10	0.35±0.06	0.38±0.03
2	0.40±0.00	0.85±0.04 (-12.50)	0.86±0.04 (-15.00)	0.86±0.04 (-15.00)	0.87±0.03 (-17.50)	0.88±0.03 (-20.00)	0.88±0.03 (-20.00)	0.88±0.03 (-20.50)	0.89±0.03 (-22.50)	0.89±0.03 (-22.50)	0.89±0.03 (-22.50)
3	0.40±0.00	0.63±0.05 (42.50)	0.55±0.06 (62.50)	0.43±0.05 (92.50)	0.58±0.10 (55.00)	0.53±0.05 (67.50)	0.48±0.03 (80.00)	0.43±0.05 (90.00)	0.40±0.00 (100.00)	0.40±0.00 (100.00)	0.40±0.00 (100.00)
4	0.35±0.10	0.65±0.10 (14.29)	0.63±0.05 (42.50)	0.50±0.00 (57.14)	0.63±0.05 (20.00)	0.55±0.06 (42.86)	0.50±0.00 (57.14)	0.50±0.00 (57.14)	0.41±0.03 (82.86)	0.40±0.00 (85.71)	0.38±0.05 (91.43)
5	0.40±0.00	0.65±0.10 (37.50)	0.60±0.08 (50.00)	0.58±0.05 (55.00)	0.63±0.05 (42.50)	0.55±0.06 (62.50)	0.54±0.05 (65.00)	0.53±0.05 (67.50)	0.48±0.03 (80.00)	0.46±0.03 (85.00)	0.44±0.03 (90.00)
6	0.48±0.05	0.65±0.06 (64.58)	0.55±0.06 (85.42)	0.55±0.06 (85.42)	0.65±0.06 (64.58)	0.63±0.05 (68.75)	0.58±0.05 (79.17)	0.56±0.05 (83.33)	0.51±0.06 (93.75)	0.50±0.07 (95.83)	0.48±0.05 (100.00)
7	0.43±0.05	0.58±0.10 (65.12)	0.53±0.05 (76.74)	0.53±0.05 (76.74)	0.63±0.05 (53.49)	0.53±0.05 (76.74)	0.50±0.00 (83.72)	0.50±0.00 (83.72)	0.48±0.03 (86.05)	0.45±0.04 (95.35)	0.44±0.03 (97.67)

Δ = change in paw diameter

Values presented as mean ± SD, n = 4, $p < 0.05$, % inhibition of oedema in parenthesis

Key:

Group 1 = 2 ml/kg of 3% Tween-80 (no formalin) Group 2 = 2 ml/kg of 3% Tween-80 + formalin

Group 3 = Dexamethazone 1mg/kg + formalin Group 4= Aspirin 100mg/kg + formalin

Group 5 = 100mg/kg of Extract + formalin Group 6 = 200mg/kg of Extract + formalin

Group 7 = 400mg/kg of Extract + formalin

3.7 Effect of Crude Methanol Extract, Partitions and Fractions of the Leaves of *S. magnificum* on Phospholipase A_2 Activity

As shown in Table 7, methanol extract of the leaves of *S. magnificum* as well as the standard drug (prednisolone) inhibited the release of haemoglobin into the medium by PLA_2. This was shown by the significant ($p < 0.05$) decreases in optical densities in the presence of varying concentrations of the extracts and prednisolone, indicating less haemoglobin. The % inhibition of haemolysis calculated relative to control showed that the highest concentration (0.8 mg/ml) had 44.19% inhibition of PLA_2 activity which exhibited the highest inhibitory activity compared with values obtained for other concentrations of extract. This is comparable with that of 0.4 mg/ml of prednisolone at 35.21%. The crude extract was partitioned with different solvents according to their polarity and its effect on PLA_2 activity showed that n-hexane partition proved more active with % inhibition of 55.81% at 0.1 mg/ml compared with aqueous methanol, ethylacetate and dichloromethane partitions with 55.43, 25.84 and 23.22% respectively at 0.1 mg/ml. An inverse % inhibition was observed between the crude extract and the partition concentrations. Fraction 1 had the highest activity of 58.05% in inhibiting PLA_2 enzyme followed by fraction 3 at 54.87%, fraction 4 at 53.56% and fraction 2 at 52.43 %.

3.8 Effect of Methanol Extract of the Leaves of *S. magnificum* on Membrane Stabilization of Human Red Blood Cell

Results in Table 8 show that the extract inhibited lyses of HRBC subjected to hypotonic stress. This is revealed by the decrease in optical densities (OD) of both isotonic and hypotonic solutions when compared with that of the control. The decrease in OD indicated less haemoglobin in the medium which implied inhibition of lyses of HRBC. Methanol extract of the leaves of *S. magnificum* had a concentration-dependent inhibition of haemolysis, increasing with increased concentration of the extract from 21.20 % at 0.1 mg/ml to 69.10 at 0.8 mg/ml. However, 0.4 mg/ml of indomethacin had 83.39 % inhibition of heamolysis which was significantly ($p < 0.05$) higher than the % inhibition of the extract.

Table 7: Effect of crude methanol extract, partitions and fractions of the leaves of *S. magnificum* on phospholipase A_2 activity

Treatment	Conc. (mg/ml)	Absorbance (crude extract)	% inhibition	Absorbance Aq.MeOH	% inhibition	Absorbance Ethylacetate	% Inhibition	Absorbance DCM	% Inhibition	Absorbance N-hexane	% inhibition
Control	-	0.534±0.001	-								
Extract	0.1	0.363±0.001*	32.02	0.238±0.001*	55.43	0.396±0.001*	25.84	0.410±0.001*	23.22	0.236±0.002*	55.81
	0.2	0.351±0.001*	34.27	0.250±0.001*	53.18	0.398±0.001*	25.47	0.416±0.001*	22.10	0.242±0.001*	54.68
	0.4	0.342±0.001*	35.96	0.254±0.001*	52.43	0.402±0.001*	24.72	0.418±0.001*	21.72	0.250±0.001*	53.18
	0.6	0.316±0.002*	40.82	0.266±0.002*	50.19	0.408±0.000*	23.60	0.436±0.002*	18.84	0.258±0.001*	51.69
	0.8	0.298±0.001*	44.19	0.274±0.001*	48.69	0.416±0.001*	22.10	0.442±0.002*	17.23	0.264±0.001*	50.56
Prednisolone	0.4	0.346±0.001*	35.21								
Fraction 1	0.4	0.224±0.005*	58.05								
2	0.4	0.254±0.004*	52.43								
3	0.4	0.241±0.003*	54.87								
4	0.4	0.248±0.002*	53.56								

Absorbance values are presented as mean ± SD, n=3.

*P<0.05 is significantly different compared with control.

Percentage inhibition of enzyme activity was calculated relative to control.

Table 8: Effect of methanol extract of the leaves of *S. magnificum* on membrane stabilization of human red blood cell

Treatment	Conc. (mg/ml)	Absorbance (418nm) Isotonic Solution	Hypotonic Solution	% Inhibition HRBC haemolysis
Control	-	0.577±0.008	1.381±0.004	0.00
Extract	0.1	0.046±0.002*	1.098±0.001*	21.20
	0.2	0.060±0.002*	1.038±0.003*	25.74
	0.4	0.061±0.002*	0.846±0.002*	40.53
	0.6	0.187±0.002*	0.746±0.002*	53.18
	0.8	0.271±0.001*	0.614±0.002*	69.10
Indomethacin	0.2	0.582±0.002*	0.863±0.003*	64.43
	0.4	0.424±0.002*	0.583±0.003*	83.39

Absorbance values are presented as mean ± SD of triplicate determinations.

*$p < 0.05$ is significantly different compared with control.

Percentage inhibition of HRBC heamolysis was calculated relative to control.

3.9 Effect of Methanol Extract of the Leaves of *S. magnificum* on Platelet Aggregation

Table 9 shows that methanol extract of the leaves of *S. magnificum* inhibited aggregation of platelet induced by $CaCl_2$ *in vitro* in a concentration and time-dependent manner. This is revealed by the decrease in optical densities across all concentrations, indicating less transmittance of light. At 150 seconds, maximal anti-platelet aggregatory activity was observed. Inhibition of platelet aggregation by the extract was comparable with that of Indomethacin.

3.10 Nitric Oxide-Scavenging Activity of the Methanol Extract of the Leaves of *S. magnificum*

Figure 5 shows that the methanol extract of the leaves of *S. magnificum* scavenged NO generated from sodium nitroprusside *in vitro*. The NO-scavenging activity of the extract were not concentration dependent but the activity significantly ($p < 0.05$) increased in higher concentrations (125, 500 and 1000 μg/ml) when compared with that of ascorbic acid whose activity was found to be higher at lower concentrations (15.63 and 31.25 μg/ml).

3.11 DPPH Radical Scavenging Activity of the Methanol Extract of the Leaves of *S. magnificum*

As shown in Table 10, the methanol crude extract of the leaves of *S. magnificum* at different concentrations (15.63 – 1000 μg/ml) had high DPPH radical-scavenging activity compared to that of standard antioxidant agent, ascorbic acid. The optimal activity was 90.95±0.45 at 250 μg/ml for the methanol crude extract and it had the highest scavenging activity as revealed by their IC_{50} values (1.09) compared with that of ascorbic acid (1.53).

Table 9: Effect of methanol extract of the leaves of *S. magnificum* on platelet aggregation

Treatment	Conc. (mg/ml)	Absorbance (520nm) at different time intervals					
		0s	30s	60s	90s	120s	150s
Control	-	0.208 ± 0.11*	0.198 ± 0.008*	0.196 ± 0.009*	0.195 ± 0.009*	0.193 ± 0.009*	0.192 ± 0.009*
Methanol Extract	0.1	0.294 ± 0.043*	0.288 ± 0.044*	0.283 ± 0.044*	0.282 ± 0.044*	0.281 ± 0.044*	0.279 ± 0.044*
	0.2	0.372 ± 0.014*	0.364 ± 0.019*	0.361 ± 0.019*	0.360 ± 0.019*	0.358 ± 0.019*	0.356 ± 0.019*
	0.4	0.485 ±0.015*	0.480 ± 0.015*	0.477 ± 0.015*	0.475 ± 0.015*	0.474 ± 0.015*	0.472 ± 0.015*
	0.6	0.511 ± 0.014*	0.495 ± 0.013*	0.494 ± 0.013*	0.491 ± 0.013*	0.487 ± 0.015*	0.485 ± 0.015*
	0.8	0.526 ± 0.008*	0.507 ± 0.004*	0.506 ± 0.004*	0.503 ± 0.003*	0.503 ± 0.003*	0.502 ± 0.002*
Indomethacin	0.2	0.358 ± 0.027*	0.345 ± 0.027*	0.343 ± 0.027*	0.342 ± 0.027*	0.341 ± 0.027*	0.340 ± 0.027*
	0.4	0.467 ± 0.035*	0.457 ± 0.035*	0.454 ± 0.035*	0.451 ± 0.035*	0.450 ± 0.035*	0.448 ± 0.035*

Values are presented as mean ± SD. *$p < 0.05$ shows significantly different compared with control

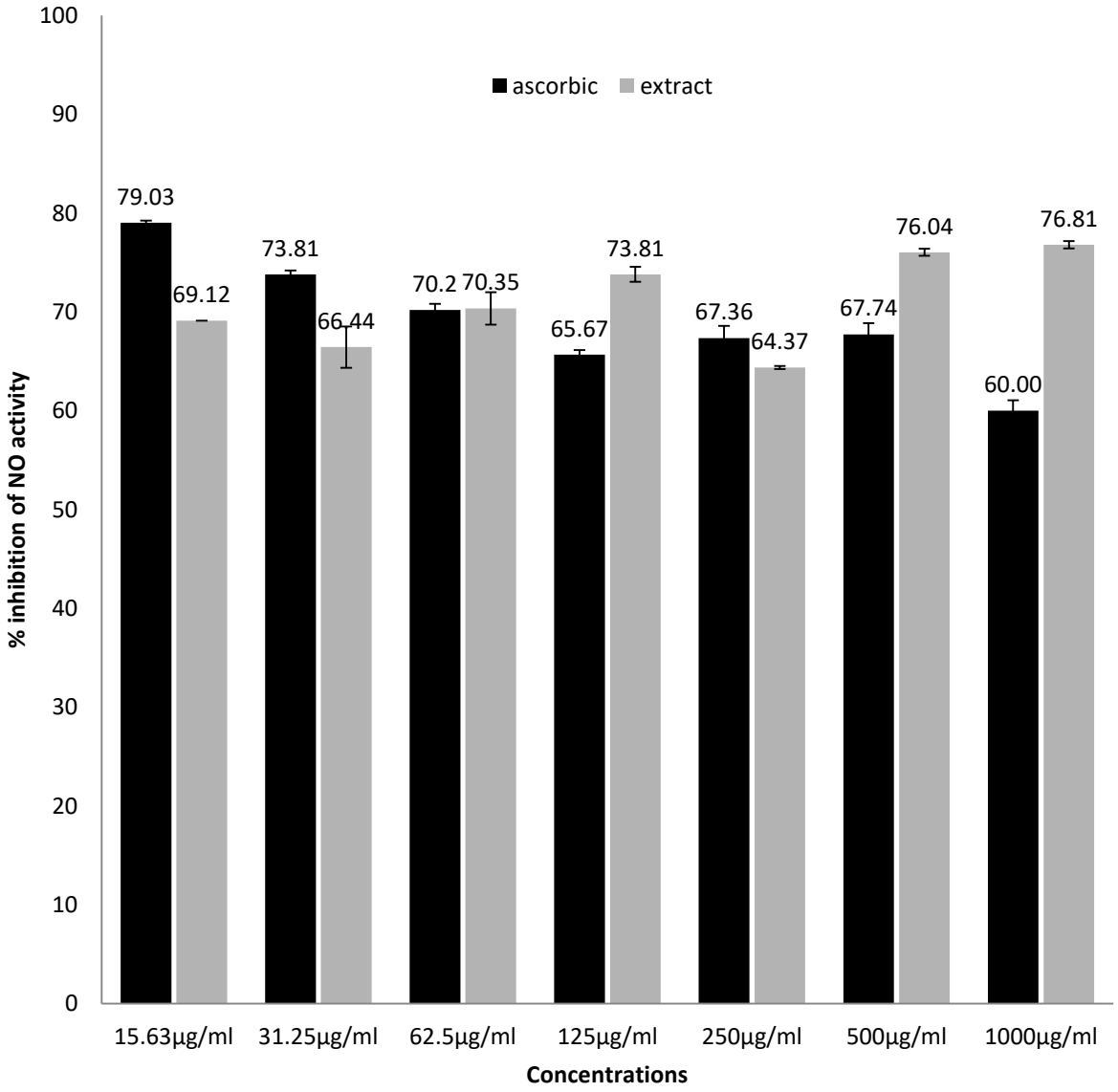


Figure 5: Nitric oxide scavenging activity of the methanol extract of the leaves of *S. magnificum*

Table 10: DPPH radical-scavenging activity of the methanol extract of the leaves of *S. magnificum*

Conc. (µg/ml)	Ascorbic acid	Methanol crude extract
15.63	62.61±1.97[a]	69.48±2.04[a]
31.25	64.19±1.34[a]	70.79±0.71[a]
62.50	63.02±0.78[a]	77.42±1.03[b]
125	63.30±0.68[a]	83.91±0.78[c]
250	64.13±0.72[a]	90.95±0.45[e]
500	62.78±0.96[a]	88.74±2.94[d]
1000	64.39±0.50[a]	84.92±4.79[cd]
IC_{50}	1.53	1.09

The values are expressed as (mean±SD) n=3

Mean values with different letters of the alphabet down the column are significantly different ($p < 0.05$) while mean values with same letters of the alphabet down the column are not significantly different ($p > 0.05$).

3.12 Ferric Reducing Power Capacity of the Methanol Extract of the Leaves of *S. magnificum*

Figure 6 shows that the methanol extract of the leaves of *S. magnificum* had low potency in terms of electron or/hydrogen donating capacity as revealed by the rapid decrease in absorbance at all the concentrations used for FRAP investigation. However, gallic acid was found to be significantly ($p < 0.05$) higher in FRAP investigation in almost all concentrations compared with the concentration of the extract.

3.13 Effect of Methanol Extract of the Leaves of *S. magnificum* on Acetic Acid-Induced Vascular Permeability

Table 11 revealed that methanol extract of the leaves of *S. magnificum* as well as indomethacin exhibited a significant ($p < 0.05$) and dose-dependent inhibition of vascular permeability induced by acetic acid. This was shown by the reduction of blue colouration and thus the less optical density values of the peritoneal fluids of treated rats. Group 5 rats treated with 400 mg/kg of the extract was found to have the highest % inhibition at 57.89% when compared with that of groups 3 and 4 treated with 100 and 200 mg/kg of the extract respectively. However, the inhibitory effect (72.37 %) observed in group 2 administered 50 mg/kg of the standard drug was found to be significantly ($p < 0.05$) higher than that of the extract-treated groups.

3.14 Effect of Methanol Extract of the Leaves of *S. magnificum* on *in vivo* Leukocyte Mobilization

Table 12 shows that administration of different doses (100, 200 and 400 mg/kg) of methanol extract of *S. magnificum* as well as indomethacin caused decreased mobilization of leukocytes into the peritoneal cavity of rats in groups 2 – 5 compared with that of group 1 administered with 2 ml/kg of 3 % Tween 80 indicating a significant ($p < 0.05$) decrease in leukocyte migration. % inhibition of leukocyte mobilization calculated relative to control revealed that the lowest dose (100 mg/kg) of the extract had the highest % inhibition at 41.17 % among the extract-treated groups. The most mobilized leukocytes were neutrophils and lymphocytes, though the number of neutrophils mobilized was found to be higher than that of lymphocytes. The neutrophils of groups 4 and 5 showed no significant ($p > 0.05$) difference when compared with that of control group (group 1). Inhibition of leukocyte mobilization into the peritoneal cavity by the extract was comparable with that of indomethacin.

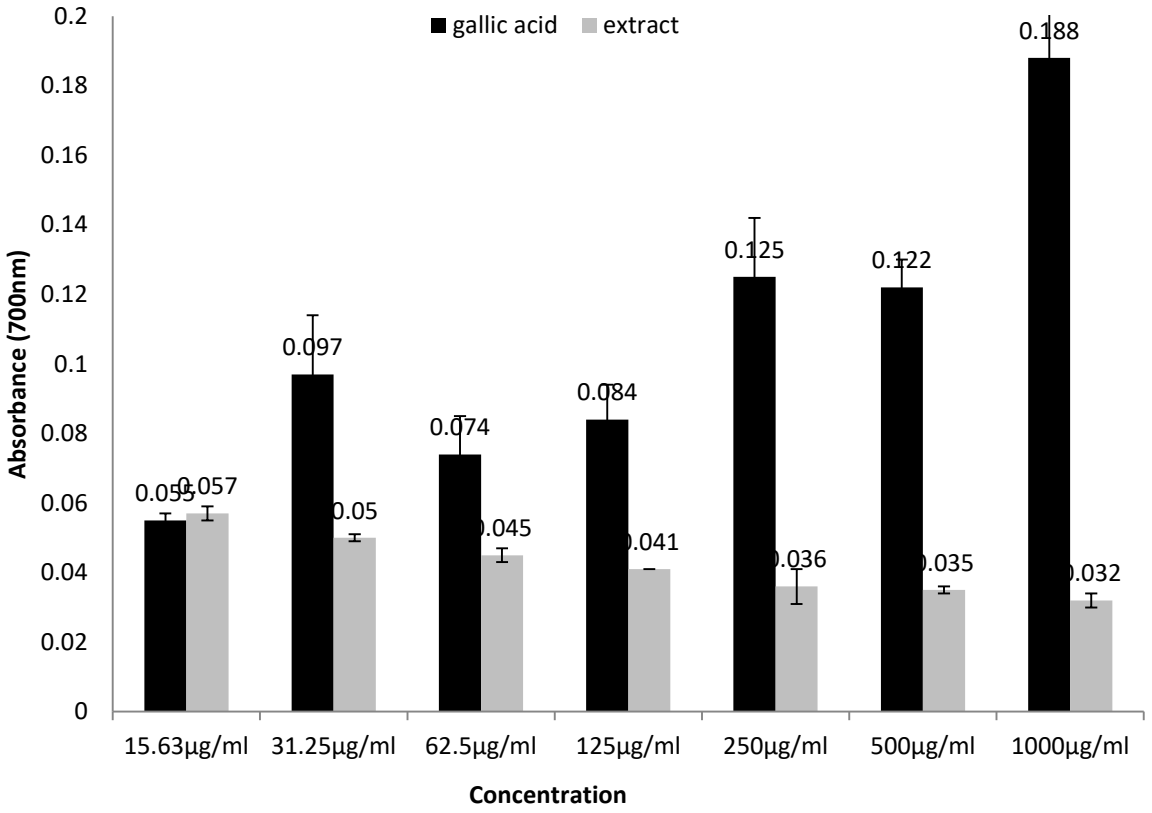


Figure 6: Ferric reducing power capacity of the methanol extract of the leaves of *S. magnificum*

Table 11: Effect of methanol extract of the leaves of *S. magnificum* on acetic acid-induced vascular permeability

Groups	Treatment	Dosage	Absorbance (610nm)	% Inhibition
1	Control	3% Tween 80 (2ml/kg)	0.076 ± 0.006^{d}	0.00
2	Indomethacin	50 (mg/kg)	0.021 ± 0.005^{a}	72.37
3	Extract	100 (mg/kg)	0.053 ± 0.007^{c}	30.26
4		200 (mg/kg)	0.045 ± 0.008^{c}	40.79
5		400 (mg/kg)	0.032 ± 0.005^{b}	57.89

Values are presented as mean ± SD, n=4

Percentage inhibition of vascular permeability was calculated relative to control

Mean values with different alphabets down the column are significantly different ($p < 0.05$) while mean values with same letters of the alphabet down the column are not significantly ($p > 0.05$) different.

Table 12: Effect of methanol extract of the leaves of *S. magnificum* on *in vivo* leukocyte mobilisation

				Differential leukocyte mobilization (%)				
Grps	**Treatment**	**TLC (10^4/mm)**	**% Inhibition**	**N**	**L**	**M**	**E**	**B**
1	2ml/kg of 3% Tween 80	88.20 ± 8.91^{d}	-	70.3 ± 7.96^{b}	26.6 ± 5.03^{a}	1.3 ± 0.22^{a}	1.1 ± 0.31^{a}	0.7 ± 0.09^{a}
2	50 mg/kg Indo.	41.71 ± 2.76^{a}	52.71	65.8 ± 9.66^{ab}	30.0 ± 1.40^{ab}	1.5 ± 0.10^{b}	1.4 ± 0.05^{ab}	$1.30.10^{b}$
3	100 mg/kg Extract	51.89 ± 3.38^{b}	41.17	58.5 ± 1.91^{a}	37.5 ± 4.39^{c}	$1.20.08^{a}$	1.5 ± 0.17^{b}	1.3 ± 0.36^{b}
4	200 mg/kg Extract	58.72 ± 3.01^{bc}	33.42	61.0 ± 4.56^{ab}	35.2 ± 1.91^{bc}	1.3 ± 0.17^{ab}	1.3 ± 0.13^{ab}	1.2 ± 0.17^{b}
5	400 mg/kg Extract	64.98 ± 3.69^{c}	26.33	65.5 ± 2.44^{ab}	30.7 ± 3.95^{ab}	1.5 ± 0.08^{b}	1.0 ± 0.33^{a}	1.3 ± 0.13^{b}

Grps. = Groups, TLC = Total leukocyte count, N = Neutrophils, L = Lymphocytes, M = Monocytes, E = Eosinophils, B = Basophils

Mean values with different letters of the alphabet down the column are significantly different ($p < 0.05$) while mean values with same letters of the alphabet down the column are not significantly different ($p > 0.05$).

Values are presented as mean ± SD, n=4

Percentage inhibition of leukocyte was calculated relative to control.

3.15 Effect of Methanol Extract of the Leaves of *S. magnificum* on MDA Concentration of Rats with Formalin-Induced Oedema

Table 13 shows that injection of formalin to induce oedema provoked significant ($p < 0.05$) lipid peroxidation as evidenced by a marked increase in the concentration of MDA in group 2 rats compared with that of group 1 rats. Treatment with varied doses (100, 200 and 400 mg/kg b.w.) of methanol extract of *S. magnificum* significantly ($p < 0.05$) decreased MDA concentration in a dose-dependent manner when compared with values for the negative control (group 2). Groups 3 and 4 treated with 1mg/kg of dexamethazone and 100mg/kg of aspirin respectively also had a significant ($p < 0.05$) decrease in MDA concentration although group 7 treated with 400 mg/kg of extract significantly ($p < 0.05$) decreased MDA concentration than the concentration of standard drug groups indicating higher inhibition of lipid peroxidation. However, no significant ($p > 0.05$) difference was revealed in standard drugs groups as well as extract-treated groups (groups 3 – 7).

3.16 Effect of Methanol Extract of the Leaves of *S. magnificum* on Some Haematological Parameters of Rats with Formalin-Induced Oedema

Oral administration of extract for 10 days caused different effects on the levels of haematological parameters as shown in Table 14. A significant ($p < 0.05$) decrease was observed in Hb, RBC, WBC and PCV but a significant ($p < 0.05$) increase in platelet in group 2 after formalin-induced oedema compared to group 1. The extract at varying concentrations significantly ($p < 0.05$) restored Hb concentration, RBC, WBC count and PCV but a significant ($p < 0.05$) decrease in platelet count was observed in all extract treated groups. This result is comparable with that obtained with group 3 and 4 treated with 1 mg/kg of dexamethazone and 100 mg/kg of aspirin respectively.

Table 13: Effect of methanol extract of the leaves of *S. magnificum* on MDA concentration of rats with formalin-induced oedema

Groups	MDA Concentration (532nm)
1	3.10 ± 0.12^{a}
2	7.31 ± 0.31^{c}
3	4.72 ± 0.94^{b}
4	4.47 ± 0.43^{b}
5	4.68 ± 0.92^{b}
6	4.52 ± 0.41^{b}
7	4.38 ± 0.64^{b}

Values are expressed as mean±SD, n=4

Mean values with different alphabets down the column are significantly different ($p < 0.05$) while mean values with same letters of the alphabet down the column are not significantly ($p > 0.05$) different.

Key:
Group 1 = 2 ml/kg of 3% Tween-80 (no formalin)
Group 2 = 2 ml/kg of 3% Tween-80 + formalin
Group 3 = Dexamethazone 1mg/kg + formalin
Group 4= Aspirin 100mg/kg + formalin
Group 5 = 100mg/kg of Extract + formalin
Group 6 = 200mg/kg of Extract + formalin
Group 7 = 400mg/kg of Extract + formalin

Table 14: Effect of methanol extract of the leaves of *S. magnificum* on some haematological parameters of rats with formalin-induced oedema

Groups	Hb (g/dl)	RBC ($x10^9$/l)	WBC ($x10^3$/l)	PCV (%)	Platelets ($x10^6$/l)
1	22.53±2.01^{d}	275.00±4.59^{g}	8161.00±29.33^{cd}	30.94±2.31^{d}	170.78±3.14^{c}
2	08.11±1.29^{a}	132.91±3.62^{a}	6825.03±21.66^{a}	20.56±1.63^{a}	185.08±3.61^{d}
3	10.01±0.20^{ab}	152.00±2.81^{b}	7981.00±23.00^{c}	24.21±1.03^{b}	174.03±1.12^{c}
4	15.20±0.81^{c}	260.00±1.28^{f}	7712.00±04.57^{b}	24.10±0.77^{b}	150.04±0.80^{a}
5	12.02±0.65^{b}	188.00±3.17^{c}	8282.00±02.19^{d}	23.18±1.68^{b}	145.50±1.28^{a}
6	15.29±1.35^{c}	217.00±2.14^{d}	8492.00±01.79^{e}	25.28±0.81^{b}	158.19±2.55^{b}
7	20.15±3.85^{d}	252.00±2.89^{e}	9121.00±01.85^{f}	28.10±1.67^{c}	175.78±8.59^{c}

Values are expressed as mean±SD, n=4

Mean values with different alphabets down the column are significantly different ($p < 0.05$) while mean values with same letters of the alphabet down the column are not significantly ($p > 0.05$) different.

Key:
Group 1 = 2 ml/kg of 3% Tween-80 (no formalin)
Group 2 = 2 ml/kg of 3% Tween-80 + formalin
Group 3 = Dexamethazone 1mg/kg + formalin
Group 4= Aspirin 100mg/kg + formalin
Group 5 = 100mg/kg of Extract + formalin
Group 6 = 200mg/kg of Extract + formalin
Group 7 = 400mg/kg of Extract + formalin

3.17 Effect of Methanol Extract of the Leaves of *S. magnificum* on the Activities of Some Liver Enzymes of Rats with Formalin-Induced Oedema

As shown in Table 15, injection of formalin caused a significant ($p < 0.05$) increase in serum AST, ALT and ALP activities of group 2 rats when compared to those of animals in those of group 1. However, treatment with methanol extract of *S. magnificum* at different concentrations significantly ($p < 0.05$) decreased AST, ALT and ALP activities compared with group 2. Highest reduction of AST and ALT activities was observed in group 7 treated with 400 mg/kg of extract while highest reduction of ALP was observed in group 6 rats treated with 200 mg/kg of extract among the extract treated groups. Similar decreases in liver enzyme activities were also observed in the standard drug treated groups.

3.18 Effect of Methanol Extract of the Leaves of *S. magnificum* on Serum Lipid Profile of Rats with Formalin-Induced Oedema

Table 16 shows that injection of formalin significantly ($P < 0.05$) altered the lipid profile indices in group 2 when compared with those of group 1. Groups 5, 6 and 7 treated with 100, 200 and 400 mg/kg of the extract respectively showed significantly ($p < 0.05$) decreased cholesterol, TAG and LDL levels when compared with those of group 2 except in HDL which showed significant ($p < 0.05$) increase. Group 7 was found to be more effective in reducing cholesterol, TAG and LDL levels amongst the extract-treated groups as well as standard drug groups.

Table 15: Effect of methanol extract of the leaves of *S. magnificum* on the activities of some liver enzymes of rats with formalin-induced oedema

Groups	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
1	48.06±2.98^{a}	33.62±4.58^{a}	57.06±4.07^{a}
2	65.39±4.87^{d}	75.82±4.28^{d}	104.80±3.14^{e}
3	62.15±2.69^{cd}	60.22±2.19^{b}	81.13±2.32^{c}
4	59.26±5.40^{bc}	58.09±2.91^{b}	91.32±2.77^{d}
5	61.65±2.78^{cd}	68.74±4.24^{c}	87.18±5.98^{cd}
6	58.10±2.04^{bc}	67.38±5.43^{c}	62.47±2.64^{ab}
7	54.47±4.73^{b}	59.83±3.72^{b}	66.04±9.53^{b}

Values are expressed as mean±SD, n=4

Mean values with different alphabets down the column are significantly different ($p < 0.05$) while mean values with same letters of the alphabet down the column are not significantly ($p > 0.05$) different.

Key:
Group 1 = 2 ml/kg of 3% Tween-80 (no formalin)
Group 2 = 2 ml/kg of 3% Tween-80 + formalin
Group 3 = Dexamethazone 1mg/kg + formalin
Group 4= Aspirin 100mg/kg + formalin
Group 5 = 100mg/kg of Extract + formalin
Group 6 = 200mg/kg of Extract + formalin
Group 7 = 400mg/kg of Extract + formalin

Table 16: Effect of methanol extract of the leaves of *S. magnificum* on serum lipid profile of rats with formalin-induced oedema

Groups	Cholesterol (mmol/l)	TAG (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
1	3.84±0.04^{a}	2.16±0.04^{a}	2.63±0.04^{e}	2.07±0.05^{a}
2	5.18±0.05^{f}	2.93±0.04^{e}	1.76±0.05^{a}	3.84±0.05^{d}
3	4.63±0.04^{e}	2.46±0.04^{bc}	2.35±0.05^{c}	2.44±0.06^{c}
4	4.57±0.04^{d}	2.53±0.04^{cd}	2.13±0.04^{b}	2.36±0.06^{b}
5	4.44±0.04^{c}	2.57±0.05^{d}	2.30±0.05^{c}	2.33±0.08^{b}
6	4.42±0.05^{c}	2.51±0.08^{cd}	2.46±0.05^{d}	2.29±0.05^{b}
7	4.29±0.04^{b}	2.42±0.05^{b}	2.37±0.09^{c}	2.31±0.03^{b}

Values are expressed as mean±SD, n=4

Mean values with different alphabets down the column are significantly different ($p < 0.05$) while mean values with same letters of the alphabet down the column are not significantly ($p > 0.05$) different.

Key:
Group 1 = 2 ml/kg of 3% Tween-80 (no formalin)
Group 2 = 2 ml/kg of 3% Tween-80 + formalin
Group 3 = Dexamethazone 1mg/kg + formalin
Group 4= Aspirin 100mg/kg + formalin
Group 5 = 100mg/kg of Extract + formalin
Group 6 = 200mg/kg of Extract + formalin
Group 7 = 400mg/kg of Extract + formalin

3.19 Effect of Methanol Extract of the Leaves of *S. magnificum* on Acetic Acid-Induced Writhing in Mice

The extract (100, 200, and 400 mg/kg) and aspirin (100 mg/kg) produced a significant ($p < 0.05$) dose-dependent reduction in the number of abdominal writhes induced by intraperitoneal injection of acetic acid in extract-treated mice when compared to the 3% Tween 80 treated mice. The effects of the extract 200 and 400 mg/kg (40.27% and 50.68%, respectively) on the treated groups were comparable to the effect of aspirin 100 mg/kg (54.25%) on the treated group (Table 17).

3.20 Effect of Methanol Extract of the Leaves of *S. magnificum* on Analgesic Pain using Hot Plate Test

A significant ($p < 0.05$) and dose-dependent elevation of the post-treatment reaction time to thermal pain was evident in the extract-treated animals as shown in Table 18. The effect of the extract (400 mg/kg) was comparable to that produced by 10 mg/kg of Pentazocine.

3.21 Effect of Methanol Extract of the Leaves of *S. magnificum* on Tail Flick Response in Mice

The results of tail flick response are shown in Table 19. The extract (100, 200, and 400 mg/kg) and pentazocine (10 mg/kg) caused a significant ($p < 0.05$) dose-dependent increase in the post reaction time (PRT) in the treated mice groups, when compared to the Tween 80 treated group. % inhibition of tail flick response to stimulus calculated relative to control showed that the standard drug group had a higher % inhibition at 46.36% more than the 400 mg/kg extract-treated group at 39.18%.

Table 17: Effect of methanol extract of the leaves of *S. magnificum* on acetic acid-induced writhing in mice

Groups	Treatment	No of writhes per 30min	% Inhibition
1	2 ml/kg of 3% Tween 80	91.25 ± 5.38^{d}	
2	100 mg/kg Aspirin	41.75 ± 6.85^{a}	54.25
3	100 mg/kg Extract	65.25 ± 7.04^{c}	28.49
4	200 mg/kg Extract	54.50 ± 3.87^{b}	40.27
5	400 mg/kg Extract	45.00 ± 2.94^{a}	50.68

Mean values with different letters of the alphabet down the column are significantly different ($p < 0.05$) while mean values with same letters of the alphabet down the column are not significantly different ($p > 0.05$).

Values are presented as mean ± SD, n=4

Table 18: Effect of methanol extract of the leaves of *S. magnificum* on analgesic pain using hot plate test

Groups	Dose	Pre-treatment (sec)	Post-treatment (sec)	% inhibition
1	2ml/kg of 3% Tween 80	2.88±0.50	2.88±0.50[a]	
2	10 mg/kg Pentazocine	3.07±0.02	9.48±0.81[c]	69.62
3	100mg/kg Extract	2.63±0.57	5.72±1.21[b]	49.65
4	200mg/kg Extract	2.85±0.50	6.89±0.88[b]	58.20
5	400mg/kg Extract	3.03±0.02	8.65±1.23[c]	66.71

Mean values with different letters of the alphabet down the column are significantly different ($p < 0.05$) while mean values with same letters of the alphabet down the column are not significantly different ($p > 0.05$).

Values are presented as mean ± SD, n=4

Table 19: Effect of methanol extract of the leaves of *S. magnificum* on tail flick response in mice

Groups	Dose	Pain Reaction Time (sec)	% inhibition
1	2 ml/kg of 3% Tween 80	2.36±0.50[a]	
2	10 mg/kg Pentazocine	4.40±0.81[d]	46.36
3	100 mg/kg Extract	3.13±1.21[b]	24.60
4	200 mg/kg Extract	3.27±0.88[b]	27.83
5	400 mg/kg Extract	3.88±1.23[c]	39.18

Mean values with different letters of the alphabet down the column are significantly different ($p < 0.05$) while mean values with same letters of the alphabet down the column are not significantly different ($p > 0.05$).

Values are presented as mean ± SD, n=4

3.22 FTIR Frequency Range and Functional Groups Present in the Most Active Fraction (Fraction 1) of the n-hexane Partition

Fraction 1 spectra (Table 20 and appendix 1) revealed strong absorption peak at a frequency of 2924.18 and 1033.88 cm^{-1} assigned to C-H of alkanes and C-O of phenol respectively. Other peaks indicate presence of amines, carboxylic acid, nitrites, esters, aldehydes, ketones and alkenes

3.23 Biologically Active Chemical Compounds of Fraction 1

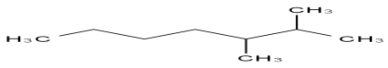
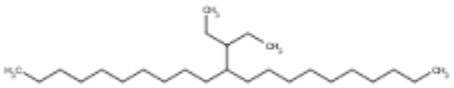
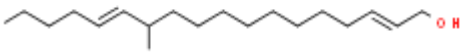
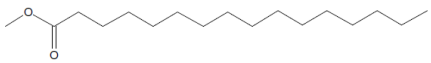
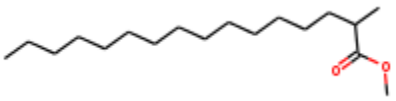
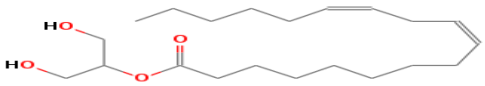
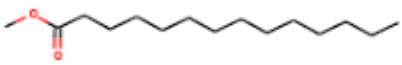
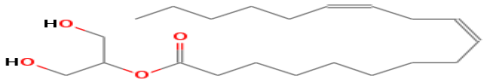
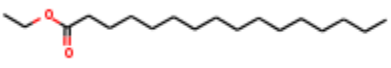
The compounds present in fraction 1 of n-hexane partition of *S. magnificum* were identified by GC-MS analysis. Spectrogram showing the peak identities of the compounds is presented in appendix 2. Active principles with their Retention Time (RT), Molecular Formula (MF), Concentration (%) are presented in Table 21. Ten compounds were identified in this fraction. Hexadecanoic acid, 2-methyl-, methyl ester is found to be responsible for the peak area (24.906%) followed by Heptane, 2,3-dimethyl- (21.669%), Hexadecanoic acid, methyl ester (13.351%), 1,5-Heptadien-3-yne (11.977%), Hexadecanoic acid, ethyl ester (8.256%), 9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (7.485%), Heptadecanoic acid, 16-methyl-, methyl ester (5.300%), 12-Methyl-E,E-2,13-octadecadien- (4.028%), 9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (2.942%) and Heneicosane, 11-(1-ethylpropyl)- (0.086%).

Table 20: FTIR frequency range and functional groups present in the most active fraction (fraction 1) of the n-hexane partition

Wave number (cm^{-1})	Functional groups (vibration mode)	Possible compounds present
3834.61, 3934.92	N-H str. (w)	Amines
3603.15, 3726.60	O-H str. (m)	Alcohols, phenols
3317.67, 3471.98	N-H str. (m)	Amines
2924.18	C-H str. (s)	Alkanes
2669.57	O-H str. (w)	Carboxylic acid
2306.94, 2422.67	NΞH str. (w)	Nitrites
1952.03	C=C=C str. (w)	Alkenes
1712.85	C=O str. (m)	Esters, aldehydes, ketones
1535.39	C=C str. (w)	Esters, aldehydes, ketones
1381.08, 1458.23	C-H deformation (m)	Alkanes
1188.19	C-O str. (w)	Phenols, esters
1033.88	C-O rocking (s)	Phenols
725.26	C-H deformation (m)	Alkenes

Key: w: weak, m: medium, s: strong, str.: stretching

Table 21: Biologically active chemical compounds of fraction 1

Name of compounds (molecular formula)	Chemical Structure	Retention time (min)	%
Heptane, 2,3-dimethyl- (C_9H_{20})		1.650	21.669
1,5-Heptadien-3-yne (C_7H_8)		2.051	11.977
Heneicosane, 11-(1-ethylpropyl)- ($C_{26}H_{54}$)		5.817	0.086
12-Methyl-E,E-2,13-octadecadien-1-ol ($C_{19}H_{36}O$)		9.953	4.028
Hexadecanoic acid, methyl ester ($C_{17}H_{34}O_2$)		10.463	13.351
Hexadecanoic acid, 2-methyl-, methyl ester ($C_{18}H_{36}O_2$)		10.839	24.906
9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester ($C_{21}H_{40}O_4$)		11.448	2.942
Heptadecanoic acid, 16-methyl-, methyl ester ($C_{19}H_{38}O_2$)		11.576	5.300
9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester ($C_{21}H_{40}O_4$)		11.790	7.485
Hexadecanoic acid, ethyl ester ($C_{18}H_{36}O_2$)		11.910	8.256

CHAPTER FOUR

DISCUSSION

Primary metabolites of the leaf of *S. magnificum* obtained showed that carbohydrate was present in high amountt while protein was moderately present. Carbohydrates are useful as they supply energy to the cells, contribute to fat metabolism, spare protein as energy source and aid assimilation of other minerals (Eze and Obinwa, 2014). The presence of carbohydrate obtained in this study is an indication that the leaf could serve as a useful source of energy. This result is in line with the works of Karabi *et al*. (2014), Ojo *et al*. (2014), Asuk *et al*. (2015) and Prabhavathi *et al*. (2016) who also reported presence of carbohydrate in *Costus specious, Cissus quadrangulari, Ficus asperifola and Jatropha curcas* leaves respectively. The presence of protein indicates that the leaves could serve as a vital tool for growth and development. It can also serve as an enzyme catalyst and cell response mediators (Ojo *et al*., 2014). Moderate presence of protein was also recorded in *Costus specious* leaves by Karabi *et al*. (2014) and *Jatropha curcas* leaves by Asuk *et al*. (2015).

Secondary metabolites have been found to possess wide range of medicinal properties, which may help in protection against various diseases (Park *et al*., 2001, Okoli and Akah 2006, Varadarajan *et al*., 2008). Interestingly, many of these phytochemicals have been discovered and even isolated from a variety of medicinal plants. Regrettably, however, not many of them have been exploited for clinical use. It is important to note that the presence or absence of any particular bioactive compound fundamentally depends on the solvent of extraction and the plant part used for the extraction (Dai and Mumper, 2010). Results of phytochemical screening (Table 2) show the presence of alkaloids, terpenoids, phenols, saponins, flavonoids and tannins.

The alkaloid content of the methanol leaf extract of *S. magnificum* was 0.440 ± 0.040mg/100g. Alkaloids have pharmacological applications as anesthetics and central nervous system stimulants (Madziga *et al.*, 2010). They produce anti-microbial, analgesic, antifungal, anti-inflammatory, anti-fibrogenic and antispasmodic effects (Ibraheem and Maimako, 2014; Renuga Devi and Krishnakumari, 2015) and as such could serve these purposes. The alkaloid content as found in this study was higher than 0.24±0.03 and 0.21±0.07mg/100g found in *E. officinalis* and *I.cylindrica* leaves respectively. However, the alkaloid content of leaves of *S. magnificum* was same as the 0.4±0.01mg/100g obtained in methanol extract of *Taraxacum officinale* leaves (Krishnaiah *et al*., 2009).

Terpeniods possess anti-inflammatory, anti-malaria, anti-cancer, sedative, insecticidal and cytotoxic activity. They are found to inhibit cholesterol synthesis, reduce blood glucose level and diastolic blood pressure (Ogugua *et al*., 2013). The terpeniod content obtained from this study was 1.720 ± 0.400mg/100g and this finding is higher than the terpeniod content of *Senna mimosoides* leaf extract reported by Ekwueme *et al*., (2015) at 0.87 ± 0.0032 mg/100g.

Phenols have a role to play in defence against pathogens (Martinez *et al*., 2008). They also have enormous ability to combat cancer and are also thought to prevent heart ailments to an appreciable degree and sometimes are anti-inflammatory agents (Kar, 2007). The phenol content of the methanol leaf extract of *S. magnificum* was 0.260 ± 0.040 mg/100g and it was found to be higher than methanol leaf extract of *Taraxacum officinale* with 0.012±0.10, *E. officinalis* with 0.037±0.19 and *I. cylindrica* with 0.05±0.25. However, phenolic content of *S. magnificum* was less than that of *H. rosa-sinensis* with 0.680±0.11 (Krishnaiah *et al*., 2009).

Concentration of saponin obtained in this study was 5.000 ± 1.000mg/100g. Saponins are important therapeutically as they are shown to have hypolipidemic effect; they do so by binding sterols of cell membrane which reduces cholesterol levels (Kar, 2007). Other therapeutic properties include antimicrobial effect (Habu and Ibeh, 2015), antioxidant effect (Shirwaikar *et al*., 2003), anticancer activity, stimulate anti-body production and anti-inflammatory agent (Sani *et al*., 2014; Abubakar *et al*., 2015). This implies that the extract could be employed as hypocholesterolemic and anti-inflammatory agent. Saponin content was found to be higher than 2.1±0.13, 2.2±0.11 and 0.38±0.01 obtained from *A.* indica, *C. asiatica* and *A. conyzoides* respectively by Krishnaiah *et al*. (2009); Okwu and Iroabuchi, (2009).

Flavonoids have been reported to exert a wide range of biological activities. These include: anti-inflammatory, antibacterial, antiviral, anti-allergic (Williams *et al*., 2004; Cushnie and Lamb, 2005), cytotoxic antitumour (Garc´ıa-Lafuente *et al*., 2009), treatment of neurodegenerative diseases, vasodilatory action (Tsuchiya, 2010). In addition, flavonoids are known to inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, cyclo-oxygenase and lipoxygenase enzyme activities. Flavonoids are well known for their ability to inhibit pain perception and anti-inflammatory properties due to inhibition of enzymes involved in inflammation, especially arachidonic acid metabolic pathway, and synthesis of prostaglandins (Serafini *et al*., 2010; Mali *et al*., 2013). They exert these effects as antioxidants, free radical scavengers, chelators of divalent cation (Middleton *et al*., 2000). This is an indication that the extract might be effective in treating inflammation. Flavonoid

content obtained in this study was 0.050 ± 0.010mg/100g. This result is in line with the findings of Krishnaiah *et al*. (2009) which revealed the flavonoid content of *C. asiatica* to be 0.052±0.20.

Tannin concentration obtained in this study was 0.860 ± 0.040mg/100g. Tannins have also shown potential antimicrobial, antiviral and anti-inflammatory effects (Akiyama *et al*., 2001; Asuk *et al*., 2015). Their microbial effects are exerted through mechanisms such as membrane disruption, binding to proteins and adhesions and enzyme activation (Ibrahim *et al*., 2014). This could explain the use of the plant in ethanomedicine for the treatment of inflammatory disorders. The tannin content was found to be higher than that obtained in the methanol extract of *U. chamae* (0.40±0.03) by Okwu and Iroabuchi. (2009).

Bioguided assay, phytochemical analyses, chromatographic techniques and spectroscopy study were used to identify the active principal. The crude extract was partitioned with n-hexane, DCM, ethylacetate and aqueous methanol (90:10) solvents with polarity index of 0.1, 3.1, 4.4 and 10.2:5.1 respectively to identify compounds for further studies. Phytochemical analyses are of vital importance in identification of active principles in plant, hence, the need to screen the four partitions for different phytochemical compositions (Table 2). Inhibition of PLA_2 activity carried out showed that the n-hexane partition was the most potent as shown on Table 7. The active partition was subjected to thin layer chromatography. This enabled the choice of solvent to be used in column chromatography. Different trials of spotting made favoured toluene:ethylacetate (80:20). The n-hexane partition was taken to column chromatography for further purification.

The acute lethal effect of *S. magnificum* extract on mice showed no death within 24 hours after treatment even at the dose of 5000 mg/kg b.w. The LD_{50}, being greater than 5000 mg/kg b.w., was thought to be safe.

Egg albumin/Carrageenan induced oedema is the most widely used primary test to screen new anti-inflammatory agents, measure the ability of a compound to reduce local oedema induced in the rat paw (Winter *et al*., 1962). It has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1 – 2 hrs) of the carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (Anosike *et al*., 2012a; Gupta *et al*., 2006;

Arunachalam *et al*., 2009). The extract at all the doses tested (100, 200 and 400 mg/kg) produced significant ($p < 0.05$) and dose-dependent inhibitory effect on oedema produced by egg albumin when compared with group 1 treated with 2 ml/kg of 3% Tween 80. The reduction in paw volume was highest at 5th hour in all extract treated groups and standard drug ibuprofen group with ibuprofen having the highest % inhibition of oedema at 74.63% followed by group 5 that was administered 400 mg/kg extract (73.13%). The extract inhibited both early and late phases of inflammation induced by egg albumin and this implies that the leaves of *S. magnificum* could be employed in treatment of inflammation. This study is in line with the works of Okoli *et al*. (2005); Mohammed *et al*. (2014); Enechi and Nwodo (2015); who also reported inhibition of egg albumin-induced oedema in rats treated with *Securidaca longipedunculata*, *Loxostylis alata* and *Buchholzia coriacea* extracts respectively.

Formalin-induced paw oedema was considered one of the most suitable test procedures to screen chronic anti-inflammatory agents, as it closely resembles human arthritis (Greenwald, 1991). Inflammation induced by formalin is biphasic, an early neurogenic component is mediated by bradykinin followed by a later tissue mediated response where histamine, 5-hydroxytryptamine and prostaglandins (PGs) are known to be involved (Kasim *et al*., 2009). Chronic inflammation is of a more prolonged duration and manifests histologically by the presence of lymphocytes and macrophages, resulting in fibrosis and tissue necrosis. The persistent chronic inflammation increases the development of the degenerative diseases such as rheumatoid arthritis, atherosclerosis, heart disease, Alzheimer (Dalgleish and O'Byrne, 2002). Vernier caliper method (Joseph *et al*., 2005) was used to show the suppressive effect of different doses of methanol extract of leaves of *S. magnificum* on formalin-induced chronic inflammation (Table 6). The extract was able to inhibit formaldehyde induced paw oedema after 10 days of treatment. A significant ($p<0.05$) decrease in paw diameter was observed in groups 3-7 treated with 100, 200 and 400 mg/kg extract respectively when compared with group 2 (untreated control). Also a relative decrease was observed in groups 3 and 4 treated with 1mg/kg Dexamethazone and 100mg/kg Aspirin respectively. A 100% inhibition was observed in group 3 on day 8, 9 and 10 and group 6 on the 10th day. The extract could have prevented the development of arthritis by inhibiting enzymatic peroxidation, which is involved in the arachidonic acid cascade and leads to the formation of PGs and leukotrienes involved in the development of arthritis (Numan *et al*., 2006). This study is in agreement with the findings of Kasim *et al*. (2009) and Naza *et al*. (2010) who reported a dose-dependent anti-inflammatory effect of Silymarin and Melatonin respectively in experimental animal model of chronic inflammation.

PLA_2 enzyme is necessary for the release of arachidonic acid. It's inhibition prevents the formation of prostaglandins, thromboxanes and leukotrienes and is the main basis for the anti-inflammatory effect (Foegh and Ramwell, 2001; Arunachalam *et al*, 2009). The extract significantly ($P<0.05$) inhibited PLA_2 activity in a concentration-related manner provoking inhibition comparable to that of prednisolone. Percentage inhibition of PLA_2 activity by the extract at 0.8 mg/ml (41.19%) was found to be significantly ($p<0.05$) higher than that of the 0.4 mg/ml standard drug (35.21%). The enzyme activity of PLA_2 was assayed using its action on erythrocyte membrane on which it creates leakage thus causing haemoglobin to flow out into the medium (Iwueke *et al*., 2006). The enzyme activity is thus directly related to the amount of haemoglobin in the medium hence the increase in absorbance (table 7) since haemoglobin absorbs maximally at 418 nm. Inhibition of PLA_2 suggests that the extract may suppress the synthesis of free fatty acids from membrane phospholipids and consequently deprive prostaglandin synthase of precursors or substrates for the production of prostaglandins. PLA_2 inhibitory activity of the extract is attributed mainly to the constituents found in the extract which other researchers have reported to be PLA_2 inhibitor. Schimmer and Parker (2001) and Nworu and Akah (2015) reported that inhibition of this enzyme by medicinal plant is mediated via a protein called lipocortine or through direct interaction with the enzyme. The synthesis of lipocortine is induced by plant steroidal hormones such as triterpenoids. According to Arnold *et al*. (2015), polyphenols unspecifically bind to proteins. The interactions between the enzyme and the hydroxyl groups present in polyphenols result in formation of stable complex which causes conformational changes that lead to inhibition of the enzymatic activity (Da Silva *et al*., 2009). Studies have shown that flavonoids inhibit eicosanoid generating enzymes including PLA_2 (Owoyele *et al*., 2005; Nworu and Akah, 2015). From the result obtained, *S. magnificum* could be in use as an anti-inflammatory drug using inhibition of PLA_2 as one of its mechanism of action. Other plants that have exhibited anti-inflammatory effects through inhibition of PLA_2 include *Vitex doniana* leaves (Iwueke *et al*., 2006), *Spiranthera odoratissima* leaves (Barbosa *et al*., 2012), *Aloe vera* water extract (Nworu and Akah, 2015), *Buchholzia coriacea* (Enechi and Nwodo, 2015).

The crude extract was purified to remove all inert and undesirable components so as to increase activity and minimize any odour, taste and colour. The purification process enables a good level of PLA_2 activity to be obtained from relatively small concentrations of the crude extract. Extract was partitioned with different solvents according to their polarity and its effect on PLA_2 activity shows that n-hexane partition proved more active with % inhibition of

55.81% at 0.1 mg/ml compared with aqueous methanol, ethylacetate and dichloromethane partitions with 55.43, 25.84 and 23.22% respectively at 0.1 mg/ml. This could be as a result of the constituents of the n-hexane partition which include alkaloids, flavonoids, steroids and terpenoids. According to Sowjanya *et al*., 2017, these compounds have been investigated in models of inflammation in rats and were found to possess significant activity in both proliferative and exudative phases of inflammation. Among the fractions, fraction 1 had the highest % inhibition of PLA_2 activity. Adebayo *et al*. (2016) reported activity of n-hexane fraction from *Peltophorum africanum* leaf on inflammation.

The result obtained from this study shows that methanol extract of *S. magnificum* protected the human erythrocyte membrane against lysis induced by hypotonic solution. Exposure of red blood cells (RBCs) to injurious substances such as hypotonic medium, heat, methyl salicylate or phenylhydrazine results in the lysis of the membranes, accompanied by haemolysis and oxidation of haemoglobin (Feirrali *et al*., 1992). The extract had a concentration-dependent inhibition of haemolysis, increasing with increased concentration of extract from 21.20% at 0.1mg/ml to 69.10% at 0.8mg/ml. However, 0.4mg/ml Indomethacin had 83.39% inhibition which was significantly ($p<0.05$) higher than that of the extract treated groups. The HRBC membrane stabilization has been used as a method to study the *in vitro* anti inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane (Shenoy *et al*., 2010) and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which cause further tissue inflammation and damage (Chaitanya *et al*., 2011). Thus, the extract perhaps stabilized the red blood cell membrane by preventing the release of lytic enzymes and active mediators of inflammation. This could be due to the presence of saponins, terpenoids and flavonoids in the extract. Hossain *et al*. (2014) and Abo-Dola *et al*. (2015) reported that these compounds exert membrane-stabilizing effect on lysosome by binding cation and other biomolecules. This finding is consistent with earlier reports of Ojogbane and Nwodo (2010); Anosike *et al*., (2012b); Akinwunmi *et al*. (2015) who also reported membrane stabilizing potentials of *Cyphostemma glaucophilla, Solanum aethiopicum* and *Cyphospenna adenocaulis* extracts respectively.

The result obtained from this study shows that methanol extract of *S. magnificum* inhibited the aggregation of platelets induced with calcium chloride. During tissue injury and

inflammation there is an increased production of thromboxane A_2, a potent platelet activator which is followed by aggregation (Strukova, 2001; Blair and Flaumenhaft, 2009). Platelets are regarded as key regulators of both haemostasis and pathogenesis of cardiovascular diseases (Bakdash and William, 2008; Lee *et al*., 2009; Fabre and Gurney, 2010). Hence, the anti-platelet activity observed in this study implies that the extract could help in preventing the onset of events that lead to cardiovascular diseases. Thus, anti-platelet activity of the extract could be attributed to the presence of tannins, flavonoids and phenolic compounds as revealed by phytochemical result. These bioactive compounds might have prevented the adhesion and aggregation of platelets, or interacted with calcium ions thereby preventing binding of other mediators involved in the aggregation cascade. These bioactive compounds have been studied extensively. The flavonoid isolated from *Urtica dioica* inhibited thrombin, ADP, epinephrine and collagen-induced platelet aggregation (El Haouari *et al*., 2006). The anticoagulant or anti-platelet aggregation activity of tannins has been demonstrated by various researchers (Mekhfi *et al*., 2006; Kee *et al.,* 2008). However, the synergistic effects of the other components cannot be ruled out, since alkaloids also have anti-platelet aggregation activity (Huang *et al*., 2008)

Free radicals are involved in a wide variety of pathological manifestations of pain, inflammation, cancer, diabetes, aging, hepatic damage, neurodegenerative, cardiovascular complications. Antioxidants fight free radicals and protect the body from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defence mechanisms (Eze, 2006; Catala, 2009; Sen and Batra, 2013). NO and DPPH assay are the most widely used methods for screening antioxidant activity of plant extracts. The evidence of the radical-scavenging potential of the extract was compared by investigating its ability to scavenge nitric oxide ($NO^{\cdot}$) production. Nitric oxide (NO) is a reactive free radical generated from sodium nitroprusside in aqueous solution at physiological pH and reacts with oxygen to form nitrite. Despite the possible beneficial effects of $NO^{\cdot}$, its contribution to oxidative damage is increasingly becoming evident (Etim *et al*., 2013). This study shows that the methanol extract of *S. magnificum* has more potent nitric oxide scavenging activity than the standard ascorbic acid. The methanol extract of *S. magnificum* was significantly higher in nitrite concentrations ($p < 0.05$) at higher concentrations (500 and 1000 μg/mL) when compared with the concentrations of ascorbic acid. Inhibition of NO could be as a result of direct competition of phytoconstituents present in the extracts with oxygen which inhibited formation of nitrite ion quantified by Griss reagent.

Results from DPPH investigation indicated definite scavenging activity of the extract in comparison with ascorbic acid as observed by the high percentage inhibition indicating high anti-oxidant potency of the extract in terms of electron/hydrogen atom-donating capacity. Table 10 also showed that the extract possessed substantial dose-dependent antioxidant activity with IC_{50} of 1.09 μg/ml compared with 1.53 μg/ml of ascorbic acid. This study, therefore, show that the methanol extract of *S. magnificum* leaves exhibited high antioxidant and free radical scavenging activities indicating that the plant is a significant source of natural antioxidant and its potential could be attributed to its phenolic content as revealed from the phytochemical result. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching single and triplet oxygen, or decomposing peroxides (Md Ekramul *et al*., 2011). The OH group found on the phenolic ring also serves as electron/hydrogen donor potentiating free radical-scavenging activity (Brewer, 2011). Flavonoids are also capable of inhibiting the expression and activation of radical generating enzymes such as nitric oxide synthase (Khanam *et al*., 2004). Inflammatory process which involves the release of excess reactive oxygen and nitrogen species from activated neutrophils and macrophages in order to eliminate antigens (Liu *et al*., 2015); the over production leads to tissue injury by damaging the biomembranes (Dandekar *et al*., 2015). These therefore reveal that, the plant could be used as a natural antioxidant source to limit free radical damage and could be employed in treatment of pathological diseases associated with oxidative stress.

FRAP assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating anti-oxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions (Krishnaraju *et al*., 2009). The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain reaction donating a hydrogen atom. They can react with free radicals to convert them into more stable products. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Md. Ekramul *et al*., 2011). The reducing capacity of methanol extract of *S. magnificum* leaves was investigated by the reduction of the Fe^{3}+/ferricyanide complex to the Fe^{2}+ form. The extract when compared with the standard gallic acid had low potency in reducing the transition state of iron and the rate which radicals are generated through fenton reaction as shown in fig. 6. Phenolic compounds may be responsible for causing the paramount antioxidant effect which is supported by previous studies (Santhakumari *et al*., 2003; Dasgupta and De, 2004; Arambewela *et al*., 2005).

Acetic acid, a vasodilator, dilates blood vessels to release histamine, prostaglandins and leukotrienes which increase vascular permeability that is sustained over 24 hours (Okoli *et al*., 2007; Ojha *et al*., 2014). Brown and Roberts, (2001) reported that vascular permeability increases due to contraction and separation of endothelial cells at their boundaries which exposes the basement membrane and becomes freely permeable to plasma proteins and fluid. Exudation which is a consequence of increased vascular permeability is considered a major feature of acute inflammation (Jain *et al*., 1995). The extract produced a dose-dependent inhibition of vascular permeability produced by acetic acid as shown in table 11. It reduced the intensity of peritoneal inflammation by reducing dye leakage induced by acetic acid in the mice peritoneum. The effect on peritoneal inflammation was attributed to the ability of the extract to inhibit the release of inflammatory mediators, which cause increase in vascular permeability of small blood vessels and enhance inflammation (Morebise *et al*., 2001). This result implies that the extract could effectively suppress the exudative phase of acute inflammation. This finding is in agreement with the studies of Morebise *et al*. (2001), Tatiya and Saluja (2011) and Mohammed *et al*. (2014) who observed inhibition of vascular permeability in experimental animals treated with *C. cyanus leaf, Machilus macrantha* seed and *Loxostylis alata* leaf extracts respectively.

Another key aspect of inflammatory response is cellular infiltration due to the pivotal role played by leukocytes. As part of their defensive roles during inflammation, these cells release their lysosomal contents such as bactericidal enzymes and proteases causing further tissue damage and inflammation (Okoli *et al*., 2008). Such injury to cell membrane will further render the cell more susceptible to secondary damage by lipid peroxidation. Increased infiltration and adhesion of leukocytes to endothelial cells could increase the inflammatory process. *S. magnificum* methanol leaf extract produced a significant ($p < 0.05$) decrease in Agar induced leukocyte mobilization. It has been observed that the chemotatic movement of neutrophils towards the foreign body is the first and the most important step in phagocytosis (Ganachari *et al*., 2004; Cunha *et al*., 2008). This activity may help to increase the general resistance of the body against microbial infections. The polymorphous neutrophils (PMNs), which engulf and eliminate invading micro organism, was the most mobilized as obtained from the result. Furthermore, the mobilization of neutrophil by the extract indicates that the extract activates the generation of respiratory burst by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which generates ROS used in killing microbes (Puga *et al*., 2012). Moreover, the extract can also mop up this ROS when they are produced in excess due to its antioxidant effect. The fact that indomethacin used decreased leukocyte

mobilization corresponds with former findings that high doses of indomethacin inhibit the accumulation of leukocytes (Bhattacherjee *et al*., 1983). It is well established that NSAIDs inhibit migration of inflammatory cells by inhibiting the release of chemical mediators, inhibiting the expression of cell adhesion molecules and inhibiting cell motility (Prempeh and Mensah-Attipoe, 2008). Considering our finding, it is possible that *S. magnificum* extract inhibited migration of the leukocyte by one or more of these mechanisms. The inhibition of leukocyte migration by *S. magnificum* extract therefore shows that the extract could also alter the action of the endogenous factors that are involved in the migration of these cells to the site of inflammation, thereby reducing the inflammatory process. This finding is line with earlier studies of Prempeh and Mensah-Attipoe (2008); Umapathy *et al*. (2010) and Anosike *et al*. (2012) who also reported inhibition of leukocytes in agar-induced leukocyte mobilization in rats.

Malondialdehyde is an endogenous genotoxic product of enzymatic and ROS-induced lipid peroxidation (LPO) whose adducts is known to exist in DNA isolated from healthy humans (Niedernhofer *et al*., 2003). Lipid peroxidation is the oxidative deterioration of lipids containing any number of carbon-carbon double bonds (El-Beltagi and Mohamed, 2013). According to Greenberg *et al.*, (2008), LPO induces disturbance of fine structures, alteration of integrity, fluidity, and permeability, and functional loss of biomembranes, modifies low density lipoprotein (LDL) to proatherogenic and proinflammatory forms, and generates potentially toxic products. Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues (El-Beltagi and Mohamed, 2013). In this study, the high concentration of MDA in group 2 shows that induction of formalin generated free radicals that induced lipid peroxidation. Increased LPO impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptor (Arulselvan and Subramanian, 2007), thereby further intensifying inflammatory damage. However, extract treated groups significantly ($p < 0.05$) decreased MDA concentration in a dose dependent manner when compared to group 2. Lipid peroxidation leads to disruption of integrity of the membrane which results in leakage of cellular contents into the serum; this has been implicated in the pathogenesis of various diseases including arthritis. Inhibition of lipid peroxidation by the extracts might be attributed to its membrane stabilizing effect. It could be that the antioxidants present in the extract scavenged free radicals thereby preventing initiation of lipid peroxidation. This result implies that the extract has the ability to hinder oxidative stress by inhibiting lipid peroxidation. Similar reduction in MDA concentration by

Talinum fruticosum plant extract after formalin induction was reported by Agnel and Shobana (2012).

A significant ($p < 0.05$) decrease was observed in Hb, RBC, WBC and PCV and an increase in platelet count in group 2 rats compared to that of group 1 rats after formalin-induced oedema. Extract-treated groups significantly ($p < 0.05$) restored Hb concentration, PCV, RBC and WBC count. A decrease in HB, RBC, WBC and PCV after formalin induction could be as a result of formaldehyde, the active ingredients of formalin which leads to variety of effects including anaemia and suppression of immune system. RBCs and related factors are major indices used to evaluate circulatory erythrocytes, diagnose anaemia and also serve as useful indices of the bone marrow capacity to produce RBCs in mammals (Ozkan *et al*., 2012). Production of some cytokines (ILI and TNF α) during inflammatory response might also impair red cell production in the bone marrow which results in anaemia. Anti-inflammatory cytokines promote anaemia by inhibiting the proliferation of erythroid progenitor cells (Agnel and Shobana, 2012). Other causes of anaemia also reduce haemoglobin production, reduce DNA synthesis, and reduce stem cell production, bone marrow infiltration, increase red cell destruction and acute and chronic blood loss (Mohammed, 2016). Treatment with *S. magnificum* extract showed a significant increase in RBC, PCV and Hb; this is usually an indication of erythropoiesis stimulation of an extract which must have increased the rate of erythropoietin release in the kidney, which is the humoral regulator of RBC production (Mishra and Tandon, 2012). The major functions of the white blood cells (WBCs) and its differentials are to fight infections, defend the body by phagocytosis against invasion by foreign organisms and to produce or at least transport and distribute antibodies (Lawal *et al*., 2015a). A significant increase in the WBC count by the extract reflect leucopoetic and possible immunomodulatory effects (Bashir *et al*., 2015), which will enhance an animal's capability to generate antibodies during phagocytosis and have high degree of resistance to diseases. The decrease in WBC count after formalin induction (group 2 rats) might be as a result of immune suppression while the observed increase in platelet might be linked to the release of cytokines which promote platelet production. A significant reduction in platelet count indicated that the anti-thrombopoetin potency of the extract and the blood clotting mechanism of the animals will be inadequate with consequent effects of high loss of blood during injury (Lawal *et al*., 2015b). Restoration of haematological indices could be attributed to some phytochemicals found in the extract such as saponins, tannins and flavonoids which have the tendency to stimulate synthesis of haemoglobin or protect haemolysis of RBC (Sani *et al*., 2014). It could also be that the

extract interfered with utilization of iron which is responsible for anaemia. From the result obtained, it could be assumed that the extract maybe effective in treating anaemia and has the ability to enhance immune system. This finding is in agreement with previous studies of Agnel and Shobana, (2012) and Udobang and Okokon (2017).

Lipid peroxidation results in damage and loss of functional integrity of the cell membrane which results in leakage of lysosomal enzymes AST, ALT and ALP in the serum (Gaurav *et al*., 2011). Formalin caused a significant ($p<0.05$) increase in serum AST, ALT and ALP activities in group 2 (treated with tween 80) compared with group 1 (normal rats) after formalin-induced oedema. The serum enzyme levels are direct measure of hepatic injury and they show the status of the liver. The elevation of enzymes induced by formalin causes hepatotoxicity which may be due to secretion of cytochrome P-450 family of enzymes. In the course of metabolism, the metabolite causes the generation of a free radical that binds to lipoprotein and leads to peroxidation of lipids of endoplasmic reticulum. The disturbance in the transport function of the hepatocytes as a result of hepatic injury causes the leakage of enzymes from cells due to altered permeability of membrane (Khatun *et al*., 2015). In this study, *S. magnificum* extract significantly suppressed the formalin-induced elevated AST, ALT and ALP. Most of the anti-inflammatory drugs exert their beneficial effect by inhibiting either release of these enzymes or by stabilizing lysosomal membrane which is one of the major events responsible for the inflammatory process (Chaitanya *et al*., 2011). Thus, it can be assumed that *S. magnificum* extract acted by either inhibiting the enzymes or stabilizing the membrane. This result indicates cell membrane/ hepatic protective effects of the extract. It also supports the results of membrane stabilization. Doshi *et al*. (2011) and Khatun *et al*. (2015) also reported significant ($p<0.05$) decrease in the activities of liver enzymes in rats treated with *Cassia auriculata* flowers extract after cotton pellet implantation and barks of *L. globosus* after formalin induced oedema respectively.

Formalin-induced oedema significantly ($p<0.05$) altered the lipid profile of rats in group 2 compared to rats in group 1. It has also been reported that patients with untreated rheumatoid arthritis and chronic systemic inflammation may have altered lipoprotein patterns that contribute to their higher risk of atherosclerosis (Choi and Seeger, 2005). A consistent pattern of reduced HDL-cholesterol levels has been observed in rheumatoid arthritis patients (Dursunoglu *et al*., 2005). However, extract-treated groups had significant ($p<0.05$) decrease in cholesterol, TAG and LDL and an increase in HDL. The increase in HDL could be attributed to the fact that antioxidant components present in the extract increased lecithin

cholesterol acyltransferase (LCAT) activity. The LCAT activity may increase the transport of cholesterol esters from peripheral tissues to liver and stimulate the production and secretion of HDL for circulation (Baragob *et al*., 2014). Saponins detected in the extract could be responsible for the high HDL and low LDL levels. Saponins aid in reducing cholesterol levels by forming complexes with cholesterol and bile acids which prevent them from being absorbed through the small intestine, hence lowers the cholesterol level in the blood and liver (Sani *et al*., 2014). A previous study had also shown that agents that can reduce serum total-cholesterol, LDL-cholesterol, VLDL-cholesterol, and triglycerides and also induce an elevation in HDL-cholesterol may confer significant protection against inflammatory-related diseases (Dursunoglu *et al*., 2005). Previous studied had associated inflammation with atherosclerosis (Li and Glass, 2002; Gonzalez-Gay *et al*., 2005). This implies that the *S. magnificum* extract may possess anti-inflammatory activity against lipid-related inflammatory diseases and could be helpful in reducing incidence of cardiovascular diseases. The result obtained is consistent with earlier report of Anyasor *et al*. (2014) who observed increase in total cholesterol, LDL and TAG in formaldehyde-induced arthritis in rat. Chandra *et al*. (2015) also reported observed increase in total cholesterol, LDL and TAG in chronic inflammation.

Acetic acid-induced writhing in mice is attributed to visceral pain and finds much attention in screening analgesic drugs (Hasan *et al*., 2010). It is a procedure to evaluate peripherally acting analgesics and pain sensation by triggering localized inflammatory response (Hassan *et al*., 2008). Such pain stimulus leads to the release of free arachidonic acid from the tissue phospholipid (Ribeiro *et al*., 2000; Franzotti *et al*., 2002). The response is thought to be mediated by peritoneal mast cells (Voilley, 2004), acid sensing ion channels (Hossain *et al*., 2006), and the prostaglandin pathways (Adzu *et al*., 2003). Acetic acid has also been postulated to act indirectly by inducing the release of endogenous mediators, which stimulate the nociceptive neurons that are sensitive to NSAIDs and narcotics (Alam *et al*., 2012). The extract evoked significant ($p<0.05$) and dose-dependent reduction in the number of acetic acid-induced writhes in mice. At 400 mg/kg, the effect of the extract was comparable to that of 100 mg/kg of aspirin (Table 17). This result implies that the extract may possess NSAID-like analgesic activities, mediated through peripheral mechanisms. NSAIDs can inhibit COX in peripheral tissues, thereby interfering with the mechanism of transduction in primary afferent nociceptors. Therefore, the analgesic action of *S. magnificum* extract could be due to a blockade of the effect or the release of endogenous substances that excite pain nerve endings such as bradykinin, similar to the mechanism of action of aspirin, which is mediated

via a peripheral mechanism. The result obtained is consistent with earlier report of Ilodigwe and Akah (2009) and Alam *et al*. (2013) who observed decrease in the number of acetic acid-induced writhes in mice by *Spathodea campanulata* and *Piper betle* leaf extract respectively.

Hot plate model which relies on nociceptive reaction against thermal stimuli, is well-established for detection of opiate (narcotic) analgesic property for centrally acting analgesics (Kumari *et al*., 2013). *S. magnificum* extract showed significant ($p < 0.05$) dose dependent analgesic effect in the hot plate test, which involves higher brain functions and consists of responses to nociceptive stimuli organized at a supraspinal level (Mohammed *et al*., 2014). Therefore, the result might imply that the extract also has a central analgesic effect. This analgesic effect of *S. magnificum* extract can be attributed in part, to its anti-inflammatory effect as in visceral pain model, the precursor releases arachidonic acid through cycloxygenase and prostaglandin biosynthesis which plays a role in the nociceptive mechanism (Franzotti *et al*., 2002). This therefore, implies that the inhibition of acute inflammation by the extract leads to their inhibitory effect on pain development process. Ilodigwe and Akah (2009) also reported increase in latency time to thermal pain by *Spathodea campanulata* leaf extract. However, the analgesic results of the acetic-induced writhing response and thermal (hot plate) experiments appear to indicate that the extract was more effective in alleviating central pain than peripheral (acetic acid) pain.

The tail-flick response is a deep pain model (Omeh and Ezeja, 2010) believed to be a spinally mediated reflex and the effectiveness of analgesic agents in the tail-flick pain model is highly correlated with relief of human pain perception. The tail-flick method is based on the observation that morphine-like compounds are selectively able to prolong the reaction time of typical tail-withdrawal effect in rats (Ponnaluri *et al*., 2016). It is mostly used to screen for the effects of the extract on the central nervous system (CNS). The extract significantly ($p<0.05$) increased the PRT of the treated mice to thermal stimulus in a dose-dependent manner when compared to Tween 80 treated mice. This implies that the extract could have increased the pain threshold in CNS of the treated mice. The extract demonstrated a weak CNS involvement when compared to the effects of pentazocine (10 mg/kg). Pentazocine is a synthetic mixed agonist-antagonist with analgesic activities similar to those of morphine (Rang *et al*., 2007). The result of the anti-nociceptive effects of *S. magnificum* leaves is in agreement with the report of Prabhavathi *et al*. (2012) and Ponnaluri *et al*. (2016). Interestingly, compounds found in the extract such as flavonoids and triterpenes in part, have been shown to possess analgesic activity as the claim made by Pritam *et al*. (2011) and Okwu

and Josiah (2006). Tannins could affect the inflammatory response via free radical scavenging properties and inhibition of iNOS in macrophages (Jeffers, 2006). Saponins, on the other hand, inhibit pain and inflammation via NO inhibition (Moharram and El-Shenawy, 2007, Hassan *et al*., 2012).

FTIR spectrum is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in compounds (Murugesh and Vino, 2017). It is an effective tool for differentiating, classifying and discriminating closely related plants (Maitera and Chukkol, 2016). The underlying mechanism of the FTIR technique is associated with transitions between quantized vibrational energy states (Griffiths and de Haseth, 2007). In FTIR analysis, absorption of IR radiation occurs when a photon transfers to a molecule and excites it to a higher energy state (Parikh and Chorover, 2005). Results of FTIR spectroscopic studies have revealed the presence of various chemical constituents in the fractions with various peak values. The peak formation in the FTIR spectrum represents the functional groups. Presence of C=O, C-H, C=C and C-O, C-C and C-O bonding structures are responsible for the formation of alkyl groups, methyl groups, alcohols, ethers, esters, carboxylic acid, anhydrides and deoxyribose (Dukor, 2002). The strong peak at 2924.18 cm^{-1} seen in fraction 1 is allocated to the C-H stretching of alkane. Alkane compounds are present in rare medicinal plants (Starlin *et al*., 2012). Other medium and weak peaks observed from the spectra (appendix 1) indicate presence of amines, phenols, carboxylic acid, esters, aldehydes and alkenes. Alkenes are used as anaesthetics. Carboxylic acids help in maintaining the cell membrane which is one of the underlining mechanisms by which plant extract inhibit inflammation (Krishna and Mohan, 2017). Phenol has anti-oxidant activity; it is used as an antiseptic and also used as a preservative in some vaccines. Phenol spray is used medically to help sore throat. Alkynes have antifungal and antitumor activities. Non appearance of any peak at 2260 cm^{-1} region indicates absence of cyanide group in the fraction which shows non-toxic nature of the plant (Ranjana and Vijay, 2015). These results agree with the work of Maobe and Nyarango, (2013) who reported that these functional groups confirm the presence of secondary metabolites and other phytoconstituents present in plants.

GC-MS analysis is a breakthrough in analysis of phytoconstituents and structure elucidation of compounds as they have a sensitivity of detecting compounds as low as 1ng (Saravanakumar *et al*., 2016). Fraction 1 was analysed by GC-MS because it showed the highest activity in inhibition of PLA_2 compared to the other fractions. GC-MS analysis was

done to determine the type(s) of compounds present. Appendix 2 showed chromatogram which is plots of the total mass eluted from GC and detected by MS as a function of time. Each peak represents a discrete chemical compound; 10 peaks were identified in fraction 1 which indicates the likely presence of 10 compounds. It however, had the compound; Hexadecanoic acid, 2-methyl-, methyl ester (24.906 %) identified as the major peak from this fraction. Hexadecanoic acid, methyl ester belongs to the fatty acid group. n-hexadecanoic acid act as antioxidant, hypocholesterolemic, nematicide, pesticide, anti androgenic flavor, hemolytic, 5-alpha reductase inhibitor (Praveen, 2014). This compound has been reported by Rajeswari *et al.* (2012) to have anti-inflammatory activities. The anti-inflammatory activity of n-hexadecanoic was revealed from structure and kinetic study carried out by Aparna *et al.* (2012) to be due to its ability to inhibit PLA_2 competitively. Ajoku *et al*. (2015) reported the ability of hexadecanoic acid, methyl ester to reduce the production of nitric oxide- a key mediator in inflammation processes in cells by inhibiting arachidonic acid which is a precursor in the biosynthesis of prostagladins and therefore, inhibit the levels of proinflammatory mediators such as interleukin-10 (IL-10), prostaglandin (PGE_2) and tumour necrosis factor-alpha (TNFα). Octadecanoic acid (oleic acid) was identified in fraction 1 as seen in Table 24. Anyasor *et al.* (2014) and Omotoso *et al.* (2014) reported that 9-octadecanoic acid has anti-inflammatory, antitumor, immunostimulory, antiandrogenic, antibacterial, antifungal, lipoxygenase inhibitory, hypocholesterolemic and cancer preventive activities. Similarly, Gnanavel and Saral, (2013) also observed antioxidant activity of this compound. This study has revealed the presence of many bioactive compounds which might be important therapeutically and pharmaceutically.

CONCLUSION

This study revealed that *S. magnificum* leaf extract possessed anti-inflammatory, antioxidant and analgesic activities, as well as the potential to restore other altered biochemical parameters generated during inflammation. The possible underlining mechanisms by which the extract inhibited inflammation include: reduction in acute (egg albumin-induced) and sub-chronic (formalin-induced) inflammation, inhibition of PLA_2 activity, inhibition of platelet aggregation and vascular permeability, stabilization of lysosomal membrane and scavenging of free radicals. This result could be attributed to the abundant phyto-constituents as well as bioactive compounds present in the plant; hence, implying that *S. magnificum* could be used in the treatment and management of pain and inflammatory-related diseases.

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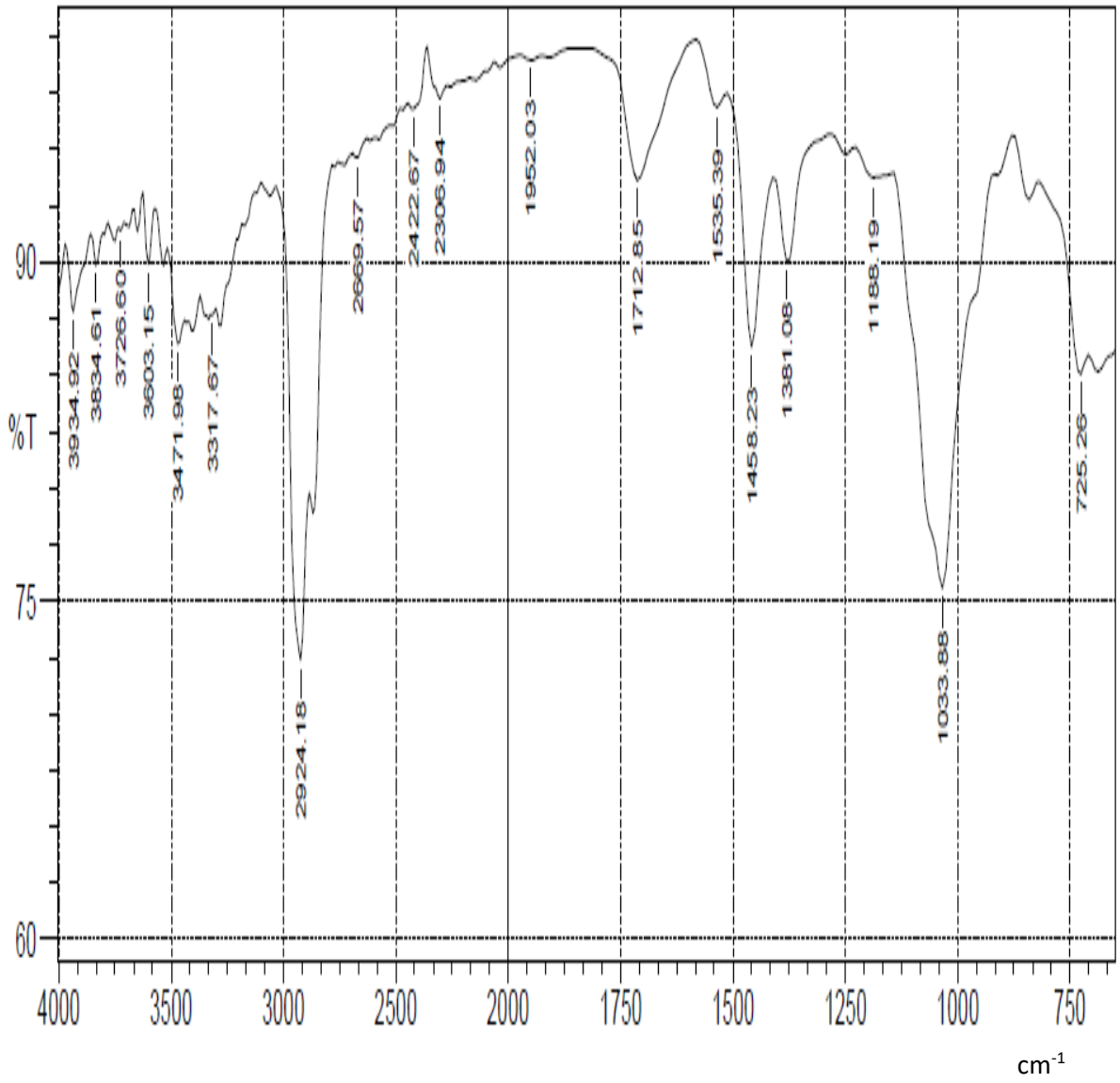
APPENDICES

Appendix 1: FTIR spectra of fraction 1 of the n-hexane partition



Appendix 2: GCMS analysis of N-hexane fraction 1