LIPID PROFILE OF PYTHON FAT AND ITS ANTI-INFLAMMATORY POTENTIALS

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NOVEMBER, 2018.

TITLE

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF MASTERS OF SCIENCE (MSc) IN BIOCHEMISTRY (INDUSTRIAL BIOCHEMISTRY) UNIVERSITY OF NIGERIA, NSUKKA.

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NOVEMBER, 2018.

CERTIFICATION

This is to certify that Sani, Shadrach Bello**,** a post graduate student with Registration Number PG/MSc/16/82370 in the Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka has satisfactorily completed the requirements for course work and research for the award of degree of Master of Science (MSc) in Biochemistry (Industrial Biochemistry). The work embodied in this report is original and has not been submitted in part or full for any other diploma or degree of this or any other university.

Dr. V. E. O. Ozougwu (Supervisor)	Prof. F. C. Chilaka (Supervisor)
Prof. F. C. Chilaka (Head of Department)	External Examiner

DEDICATION

This work is dedicated to God Almighty.

ACKNOWLEDGEMENTS

I am grateful to the God Almighty who granted me good health, protection and all needed to start and accomplish this work.

I want to acknowledge everyone who contributed to making this work a reality. My sincere gratitude goes to my supervisors; Dr. V.E.O Ozougwu and Prof. F.C. Chilaka for their guidance, outstanding supervision and immeasurable assistance throughout the period of this study. My distinguished lecturers: Prof. L. U. S. Ezeanyika, Prof. O. U. Njoku, Prof. I. N. E. Onwurah, Prof. H. A. Onwubiko, Prof. B. C. Nwanguma, Prof. S. O. O. Eze, Prof. V. N. Ogugua, Dr. P. E. Joshua, Dr. C. S. Ubani, Dr. O. C. Enechi, Dr. A. C. Ezugwu, Dr. (Mrs.) C. A. Anosike and Dr. (Mrs.) O. U. Njoku, amongst others, for the knowledge they imparted to me. I also want to acknowledge the entire staff (academic and non-academic) of the Department of Biochemistry, University of Nigeria, Nsukka. The input of Mr. Kingsley Omeje, Mr. Chibuogu Christian, Mr. Iroha Okechukwu., in this research work cannot be over emphasized, God bless you.

My utmost gratitude goes to my parents, Mr and Mrs Sani, Bello for all they have done for me: They have been my anchor spiritually, physically, financially and morally. Thank God for the gift of them. I also want to acknowledge my siblings, Mr. Shamsudeen, Miss Sadiya and Master Barnabas. In addition, I acknowledge my colleagues, Ganyam Martin, Joseph Verlumun Willinson. To all of you including those not mentioned, the I am sincerely grateful.

ABSTRACT

Ethnozoological studies have shown that *Python sebae*, is associated with medicinal uses in Nigeria. This study was designed to determine the chemical composition of the oil extracted from the body fat of *Python sebae* and to evaluate its anti-inflammatory potentials. The Python fat used in the study were obtained from two (2) locations and were tagged: Fat Python A (FPA) and Fat Python D (FPD). The fatty acid profile was identified using GC-MS. The anti-inflammatory activity of the fat was tested using egg albumin-induced paw oedema in rats and membrane stabilization (heat-induced and hypotonicity-induced haemolysis of human red blood cells (HBRCs). The physicochemical properties of FPA and FPD showed peroxide value (2.25 ± 0.21 and 0.90 ± 0.14), acid value (34.08 ± 0.17and 15.82 ± 0.16 mg KOH/g), saponification value (160.88 ± 0.88 and 179.05 ± 0.66 mg KOH/Kg) for FPA and FPD, respectively and the fatty acid composition of the fixed oils of *Python sebae* identified 12 constituents. The percentages of saturated and unsaturated fatty acids were 22.78 % and 44.06 % for FPA, respectively and 17.91 % and 74.76 % for FPD, respectively. The most abundant components being oleic (40.19 %) and linoleic (34.54 %). The *Python sebae* fat samples (FPA and FPD) were found to significantly ($p < 0.05$) reduce the oedema induced by the phlogistic agent in rats in a dose-related manner and the reduction was during the early and later phases of the inflammation. Also, FPA and FPD exhibited membrane-stabilizing property, as it significantly ($p < 0.05$) reduced the levels of haemolysis of HRBCs exposed to heating and hypotonic solution. Fat extracted from the *Python sebae* revealed a potent anti-inflammatory activity due to its fatty acid compositions and can be exploited for pharmaceutical and industrial applications.

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LIST OF ABBREVIATIONS

FPA:	Fat Python A
FPD:	Fat Python D
GC-MS:	Gas Chromatography Mass Spectrometry
EPA:	Eicosapentaenoic Acid
DHA:	Docosahexaenoic Acid
AA:	Arachidonic Acid
LA:	Linoleic Acid
n-3:	Omega-3
n-6:	Omega-6
TFA:	Total fatty acid
TSFAs:	Total saturated fatty acid
TUFAs:	Total unsaturated fatty acid
TPUFAs:	Total polyunsaturated fatty acids
ND:	Not detected
COX:	Cyclooxygenase
min:	Minutes
h:	Hour
HRBCs:	Human Red Blood Cells
RBCs:	Red Blood Cells
NSAIDs:	Non-Steroidal Anti-Inflammatory Drugs
OD:	Optical Density

CHAPTER 1

INTRODUCTION

Inflammation is a complex process, which is frequently associated with pain and involves occurrences such as the increase of vascular permeability, increase of protein denaturation and membrane alteration (Medzhitov, 2010). Inflammation underlies a wide variety of physiological and pathological processes as it has been implicated in the development of many complex diseases and disorders including autoimmune diseases, metabolic syndrome, neurodegenerative diseases, cancers, and cardio- vascular diseases (Leelaprakash and Mohan-Dass, 2011). Steroidal and non-steroidal anti-inflammatory drugs are known to treat inflammation and pain. However, their prolonged use often leads to serious side-effects such as gastrointestinal tract dyspepsia, peptic ulceration, haemorrhage and perforation leading to death in some patients (Griffin, 1998). The use of plant and animal resources constitutes an important therapeutic alternative for many populations (Alves *et al*., 2007), and they have been reported to be used against many illnesses (Salvagnini *et al*., 2008). Although there is no established scientific basis for the use of a particular animal or plant material in the treatment of a defined ailment, alternative medicine practice (traditional medicine) continues to be popular due to effectiveness, prohibitive costs of modern medical facilities and the ease with which the practitioners can be reached (Okafor, 2003). Python fat is one of such products used for therapeutic purposes. It is extracted from the wild Python s, with species including *Python sebae, Python molurus, Python tigris* (McDiarmid *et al*., 1999). It is golden yellow in colour and melts to pale yellow oil on standing. Traditionally in Nigeria (especially in the Southern and Western parts), Python fat has been used in the treatment of rheumatism, boils, keloids and broken bones (Adeola, 1992). Oils of plant and animal origin have been studied for their pharmacological properties including antimicrobial (Nissen *et al*., 2010), anti-inflammatory, antioxidant (Sepahvand *et al*., 2014), and anticancer (Nikolakopoulou *et al*., 2013) properties. These properties are often due to the fatty acids that make up these oils or fats, especially polyunsaturated fatty acids (Black and Rhodes, 2006). This study was therefore, aimed to elucidate the lipid profile and anti-inflammatory properties of Python fat.

1.1 Inflammation

Inflammation is the body's immediate response to damage to its tissues and cells by pathogens, noxious stimuli such as chemicals, or physical injury (Medzhitov, 2008). It is derived from the Latin word, "*inflammatio*" meaning to set on fire. It could also be referred to as part of the

complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants (Ferrero-Miliani *et al*., 2007). Inflammation is a protective response that involves immune cells, blood vessels, and a host of molecular mediators. The mechanisms involved in the inflammatory process are common to all, regardless of the triggering factor. It involves a cascade of biochemical events comprising of the local vascular system and the immune system (Da Silveira e Sá *et al*., 2013). It also involves the production of factors that could cause damage to tissues when not properly regulated. As a consequence, genes that play key roles in effector functions of inflammatory responses are actively repressed under normal conditions and are only induced when cells sense evidence of a triggering factor (Nathan, 2002). The primary functions of inflammation are to eliminate the initial cause of cell injury, clear necrotic cells and tissue damaged from the original insult and inflammatory process, initiate the process of repair and then restore tissue homeostasis (Soehnlein and Lindbom, 2010).

1.1.1 Signs of Inflammation

Inflammatory signs are secondary to one primary pathophysiological event, which is the enhancement of vascular permeability. Inflammation is defined by the presence of five signs, which includes:

- **Redness *(Rubor)***: This is due to local hyperaemia as red-haemoglobin containing erythrocytes accumulate in the course of vascular stasis and arteriolar dilation (Werner, 2009).
- **Heat *(Calor)***: This is also due to local hyperaemia as enhanced blood flow into the inflamed tissue elevates its temperature by heat conduction from the warmer blood to the affected tissue (Stankov, 2012).
- **Swelling *(Tumour)***: This is also known as oedema. It occurs as a result of increased passage of fluid from dilated blood vessels into the surrounding tissues, and to a much lesser extent, from the infiltration of inflammatory cells into the damaged area (Klos *et al*., 2009). In prolonged inflammatory responses, it is caused as a result of deposition of connective tissue. Mediators responsible for oedema include histamine, serotonin, kinins, PAF, PGs and the complement anaphylatoxins, which act on mast cells to release histamine. Localised oedema is the only specific, macroscopic sign of inflammation (Stankov, 2012).
- **Pain *(Dolor)***: Pain is the intensive sensation of a noxious stimulus. It is due to the direct effects of mediators of inflammation, especially bradykinin and some of the PGs due

to their sensitizing effects. It also results from direct mechanical pressure of oedematous fluid on adjacent nerves endings leading to the stretching and distortion of the nerves (Vogel and Berke, 2009).

- **Loss of Function *(Functio laesa)***: This is the impaired function of the affected organ due to oedema and pain, as extensive oedema development can result in increased hydrostatic pressure that can seriously impair organ function. It is also due to the replacement of functional cells with scar tissue (Rather, 1971).

1.1.2 Types of Inflammation

The two major types of inflammation are acute and chronic inflammation. Inflammation can be classified as either *acute* or *chronic*. *Acute inflammation* is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A series of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as *chronic inflammation*, leads to a progressive shift in the type of cells present at the site of inflammation, such as mononuclear cells, and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.

1.1.2.1 Acute Inflammation

Acute inflammation is a short-term response that usually results in healing: leukocytes infiltrate the damaged region, removing the stimulus and repairing the tissue. It involves a coordinated and systemic mobilization response locally of various immune, endocrine and neurological mediators of acute inflammation. In a normal healthy response, it becomes activated, clears the pathogen and begins a repair process and then ceases (Kumar *et al.*, 2004). It is characterized by the five cardinal signs of inflammation (Parakrama and Clive, 2005).

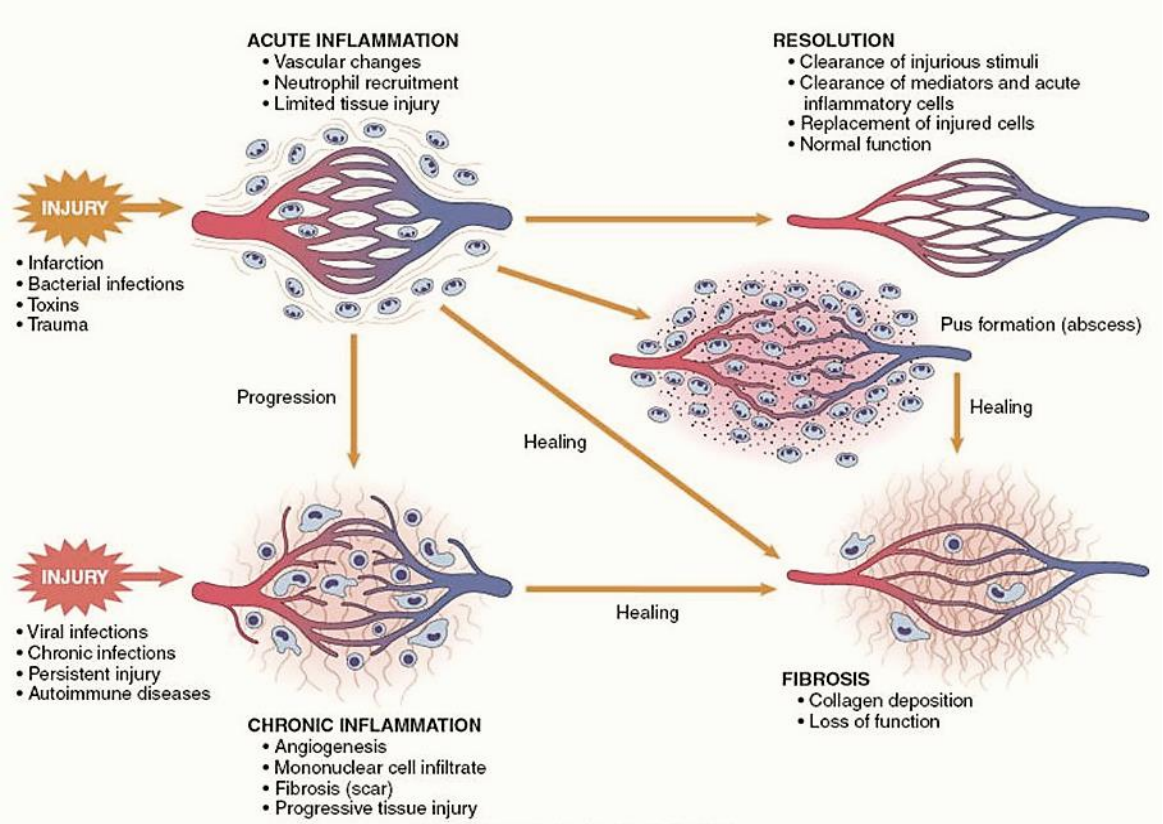

Figure 1: Acute inflammation and Chronic inflammation **(**McGhan and Jaroszewski, 2011**)**

1.1.2.1.1 Process of Acute Inflammation

The process of acute inflammation is initiated by resident immune cells present in the tissue involved, mainly resident macrophages, dendritic cells, histiocytes, Kupffer cells and mast cells. These cells possess surface receptors known as pattern recognition receptors (PRRs), which recognize (i.e., bind) two subclasses of molecules: pathogen associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Bianchi, 2007). PAMPs are compounds that are associated with various pathogens, but which are distinguishable from host molecules. DAMPs are compounds that are associated with host-related injury and cell damage. At the onset of an infection, burn, or other injuries, these cells undergo activation (one of the PRRs recognize a PAMP or DAMP) and release inflammatory mediators responsible for the clinical signs of inflammation (McGhan and Jaroszewski, 2011). Vasodilation and its resulting increased blood flow causes the redness (*rubor*) and increased heat (*calor*). Increased permeability of the blood vessels results in an exudation (leakage) of plasma proteins and fluid into the tissue (oedema), which manifests itself as swelling (*tumor*). Some of the released mediators such as bradykinin increase the sensitivity to pain (hyperalgesia, *dolor*). The mediator molecules also alter the blood vessels to permit the

migration of leukocytes, mainly neutrophils and macrophages, outside of the blood vessels (extravasation) into the tissue. The neutrophils migrate along a chemotactic gradient created by the local cells to reach the site of injury. The loss of function (*functio laesa*) is probably the result of a neurological reflex in response to pain (Osterloh *et al*., 2009). In addition to cell-derived mediators, several acellular biochemical cascade systems consisting of preformed plasma proteins act in parallel to initiate and propagate the inflammatory response. These include the complement system activated by bacteria and the coagulation and fibrinolysis systems activated by necrosis, e.g. a burn or a trauma. Acute inflammation may be regarded as the first line of defence against injury. Acute inflammatory response requires constant stimulation to be sustained. Inflammatory mediators are short-lived and are quickly degraded in the tissue. Hence, acute inflammation begins to cease once the stimulus has been removed (Cotran *et al.*, 1998).

1.1.2.1.2 Responses in Acute Inflammation

Acute inflammation involves vascular and cellular responses. The vascular responses are vasodilatation and increased vascular permeability, while the cellular component involves the emigration of leukocytes from the vascular to the extravascular compartment.

1.1.2.1.2.1 Vasodilatation

This involves changes in the vascular calibre leading to increased blood flow to tissues. It is caused by arteriolar dilation which sometimes occurs after transient vasoconstriction, resulting in heat and redness. It is also due to the opening of new capillaries. It occurs primarily as a result of histamine and NO acting on the vascular smooth muscle (Ferrero-Miliani *et al*., 2007).

1.1.2.1.2.2 Increased Vascular Permeability

This involves the contraction of endothelial cells, leading to the escape of plasma protein-rich fluid known as exudates from the vascular compartment into the extravascular space. This process is called exudation. It leads to increased extravascular osmotic pressure leading to oedema (Stankov, 2012). This vascular leakage of exudates can be chemically mediated or injury induced. Chemical mediation involves the retraction of endothelial cells by mediators of acute inflammation such as histamine, bradykinins, LTs and PGs released by mast cells and tissue resident macrophages. These mediators bind to their receptors on endothelial cells leading to vasodilatation, contraction of endothelial cells and widening of intercellular

junctions (Da Silveira e Sá *et al*., 2013). Injury induced vascular leakage is an abnormal leakage caused by toxins and physical agents as they may cause necrosis of the vascular endothelium.

1.1.2.1.2.3 Neutrophil Extravasation

This involves the movement of leukocytes from the circulation to the site of inflammation. All granulocytes and monocytes respond to chemotactic factors and move along a concentration gradient (Cicchetti *et al*., 2002). Like the leakage of fluids, the delivery of leukocytes to inflammatory sites is assisted by the release of vasodilators, such as histamine and PGs. Neutrophil extravasation involves, margination, tethering and rolling, stable arrest and adhesion, diapedesis and migration in interstitial tissues (Kolaczkowska and Kubes, 2013; Zarbock *et al*., 2011).

1.1.2.1.3 Resolution of the Acute Inflammatory Response

Resolution of the acute inflammatory response is an active biochemical process that is important to protect the host from overt tissue damage and amplification of the acute inflammatory response towards chronicity (Serhan, 2010). It is also critical for successful repair after tissue injury. After the first few hours of inflammation, a coordinated program of resolution is set into motion by tissue-resident and recruited macrophages. The activation of macrophages during the inflammatory process must be tightly regulated in order to avoid unrestrained inflammatory process through inappropriate release of cytokines such as TNF-α, IFN-γ and IL-1 (Sakic *et al*., 2011). Macrophages therefore liberate the anti-inflammatory cytokine, TGF-β which inhibits the production of TNF-α. Resolution of inflammation also involves upregulating anti-inflammatory molecules, like IL-1 receptor antagonist or soluble TNF-α receptor (Eming *et al*., 2007). Cytokines such as interleukin-4 (IL- 4), interleukin-10 (IL-10) and interleukin-12 (IL-12) in very low concentrations are inducible, and act as powerful anti-inflammatory mediators by stabilizing Iκβ, which blocks NF-κβ activation (Shaikh, 2011). These regulatory cytokines therefore greatly diminish the production of pro inflammatory mediators, downregulate the expression of chemokines and thus reduce the numbers of leukocytes accumulating in tissues (Iwalewa *et al*., 2007). Lipoxins (LXs), produced from arachidonic acid through the 5-LOX pathway are anti-inflammatory. During the onset of acute inflammation, tissue-resident and recruited macrophages in the inflamed tissue, including PMNs produce pro-inflammatory lipid mediators (e.g. PGE2 and LTB4). With time, a lipid mediator switch occurs, and these cells begin to produce lipoxin A4 (LXA4) which is a pro resolving mediator (Serhan *et al*., 2008; Serhan, 2010). Lipoxin A4 blocks neutrophil

recruitment, initiates non-phlogistic recruitment of monocytes and the phagocytosis of apoptotic neutrophils by macrophages (Kolaczkowska and Kubes, 2013). Closely related to LXA4 are lipids mediators synthesized from omega-3 polyunsaturated fatty acids (PUFAs) that stop neutrophil migration. They include resolvins, protectins and maresins. Resolvins and protectins are produced by neutrophils while maresins are produced by macrophages (Serhan, 2010). Leukocytes are cleared in tissues via lymphatics or by apoptosis. This is because apoptotic PMNs releases signals that attract monocytes which phagocytose the PMNs, cellular debris and red blood cells (RBCs) in the extravascular compartment (Soehnlein and Lindbom, 2010). Apoptosis of neutrophils is promoted by resolvins and protectins. Some of the neutrophils do not die in the tissue but are reverse-transmigrated (re-enter the vasculature) because they are more resistant to apoptosis compared with other extravasated neutrophils. Reverse-transmigrating neutrophils could be a way of preserving neutrophils when they are not needed to fight infection (Kolaczkowska and Kubes, 2013). Increased vascular permeability is reversed due to closure of the open endothelial cell-cell junctions, ceasing the escape of exudates and leukocyte emigration from the blood compartment (Serhan and Savil, 2005). Exudates are also cleared by the uptake of the fluids together with inflammatory cells into the draining lymphatics (Ward, 2010) or they pinocytose into macrophages. They could also be cleared through the hydrolysis of kinins by kininases, thereby preventing their vasopermeability effect. Thrombosis within vessels can be cleared by the activation of the fibrinolytic system.

1.1.2.2. Chronic Inflammation

In inflammation, if the injurious agent persists then chronic inflammation will ensue (Eming *et al*., 2007). This process, marked by inflammation lasting many days, months or even years, may lead to the formation of a chronic wound. Chronic inflammation is characterised by the dominating presence of macrophages in the injured tissue. These cells are powerful defensive agents of the body, but the toxins they release (including reactive oxygen species) are injurious to the organism's own tissues as well as invading agents. As a consequence, chronic inflammation is almost always accompanied by tissue destruction (Ferrero-Miliani *et al*., 2007).

Chronic inflammation is associated with many human diseases including, allergy, cancer, arthritis, diabetes, cardiovascular, neurological and autoimmune diseases (Glass *et al*., 2010). It is often due to infections with higher order organisms such as mycobacteria, fungi and metazoan parasites which are resistant to killing and clearing by the body. It is also due to

immune complexes in autoimmune diseases and prolonged exposure to toxins. It is associated with the infiltration of mononuclear cells such as lymphocytes, macrophages and plasma cells (Vishal *et al*., 2014) due to the persistent stimulus. Plasma cells produce antigen-specific antibodies in a bid to clear the persistent antigen. Apart from recruited macrophages, there is also local proliferation of macrophages at the inflammatory site leading to their accumulation. These macrophages are activated by endotoxin, ECM proteins and chemical mediators. Activated macrophages present processed antigen fragments on their surface and interact with T-lymphocytes leading to the activation of the lymphocytes (Dalgleish and O'Byrne, 2002). Monokines from activated macrophages also activate lymphocytes. Activated lymphocytes releases IFN-γ which activates additional macrophages, leading to persistence of the inflammatory response.

Chronic inflammation is also associated with attempts at healing by tissue regeneration or with fibrosis (Kumar *et al*., 2013). Fibrosis involves fibrous connective tissue replacement of damaged tissue when regeneration cannot be accomplished. It involves angiogenesis, migration and proliferation of fibroblasts, and deposition of ECM especially collagen. This is followed by the appearance of granulation tissue and the subsequent formation of scar tissue. Activated macrophages lead to repair due to the production of growth factors (PDGF, bFGF, and TGF β), fibrogenic cytokines, angiogenesis factors and remodelling collagenases (Eming *et al*., 2007).

1.1.3 Inflammatory Mediators

Inflammatory mediators are substances triggered by inflammatory stimuli. They are derived from inflammatory cells, or released as plasma proteins (Vishal *et al*., 2014). Most mediators bind to specific target receptors on the cells to elicit their effects. Exceptions are lysosomal enzymes that have a direct enzymatic effect and reactive oxygen species (ROS) that have a direct toxic effect. Mediators can stimulate target cells to release secondary mediators.

1.1.3.1 Cell Derived Mediators

These mediators are derived from stimulated inflammatory cells. They are either pre-formed or stored in the granules of these cells and, may be synthesized and secreted when needed (Stone *et al*., 2010).

1.1.3.1.1 Vasoactive Amines

Vasoactive amines (histamine and serotonin) are produced in an all-or-none manner when mast cells and platelets degranulate. They have complex effects on the vasculature, causing increased vascular permeability and vasodilation, or vasoconstriction, depending on the context. The immediate consequences of their release by mast cells can be highly detrimental in sensitized organisms, resulting in vascular and respiratory collapse during anaphylactic shock (Medzhitov, 2008).

1.1.3.1.2 Cytokines

These are soluble proteins of low molecular weight that modulate the differentiation, proliferation, and function of immune cells, and coordinate inflammatory responses (Ashley *et al.,* 2012). They are secreted primarily by activated tissue macrophages, lymphocytes and endothelial cells. Their main effects are to induce the acute phase reaction and to activate vascular endothelium, leukocytes, platelets and fibroblasts, thus, initiating the cascade of vascular, cellular and humoural events which together comprise the inflammatory response (Tan *et al*., 1999). Several cytokines play essential roles in orchestrating the inflammatory process, especially interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α) which are monokines, produced by monocytes and macrophages (Simopoulos, 2002). Lymphokines such as interferon-gamma (IFN-γ) are produced by lymphocytes and they play an important role in the first line of defence against viral infections.

1.1.3.1.3 Chemokines

Chemokines are produced by many cell types in response to inducers of inflammation. They control leukocyte extravasation and chemotaxis towards the affected tissues (Kolaczkowska and Kubes, 2013). Chemokines are inducible molecules. They include, C-X-C (or α) chemokines and C-C (or β) chemokines. C-X-C chemokines are so-called because they have an intervening amino acid between the first two of the four conserved cysteine residues at their amino terminal. C-C chemokines on the other hand, do not have an intervening amino acid between their first two amino-terminal cysteine residues (Tan *et al*., 1999).

1.1.3.1.4 Nitric Oxide

Nitric oxide is a soluble gas generated by macrophages, endothelial cells and some neurons. It is also generated through the action of nitric oxide synthase (NOS), as it oxidizes the terminal guanidine nitrogen atom of L-arginine (Kumar *et al*., 2013). Nitric oxide is toxic to bacteria

and directly inhibits viral replication. It also combines with ROS to yield peroxynitrate radicals which have potent antimicrobial activity (Chukwuka *et al*., 2011). Additionally, it is a potent vasodilator, increases vascular permeability, stimulates relaxation of smooth muscle cells within the vessel walls and inhibits platelet aggregation.

1.1.3.1.5 Prostaglandins

Are derived from phospholipids, such as phosphatidylcholine, that are present in the inner leaflet of cellular membranes. After activation by intracellular $Ca2^{+}$ ions, cytosolic phospholipase A2 generates arachidonic acid and lysophosphatidic acid, the precursors of the two classes of lipid mediator listed above, from phosphatidylcholine. Arachidonic acid is metabolized to form eicosanoids either by cyclooxygenases (COX1 and COX2), which generate prostaglandins and thromboxanes, or by lipoxygenases, which generate leukotrienes and lipoxins (Kumar *et al.*, 2003). The prostaglandins PGE_2 and PGI_2, in turn, cause vasodilation, and PGE_2 is also hyperalgesia and a potent inducer of fever and pain (Moreno, 2017).

1.1.3.1.6 Thromboxane A2 and Prostacyclin

Thromboxane's and Prostacyclin's are also produced through the action of COX on arachidonic acid. Thromboxane A2 (TxA2) is a potent platelet-aggregating agent and a vasoconstrictor. It is unstable and is rapidly converted to inactive Thromboxane B2 (TXB2). Prostacyclin or prostaglandin I2 (PGI2) on the other hand is a vasodilator and a potent inhibitor of platelet aggregation (Pilotto *et al*., 2010). It increases blood flow as well as blood vessel permeability by assisting in the release of NO from the endothelium (Vishal *et al*., 2014).

1.1.3.1.7 Leukotrienes

Leukotrienes (LTs) like prostanoids, are released from excess arachidonic acid due to the activity of 5-lipoxygenase (5-LOX). The lipoxygenase (LOX) pathway is a parallel inflammatory pathway to the COX pathway (George *et al*., 2014). Leukotrienes are released from leukocytes and mast cells and are generally pro-inflammatory. Leukotriene B4 (LTB4) is a potent chemotactic agent for neutrophils. It also promotes their adhesion to vascular endothelial cells, their trans-endothelial migration and stimulates the synthesis of pro inflammatory cytokines from macrophages and lymphocytes (Dalgleish and O'Byrne, 2002; Medzhitov, 2008). Leukotriene B4 also promotes degranulation and the generation of ROS. Cysteinyl-containing leukotriene C4 (LTC4), leukotriene D4 (LTD4) and leukotriene E4

(LTE4) cause intense vasoconstriction, bronchospasm and increased vascular permeability in venules.

1.1.3.1.8 Platelet-activating Factor

These are a class of lipid mediator, platelet-activating factors, are generated by the acetylation of lysophosphatidic acid and activate several processes that occur during the inflammatory response, including recruitment of leukocytes, vasodilation and vasoconstriction, increased vascular permeability and platelet activation (Moreno, 2017).

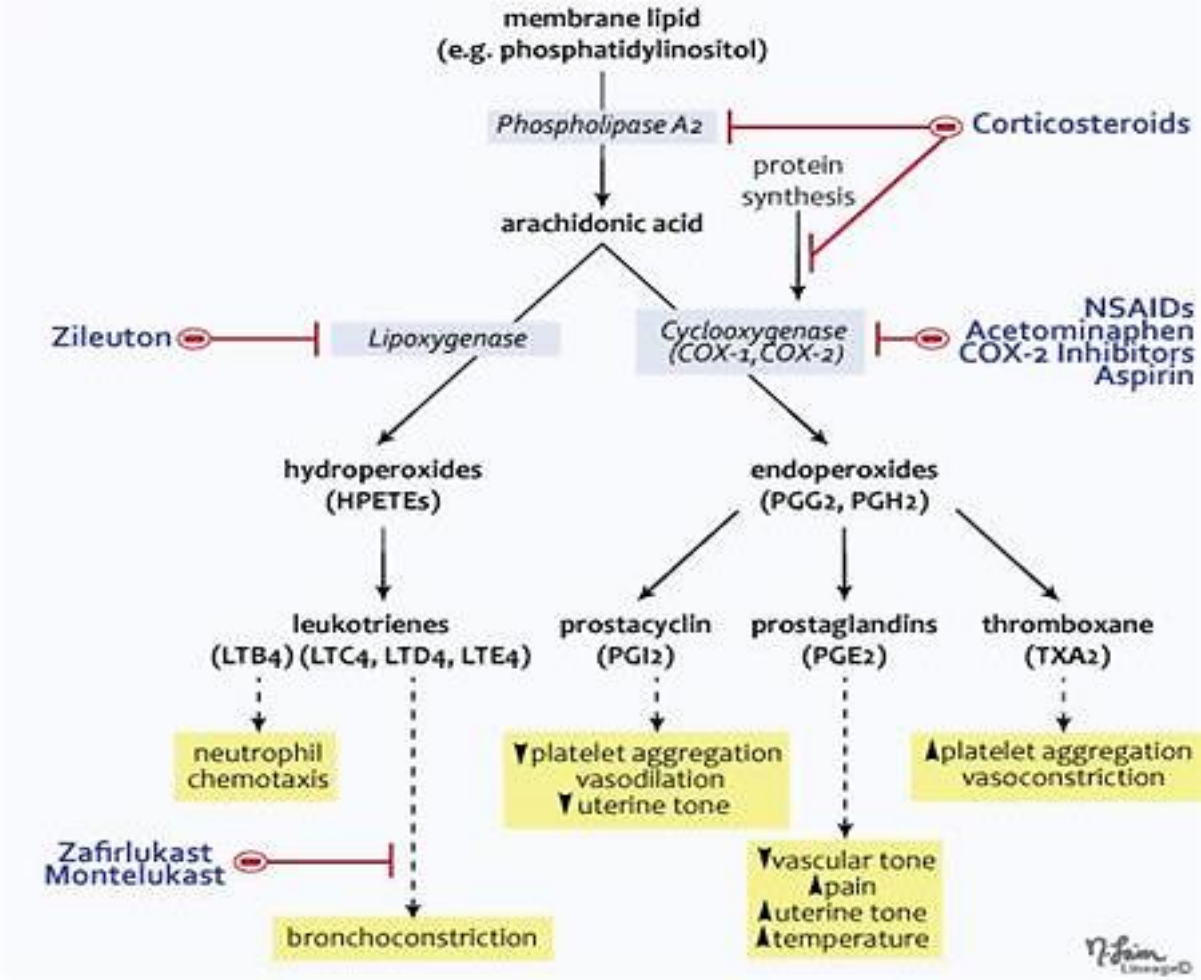

Figure 2: Generation of arachidonic acid metabolites and their roles in inflammation (Tikhonovich *et al*., 2018)

1.1.3.2 Plasma Derived Mediators

These are proteins that circulate in the plasma as inactive precursors. They undergo proteolytic cleavage to become active. Mediator-producing systems in plasma include: complement, clotting, fibrinolytic and kinin systems.

1.1.3.2.1 Complement System

The complement system is an important part of the innate immune system conferring protection against invading infectious agents, such as, bacteria, viruses and protozoa. Its role is to generate biologically active products from the pathways of complement activation which include: classical, lectin, alternative, properdin and thrombin pathways (Neher *et al*., 2011). Peptides generated by complement activation play a critical role in the elimination of invading pathogens. The complement fragments C3a, C4a and C5a (also known as anaphylatoxins) are produced by several pathways of complement activation. C5a (and to a lesser extent C3a and C4a) promote granulocyte and monocyte recruitment and induce mast-cell degranulation, thereby affecting the vasculature. They also induce the expression of adhesion molecules on endothelial cells and cause smooth-muscle contraction (Klos *et al*., 2009).

1.1.3.2.2 Fibrinolytic System

The fibrinolytic system acts in opposition to the coagulation system, to counterbalance clotting. It is activated by urinary plasminogen activator (uPA) and tissue plasminogen activator (tPA) which converts plasminogen to plasmin. Plasmin directly interacts with fibrin to bring about the breakdown of fibrin clots as they are formed within the intravascular compartment (Ward, 2010).

1.1.3.2.3 Kinin System

Kinins are vasoactive peptides generated through the kinin-generating cascade. The most important product of this cascade is the plasma protein, bradykinin. Prekallikrein is converted to the protease, kallikrein by factor XIIa from the clotting cascade. Kallikrein interacts with high molecular- weight kininogen (HMWK) to bring about the hydrolysis and release of bradykinin. Bradykinin is a powerful vasopermeability agent. It also causes pain, vasodilatation and oedema, all contributing to inflammation (Fukaya *et al.*, 2014).

1.1.4 Cellular Components of Inflammation

The principal cellular components of the inflammatory response involve leukocytes. They originate from pluripotent hemopoietic stem cells and are found in tissues during acute inflammatory process and in the superficial aspects of a lesion during sub-acute or chronic inflammation (Anosike *et al*., 2012). Leukocytes also release inflammatory mediators that develop and maintain the inflammatory response. They include the granulocytes and

agranulocytes. Other cells involved in inflammation are platelets, vascular endothelial cells, fibroblasts and plasma cells.

1.1.4.1 Granulocytes

These are a type of white blood cells (WBCs) characterised by the presence of granules within their cytoplasm. Granulocytes comprise of neutrophils, eosinophils, basophils and mast cells, distinguished by the colour their granules stain when treated with a compound dye.

1.1.4.2 Neutrophils

Neutrophil granulocytes (also known as neutrophils) are the most abundant (40 % to 75 %) type of white blood cells in most mammals and form an essential part of the innate immune system. Functionality varies in different animals (Ermert *et al*., 2013). They are formed from stem cells in the bone marrow. They are short-lived and highly motile. Neutrophils may be subdivided into segmented neutrophils and banded neutrophils (or bands). They form part of the polymorphonuclear cell family (PMNs) together with basophils and eosinophils (Nathan, 2006). During the beginning (acute) phase of inflammation, particularly as a result of bacterial infection, environmental exposure (Jacobs *et al.*, 2010) and some cancers (Waugh and Wilson, 2008), neutrophils are one of the first-responders of inflammatory cells to migrate towards the site of inflammation. They migrate through the blood vessels, then through interstitial tissue, following chemical signals such as Interleukin-8 (IL-8), C5a, fMLP and Leukotriene B4 in a process called chemotaxis. They are the predominant cells in pus, accounting for its whitish/yellowish appearance. Neutrophils are recruited to the site of injury within minutes following trauma, and are the hallmark of acute inflammation (Cohen and Burns, 2002). However, due to some pathogens being indigestible, they can be less useful alone (Zucker-Frankli*et al*., 1988). Also, neutrophil antimicrobial products can also damage host tissues, their short life limits damage to the host during inflammation. Neutrophils undergo a process called chemotaxis, which allows them to migrate toward sites of infection or inflammation. Cell surface receptors allow neutrophils to detect chemical gradients of molecules such as interleukin-8 (IL-8), interferon gamma (IFN gamma), C3a, C5a, and Leukotriene B4, which these cells use to direct the path of their migration. Being highly mobile, neutrophils quickly congregate at a focus of infection, attracted by cytokines expressed by activated endothelium, mast cells, and macrophages. Neutrophils express and release cytokines, which in turn amplify inflammatory reactions by several other cell types (Ear and McDonald, 2008).

1.1.4.3 Eosinophils

Eosinophil granulocytes are white blood cells and one of the immune system components responsible for combating multicellular parasites and certain infections in vertebrates. Along with mast cells, they also control mechanisms associated with allergy and asthma. They are granulocytes that develop during haematopoiesis in the bone marrow before migrating into blood. These cells are eosinophilic or 'acid-loving' as shown by their affinity to coal tar dyes: Normally transparent, it is this affinity that causes them to appear brick-red after staining with eosin, a red dye, using the Romanowsky method. The staining is concentrated in small granules within the cellular cytoplasm, which contain many chemical mediators, such as histamines and proteins such as eosinophil peroxidase, ribonuclease (RNase), deoxyribonucleases (DNase), lipase, plasminogen, and major basic protein. These mediators are released by a process called degranulation following activation of the eosinophil, and are toxic to both parasite and host tissues. In normal individuals, eosinophils make up about 1 - 6 % of white blood cells, and are about 12-17 micrometres in size (Young *et al*., 2006). They are found in the medulla and the junction between the cortex and medulla of the thymus, and, in the lower gastrointestinal tract, ovary, uterus, spleen, and lymph nodes, but not in the lung, skin, oesophagus, or some other internal organs under normal conditions. The presence of eosinophils in these latter organs is associated with disease. These leukocytes are critically involved in the initiation and maintenance of inflammation. These cells must be able to get to the site of injury from their usual location in the blood, therefore mechanisms exist to recruit and direct leukocytes to the appropriate place (Young *et al*., 2006).

1.1.4.4 Mast Cells

A mast cell is a cell that is derived from the myeloid stem cell. It contains many granules rich in histamine and heparin. They are known to be involved in wound healing, angiogenesis, defence against pathogens and blood-brain barrier function (da Silva *et al*, 2014). They are also known to play roles in allergy and anaphylaxis. Mast cells look very much like basophils but are different cells as they develop from different hematopoietic stem cells. Unlike basophils, which leave the bone marrow in the mature form, mast cells circulate in an immature form and only mature when they get to their target tissue site (Prussin and Metcalfe, 2003). They are seen in tissues surrounding blood vessels and nerves and also in the skin, mucosa of the lungs, digestive tract, conjunctiva, nose and mouth (Prussin and Metcalfe, 2003). Mast cells play important roles in the inflammatory process. They release histamine which dilates post-

capillary venules, activates the endothelium, increases blood vessel permeability leading to local oedema and also depolarizes the nerve endings leading to pain or itching (Polyzoidis *et al*, 2015).

1.1.4.5 Basophils

Basophils are the least common granulocytes with granules rich in mediators like histamine, serotonin and leukotrienes (Stock and Hoffman, 2000). Together with mast cells, they do not die after releasing their granules, unlike PMNs. They emigrate into extravascular tissues at inflammatory sites, and are predominant when inflammation is initiated by immediate allergic reactions or parasites (Fehervari *et al*., 2005). Basophils, like mast cells produce LTC4 and its peptidolytic products LTD4 and LTE4 after activation. Unlike mast cells, they do not produce PGD2.

1.1.4.6 Agranulocytes

Agranulocytes are also referred to as mononuclear leukocytes. These leukocytes are characterised by the apparent absence of granules in their cytoplasm. They include lymphocytes, monocytes and macrophages (Stone *et al*., 2010).

1.1.4.7 Lymphocytes

Lymphocytes represent about a third of the WBCs in the peripheral blood (Stock and Hoffman, 2000). Two thirds are T cells while the remainder are B cells. T cells participate in cell-mediated immune responses. They influence the healing response by direct cell-cell interactions with resident and non-resident cells at the wound site (Eming *et al*., 2007). They recognise pathogens only after antigen processing cells (APCs) such as a dendritic cell or a macrophage digests it into antigen fragments and presents the fragments on proteins known as major histocompatibility complex (MHC) molecule on the surface of the APC. Two main types of T cells are cytotoxic T cell and the helper T cell. B cells are programmed to produce antibodies in the humoural immune response. Lymphocytes are prominent cells in chronic inflammation and viral infections.

1.1.4.8 Monocytes

Monocytes are a type of white blood cells (leukocytes). They are the largest of all leukocytes. They are part of the innate immune system of vertebrates including all mammals (humans included), birds, reptiles, and fish. They are amoeboid in shape, having agranulated cytoplasm.

Monocytes have unilobar nuclei, which makes them one of the types of mononuclear leukocytes (containing azurophil granules). The archetypal idea of the nucleus is that it is bean-shaped or kidney-shaped, although the most important distinction is that it is not deeply furcated into lobes, as occurs in polymorphonuclear leukocytes. Monocytes constitute 2 % to 10 % of all leukocytes in the human body. They play multiple roles in immune function. Such roles include replenishing resident macrophages under normal states; and in response to inflammation signals, monocytes can move quickly (approximately 8 – 12 hours) to sites of infection in the tissues and divide/differentiate into macrophages and dendritic cells to elicit an immune response. Half of them are stored in the spleen (Swirski *et al*., 2009).

1.1.4.9 Macrophages

Macrophages are white blood cells that engulf and digest cellular debris, foreign substances, microbes, cancer cells, and anything else that does not have the types of proteins specific to the surface of healthy body cells on its surface in a process called phagocytosis. They are found in essentially all tissues (Ovchinnikov, 2008) where they patrol for potential pathogens by amoeboid movement. They play a critical role in non-specific defence (innate immunity), and also help initiate specific defence mechanisms (adaptive immunity) by recruiting other immune cells such as lymphocytes. In humans, dysfunctional macrophages cause severe diseases such as chronic granulomatous disease that result in frequent infections. Beyond increasing inflammation and stimulating the immune system, macrophages also play an important anti-inflammatory role and can decrease immune reactions through the release of cytokines. Macrophages that encourage inflammation are called M1 macrophages, whereas those that decrease inflammation and encourage tissue repair are called M2 macrophages (Mills, 2012). This difference is reflected in their metabolism, M1 macrophages have the unique ability to metabolize arginine to the "killer" molecule nitric oxide, whereas M2 macrophages have the unique ability to metabolize arginine to the "repair" molecule ornithine. Macrophages are essential for wound healing. They replace Polymorphonuclear neutrophils as the predominant cells in the wound by two days after injury. Attracted to the wound site by growth factors released by platelets and other cells, monocytes from the bloodstream enter the area through blood vessel walls. Numbers of monocytes in the wound peak one to one and a half days after the injury occurs. Once they are in the wound site, monocytes mature into macrophages. Macrophages also secrete a number of factors such as growth factors and other cytokines, especially during the third and fourth post-wounding days. These factors attract cells involved

in the proliferation stage of healing to the area. Macrophages may also restrain the contraction phase (Newton *et al*., 2004).

1.1.4.10 Vascular Endothelial Cells

Vascular endothelial cells line the interior surface of blood vessels, forming a vast surface area of interface between the components of the circulation including leukocytes, platelets and plasma proteins in the lumen and all of the body's tissues (Tan *et al*., 1999). As such, un-activated vascular endothelium regulates the traffic of water, solutes, bioactive proteins, lipids and peripheral blood leukocytes between the circulation and interstitial tissues. Un-activated endothelium is also antithrombotic as it forms an insulating barrier between thrombogenic subendothelial matrix components such as von Willebrand factor (vWF) and platelets in the circulation (Kee *et al*., 2008). It also secretes PGI2 and NO which inhibit platelet aggregation as well as tPA which inhibit coagulation. Vascular endothelium becomes locally activated at the sites of injury and inflammation either by the injurious stimulus itself, or by cytokines generated in response to the injurious stimulus (Simopoulos, 2002). Vascular endothelium is also activated by histamine and thrombin, as they stimulate the redistribution of P-selectin and vWF from endothelial granules, known as Weibel- Palade bodies to the cell surface. Activated vascular endothelium is thus prothrombotic in response to vascular injury, as vWF facilitates platelet aggregation at sites of injury. It is also pro-inflammatory as P-selectin is a cell adhesion molecule (CAM) important in the adhesion of leukocytes to endothelial cells. Vascular endothelium also expresses and secretes PAF and IL-8 which may activate adherent leukocytes and direct their migration across the endothelium and within the inflamed tissues (Ley *et al*., 2007).

1.1.4.11 Platelets

Platelets, also known as thrombocytes are a component of blood, whose function along with the coagulation factors is to contribute to haemostasis through clumping and clotting of blood vessel injuries (Tan *et al*., 1999). They are crucial to the body's normal inflammatory response because of their ability to form haemostatic platelet plugs very rapidly at sites of vascular injury (Sobotková *et al*., 2009). Vascular injury leads to damage to the endothelial lining of the blood vessel in which thrombogenic sub-endothelial matrix components such as collagen, fibronectin and vWF are exposed to the blood (Tan *et al*., 1999). Platelets adhere to these sub-endothelial matrix proteins and become activated. Activated platelets lead to the formation of TXA2 which is a potent vasoconstrictor and stimulus of platelet aggregation. Thromboxanes are known to

induce blood vessel constriction and platelet aggregation by increasing intracellular calcium ion (Ca2+), which promote fusion of dense and alpha platelet granules with the platelet membrane, thus, releasing their contents. Adenosine diphosphate (ADP) which also mediates platelet aggregation is released. Activated platelets also trigger the coagulation cascade leading to the formation of thrombin. Thrombin and other platelet agonists such as, ADP, PAF and adrenaline bind to specific receptors on platelet membrane and stimulate aggregation in which more platelets are recruited and begin to adhere to each other. This forms haemostatic plug onto which fibrin may be deposited, thus, stabilizing the clot formed. The degranulation of platelets also releases P-selectin and histamine. Histamine increases vascular permeability while P-selectin facilitates leukocyte migration from blood to tissues (Zarbock *et al*., 2006).

1.1.5 Anti-inflammatory Agents

Anti-inflammatory agents refer to substances that are used in the treatment of inflammation or swelling (Dinarello, 2010), they make up about half of analgesics, remedying pain. These include: corticosteroids, non-steroidal anti-inflammatory drugs (NASIDs), disease-modifying antirheumatic drugs (DMARDs), anti-leukotrienes, Immune selective anti-inflammatory Derivatives (ImSAIDs), anti-cytokine agents and signal transduction inhibitors (Rainsford, 2007). They are also Grouped into two (2). The steroidal (corticosteroids) and the non-steroidal anti-inflammatory drugs (Tikhonovich *et al*., 2018).

1.1.5.1 Corticosteroids

Corticosteroids are a class of steroid hormones that are produced in the adrenal cortex of vertebrates, as well as the synthetic analogues of these hormones. Two main classes of corticosteroids, glucocorticoids and mineralocorticoids, are involved in a wide range of physiologic processes, including stress response, immune response, and regulation of inflammation, carbohydrate metabolism, protein catabolism, blood electrolyte levels, and behaviour (Nussey and Whitehead, 2001) Anti-inflammatory effects are mediated by blocking the action of inflammatory mediators (transrepression) and inducing anti-inflammatory mediators (transactivation) (Liu *et al.*, 2013). Vasoconstrictive effects are mediated by inhibiting the action of inflammatory mediators such as histidine Topical formulations are also available for the skin, eyes (uveitis), lungs (asthma), nose (rhinitis), and bowels. They inhibit PLA2 activity which in turn reduces arachidonic acid release. This they do indirectly by causing the release of the inhibitory protein, lipocortin which binds to the cell membrane, preventing PLA2 from coming in contact with arachidonic acid. By inhibiting PLA2 activity,

corticosteroids neutralize the two main pathways of the arachidonic acid cascade (COX and LOX pathways) thereby preventing the formation of PGs, TXs and LTs which are inflammatory mediators.

An example of corticosteroid is Prednisone. Prednisone is the active metabolite of prednisone (Czaja, 2012). It is an anti-inflammatory and a potent immunosuppressive agent. Corticosteroids principally exert their effect by binding to the specific glucocorticoid receptor (GR) within the cytosol causing the dissociation of chaperone proteins from the GR. This complex then translocates to the nucleus where it is recognized by and binds to specific DNA sequences known as glucocorticoid response elements (GREs). The major effect of binding to DNA is the suppression of transcription by opposing the activation of the transcription factors AP-1 and NF- κβ (Dinarello, 2010; Czaja, 2012). Prednisone specifically has an intracytoplasmic effect on the activity of NF-κβ by stimulating the production of Iκβ, thereby preventing the expression of genes encoding nearly all pro-inflammatory cytokines, thus suppressing pro-inflammatory and immune-stimulatory actions. The suppression of pro inflammatory cytokines production in turn reduces the expression of genes encoding COX-2, iNOS, and ICAM-1.

Some side-effects associated with the use of corticosteroids which are often severe or irreversible include, Neuropsychiatric (steroid psychosis, anxiety and depression), hypertension, hyperglycaemia, insulin resistance, steroid-induced osteoporosis, bone damage, skin thinning, immunosuppression and propensity to infection, gastrointestinal ulcers and bleeding, and topical steroid addiction (Martinek *et al.*, 2010; Fukaya *et al.*, 2014).

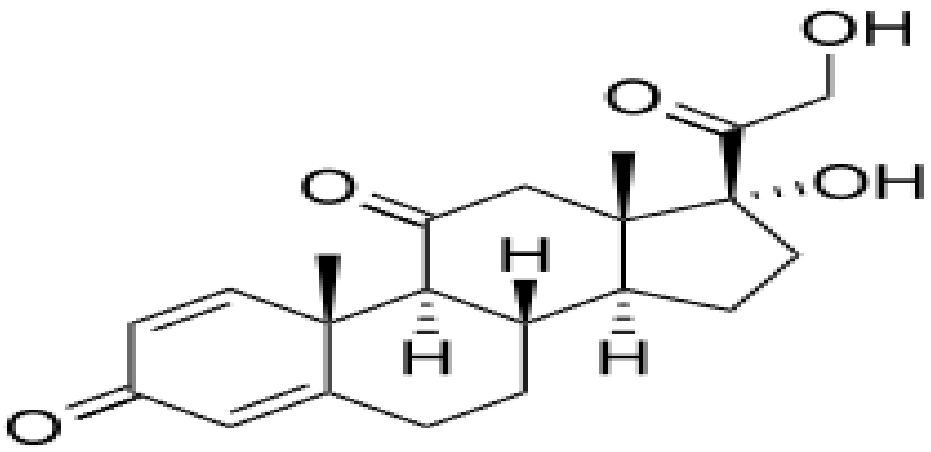

Figure 3: Structure of Prednisone (Buttgereit and Gibofsky, 2013)

1.1.5.2 Non-Steroidal Anti-inflammatory Drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a drug class that reduce pain, decrease fever, prevent blood clots and, in higher doses, decrease inflammation (Bally *et al.*, 2017). The term nonsteroidal distinguishes these drugs from steroids, which while having a similar eicosanoid-depressing, anti-inflammatory action, have a broad range of other effects. First used in 1960, the term served to distance these medications from steroids (Buer, 2014). NSAIDs work by inhibiting the activity of cyclooxygenase enzymes (COX-1 and/or COX-2). In cells, these enzymes are involved in the synthesis of key biological mediators, namely prostaglandins which are involved in inflammation, and thromboxane's which are involved in blood clotting. There are two types of NSAID available non-selective and COX-2 selective (Brater *et al.,* 2001). Most NSAIDs are non-selective, and inhibit the activity of both COX-1 and COX-2. These NSAIDs, while reducing inflammation, also thin the blood (especially aspirin) and increase the risk of gastrointestinal ulcers/bleeds. COX- 2 selective inhibitors have less gastrointestinal side effects, but promote thrombosis and substantially increase the risk of heart attack. As a result, COX-2 selective inhibitors are generally contraindicated due to the high risk of undiagnosed vascular disease. These differential effects are due to the different roles and tissue localisations of each COX isoenzyme. The most prominent NSAIDs are aspirin, ibuprofen and naproxen, all available over the counter in most countries (Warden, 2010). Paracetamol (acetaminophen) is generally not considered an NSAID because it has only little anti-inflammatory activity. It treats pain mainly by blocking COX-2 mostly in the central nervous system, but not much in the rest of the body (Hinz *et al.,* 2008). The widespread use of NSAIDs has meant that the adverse effects of these drugs have become increasingly common. Use of NSAIDs increases risk of a range of gastrointestinal (GI) problems, kidney disease and adverse cardiovascular events (Rostom *et al.,* 2002) NSAIDs, aside from aspirin, increase the risk of myocardial infraction and stroke, erectile dysfunction (Trelle *et al.,* 2011; Bally *et al.,* 2017).

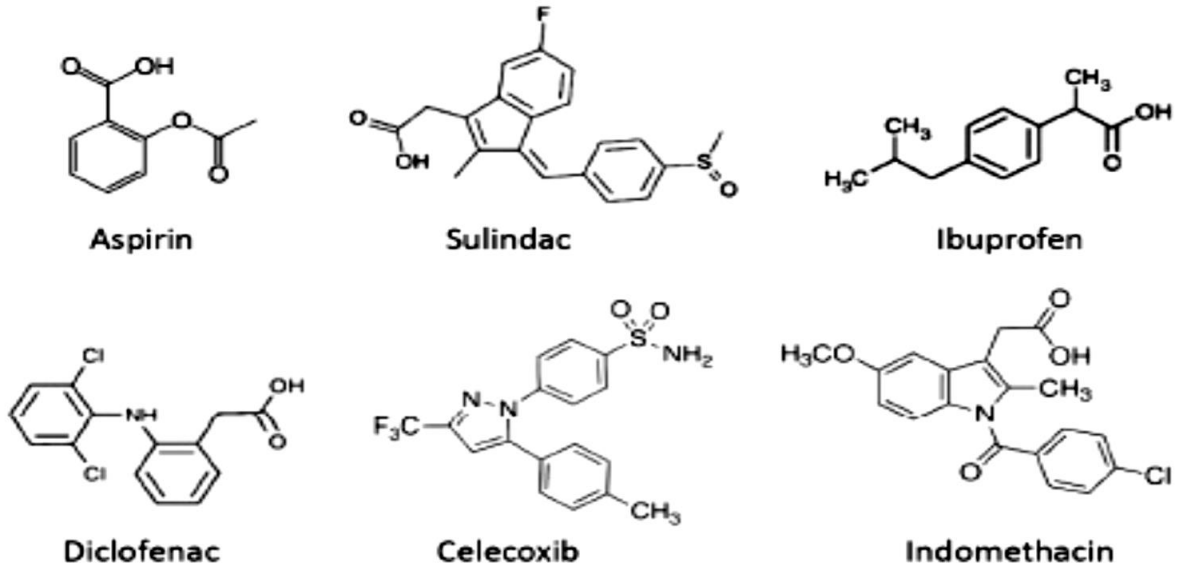

Figure 4: Chemical structures of common non-steroidal anti-inflammatory drugs (Trelle *et al.*, 2011)

1.2 *Python Sebea*

The African rock Python (Python sebae*)* is a large, nonvenomous snake of sub-Saharan Africa. It is one of 11 living species in the genus *Python.* It is found in Central and Western Africa and in Southern Africa. Africa's largest snake and one of the six largest snake species in the world (along with the green anaconda, reticulated Python, Burmese Python, Indian Python, and amethystine Python), specimens may approach or exceed 6 m (20 ft.). The southern subspecies is generally smaller than its northern relative. The snake is found in a variety of habitats, from forests to near deserts, although usually near sources of water. The snake becomes dormant during the dry season. The African rock Python kills its prey by constriction and often eats animals up to the size of antelope, occasionally even crocodiles. The snake reproduces by egg-laying. Unlike most snakes, the female protects her nest and sometimes even her hatchlings. The snake is widely feared, though it very rarely kills humans. Although the snake is not endangered, it does face threats from habitat reduction and hunting (McDiarmid *et al*., 1999).

1.2.1 Scientific Classification

Kingdom: Animalia

Phylum: Chordata

Class: Reptilia

Order: Squamata

Suborder: Serpentes

Family: Python idae

Genus: *Python*

Species: *P. sebae*

1.2.2 Taxonomy and Etymology

The African rock Python is in the genus *Python,* large constricting snakes found in the moist tropics of Asia and Africa. The species *P. sebae* is divided into two subspecies, *P. s. sebae* (the nominate subspecies) and *P. s. natalensis* (the Southern African rock Python) (O'Shea,2007). Some consider the more southerly population of this snake to be a separate species, *Python natalensis*, (Schmidt, 2006) while others consider this population to be a subspecies (Bartlett and Wanger, 2009). *P. sebae* was first described by Johann Friedrich Gmelin, a German naturalist, in 1788. Therefore, he is also the taxon author of the nominate subspecies. The southern subspecies was first identified by South African zoologist Sir Andrew Smith, in 1833 (Mehrtens, 1987). The generic name, *Python,* is a Greek at Delphi slain by Apollo in Greek mythology. The specific name, *sebae,* is a Latinization of the surname of Dutch zoologist, Albertus Seba (Beolens *et al*., 2011). The subspecific name, *natalensis*, refers to the Natal region of South Africa. Common name usage varies with both the species and northern subspecies referred to as African rock Python or simply rock Python. The southern African rock Python is sometimes referred to as the Natal rock Python or the African Python (Ditmars, 1983).

1.2.3 Description

Africa's largest snake species and one of the world's largest (Alden *et al*., 1996), the typical African rock Python adult measures 3 to 3.53 m (9 ft. 10 in to 11 ft. 7 in) in total length (including tail), with only unusually large specimens likely to exceed 4.8 m (15 ft. 9 in). Reports

of specimens over 6 m (19 ft. 8 in) are considered reliable, although larger specimens have never been confirmed (Murphy and Henderson, 1997). Weights are reportedly in the range of 44 to 55 kg (97 to 121 lb), per one study adults are expected to weigh only up to 32.2 kg (71 lb). Exceptionally large specimens may weigh 91 kg (201 lb) or more (Spawls and Branch, 1995). On average, large adults of African rock Python s are quite heavily built, perhaps more so than most specimens of the somewhat longer reticulated as well as Indian and Burmese Python s and far more so than the Amethystine Python, although the species is on average less heavily built than the green anaconda. The African species may be the second heaviest living snake with some authors agreeing that it can exceptionally exceed 90 kg (200 lb) (Vincent *et al*., 2006; Bodson, 2003).

The snake varies considerably in body size between different areas. In general, it is smaller in highly populated regions, such as in southern Nigeria, only reaching its maximum length in areas such as Sierra Leone, where the human population density is lower. Males are typically smaller than females (Starin and Burghardt, 1992). The African rock Python 's body is thick and covered with coloured blotches, often joining up in a broad, irregular stripe. Body markings vary between brown, olive, chestnut, and yellow, but fade to white on the underside (Alden *et al*., 1996). The head is triangular and is marked on top with a dark brown spear head" outlined in buffy yellow. Teeth are many, sharp, and backwardly curved (Schmidt, 2006), Under the eye, there is a distinctive triangular marking, the sub ocular mark. Like all Python s, the scales of the African rock Python are small and smooth (Branch, 1998). Those around the lips possess heat-sensitive pits, which are used to detect warm-blooded prey, even in the dark (Halliday and Adler, 2002). Python s also possess two functioning lungs, unlike more advanced snakes which have only one, and also have small, visible pelvic spurs, believed to be the vestiges of hind limbs.

1.2.4 Distribution and Habitat

The African rock Python is found throughout almost the whole of sub- Saharan Africa, from Senegal east to Ethiopia and Somalia and south to Namibia and South Africa. Python sebae *sebae* ranges across central and western Africa, while P. s. *natalensis* has a more eastern and southerly range, from southern Kenya to South Africa (Luiselli *et al*., 2007). In 2009, an African rock Python was found in the Florida Everglades. It is feared to be establishing itself as an invasive species alongside the already established Burmese Python. Feral rock Python s were also noted in the 1990s in the Everglades (Murphy and Henderson, 1997). The African

rock Python inhabits a wide range of habitats, including forest, savanna, grassland, semidesert, and rocky areas. It is particularly associated with areas of permanent water, and is found on the edges of swamps, lakes, and rivers (Alden *et al*., 1996). The snake also readily adapts to disturbed habitats, so is often found around human habitation, especially cane fields (Branch and Hacke, 1980).

Table 1: Use of Python and its Body Parts in Traditional Medicine (Sanghamitra *et al*., 2017)

Body Part/ Secretion	**Condition**	**Country of Origin**
Blood	Increase sexual virility, arthritis, posts–surgical complications, male impotency, fatigue, replenished human blood, asthma, eczema, skin nourishing activity, near sightedness, baldness, restoration of olfactory sensation.	India, China, Jakarta
Plasma	Cardio–protective activity	Unknown
Serum	Anti–tumour activity	African
Meat	Skin disease, pain, intestinal haemorrhage, bone disorder, augment appetite, obesity, hypertension, wound healing, nervous breakdown, allergy, diabetes, impotency, cold flues, epidemics, foot and mouth disease of cattle, urination, eye sight improvement.	China, India
Fat	Anti-inflammatory, antimicrobial, joint pain, migraine, bone disorder, impotency, paralysis, rheumatism, joint pain, prevent excessive hair loss, migraine, fractured bone, migraine, fractured bone, asthma rheumatism, erectile dysfunction, rheumatic pain, head ache, furuncle, wound, stings, male impotency, whooping cough, high fever, convulsion, hemiplegia, haemorrhoids, gum bleeding, skin infections, antitussive action	Unknown, India, Europe, Nigeria, Brazil, Africa
Venom	Arthritis, joint pain, skin disorders, bed sores, boils, gall stones, gums, bleeding, head ache, haemorrhoids, menopause	India, Nigeria
Bone	Weakness, gangrene, burns, infection, malaria, dengue, cancer, AIDS	Slovenia, India

1.2.5 Python Fat

Python fat is a lipid obtained from the intestinal walls of *Python sebae*. It is golden yellow in colour and melts to pale yellow oil on standing (Ugwudike *et al*., 2013). The use of Python fat and snake oil originally came from China where it was used as a remedy for inflammation and pain in rheumatoid arthritis, bursitis and other similar condition (Young and Scrimshaw, 1971). It is still used as a pain reliever in China (Andrade *et al*., 2004). It is also used locally in Nigeria (especially in the Southern and Western parts) for the treatment of burns and inflammatory conditions. Fats and oils from snakes are higher in eicosapentaenoic acid (EPA) than other sources which are good anti-inflammatory agents. Python fat is known to be a rich source of monounsaturated fatty acid which is known to reduce the chances of heart diseases (Wang *et al*., 2006). Snake fat also played a role in ancient Egyptian medicine, mixed with the fat of lion, hippopotamus, crocodile, tomcat, and *Nubian ibex* into a homogenous mass believed to cause bald men to grow hair.

The fat is referred to as 'Abuba Eke' in Igbo language of South-eastern Nigeria and 'Ora Ere' in the Yoruba language of Southwestern Nigeria. The Python fat is usually sold by local women who trade in ingredients of folklore medicine in rural markets in Southwestern Nigeria. *Python sebae* is a vulnerable specie which is usually hunted for subsistence, leather and many other traditional use in ethno-medicine (Murphy and Henderson, 1997; Luiselli *et al*., 2001).

Figure 5: Sacks of *Python Fat Sebae* (Moussounga *et al*., 2018)

1.2.5.1 Physicochemical Composition of Python Fat

The physiochemical properties include acid value, peroxide value, saponification value, specific gravity, iodine value. They are used in establishing standards for drugs and compounds (CODEX STAN 19-1981). It also establishes whether a fat is rancid due to peroxidation, length and mode of storage and, other environmental conditions. Whether it will be a good raw material for cosmetic and soap production and other industrial applications (Eromosele *et al.*, 1994). The acid values obtained from literature for Python sebae and *regius* fat vary in average from 2.38 to 127.59 mg KOH / g (Moussounga *et al*., 2018; Okere *et al.*, 2014). Falodun *et al*. (2008) reported acid value of boa constrictor fat (310.73 mgKOH / g). Python fat has been reported to have peroxide values on an average between 0.28 and 5.75 meq. O2 / kg (Moussounga *et al*., 2018; Okere *et al.*, 2014; Datubo–Brown and Blight, 1990; Kunin, 1989). Moussounga *et* al. (2018), reported saponification indices of *Python sebae* in Congo, it varies on average from 169.25 to 178.73 mg KOH / g of fat and Okere *et al*. (2014) reported on *Python* regius fat (164.09 mgKOH / g). High values in the saponification index means that Python *fat* have long chains of carbon and can be applied in the field of soap.

There is no standard for the fatty acid composition of *Python fat* as it varies among species, environmental conditions, habitat and feeding habit of the reptile. Palmitic, stearic, oleic, and linoleic acids are major components. Myristic, arachidic, linolenic and higher unsaturated acids are present as minor components (Kunin, 1989). *Boa constrictor* fat contains 62 % unsaturated and 38 % of saturated fatty acids (Falodun *et al*. 2008), 60.60 % unsaturated and 39.19 % saturated fatty acids ((Moussounga *et al*., 2018), 72.30 % unsaturated and 27 % saturated fatty acids (Okere *et al*., 2014). Python fat has been reported to have fatty acid profiles rich in unsaturated fatty acids, and they have any ethnopharmacological activity (Khunsap *et al*., 2016; Dias *et al*., 2013; Okere *et al*., 2014), as they show antibacterial activity by modifying the endogenous synthesis of bacterial fatty acids (Ferreira *et al.*, 2011). Fats and oils from snakes are higher in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) than other sources which are good anti-inflammatory agents (Wang *et al*., 2006).

1.2.5.2 Benefits of Python Fat

Traditionally in Nigeria (especially in the Southern and Western parts), Python fat has been used in the treatment of rheumatism, boils, keloids and broken bones (Adeola, 1992).
Python fat is used to treat impotency, paralysis (Joshi and Joshi 2010). *Boa constrictor* fat possesses anti-inflammatory and antimicrobial activity against *S. aureus* and *S. pyrogenes*

organisms (Falodun *et al.,* 2008). *Python morulus* fat is used to treat rheumatic pain, burn wounds. *Boa constrictor* fat contains 62 % unsaturated (palmitoleic acid, oleic acid, vaccenic acid, arachidonic acid, linoleic acid) and 38 % of saturated fatty acids (myristic acid, palmitic acid, stearic acid), the unsaturated fatty acids can show antibacterial activity by modifying the endogenous synthesis of bacterial fatty acids (Ferreira *et al.*, 2011). In traditional Indian medicine *P. tyasmucosus* fat is used in the treatment of joint pain. *Naja tripudians* and cobra fat is used in the management of excessive hair loss, topically applied on head to cure migraine and fractured bone. Topical application of boa snake's fat is use to cure asthma (Joshi and Joshi 2010). In India cobra Python, viper fats are used to cure tuberculosis, asthma, rheumatism, erectile dysfunction, joint pain. The fats are usually cooked and consumed or warmed and massaged externally or are stored in bamboo containers and are externally applied. Tribal populations of Tamil Nadu also practice the use Python fat in the treatment of leprosy (Chakravorty *et al.,* 2011). Rattle snake, *Boa sp*. and anaconda fat are used to treat rheumatoid arthritis (Gomes *et al.,* 2011). In Brazilian folk medicine *Crotalus durissus*, *Bothrop sleucurus, Lachesis muta Boa constrictor*, *Epicrates cenchria*, *Eunectes murinus* fats are used in treatment of rheumatic pain, head ache, ear ache, sore throat, tooth ache, pain in the spine, furuncle, wounds, asthma, stings of insects, dogs bite, masculine impotence, tumour. Datubo–Brown and Blight (1990) for the first time reported the presence cytotoxic activity in the snake oil. They scientifically established that the oil from *Boa constrictor* had growth inhibitory activity on human fibroblast: both keloid and normal dermal fibroblasts in *in vitro* model. They postulated that the fatty acids are the major ingredients present in the snake oil that caused the inhibition of the fibroblasts.

1.2.5.3 Side Effects of Python

There are a few side effects of snake oil, such as inflammation of the skin, allergic reactions, and gastrointestinal distress, but these are mainly caused by improper consumption of the oil or the use of low-quality oil. Despite the historical connotation of "snake oil", there are many legitimate forms of true snake oil that can provide health benefits (Okere *et al*., 2014).

1.3 Lipids

Lipids are biological molecules characterized by their solubility in nonpolar solvents. Most lipids are amphipathic molecules, which interact with other molecules and with aqueous solvents via hydrogen bonds and electrostatic interactions (Fahy *et al.,* 2009). The most abundant plant lipids are acyl (fatty acid containing) molecules such as phospholipids,

glycolipids, and triacylglycerol's. Other important lipids include sterols, carotenoids, tocopherols, phytol's, and waxes (Watson, 2006). Biological lipids originate entirely or in part from two distinct types of biochemical subunits or "building-blocks": ketoacyl and isoprene Groups. Using this approach, lipids may be divided into eight categories: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides (derived from condensation of ketoacyl subunits); and sterol lipids and prenol lipids (derived from condensation of isoprene subunits) (Fahy *et al.*, 2009).

Although the term "lipid" is sometimes used as a synonym for fats, fats are a sub Group of lipids called triglycerides. Lipids also encompass molecules such as fatty acids and their derivatives (including tri-, di-, monoglycerides, and phospholipids), as well as other sterol-containing metabolites such as cholesterol (Michelle *et al.*, 1993). Although humans and other mammals use various biosynthetic pathways both to break down and to synthesize lipids, some essential lipids can't be made this way and must be obtained from the diet.

1.3.1 Categories of Lipids

Lipids are divided into eight categories: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides (derived from condensation of ketoacyl subunits); and sterol lipids and prenol lipids (derived from condensation of isoprene subunits) (Fahy *et al.*, 2009).

1.3.1.1 Fatty Acids

The fatty acid structure represents the major lipid building block of complex lipids and therefore is one of the most fundamental categories of biological lipids. The fatty acyl Group in the fatty acids and conjugates class is characterized by a repeating series of methylene Groups that impart hydrophobic character to this category of lipids. The first subclass includes the straight-chain saturated fatty acids containing a terminal carboxylic acid. It could also be considered the most reduced end product of the polyketide pathway. Variants of this structure have one or more methyl substituents and encompass quite complex branched-chain fatty acids, such as the mycolic acids. The longest chain in branched-chain fatty acids defines the chain length of these compounds. A considerable number of variations on this basic structure occur in all kingdoms of life (Vance and Vance, 2002; Small, 1986; Brennan and Nikaido, 1995; Ohlrogge, 1997) including fatty acids with one or more double bonds and even acetylenic (triple) bonds. Heteroatoms of oxygen, halogen, nitrogen, and sulphur are also linked to the

carbon chains in specific subclasses. Cyclic fatty acids containing three to six carbon atoms as well as heterocyclic rings containing oxygen or nitrogen are found in nature. The cyclopentenyl fatty acids are an example of this latter subclass. The thia fatty acid subclass contains sulphur atom(s) in the fatty acid structure and is exemplified by lipoic acid and biotin. Thiols and thioethers are in this class, but the thioesters are placed in the ester class because of the involvement of these and similar esters in fatty acid metabolism and synthesis. Separate classes for more complex fatty acids with multiple functional Groups (but nonbranched) are designated by the total number of carbon atoms found in the critical biosynthetic precursor. These include octadecanoids and lipids in the jasmonic acid pathway of plant hormone biosynthesis, even though jasmonic acids have lost some of their carbon atoms from the biochemical precursor, 12 oxophytodienoic acid (Agrawal *et al*.,2004). Eicosanoids derived from arachidonic acid include prostaglandins, leukotrienes, and other structural derivatives (Murphy and Smith, 2002). The docosanoids contain 22 carbon atoms and derive from a common precursor, docosahexaenoic acid (Bazan, 1989). Many members of these separate subclasses of more complex fatty acids have distinct biological activities. Other major lipid classes in the fatty acyl category include fatty acid esters such as wax monoesters and diesters and the lactones. The fatty ester class also has subclasses that include important biochemical intermediates such as fatty acyl thioester-CoA derivatives, fatty acyl thioester-acyl carrier protein (ACP) derivatives, fatty acyl carnitines (esters of carnitine), and fatty adenylates, which are mixed anhydrides. The fatty alcohols and fatty aldehydes are typified by terminal hydroxy and oxo Groups, respectively. The fatty amides are also *N* -fatty acylated amines and unsubstituted amides, and many simple amides have interesting biological activities in various organisms. Fatty acyl homoserine lactones are fatty amides involved in bacterial quorum sensing (Roche *et al*., 2004). Hydrocarbons are included as a class of fatty acid derivatives because they correspond to six electron reduction products of fatty acids that may have been generated by loss of the carboxylic acid from a fatty acid or fatty acyl moiety during the process of diagenesis in geological samples. Long-chain ethers also have been observed in nature.

1.3.1.2 Glycerolipids

The glycerolipids essentially encompass all glycerol-containing lipids. They are abundant and important as membrane constituents, metabolic fuels, and signalling molecules. The glycerolipid category is dominated by the mono-, di- and tri-substituted glycerol, the most well-known being the fatty acid esters of glycerol (acylglycerols) (Stam *et al*., 1987; Coleman and Lee, 2004). Additional subclasses are represented by the glycerolglycans, which are

characterized by the presence of one or more sugar residues attached to glycerol via a glycosidic linkage (Pahlsson *et al*., 1998). Macrocyclic ether tab lipids also occur as glycerolipids in the membranes of archaebacteria (Koga *et al*., 1983)

Table 2: Name and Symbol of some Fatty Acids (Calder, 2011).

Systematic name	**Trivial name**	**Shorthand Notation**
Octanoic	Caprylic	8:0
Decanoic	Capric	10:0
Dodecanoic	Lauric	12:0
Tetradecanoic	Myristic	14:0
Hexadecanoic	Palmitic	16:0
Octadecanoic	Stearic	18:0
Cis 9-Hexadecenoic	Palmitoleic	16:1n-7
Cis 9-Octadecenoic	Oleic	18:1n-9
Cis 9, cis 12-Octadecadienoic	Linoleic	8:2n-6
Cis 9, 12, 15-Octadecatrienoic	α-Linolenic	18:3n-3
Cis 6, 9, 12-Octadecatrienoic	γ-Linolenic	18:3n-6
Cis 8, 11, 14-Eicosatrienoic	Dihomo-γ-linolenic	20:3n-6
Cis 5, 8, 11, 14-Eicosatetraenoic	Arachidonic	20:4n-6
Cis 5, 8, 11, 14, 17-Eicosapentaenoic	Eicosapentaenoic	20:5n-3
Cis 7, 10, 13, 16, 19-Docosapentaenoic	Docosapentaenoic	22:5n-3
Cis 4, 7, 10, 13, 16, 19-Docosahexaenoic	Docosahexaenoic	22:6n-3

1.3.1.3 Glycerophospholipids

The glycerophospholipids are ubiquitous in nature and are key components of the lipid bilayer of cells. Phospholipids may be subdivided into distinct classes based on the nature of the polar "head Group" at the *sn*- 3 position of the glycerol backbone in eukaryotes and eubacteria or the *sn* -1 position in the case of archaebacteria (Pereto *et al*.,2004). In the case of the glycerophosphoglycerols and glycerophosphoglycerophosphates, a second glycerol unit constitutes part of the head Group, whereas for the glycerophosphoglycerophosphoglycerols (cardiolipins), a third glycerol unit is typically acylated at the *sn*- 1 and *sn*- 2 positions to create a pseudo symmetrical molecule. Each head Group class is further differentiated on the basis of the *sn*- 1 and *sn*- 2 substituents on the glycerol backbone. Although the glycerol backbone is symmetrical, the second carbon becomes a chiral centre when the *sn* -1 and *sn* -3 carbons have different substituents. A large number of trivial names are associated with phospholipids. In the systematic nomenclature, mono/di-radylglycerophospholipids with different acyl or alkyl substituents are designated by similar conventions for naming of classes (see below) and are Grouped according to the common polar moieties (i.e., head Groups). Typically, one or both of these hydroxyl Groups are acylated with long-chain fatty acids, but there is also alkyl- linked and 1 *Z* -alkenyl linked (plasmalogen) glycero-phospholipids, as well as dialkylether variants in prokaryotes. The main biosynthetic pathways for the formation of GPCho and GPEtn were elucidated through the efforts of Kennedy and co-workers (Kennedy, 1962) in the 1950s and 1960s, and more detailed interconversion pathways to form additional classes of phospholipids were described more recently. In addition to serving as a primary component of cellular membranes and binding sites for intracellular and intercellular proteins, some glycerophospholipids in eukaryotic cells are either precursors of, or are themselves, membrane-derived second messengers. A separate class, called oxidized glycerophospholipids, is composed of molecules in which one or more of the side chains have been oxidized. Several overviews are available on the classification, nomenclature, metabolism, and profiling of glycerophospholipids (Cevc,1993; Forrester *et al*., 2004; Ivanova *et al*., 2004; Cronan, 2003).

1.3.1.4 Sphingolipids

Sphingolipids are a complex family of compounds that share a common structural feature, a sphingoid base backbone that is synthesized de novo from serine and a long chain fatty acyl CoA, then converted into ceramides, phosphosphingolipids, glycosphingolipids, and other species, including protein adducts (Merrill and Sandhoff, 2002; Taniguchi *et al*., 2002). A

number of organisms also produce sphingoid base analogues that have many of the same features as sphingolipids (such as long-chain alkyl and vicinal amino and hydroxyl Groups) but differ in other features. These have been included in this category because some are known to function as inhibitors or antagonists of sphingolipids, and in some organisms, these types of compounds may serve as surrogates for sphingolipids. Sphingolipids can be divided into several major classes: the sphingoid bases and their simple derivatives (such as the 1-phosphate), the sphingoid bases with an amide-linked fatty acid (e.g., ceramides), and more complex sphingolipids with head Groups that are attached via phosphodiester linkages (the phosphosphingolipids), via glycosidic bonds (the simple and complex glycosphingolipids such as cerebrosides and gangliosides), and other Groups (such as phosphono- and arseno-sphingolipids). The IUPAC has recommended a systematic nomenclature for sphingolipids (3). The major sphingoid base of mammals is commonly referred to as "sphingosine," because that name was affixed by the first scientist to isolate this compound (Thudichum, 1884).

1.3.1.5 Sterol Lipids

The sterol category is subdivided primarily on the basis of biological function. The sterols, of which cholesterol and its derivatives are the most widely studied in mammalian systems, constitute an important component of membrane lipids, along with the glycerophospholipids and sphingomyelins (Bach and Wachtel, 2003). There are many examples of unique sterols from plant, fungal, and marine sources that are designated as distinct subclasses in this schema. The steroids, which also contain the same fused four ring core structure, have different biological roles as hormones and signalling molecules (Tsai and O'Malley, 1994). These are subdivided on the basis of the number of carbons in the core skeleton. The C18 steroids include the estrogen family, whereas the C19 steroids comprise the androgens such as testosterone and androsterone. The C21 subclass, containing a two-carbon side chain at the C17 position, includes the progestogens as well as the glucocorticoids and mineralocorticoids. The secosteroids, comprising various forms of vitamin D, are characterized by cleavage of the B ring of the core structure, hence the "seco" prefix (Jones *et al*., 1998). Additional classes within the sterols category are the bile acids (Russell *et al*., 2003) which in mammals are primarily derivatives of cholan24-oic acid synthesized from cholesterol in the liver and their conjugates (sulfuric acid, taurine, glycine, glucuronic acid, and others).

1.3.1.6 Prenol Lipids

Prenols are synthesized from the five carbon precursors isopentenyl diphosphate and dimethylallyl diphosphate that are produced mainly via the mevalonic acid pathway (Kuzuyama and Seto *et al*., 2003). In some bacteria (e.g., *Escherichia coli*) and plants, isoprenoid precursors are made by the methylerythritol phosphate pathway (Rodriguez-Concepcion, 2004). Because the simple isoprenoids (linear alcohols, diphosphates, etc.) are formed by the successive addition of C5 units, it is convenient to classify them in this manner, with a polyterpene subclass for those structures containing more than 40 carbons (8 isoprenoid units) (Porter and Spurgeon, 1981). Note that vitamin A and its derivatives and phytanic acid and its oxidation product pristanic acid are Grouped under C20 isoprenoids. Carotenoids are important simple isoprenoids that function as antioxidants and as precursors of vitamin A (Demming-Adams and Adams, 2002). Another biologically important class of molecules is exemplified by the quinones and hydroquinones, which contain an isoprenoid tail attached to a quinonoid core of nonisoprenoid origin. Vitamins E and K (Ricciarelli *et al*., 2001; Meganathan *et al*., 2001) as well as the ubiquinones (Raetz and Whitfield *et al*., 2002) are examples of this class. Polyprenols and their phosphorylated derivatives play important roles in the transport of oligosaccharides across membranes. Polyprenol phosphate sugars and polyprenol diphosphate sugars function in extracytoplasmic glycosylation reactions (Lazar and Walker *et al*., 2002), in extracellular polysaccharide biosynthesis for instance, peptidoglycan polymerization in bacteria (Schenk *et al*., 2001), and in eukaryotic protein *N*-glycosylation (Helenius and Aebi *et al*., 2001). The biosynthesis and function of polyprenol phosphate sugars differ significantly from those of the polyprenol diphosphate sugars; therefore, we have placed them in separate subclasses. Bacteria synthesize polyprenols (called bactoprenols) in which the terminal isoprenoid unit attached to oxygen remains unsaturated, whereas in animal polyprenols (dolichols) the terminal isoprenoid is reduced. Bacterial polyprenols are typically 10 to 12 units long (Schenk *et al*., 2001), whereas dolichols usually consist of 18 to 22 isoprene units. In the phytoprenols of plants, the three distal units are reduced.

1.3.2 Fatty Acids and Inflammation

Fatty acids are naturally occurring constituents of the diet. They have metabolic, structural and functional roles within the body, where they act as important sources of energy, major components of all cell membranes, precursors to signalling molecules and regulators of cellular responses (Calder and Burdge, 2004). Although all fatty acids can be used as energy sources,

different fatty acids have different, often unique, structural and functional roles and biological activities. Indeed, several fatty acids have roles and activities that oppose one another, indicating that the overall biological outcome will be the result of interactions among several fatty acids. With regard to inflammation it is often considered that it is the PUFA of the n-6 and n-3 families that are most important, these often acting to oppose one another's actions (Calder, 2009). However, it is now recognised that other fatty acids and fatty acid families are likely also involved in inflammation, with fairly recent work focusing on SFA (Wong *et* al., 2009). There are a number of general mechanisms by which fatty acid exposures could affect inflammatory cell function, and so inflammatory processes. They include:

- Action directly via surface or intracellular 'fatty acid receptors'.
- Incorporation into the phospholipids of inflammatory cell membranes, where the fatty acids play important roles assuring the correct environment for membrane protein function, maintaining membrane order ('fluidity'), influencing lipid raft formation and modifying membrane-generated intracellular signalling cascades.
- Acting as precursors of extracellular signalling molecules such as PG.

1.3.2.1 Fatty acids, PPARγ and inflammation

PPARγ is a transcription factor that acts in an anti-inflammatory inflammatory gene expression, but it also interferes with the activation of the prototypical pro-inflammatory transcription NF-kB (Van den Berghe *et al*., 2003). PUFA and their derivatives are endogenous ligands for PPARγ. The n-3 PUFA DHA induced PPARγ in dendritic cells and this was associated with reduced production of the pro-inflammatory cytokines TNFa and IL 6 following endotoxin stimulation (Kong *et* al., 2010). In addition, DHA induced a number of known PPARγ target genes in dendritic cells, suggesting this as an important anti-inflammatory mechanism of action (Zapata-Gonzalez *et al*, 2008).

1.3.2.2 Fatty acids, GPR120 and inflammation

The cell surface G-protein coupled receptor termed GPR120 is highly expressed on inflammatory macrophages, and a GPR120 agonist GW9508 inhibited responsiveness of macrophages to LPS (Oh *et al*., 2010). This involved reduced phosphorylation of the IkB and its maintenance in the cytosol (phosphorylated IkB is degraded) and reduced TNFa and IL-6 production. These observations suggest that GPR120 is anti-inflammatory. DHA and another n-3 PUFA, EPA, but not arachidonic, palmitic or myristic acids, promoted GPR120-mediated

gene activation, although they were much less potent than GW9508. The effects of DHA were further explored (Oh *et al*., 2010). Its inhibitory effects on LPS-induced IkB phosphorylation, IkB degradation and TNFa, IL-6 and also on monocyte chemotactic protein-1 production did not occur in GPR120 knockdown cells.

1.3.2.3 Modification of Inflammatory Cell Membrane Fatty Acid Composition and Consequent Alteration of Lipid Mediator Profiles

1.3.2.3.1 Modification of Inflammatory Cell Membrane Fatty Acid Composition

PUFA are important constituents of the phospholipids of the membranes of inflammatory cells. Typically, these contain a relatively high proportion of the n-6 PUFA, arachidonic acid; this is seen in both laboratory animals (Lokesh *et al*.,1986; James *et al*., 1991) and human subjects. Increased oral supply of the n-3 PUFA EPA and DHA results in an increase in the amount of those fatty acids in inflammatory cells, seen in both laboratory animals and human subjects. The increase in content of EPA and DHA happens over the course of days to weeks, occurs in a dose–response manner and is accompanied by a decrease in content of arachidonic acid (Sperling *et al*., 1993; Gibney *et al*., 1993).

1.3.2.3.2 Fatty Acid Modification of Eicosanoid Profiles

Eicosanoids, which include PG, thromboxanes and leukotrienes, are long-recognised mediators and regulators of inflammation. They are formed from C20 PUFA, typically arachidonic acid, by the COX and lipoxygenase enzymes. In general, arachidonic acid-derived eicosanoids act in a pro inflammatory way, although this is an oversimplification since it is now recognised that PGE2, for example, has both pro- and anti-inflammatory effects (Calder, 2009), and that another eicosanoid derived from arachidonic acid, lipoxin A4, is anti-inflammatory (Levy *et al*., 2001; Vachier *et al*., 2002; Gewirtz *et al.*, 2002; Serhan *et al*., 2003). The decrease in arachidonic acid content of inflammatory cell membranes that occurs with incorporation of the n-3 PUFA reduces the availability of the usual eicosanoid substrate and so the production of the major 2-series PG and 4-series leukotrienes is decreased (Peterson *et al*., 1998; Von Schacky *et al*., 1993). EPA is also a substrate for the COX and lipoxygenase, but the mediators produced have a different structure from the arachidonic acid-derived mediators, and this often influences their potency (Wada *et al*., 2007). For example, EPA-derived leukotriene B5 is ten- to 100-fold less potent as a neutrophil chemoattractant compared with leukotriene B4 (Goldman *et al*., 1983; Lee *et al*., 1984). Furthermore, EPA-derived eicosanoids

may antagonise the action of those produced from arachidonic acid, as was recently demonstrated for PGD3 v. PGD2 (Tull *et al*., 2009).

1.3.2.3.3 Novel Anti-Inflammatory and Inflammation Resolving Mediators Produced from EPA and DHA: Resolvins and Protectins

EPA and DHA are substrates for synthesis of fairly recently discovered lipid mediators that are potent anti-inflammatory and inflammation resolving agents. These include resolvins and protectins, which are produced through pathways involving COX and lipoxygenase enzymes (Serhan *et al*., 2000; Serhan *et al*., 2002). Examples of the activities are these compounds include the inhibition of transendothelial migration of neutrophils by resolvin E1, resolvin D1 and protectin D1, and inhibition of TNFa and IL-1b production by protectin D1(Serhan *et al*., 2008).

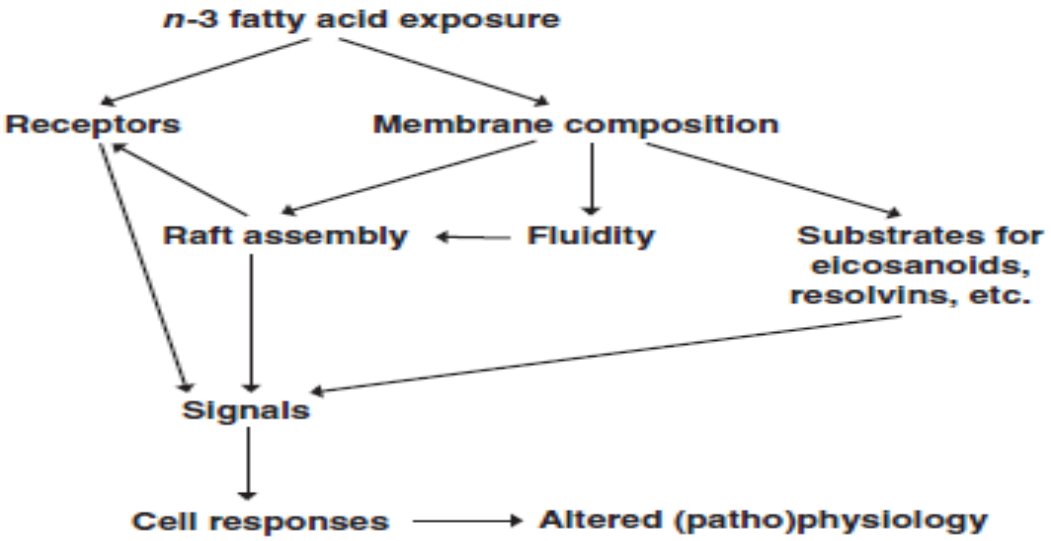

Figure 6: Overview of the mechanisms by which fatty acids can influence inflammatory cell function (Calder, 2012)

1.3.2.4 Therapeutic Benefits of The Anti-Inflammatory Actions of Fatty Acids

A number of human conditions and diseases have an inflammatory component, and it seems that, irrespective of the body compartment(s) involved, these conditions and diseases all involve excessive or inappropriate production of inflammatory mediators including eicosanoids and cytokines (Calder *et al*., 2009). It is evident that the n-3 PUFA EPA and DHA act through multiple interconnected mechanisms (Calder, 2011) to reduce production of inflammatory eicosanoids and cytokines and to enhance production of anti-inflammatory and

inflammation resolving resolvins and protectins. In these ways, n-3 PUFA act to oppose the proinflammatory actions of SFA and of n-6 PUFA. The roles of n-3 PUFA in shaping and regulating inflammatory processes and responses suggest that the level of exposure to these fatty acids might be important in determining the development and severity of inflammatory diseases. The recognition that n-3 PUFA have anti-inflammatory actions has led to numerous studies supplementing the diet of patients with inflammatory diseases to evaluate clinical benefit. Studies in patients with rheumatoid arthritis have been the most successful among those in patients with an overt inflammatory disease, with a number of trials reporting clinical benefits (Calder, 2008a), these benefits being supported by meta-analyses (Goldberg *et al*., 2007). Studies in patients with inflammatory bowel diseases (Crohn's disease and ulcerative colitis) provide equivocal findings with some showing some benefits and others not (Calder, 2008b). Similarly, studies conducted in patients with asthma do not provide a clear picture with most studies conducted in adults not showing a clinical benefit, although there are indications of benefits of n-3 PUFA in children and adolescents (Calder, 2006). In most other inflammatory diseases and conditions there are too few studies to draw a clear conclusion of the possible efficacy of n-3 PUFA. One reason for these discrepancies may be that the dose of n-3 PUFA required to treat different inflammatory conditions is not known, although it is evident that the anti-inflammatory effects of these fatty acids are dose-dependent (Rees *et al*., 2006).

1.4 Aim of the Study

The study was aimed at investigating the lipid profile of the Python fat and its anti-inflammatory properties.

1.5 Specific Objectives of Study

This research was designed to:

- Determine the physicochemical properties of the Python fat.
- Determine the fatty acid profile of the Python fat.
- Determine the effect of the Python fat on egg albumin-induced rat paw oedema.
- Determine the membrane stabilization effect of the Python fat.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Python Fat

The fat extracted from the *Python sebae* were purchased and authenticated by a hunter at the traditional medicine area in Ogige Nsukka and Ogbete Main Market Enugu, Enugu State, Nigeria and stored at 4˚C.

2.1.2 Animals

Animals used for this study were male Wistar rats weighing between 100 and 150 g. They were purchased from the animal house of the Department of Zoology and Environmental Biology, University of Nigeria, Nsukka. The animals were acclimatised for one week prior to the commencement of experiment and maintained on a regular feed (commercial chicken growers' mash) and water *ad libitum*. The animals received humane care in line with provisions of the University of Nigeria, Nsukka on care and management of laboratory animals.

2.1.3 Blood Samples

This was obtained from adult human volunteers who were free from drugs for at least two weeks before sample collection.

2.1.4 Chemicals and Reagents

The chemicals and reagents used for this study were of analytical grade and products of Karmel India, Sigma Aldrich Germany, JHD Guanghua Guangdong China, Qualikems, and Cultivalae chemicals Ltd., others were purchased from standard chemical and reagent stores within Enugu State.

2.1.5 Equipment, Apparatus and Instruments

Weighing balance (Vickas Ltd, England), Whatman 1 filter paper, water bath (Precision CIR19), Spectrophotometer (Jenway 6305, China), Centrifuge, GC-MS QP2010 PLUS (Shimadzu).

2.2 Methods

2.2.1 Preparation of Python Fat

The lipids were extracted simply by heating the small pieces of fat from fat sack of fat extracted from the belly of Python between 37 and 50 ˚C until complete dissolution of these fats. Lipids were kept at 4˚C until use (Khunsap *et al*. 2016). Fats from Ogige Nsukka was tagged (FPA) and that from Ogbete Enugu (FPD).

2.2.2 Physicochemical Analyses

2.2.2.1 Determination of Chemical Indices

The different indices measured in this study were determined according to the following official AOAC standards: Acid Index (AOAC, 969.17, 1997); Iodine Number (AOAC, Standard 993.20, 1997); Peroxide Index (AOAC, Standard 965.33, 1997); Saponification index (AOAC, standard 920.160.1997) (AOCS, 1997; Kyari M.Z., 2008).

2.2.2.1.1 Determination of Acid Value

The acid value is the number of mg of potassium hydroxide (KOH) required to neutralize 1 g of a sample.

Procedure

A total of 1 g of sample was weighed accurately; 50 ml of an ethanol-ether mixture was added (1:1). It was dissolved while heating and the solution was used as test solution. It was allowed to cool, few drops of phenolphthalein TS was added, titrated with 0.1 M ethanolic potassium hydroxide until the pink colour of the solution persists for 30 seconds, and the acid value was calculated by the formula below. Before using the solvent, 0.1 M ethanolic potassium hydroxide was added to the solvent until its pink colour persists for 30 seconds, using phenolphthalein TS as an indicator.

$$\text{Acid value} = \frac{\text{Vol. (ml) of 0.1 Mol/L ethanolic potassium hydroxide consumed} \times 5.611}{\text{Weight (g) of the sample}}$$

Where 5.611 is a constant

2.2.2.1.2 Determination of Peroxide Value

Peroxide value is usually applied to edible product including tallow and associated product such as margarine shortenings and fried fats. It is also applied to the fat in meat and meat product. It is used as an indicator of the extent of oxidative rancidity. It is affected by the age

of raw material as well as oxidation of fat during processing and storage. It reflects the palatability of the oil.

Procedure

1.0 g of melted fat was added in a 250 ml stoppered conical flask. While the fat was still liquid, 30 ml of acetic acid-chloroform solution was added and the flask was swirled to dissolve the fat in the solvent. 0.5 M of saturated KI solution was added from a 1 ml graduated pipette. Stopper the flask and allow the mixture to stand with occasional shaking for exactly 1 min. After 1 min, few drops of distilled water were added. About 5 drops of starch indicator solution was also added. Titrate with 0.01 M $Na_2S_2O_4$ solution. The flask was shaked vigorously during the titration to extract iodine from the chloroform layer. The end point occurred when the purple colour disappeared. For peroxide values > 10 meq/1000 g, it was titrated with 0.1 M $Na_2S_2O_4$. A blank determination of the reagents was performed. The blank titration should not exceed 0.1 M of 0.01 M $Na_2S_2O_4$. The calculation is shown below:

Peroxide value (meq/1000 g) = $\frac{(A - B) \times M \times 1000}{W}$

Where:

Titration value of sample (ml of thiosulphate) = A

Titration value of blank (ml of thiosulphate) = B

Molarity of $Na_2S_2O_4$ = M

Weight of sample (g) = W

2.2.2.1.3 Determination of Saponification Value

The saponification value is the number of mg of potassium hydroxide (KOH) to saponify the esters in 1 g of the sample and neutralize the free acids in 1 g of a sample.

Procedure

A total of 1 g of sample was weighed accurately and transferred into a conical flask, added with 40 ml of ethanol, and dissolved while warming if necessary. 20 ml of ethanolic potassium hydroxide was added. The flask was equipped with a reflux condenser, and heated in a water bath for 30 minutes while shaking the flask occasionally. It was allowed to cool, few drops of phenolphthalein TS was added, and immediately titrated using excess potassium hydroxide and 0.5 M hydrochloric acid. A blank test was performed, and the saponification value was calculated by the formula:

$$\text{Saponification value} = \frac{(a - b) \times 28.05}{\text{Weight (g) of the sample}}$$

Where

a = volume of 0.5 M hydrochloric acid consumed in the blank test;

b = volume (ml) of 0.5 M hydrochloric acid consumed in the test.

2.2.3 Determination of the Fatty Acid Composition

2.2.3.1 Preparation of Fatty Acid Methyl Esters (FAMEs)

Methyl-esterification of samples used in the analyses was performed by boron trifluoride-methanol method after alkaline hydrolysis. To 20 mg of sample oils were added 2 ml of 0.5 mol/L NaOH-methanol solutions, and the mixture was heated at 100 ^{0}C for 7 min. After cooling, 3 ml of 14 % boron trifluoride-methanol reagent was added, and the vessel was sealed and heated at 100 ^{0}C for 5 min. After cooling, 2 mL of hexane and 7 mL of saturated NaCl solution were added, followed by a thorough shaking. The resulting hexane layer (2 mL) was used as a sample solution for GC (Shirasawa *et al*., 2007; Moigradean *et al*., 2013).

2.2.3.2 GC-MS Quantification Method

Analysis of FAME was performed by using GC-MS (QP2010 PLUS Shimadzu) equipped with a split injector. Separations were achieved using an SP^{TM} 2560 Sigma Aldrich capillary column (100 m × 0.25 mm ID, 0.20 μm film thickness). Helium was used as the carrier gas at flow rates of 1.80 mL/min and a split ratio of 20:1. The injector temperature was 250 °C. The oven temperature was programmed at 70 °C for a hold time of 1min and increased to 280 °C for a hold time of 5 min. The injection volume was 2μL. MS spectra were obtained at a scan range of 30-400 m/z, interface temperature of 250 °C, ion source temperature of 200 °C, solvent cut time of 2.50 min, event time of 0.50 secs, and scan speed of 666 u/sec. Ionization energy (EI) of 70 eV was used for the mass spectroscopy detector at a scan rate of 1 sec. FAME peaks were identified by comparing their mass spectra with those of compounds obtained from the NIST mass spectral library (NIST 05) database.

2.2.4 *In vivo* Anti-inflammatory Study

2.2.4.1 Determination of the Effect of the Python Fat on Egg Albumin Rat Paw Oedema

This was done according to the method of Winter *et al*. (1962). The increase in the right hind paw size of the rats induced by the sub-plantar injection of fresh egg albumin was used as a

measure of acute inflammation (Akah and Nwambie, 1994).

Principle

Fresh egg albumin releases mediators of acute inflammation responsible for causing oedema. The ability of the Python fat to inhibit this release of mediators is a measure of the anti-inflammatory effect of the fat.

Experimental Design

A total of eighteen (18) male Wistar albino rats were used for the study. They were divided into six (6) Groups of three (3) rats each and treated as follows:
Group 1: Received Petroleum Jelly (vehicle)
Group 2: Received 4% Indomethacin (w/v in petroleum jelly)
Group 3: Received FPA, 2% (v/v in petroleum jelly)
Group 4: Received FPA, 5% (v/v in petroleum jelly)
Group 5: Received FPD, 2% (v/v in petroleum jelly)
Group 6: Received FPD, 5% (v/v in petroleum jelly)

Procedure

Rats were fasted for 18 h before the experiment to ensure uniform hydration and minimize variability in oedematous response, after which the right hind paw size of the rats at time zero (before the induction of oedema) was measured using a digital Vernier calliper. This was followed by topical application of test substances as outlined above. Three (3) hours after application of ointment, acute inflammation was induced by injecting 0.1 ml of undiluted fresh egg albumin into the sub-plantar surface of the right hind paw of rats. The increase in the right hind paw size of rats was subsequently measured at 0.5, 1, 2, 3 and 4 h after fresh egg albumin injection. The difference between the paw size of the injected paws at time zero and at different times after fresh egg albumin injection was used to assess the formation of oedema. Cardboard collars were placed about their necks before application of the preparation to prevent ingestion of the topically applied preparation (ointment). These values were used in the calculation of the percentage inhibition of oedema for each dose of the Python fat and for indomethacin at the different time intervals using the relation below:
Paw oedema = $(V_t - V_o)$
V_o = Paw oedema at time zero

V_t = Paw oedema at time t (0.5, 1, 2, 3 and 4 h)

Percentage inhibition of oedema = $\frac{(Vt-Vo)control-(Vt-Vo)treated\ Groups}{(Vt-Vo)control} \times 100$

2.2.5 *In vitro* Anti-inflammatory Studies

2.2.5.1 Determination of the Effect of the Python Fat on Membrane Stabilization

The effect of the Python fat on membrane stabilization was determined using both heat and hypotonicity induced haemolysis of human red blood cells (HRBCs)

Principle

This test is based on the susceptibility of RBCs membrane to oxidative damage, made possible as a result of the preponderance of PUFAs in the membranes leading to haemolysis. Haemoglobin and other internal cell components are thus released into the surrounding fluids. Absorbance readings at 418 and 540 nm are thus, reflection of the amount of haemoglobin in the medium. High absorbance values thus imply high haemolysis and less stability of the RBCs membranes.

2.2.5.1.1 Determination of the Effect of the Python Fat on Heat-induced Haemolysis of Human Red Blood Cells

This was evaluated using modified method of Shinde *et al*. (1989).

Preparation of Human Red Blood Cells Suspension

Fresh blood sample (3 ml) was collected from a healthy adult volunteer into an EDTA bottle and centrifuged at 3,000 rpm for 10 min and the supernatant discarded. A volume of normal saline equivalent to that of the supernatant was used to resuspend the red blood cells. The volume of the resuspended red blood cells was measured and reconstituted as a 40% (v/v) suspension with normal saline. The reconstituted red blood cells were used as such.

Procedure

Samples of the Python fat and indomethacin used were dissolved in 3% tween 80 (dissolved in normal saline). A set of test tubes containing respectively, 5 ml graded doses of the Python Fat (0.1 mg/ml, 0.2 mg/ml and 0.5 mg/ml) were arranged in quadruplicate (4 sets per dose). Control tubes contained 5 ml of the vehicle and 5 ml of 0.2 mg/ml indomethacin, respectively. HRBCs suspension (0.1 ml) was added to each of the tubes and mixed gently. A pair of the tubes was

incubated in a regulated water bath at 54 ^{0}C for twenty minutes while the other pair was maintained in the freezer for twenty minutes. Afterwards, the tubes were centrifuged at 1300 rpm for 3 min and the haemoglobin content of the supernatant was estimated using a spectrophotometer at 540 nm. The blanks contained varying concentration of the Python Fat, indomethacin and vehicle (for control) without HRBCs suspension. The percentage (%) inhibition of haemolysis by the chloroform Python Fat and indomethacin was calculated thus:

% Inhibition of Haemolysis = $\left(1 - \frac{OD_2 - 0D_1}{OD_3 - OD_1}\right) \times 100$

Where OD1 = Absorbance of test sample (Python Fat, indomethacin) unheated

OD2 = Absorbance of test sample heated

OD3 = Absorbance of control sample heated

2.2.5.1.2 Determination of the Effect of the Python Fat on Hypotonicity-Induced Haemolysis of Human Red Blood Cells

This was determined using modifications of the method of Oyedepo *et al*. (1997).

Preparation of Human Red Blood Cells Suspension

Blood sample (3 ml) obtained from a healthy volunteer was placed into an EDTA bottle, centrifuged at 3,000 rpm for 10 min and washed three times with equal volume of normal saline. The blood volume was measured and reconstituted as a 40% (v/v) suspension with normal saline.

Procedure

Samples of the Python Fat and indomethacin used were dissolved in 3 % tween 80 (dissolved in Tween 80) which served as the hypotonic solution. An aliquot (1 ml) of varying concentrations of the Python Fat (0.1 mg/ml, 0.2 mg/ml and 0.5 mg/ml) was put into each of a set of test tubes. Another tube contained 1 ml of 0.2 mg/ml indomethacin. The contents of the respective tubes were made up to 4.9 ml with the vehicle (3 % tween 80 dissolved in distilled water). Two control tubes were used for this test. A control tube contained 4.9 ml of the vehicle while another contained 4.9 ml of 3 % tween 80 dissolved in normal saline (isotonic solution). HRBCs suspension (0.1 ml) was added to each tube, and after gentle mixing, the mixtures were incubated for 1 h at room temperature (37 ^{0}C). After incubation, the reaction mixture for each tube was centrifuged at 3,000 g for 10 min and the absorbance of the supernatant measured at 418 nm using a spectrophotometer. The tests were carried out in triplicates. Reaction media containing 1 ml varying concentrations of Python Fat or indomethacin made up to 5.0 ml with

3 % tween 80 in normal saline, without HRBCs suspension were used as the respective blank for each test. The blank for the control tubes contained 3 % tween 80 in normal saline also without HRBCs suspension. The percentage inhibition of haemolysis was calculated using the relation below:

% Inhibition of Haemolysis = $\left(1 - \frac{OD_2 - 0D_1}{OD_3 - OD_1}\right) \times 100$

Where

OD1 = Absorbance of control I (isotonic solution)

OD2 = Absorbance of test sample

OD3 = Absorbance of control II (hypotonic solution)

2.2.6 Statistical Analysis

The data obtained were analysed and results expressed as mean ± standard deviation (SD) using Statistical Product and Service Solutions (SPSS), version 22. Tests of statistical significance were carried out using one-way Analysis of Variance (ANOVA). Mean values with ($p < 0.05$) were considered statistically significant.

CHAPTER 3

RESULTS

3.1 Chemical Properties of Python Fat

The results of the chemical properties determined on the two fat samples were presented in Table 3. The samples peroxide (2.25 ± 0.21 and 0.90 ± 0.14) values, acid values (34.08 ± 0.17 and 15.82 ± 0.16 mg KOH/g), saponification value (160.88 ± 0.88 and 179.05 ± 0.66 mg KOH/Kg) for FPA and FPD, respectively.

3.2 Fatty Acid Profile of Python Fat

The relative percentage composition of the fatty acids of the studied Python fat is shown in Table 4. The Python fats showed a high percentage of unsaturated fatty acids (44.06 and 74.76%) for FPA and FPD, respectively.

Table3: Chemical Indices of Python Fat

Parameter	**Samples**	
	FPA	**FPD**
Acid Value (mg KOH/g)	34.08 ± 0.17	15.82±0.16
Peroxide Value (Meq.O_2/Kg)	2.25 ± 0.21	0.90 ± 0.14
Saponification Value (mg KOH/g)	160.88±.88	179.05±.66

Values are presented as mean ± standard deviation for duplicates replication

Table 4: Fatty Acid Profile of Python Fat

		Samples	
Fatty acid	**Symbol**	**FPA (%)**	**FPD (%)**
Capric acid	10:0	2.08	ND
Lauric acid	12:0	4.33	ND
Myristic acid	14:0	2.15	5.36
Palmitic acid	16:0	14.22	7.60
Stearic acid	18:0	ND	4.95
Oleic acid	18:1	40.19	2.79
Ricinoleic aicd	18:1	ND	5.31
Vaccenic acid	18:1	ND	7.87
Erucic acid	22:1	ND	2.97
Linoleic acid	18:2	3.87	34.54
Linolenic acid	18:3	ND	3.32
Eicosadienoic acid	20:2	ND	17.96
TFAs		66.84	92.67
TSFAs		22.78	17.91
TUFAs		44.06	74.76
TPUFAs		3.87	55.82
TUFA/TSFA		1.93	4.17

TFAs: Total fatty acid, TSFAs: Total saturated fatty acid, TUFAs: Total unsaturated fatty acid, TPUFAs: Total polyunsaturated fatty acids, ND: Not detected

3.3 Effect of Python Fat on Fresh Egg Albumin-Induced Paw Oedema of Rats

Table 5 shows the effect of Python fat on rat paw oedema induced in rats with 0.1ml undiluted fresh egg albumin. It shows that egg albumin induced oedema in the rat paw was sustained over a period of 4 hours (h). Groups treated with 2 % and 5 % of both Python fat samples (FPA and FPD) and 4 % of indomethacin showed a significant ($p < 0.05$) decrease in paw oedema when compared with the control. There was significant ($p < 0.05$) decrease in paw oedema of Groups treated with Python fat when compared with the indomethacin Group at 30 minutes (min). The paw size of animals treated with increasing doses of Python fats and indomethacin significantly ($p < 0.05$) decreased with time. The percentage oedema inhibition for rats treated with FPA 2 % after 30 min, 1 h, 2 h, 3 h and 4 h were 15.89 %, 45.27 %, 42.43 %, 35.25 % and 40.43 %, respectively. The percentage oedema inhibition for rats treated with FPA 5 % after 30 min, 1 h, 2 h, 3 h and 4 h were 22.19 %, 38.51 %, 20.18 % and 15.74 %, respectively. The percentage oedema inhibition for rats treated with FPD 2 % after 30 min, 1 h, 2 h, 3 h and 4 h were 8.77 %, 34.46 %, 22.44 %, 2.68 % and 0.85 %, respectively. The percentage oedema inhibition for rats treated with FPD 5 % after 30 min, 1 h, 2 h, 3 h and 4 h were 31.78 %, 52.93 %, 46.29 %, 41.29 % and 40.85 %, respectively. The percentage oedema inhibition for rats treated with indomethacin 4 % after 30 min, 1 h, 2 h, 3 h and 4 h were -22.19 %, 27.48 %, 26.11 %, 26.05 % and 23.83 %.

Table 5: Effect of Python Fat on Egg Albumin-Induced Rats Paw Oedema

Group	**Dose**	**Mean Paw Oedema (mm)**				
		30min	**1h**	**2h**	**3h**	**4h**
Group 1		8.71 ± 0.32^{bC}	9.50 ± 0.07^{bD}	8.43 ± 0.21^{bC}	7.67 ± 0.43^{bB}	7.41 ± 0.54^{bB}
Group 2	4%	8.38 ± 0.44^{bD}	7.14 ± 0.53^{aC}	6.41 ± 0.60^{aBC}	5.85 ± 0.58^{aB}	5.71 ± 0.55^{aB}
		◊ -22.19	◊ 27.48	◊ 26.11	◊ 26.05	◊ 23.83
Group 3	2%	7.40 ± 0.54^{aD}	6.76 ± 0.55^{aCD}	6.27 ± 0.26^{aBC}	6.02 ± 0.20^{aB}	5.73 ± 0.30^{aB}
		◊ 15.89	◊ 45.27	◊ 42.43	◊ 35.25	◊ 40.43
Group 4	5%	6.58 ± 0.80^{aB}	6.47 ± 0.70^{aB}	6.43 ± 0.55^{aB}	5.78 ± 0.49^{aB}	5.72 ± 1.34^{aB}
		◊ 22.19	◊ 38.51	◊ 20.18	◊ 21.84	◊ 15.74
Group 5	2%	7.26 ± 0.56^{aB}	6.84 ± 0.34^{aB}	6.54 ± 0.40^{aB}	6.47 ± 0.74^{aB}	6.26 ± 0.72^{abB}
		◊ 8.77	◊ 34.46	◊ 22.55	◊ 2.68	◊ 0.85
Group 6	5%	6.79 ± 0.29^{aC}	6.39 ± 0.41^{aBC}	6.11 ± 0.54^{aBC}	5.82 ± 0.38^{aB}	5.69 ± 0.44^{aB}
		◊ 31.78	◊ 52.93	◊ 46.29	◊ 41.76	◊ 40.85

Results are expressed as mean ± standard deviation; n = 3; ◊ = Percentage inhibition of oedema calculated relative to control
Mean values having different lower-case letters as superscripts from top to bottom of the column and those having different upper-case letters as superscripts across the row are considered significant ($p < 0.05$)

Group 1: Normal control received petroleum jelly topically
Group 2: Standard control treated with 4% indomethacin (in petroleum jelly)
Group 3: Treated with FPA 2% (in petroleum jelly)
Group 4: Treated with FPA 5% (in petroleum jelly)
Group 5: Treated with FPD 2% (in petroleum jelly)
Group 6: Treated with FPD 5% (in petroleum jelly)

3.4 Effect of the Python Fat on Membrane Stabilization

3.4.1 Effect of Python Fat on Heat-induced Haemolysis of Human Red Blood Cells

At various concentrations (0.1 mg/ml, 0.2 mg/ml and 0.5 mg/ml), the Python fat like indomethacin (0.2 mg/ml) significantly ($p < 0.05$) inhibited heat-induced haemolysis of HRBCs compared to the control. The highest percentage inhibition (61.78 % and 71.07 %) of haemolysis was obtained at 0.2 mg/ml for both Python fat samples (Table 6).

Table 6: Effect of Python Fat on Heat Induced Red Blood Cell Haemolysis

Treatment	Concentrations	OD_{540nm}			Difference in OD Values		Percentage Inhibition of Haemolysis (%)
		Heated Solution (OD_3)	Heated Solution (OD_2)	Unheated Solution (OD_1)	(OD_2-OD_1)	(OD_3-OD_1)	
Control		0.649 ± 0.001^{g}					
FPA	0.1mg/ml		0.392 ± 0.011^{e}	0.022 ± 0.000^{d}	0.370	0.627	40.989
FPA	0.2mg/ml		0.261 ± 0.001^{b}	0.021 ± 0.001^{c}	0.240	0.628	61.783
FPA	0.5mg/ml		0.311 ± 0.002^{c}	0.019 ± 0.001^{a}	0.292	0.630	53.651
FPD	0.1mg/ml		0.371 ± 0.007^{de}	0.020 ± 0.000^{bc}	0.351	0.629	44.197
FPD	0.2mg/ml		0.202 ± 0.052^{a}	0.020 ± 0.001^{abc}	0.182	0.629	71.065
FPD	0.5mg/ml		0.332 ± 0.002^{cd}	0.019 ± 0.000^{ab}	0.314	0.631	50.238
Indomethacin	0.2mg/ml		0.462 ± 0.003^{f}	0.030 ± 0.001^{e}	0.432	0.619	30.210

Results are expressed in means ± standard deviation; n = 2
Mean values having different letters as superscripts from top to bottom of the column are considered significant ($p < 0.05$)

3.4.2 Effect of Python Fat on Hypotonicity-induced Haemolysis of Human Red Blood Cells

Effect of the Python Fat on Hypotonicity-induced Haemolysis of Human Red Blood Cells is shown in Table 7. The Python fat significantly ($p < 0.05$) protected the human erythrocyte membrane against lyses induced by hypotonic solution compared to the control. Like that of heat-induced haemolysis (Table 6), there were significantly ($p < 0.05$) higher inhibitions of hypotonicity-induced haemolysis of HRBCs at a concentration of (0.2mg/ml) of the Python fat compared to indomethacin. Also, a non-significant ($p > 0.05$) inhibition was observed with the (0.1 mg/ml and 0.5 mg/ml) concentrations of the Python fat compared to indomethacin. The highest percentage inhibition (53.846 % and 55.769 %) of haemolysis in this case was obtained at 0.2 mg/ml for each Python fat sample.

Table 7: Effect of Python Fat on Hypotonicity Induced Haemolysis of Human Red Blood Cell

Treatment	Concentrations	OD_{418nm}			Difference in OD Values	Percentage Inhibition of Haemolysis (%)
		Hypotonic Solution (OD_3)	Hypotonic Solution (OD_2)	Isotonic Solution (OD_1)	(OD_2-OD_1)	
Hypotonic Solution		0.835 ± 0.212^{e}				
Isotonic Solution				0.315 ± 0.494^{a}		
FPA	0.1mg/ml		0.675 ± 0.212^{d}		0.360	30.769
FPA	0.2mg/ml		0.555 ± 0.494^{b}		0.240	53.846
FPA	0.5mg/ml		0.580 ± 0.014^{bc}		0.265	49.038
FPD	0.1mg/ml		0.670 ± 0.035^{d}		0.355	31.731
FPD	0.2mg/ml		0.545 ± 0.017^{b}		0.230	55.769
FPD	0.5mg/ml		0.575 ± 0.035^{bc}		0.260	50.000
Indomethacin	0.2mg/ml		0.650 ± 0.028^{cd}		0.520	35.577

Results are expressed in means ± standard deviation; n = 2
Mean values having different letters as superscripts from top to bottom of the column are considered significant ($p < 0.05$)

CHAPTER 4

DISCUSSION

The physiochemical properties are used in establishing standards for drugs and compounds. They are used to determine the quality of substances and compounds. The acid values obtained in this study were 34.08 ± 0.17 and 15.82 ± 0.16 mg KOH/g for FPA and FPD, respectively. According to the Codex Alimentarius (CODEX STAN 19-1981), a good quality fat must have an acidity level of 4 mg KOH/g of fat. Both fat samples in this study did not conform to the standard. The high acidity of the sample could be due to poor storage. This also indicates high amount of fatty acids hydrolysed from triglycerides which is related to increase in acid number (Falodun *et al.,* 2008). Although Folodun *et al*. (2008) recorded an acid value of 310.73 mg KOH/g. For soap production higher acid values are required, consequently, the oil is suitable for soap production with respect to its acid values (Eromosele *et al.*, 1994). From the oxidation point of view, the peroxide value of the fats analysed were 2.25 ± 0.21 and 0.90 ± 0.14 Meq.O_2/kg for FPA and FPD, respectively. These values were in accordance with the Codex Alimentarius (CODEX STAN 19-1981) which sets the lower value up to 10 Meq.O_2/kg of fat. This means the fats used in were of good quality. Low peroxide value obtained was an indication that the oil was not likely to be liable to oxidative rancidity at room temperature (Anyasor *et al*., 2009). Saponification value is a measure of the average molecular weight (or chain length) of all the fatty acids present in the fat (Odoemelan, 2005). The saponification values obtained for the python fat in this study were 160.875 ± 0.88 and 179.050 ± 0.66 mg KOH/g for FPA and FPD, respectively. These values were close to those found by Okere *et al*. (2014) on *Python regius* fat (164.09 mgKOH/g) and 178.73 mgKOH/g by Moussounga *et al*. (2018). These high values in the saponification value shows that the fats used in this study have high molecular weight triglycerides and long chains of carbon which means the fats can be applied in the field of soap making (Moussounga *et al.,* 2018).

Python sebae fats gave a high probability of unsaturated fatty acids 44.06 % and 74.76 % for FPA and FPD, respectively. The most represented being oleic acid (40.19 %) and linoleic acid (34.54 %). Saturated fatty acids represent 22.78 % and 17.913 % for FPA and FPD of total fatty acids with a significant proportion (14.22 % and 7.60 %) of palmitic acid for FPA and FPD, respectively. The ratio of unsaturated fatty acids to saturated fatty acids was 1.93 and 4.17 for FPA and FPD, respectively. The ratio for FPA was similar to that reported by

Moussounga *et al.* (2018) of 1.55 and lower to the result obtained for FPD in this study. The percentage of unsaturated and saturated fatty acid in this study is similar to that reported by Okere *et al.* (2014); Khunsap *et al.* (2016) (55.16 % and 26.29 %) on Cobra Oil, Oliveira *et al.* (2014) (61.38 % and 33.59 %) on *Spilotes pullatus* for total unsaturated and total saturated fatty acids, respectively. Although a deviation from this study was reported by Victoria (2015) who recorded a higher percentage of total saturated fatty acid to total unsaturated fatty acid (61.6 % and 37.5 %) for total saturated fatty acid and total unsaturated fatty acid, respectively.

Palmitic acid ($C_{16:0}$) was the major saturated fatty acid for all the fat samples analyzed. Amounts of capric ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$) acids were observed in FPA and myristic ($C_{14:0}$) and stearic ($C_{18:0}$) in FPD. These findings were similar to those reported by (Moussounga *et al.* (2018) and Okere *et al.* (2014). The presence of capric acid ($C_{10:0}$) and lauric acid ($C_{12:0}$) was found only in FPA oil at 2.08 % and 4.33 %, respectively. Myristic acid ($C_{14:0}$) was found in both FPA and FPD at (2.08 % and 5.36 %), respectively. Stearic acid ($C_{18:0}$) was found in FPD only at 4.95 %. These findings are very close to the results presented by Moussounga *et al.* (2018). All the samples analyzed presented total SFA content of less than total unsaturated fatty acid which was more than half of the total fatty acid content (saturated and unsaturated fatty acids). This finding was in disagreement with the results presented by Victoria (2015) where the total saturated fatty acid content of fat analyzed was found to be greater than 50 % of the total fatty acid content. The presence of these saturated fatty acids also explains the high saponification value of 160.875 ± 0.88 and 179.050 ± 0.66 mg KOH/g for FPA and FPD, respectively, obtained from the chemical analysis and makes both samples suitable for production of soaps, cosmetics and release agents.

The total unsaturated fatty acid content of FPA and FPD were 44.02 % and 74.19 %, respectively shown in Table 4. Linoleic ($C_{18:2}$) acid and oleic ($C_{18:1}$) acid were the major unsaturated fatty acids present in the samples. FPD had 5.31 % ricinoleic acid, 3.32 % linolenic acid, 7.87 % vaccenic acid, 17.96 % eicosadienoic acid and 2.97 % erucic acid, which were not in FPA. Python fat used for this study possessed 44.02 % total unsaturated fatty acid with a predominant 40.19% oleic acid for FPA. FPD had 74.19 % unsaturated fatty acid with linoleic acid at 34.54 % and eicosadienoic acid at 17.96 % the predominant. Polyunsaturated fatty acid (PUFA) from the samples were linolenic acid, linoleic acid, eicosadienoic acid. FPA had only two unsaturated fatty acid types; Oleic acid, a monounsaturated fatty acid (MUFA) comprising 40.19 % and linoleic acid a polyunsaturated fatty acid (PUFA) comprising 4.33 % of the total fatty acid content of the sample.

Capric acid is used in the manufacture of esters for artificial fruit flavours and perfumes. It is also used as an intermediate in chemical syntheses. It is used in organic synthesis and industrially in the manufacture of perfumes, lubricants, greases, rubber, dyes, plastics, food additives and pharmaceuticals. *In vitro* experiments have suggested that some fatty acids including lauric acid could be a useful component in a treatment for acne, but no clinical trials have yet been conducted to evaluate this potential benefit in humans (Nakatsuji *et al.*, 2009; Yang *et al.*, 2009).

The principal use of oleic acid is as a component in many foods, in the form of its triglycerides. It is a component of the normal human diet as a part of animal fats and vegetable oils. Oleic acid, a monounsaturated (omega-9) fatty acid readily finds application in cosmetic and pharmaceutical formulations as an excipient. It serves as penetration enhancer in transdermal formulations for the bioavailability of poorly hydrophilic active ingredients (Morgan *et al.*,1998). Oleic acid as its sodium salt is a major component of soap as an emulsifying agent. It is also used as an emollient (Carrasco, 2009). Small amounts of oleic acid are used as an excipient in pharmaceuticals, and it is used as an emulsifying or solubilizing agent in aerosol products (Smolinske, 1992).

The omega-3 (n-3) fatty acid content in python fat samples was linolenic acid, which is a precursor eicosapentaenoic (EPA) acid; and snake oils have been shown to be enriched in EPA (Kunin, 1989). While the full mechanism for n-3 fatty acid inhibition of cell growth is not known, studies n-3 fatty acids have been implicated in down-regulating anti-apoptotic signals and in blocking pro-inflammatory signals that lead to increased cell proliferation. (Lim *et al.*, 2009; Ghosh-Choudhury *et al.*, 2009). Omega-3 polyunsaturated fatty acids (PUFA) and n-6 polyunsaturated fatty acids compete for metabolism to eicosanoids, and it has been shown that dietary n-3 polyunsaturated fatty acid supplementation is associated with increased incorporation into skin and with reduced basal and UVB-induced skin prostaglandin E2 (PGE2) levels (Ziboh, 1989; Rhodes *et al.*, 1984). Omega-3 polyunsaturated fatty acid supplementation has also been shown to reduce secretion of pro-inflammatory cytokines, tumour necrosis factor (TNF-α), and interleukin (IL-1β) in circulating monocytes (Endres *et al.*, 1989; Blok *et al.*, 1996). PUFAs reduce, for example, the effect of TNF-α, which suggests a wider range of anti-inflammatory properties for these agents (Storey *et al.*, 2005) and may induce PGE3, which in turn may subdue cellular response to mitogenic and inflammatory stimuli (Bagga *et al.*, 2003). Administration of PUFAs has been reported to be beneficial in a range of immune-mediated conditions (Ergas *et al.*, 2002) and local application of linoleic acid

to burn wounds has been shown to improve healing by decreasing hypertrophic scarring (Shakespeare and Strange, 1982). Louw (2000) observed decreased levels of omega-6 fatty acids: linoleic acid (LA), γ-linolenic acid (GLA), dihomo-glinolenic acid (DGLA), n-3 fatty acids (α-linolenic acid [ALA] and eicosapentaenoic acid) in combination with enhanced arachidonic acid (AA) levels in scar tissues. This is in agreement with a previous work by Shakespeare and Strange (1982) who found a significantly lower level of essential fatty acids (Linoleic acids C 18:2) in phospholipids isolated from swabs of hypertrophic scars. Ricinoleic acid exerts analgesic and anti-inflammatory effects (Vieira *et al.*, 2000). Ricinoleic acid specifically activates the EP3 prostanoid receptor for prostaglandin E2 (Tunaru *et al.*, 2012). The fatty acid composition of the FPA and FPD showed that the fat has potential to be used for both industrial and pharmaceutical applications.

Swelling (tumour/oedema) is one of the classical signs of inflammation. Oedema is caused by accumulation of fluid (Cotran, 1998). Increased permeability of the blood vessels results in an exudation (leakage) of plasma proteins and fluid into the tissue (oedema), which manifests itself as swelling (Parakrama and Clive, 2005). The topical anti-inflammatory activity of the python fat (FPA and FPD) was confirmed by measuring its ability to reduce local oedema induced in the rat paw by injection of an irritant/phlogistic agent (Omkar *et al.*, 2007). Subcutaneous injection of fresh egg albumin into the rat paw produced oedema resulting from protein rich fluids exudation along with neutrophil extravasation (Yankanchi and Koli, 2010). Carrageenan induction of inflammation involves three distinct phases of mediators' release including histamine and 5-hydroxytryptamine in the first phase, kinins in the second phase and prostaglandin in the third phase (Di Rosa *et al.*, 1971). Prostaglandin in particular, is known to cause or enhance the cardinal signs of inflammation. Fresh egg albumin-induced inflammation appears to be similar to carrageenan induced inflammation in rats (Nwafor and Okwuasaba, 2003). Many clinically useful non-steroidal drugs have been demonstrated to possess anti-inflammatory and analgesic actions when given orally, some studies have also shown considerable bioavailability and efficacies when indomethacin (Naito and Tsai, 1981) and aspirin (Poisson *et al.*, 1985) are used topically in animal models. These served as bases for the selection of the standard drugs as references in this study. The python fat samples (FPA and FPD) at 2 % and 5 % showed good anti-inflammatory activity as it significantly ($p < 0.05$) inhibited the increase in paw circumference from 30 minutes (min) to 4 hour (h) which covers the three phases for carrageenan induced oedema namely, histamine and 5-hydroxytryptamine (5- HT) release in the first (early) phase, kinins release in the second phase and prostaglandins

release in the third (late) phase (Di Rosa *et al.*, 1971; Flower *et al.*, 1972). This shows that the extract inhibited all the phases of the inflammatory response. FPA and FPD at 2 % and 5 % showed a higher significant ($p < 0.05$) decrease of oedema compared to indomethacin 4 % after 30 min of induction of oedema but had non-significant ($p < 0.05$) difference from 1 h to 4 h in the inflammation model tested. The suppression of oedema in the second and third phase of inflammation suggests that the anti-inflammatory activity of the extract may also be due to the suppression of kinin and prostaglandin formation induced by egg albumin within this period. The python fat samples reduce vascular permeability and fluid exudation, probably by preventing the contraction of endothelial cells, thus supressing oedema. The inhibition of oedema by the extract might be due to the presence of MUFAs ricinoleic aicd and PUFAs linolenic acid and eicosadienoic acid with anti-inflammatory properties and have been implicated in down-regulating anti-apoptotic signals and in blocking pro-inflammatory signals that lead to increased cell proliferation (Vieira *et al.*, 2000; Tunaru *et al.*, 2012; Lim *et al.*, 2009; Ghosh-Choudhury *et al.*, 2009). Omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids compete for metabolism to eicosanoids, prostaglandins produced from n-3 PUFA constitute a minor group compared to eicosanoids produced from arachidonic acid (AA) and may be as much as one hundred-fold less potent biologically for inducing pro-inflammatory cellular responses than those derived from AA (Robinson *et al.*, 1988). n-3 PUFA (LA, EPA and DHA) also lower blood triglyceride concentrations (Harris, 1997) and are substrates for the synthesis of resolvins, which are believed to play a key role in terminating inflammatory processes (Kohli and Levy, 2009). Thus, these n-3 fatty acids could be important in treating inflammatory diseases (Mori and Beilin, 2004). Omega-3 polyunsaturated fatty acid supplementation has also been shown to reduce secretion of pro-inflammatory cytokines, tumour necrosis factor (TNF-α), and interleukin (IL-1β) in circulating monocytes (Endres *et al.*, 1989; Blok *et al.*, 1996). Administration of PUFAs has been reported to be beneficial in a range of inflammatory conditions (Ergas *et al.*, 2002). PUFAs reduce, for example, the effect of TNF-α, which suggests a wider range of anti-inflammatory properties for these agents (Storey *et al.*, 2005) and may induce PGE3, which in turn may subdue cellular response to mitogenic and inflammatory stimuli (Bagga *et al.*, 2003). Also, local application of linoleic acid to burn wounds has been shown to improve healing by decreasing hypertrophic scarring (Shakespeare and Strange, 1982).

Leakage of lytic enzymes, serum proteins, fluids and other mediators of inflammation from the lysosomal membrane is one of the processes involved during inflammation (Cunha *et al.*,

2007). HRBCs are analogous to lysosomal membrane (Mounnissamy *et al*., 2008). The HRBCs membrane is made up of majorly polyunsaturated fatty acids which makes the cells highly susceptible to oxidative damage (Nagamani *et al.*, 2012) leading to haemolysis by which haemoglobin and other internal cell components are released into the surrounding fluids. Red blood cell membrane can be lysed due to its exposure to injurious substances such as hypotonic medium, heat, methyl salicylate or phenylhydrazine. This study demonstrated the capability of the python fat samples to inhibit the lyses of HRBCs membrane induced by hypotonic solution and heat. The python fat samples at concentrations of 0.1 mg/ml, 0.2 mg/ml and 0.5 mg/ml were able to significantly ($p < 0.05$) stabilize HRBCs membrane, which is an indication of the ability of the fat to prevent haemolysis. The stabilization of erythrocyte membranes by the fat samples thus, implies that it may as well stabilize lysosomal membranes. HRBCs are analogous to lysosomal membrane (Mounnissamy *et al*., 2008; Mohammedi and Atik, 2014) and stabilization of lysosomal membranes is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated leukocytes such as, bactericidal enzymes and proteases, which upon extracellular release cause further tissue inflammation and damage (Borregaard, 2010; Anosike *et al.*, 2012). This result thus provides evidence for membrane stabilization as an additional mechanism of the anti-inflammatory effect of python fat. The haemolytic effect of hypotonic solution could be related to osmotically coupled water uptake by the cells, resulting in swelling and the subsequent rupturing of its membrane, releasing intracellular electrolyte and fluid components. This implies that the fats perhaps stabilized the RBC membrane by inhibiting processes which stimulate or enhance the efflux of these intracellular components such as, by preventing the release of lytic enzymes and active mediators of inflammation. Stabilization of the membrane also prevents the leakage of serum protein and fluids into the tissues during a period of increased vascular permeability caused by inflammatory mediators (Chaitanya *et al.*, 2011).

4.2 CONCLUSION

This study suggests that the python fat (FPA and FPD) possess anti-inflammatory effect. The fatty acid profile of the fats showed high amount of unsaturated fatty acids which have been reported to have anti-inflammatory, skin repair and wound healing property. The anti-inflammatory mechanism of these fatty acids could be through the inhibition of the synthesis of inflammatory mediators and through the synthesis of anti-inflammatory mediators, and inflammatory resolving lipids. The fat could also act by stabilizing the lysosomal membrane. The python fat from this study showed high saponification value, which makes it a good raw material for cosmetic and soap production. This suggest that the fat sample can be used for industrial and pharmaceutical applications. This research, thus, consolidates the use of the python fat in ethno-medicine for treating inflammatory conditions, burns, scar and wound.

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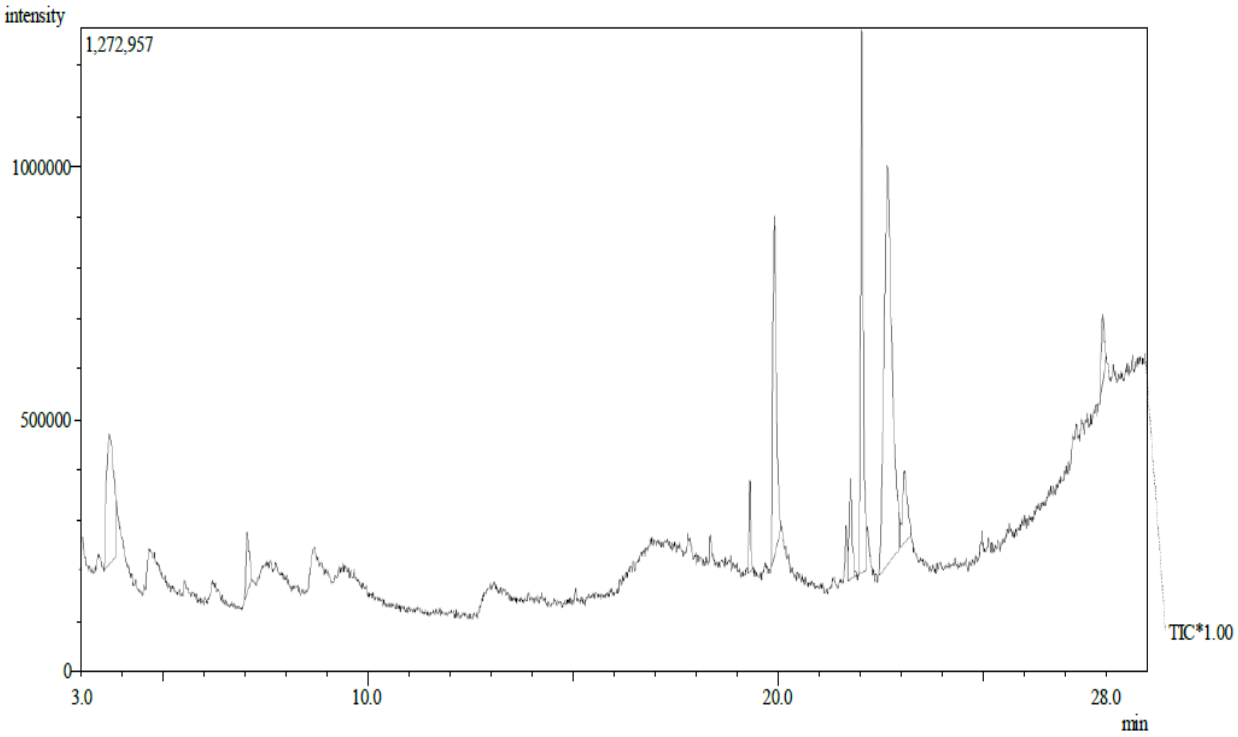
APPENDICES

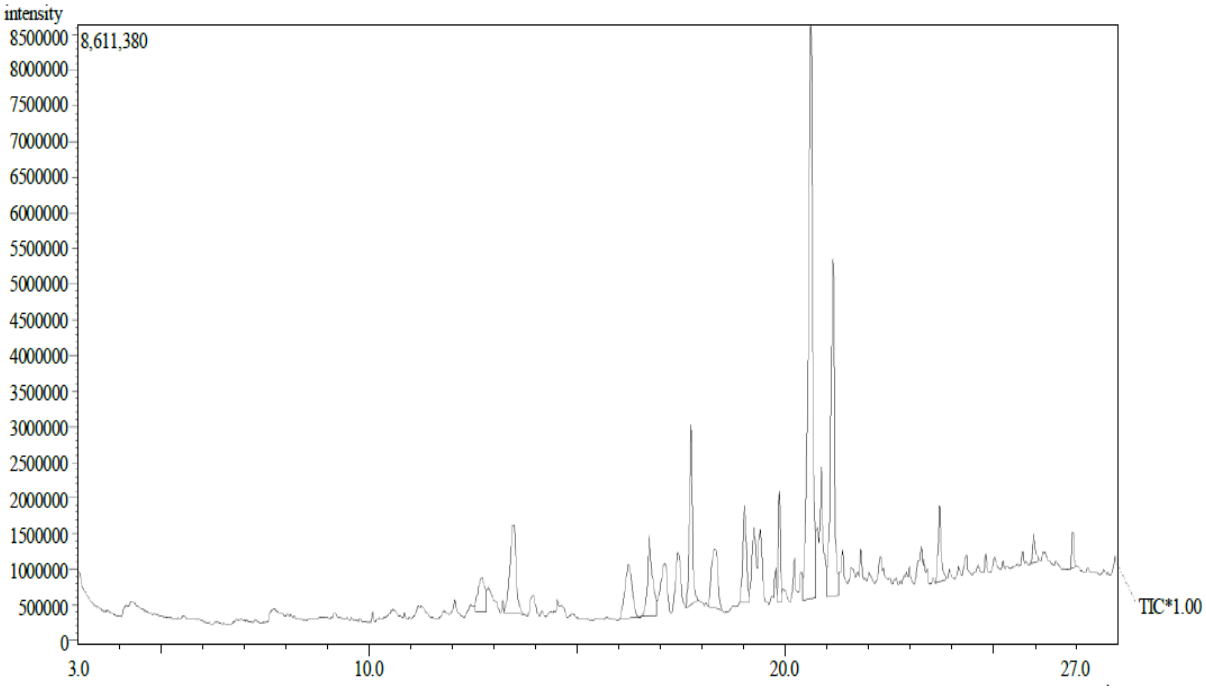
Appendix 1: Chromatogram of FPA



Appendix 2: Peak Report FPA

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H
1	3.676	3.542	3.850	3362880	12.39	260808	7.24	12.89
2	7.037	6.975	7.158	686540	2.53	122963	3.41	5.58
3	19.329	19.283	19.408	564286	2.08	185347	5.15	3.04
4	19.926	19.842	20.092	3858883	14.22	677254	18.80	5.70
5	21.791	21.725	21.900	1049099	3.87	203970	5.66	5.14
6	22.068	21.900	22.183	4946274	18.23	1080020	29.99	4.58
7	22.686	22.492	22.983	10905056	40.19	795441	22.08	13.71
8	23.115	23.008	23.242	1175558	4.33	143940	4.00	8.17
9	27.959	27.883	28.042	582943	2.15	132091	3.67	4.41
				27131519	100.00	3601834	100.00	

Appendix 3: Chromatogram of FPD



Appendix 4: Peak Report FPD

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H
1	12.721	12.558	12.825	5495424	3.23	481426	1.95	11.41
2	13.484	13.258	13.733	13391072	7.87	1247079	5.06	10.74
3	16.245	16.042	16.475	9037488	5.31	747735	3.03	12.09
4	16.751	16.475	16.925	9126757	5.36	1135536	4.61	8.04
5	17.765	17.633	17.925	12932268	7.60	2523818	10.24	5.12
6	18.325	18.167	18.508	8764266	5.15	836232	3.39	10.48
7	19.047	18.917	19.158	8418070	4.95	1368356	5.55	6.15
8	19.885	19.833	19.942	4750051	2.79	1561549	6.34	3.04
9	20.646	20.450	20.767	58782204	34.54	8030612	32.59	7.32
10	21.180	21.025	21.308	30567738	17.96	4735913	19.22	6.45
11	23.744	23.650	23.883	5046428	2.97	1059933	4.30	4.76
12	26.010	25.933	26.108	1951927	1.15	403113	1.64	4.84
13	26.954	26.883	27.042	1902234	1.12	513573	2.08	3.70
				170165927	100.00	24644875	100.00	